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Contents

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Contents

World Journal of Clinical Oncology
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REVIEW

Therapeutic management options for stage III non-small cell lung cancer

Stephanie M Yoon, Talha Shaikh, Mark Hallman

Stephanie M Yoon, Talha Shaikh, Mark Hallman, Department of Radiation Oncology, Fox Chase Cancer Center, Philadelphia, PA 19111, United States

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Correspondence to: Talha Shaikh, MD, Department of Radiation Oncology, Fox Chase Cancer Center, 333 Cottman Avenue, Philadelphia, PA 19111, United States. talha.shaikh@fccc.edu
Telephone: +1-215-7282581
Fax: +1-215-2144038

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Abstract

Lung cancer is the leading cause of cancer death worldwide. Majority of newly diagnosed lung cancers are non-small cell lung cancer (NSCLC), of which up to half are considered locally advanced at the time of diagnosis. Patients with locally advanced stage III NSCLC consists of a heterogeneous population, making management for these patients complex. Surgery has long been the preferred local treatment for patients with resectable disease. For select patients, multi-modality therapy involving systemic and radiation therapies in addition to surgery improves treatment outcomes compared to surgery alone. For patients with unresectable disease, concurrent chemoradiation is the preferred treatment. More recently, research into different chemotherapy agents, targeted therapies, radiation fractionation schedules, intensity-modulated radiotherapy, and proton therapy have shown promise to improve treatment outcomes and quality of life. The array of treatment approaches for locally advanced NSCLC is large and constantly evolving. An updated review of past and current literature for the roles of surgery, chemotherapeutic agents, radiation therapy, and targeted therapy for stage III NSCLC patients are presented.

Key words: Non-small cell lung cancer; Chemo-radiotherapy; Multi-modality; Targeted therapy; Dose-escalation

Core tip: Locally advanced non-small cell lung cancer consists of a heterogeneous population making management challenging. Multiple strategies are being developed to maximize survival and disease control. The role of surgery is being re-evaluated given new insight into the efficacy chemotherapy and radiation. Multi-modality therapy is playing an increasingly important role for both resectable and unresectable stage III patients. Chemoradiation plays a large role in the management of inoperable or unresectable patients. Third generation

chemotherapy and other targeted therapies are being incorporated into chemoradiation. Radiation dose-escalation, alternative fractionation schedules, intensity-modulated radiotherapy, and proton therapy are evaluated to improve outcomes from chemoradiation.

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INTRODUCTION

Lung cancer is the leading cause of cancer death in the United States and worldwide. In 2016, approximately 224390 Americans are estimated to be newly diagnosed with lung cancer, and 158080 will die from this disease[1]. About 80% of lung cancer cases are non-small cell lung cancer (NSCLC), of which up to half are locally advanced at the time of diagnosis[2]. According to guidelines, locally advanced NSCLC is often defined as the 7th edition AJCC staging classification stage III NSCLC[3,4].

Stage IIIA and IIIB are two subsets within this classification, and the distinction is made because prognosis, treatment options, and long-term outcomes differ from one another. Furthermore, stage IIIA disease must be differentiated as resectable or unresectable at time of diagnosis. Stage IIIA (T1-3 N2, T3-T4 N1, T4 N0) disease involves hilar or mediastinal lymph nodes limited to the ipsilateral mediastinum, and a subset of these patients are amenable to surgery[3,4]. However, Stage IIIB (T1-4 N3, or T4 N2) involves lymph node metastasis in the contralateral thorax or supraclavicular fossa and/or an unresectable primary tumor, making patients with this disease not ideal candidates for surgical resection[3,4]. With such a heterogeneous population, a multi-modality approach involving surgery, radiation, and systemic agents is most commonly employed. A standard treatment option for unresectable or inoperable stage IIIA and stage IIIB disease is concurrent chemoradiation, while management of IIIA is more complex and controversial[5]. Treatment options for IIIA disease includes surgery with neoadjuvant or adjuvant chemotherapy, radiation, or both; as well as definitive chemoradiation[3,5,6]. Long-term outcomes are poor, with baseline 5-year overall survival (OS) of 15%-35% for stage IIIA and 5%-10% for stage IIIB[7]. The appropriate combination, timing, and sequence of individual treatment components in order to improve outcomes are under active research for both disease subsets. The aim of this review is to provide an overview of current and future treatment options for the management of locally advanced NSCLC.

MANAGEMENT OPTIONS FOR RESECTABLE STAGE IIIA NSCLC

Surgery

Up to 30%-50% of stage III NSCLC are locally advanced and inoperable at time of diagnosis[2,8]. Accurate pre-operative staging, particularly of mediastinal lymph nodes, is imperative as it dictates further management. Lymph node evaluation techniques include endobronchial ultrasound, endoscopic ultrasound-guided biopsy, cervical mediastinoscopy, or transthoracic needle aspiration. Positron emission tomography/computed tomography (PET/CT) scans have improved the accuracy of lymph node staging by improved detection of subclinical micro- and macro-metastases[9]. For patients who are deemed to have resectable disease, surgery plays an important role in their treatment. Generally, those with limited mediastinal lymphadenopathy are considered potentially more favorable candidates for resection than those with multistation or bulky mediastinal involvement, as it is associated with a higher rate of micro-metastasis. However, there are no specific guidelines to determine to what extent lung tumors should be considered "resectable"[6]. In fact, data have shown that a substantial proportion of stage IIIA-N2 patients who were considered resectable ultimately had an R1, 2 resection[10].

Pre- and post-operative chemotherapy

While surgery is an important aspect in the management for resectable stage IIIA patients, surgery alone continues to have poor outcomes, and as many as 30%-70% of resected patients experience recurrence or death[11,12]. The addition of post-operative chemotherapy has been extensively studied, and shown to improve treatment outcomes in patients with locally advanced disease[13-15]. In an analysis by the NSCLC Meta-analysis Collaborative Group[13] in which a meta-analyses totaling 34 trials and 8447 patients were evaluated, adjuvant chemotherapy was shown to have an absolute 5-year overall survival benefit of 4%, increasing OS rate from 60% to 64%, for patients with stage I-III disease. More specifically, a 5% absolute improvement in 5-year survival for stage III disease was observed, increasing 5-year OS rates from 30% to 35%. Other recent studies[14,15] have shown similar results, in which post-operative chemotherapy increased median survival from 45 mo from surgery alone to 54 mo[14]. These studies also demonstrated adjuvant chemotherapy increased 5-year progression free survival (PFS) by approximately 5%[14,15]. Because post-operative chemotherapy has been shown to significantly improve treatment outcomes, it is the standard of care for resectable locally advanced disease[3].

While surgical resection followed by chemotherapy is commonly employed, induction chemotherapy followed by surgical resection has also been studied[7,16-19]. Indu-

ction chemotherapy has the potential to eradicate micrometastases prior to resection, reduce tumor size, and increase the likelihood of resection. However, a concern with induction chemotherapy would be to delay a potentially curative surgery due to disease progression or declining health of the patient. The same NSCLC Meta-analysis Collaborative Group recently summarized the findings of 15 randomized controlled trials totaling 2385 patients on the effects of administering chemotherapy prior to surgical resection for patients with stage IB-IIIA disease[16]. In this analysis, pre-operative chemotherapy increased 5-year survival from 20% to 25%. Similar to adjuvant chemotherapy, induction chemotherapy also reduced relative risk of death by 13%. Five-year PFS improved from 30% to 36% with induction chemotherapy, and the time to distant recurrence also improved by 10% at 5-year. Results from older studies have shown that induction chemotherapy improved median survival from 11 mo to anywhere between 22 to 64 mo[17-19]. The NSCLC Meta-analysis Collaborative Group did not note a difference in complete resection rates between surgery *vs* preoperative chemotherapy with surgery, suggesting that the delay for induction chemotherapy does not significantly reduce chances of a potentially curative resection[16].

There does not seem to be a difference in survival or recurrence between adjuvant and induction chemotherapy. In a phase III trial, Felip *et al*[20] randomized 624 stage IA to IIIA patients to surgery alone, three cycles of preoperative carboplatin-paclitaxel followed by surgery, or surgery followed by three cycles of adjuvant carboplatin-paclitaxel. There was no difference in 5-year OS or PFS rates between induction and adjuvant chemotherapy regimens compared to surgery alone, though there was a non-significant trend towards longer PFS in the preoperative arm. Given that pre- and post-operative chemotherapy yields similar outcomes, induction chemotherapy could be reserved for patients with larger, more advanced tumors or those unable to tolerate chemotherapy while recovering after surgery[16]. Adjuvant chemotherapy could be utilized for patients with better prognosis and earlier disease stages[16].

Post-operative radiotherapy

Despite having complete resection and adjuvant chemotherapy, up to 40% of resectable stage IIIA patients experience local tumor recurrence[21,22]. In order to improve local tumor control and survival, post-operative radiotherapy (PORT) has long been utilized to intensify local therapy. Yet the ideal candidate for PORT has been controversial with conflicting results from different trials and series. Historical randomized control trials demonstrated that PORT significantly reduced local recurrence without any impact on overall survival[23-25]. One trial demonstrated a detrimental effect of PORT on survival compared to surgery alone, in which 5-year OS rates were 30% and 43% respectively[23]. The PORT Meta-analysis[26] demonstrated that PORT had an adverse effect on survival by increasing the relative risk of death by 21%, translating to a 7% reduction in 2-year OS from 55% to 48%. Subgroup analysis indicated a detriment in OS for patients with stage I/II N0-1 due to excess of toxicity from PORT. However, PORT for stage III-N2 disease trended toward, but did not reach, a significant survival benefit, suggesting a need for further investigation. A significant flaw of the PORT Meta-analysis was the inclusion of historical series with patients treatments utilizing antiquated techniques that were potentially more toxic than modern radiation delivery with image guidance, respiratory motion assessment, and higher dose conformality.

A recent retrospective analysis of the SEER database analyzing 7465 stage II-III patients receiving PORT following lobectomy or pneumonectomy demonstrated that PORT significantly increased survival for patients with N2 disease and associated with worse survival for N0-1 disease[27]. Among N2 patients, PORT improved 5-year OS from 20% to 27% (HR = 0.85), while reducing 5-year OS by 10% (HR = 1.2) and 4% (HR = 1.1) among N0 and N1 patients respectively[27]. The survival benefit for N2 disease was not observed until 2.5 years after PORT, while the lack of benefit for N0-1 disease was evident within one year of receiving PORT. A similar population-based series from the National Cancer Database also demonstrated an improvement in median OS from 45 mo with PORT *vs* 41 mo without PORT[28]. These results were consistent with a separate subset analysis from the Adjuvant Navelbine International Trialists Association trial[29]. In this trial 850 patients were randomized to adjuvant cisplatin and vinorelbine or observation following complete resection. The decision to provide PORT was left to the discretion of the participating institutions but was suggested for patients with node-positive disease. PORT was delivered to 232 patients. Median survival (MS) improved after PORT among patients with N2 disease receiving either adjuvant chemotherapy (from 23.8 to 47.4 mo) or observation (from 12.7 to 22.7 mo) following surgical resection. This analysis also confirmed that PORT reduced local recurrence regardless of nodal status. However, patients that received PORT and adjuvant chemotherapy with stage N1 disease experienced worse MS compared with chemotherapy alone (46.6 mo *vs* 96.6 mo) and 5-year OS (40% *vs* 56.3%), respectively. This study suggests that PORT may be influenced by the use of adjuvant therapy and extent of nodal involvement.

Since the PORT Meta-analysis, further prospective trials for PORT have drastically declined. However, this series may not be as relevant today since cobalt-60 sources and older treatment delivery systems were used for patient treatment[27]. Today's technology has significantly improved radiation delivery. There is a need for updated PORT studies using modern techniques since more conformal radiotherapy could improve local control while reducing cardiac and pulmonary toxicities observed in PORT Meta-analysis[30,31]. The LungART trial is a large European Phase III multi-institutional

prospective study of PORT using modern staging and treatment planning among N2 patients who have undergone complete resection. This trial is currently being conducted, and results are highly anticipated[32].

Post-operative radiotherapy and concurrent chemotherapy

The benefits of post-operative concurrent chemoradiation continue to be under debate. The Intergroup 0115 (ECOG 3590, RTOG 9501)[33] was a trial of 488 stage II-IIIA patients randomized to PORT alone or with four cycles of cisplatin and etoposide. A total of 50.4 Gy was delivered in 28 daily fractions to both groups. After median follow-up time of 44 mo, no survival benefit of concurrent chemoradiotherapy was observed. MS was not different in the post-operative chemoradiation group (38 mo) *vs* those in PORT group (39 mo) with a relative likelihood of survival to be 0.93. Intrathoracic disease recurrences within the irradiated field were 12% and 13%, respectively and was not significantly different. Compared to these results, the RTOG 9705 trial[34] found more favorable OS and PFS benefit with the addition of adjuvant chemotherapy to PORT. However, this was a phase II non-randomized study. In this trial, 88 stage II-III NSCLC patients received concurrent radiotherapy at 50.4 Gy in 28 daily fractions, carboplatin, and paclitaxel with a MS of 56.3 mo. The 3-year OS and PFS rates in this study were 61% and 50% respectively, while intrathoracic recurrence rate was similar to that observed in INT 0115 at 15%. To date, there remains no evidence supporting concurrent delivery of adjuvant chemotherapy with PORT.

Neoadjuvant radiation and multi-modality therapy

Thus far, treatment strategies incorporating surgical resection have demonstrated the best local control for operable NSCLC, and outcomes may be improved by managing distant metastases by induction or adjuvant therapy. However, OS and local control remains low. In an attempt to further improve resectability, local regional control, and survival for select patients with potentially resectable disease, combinations involving all three treatment modalities have been studied. An international multi-centered European trial[35] sought to compare the benefits of neoadjuvant chemoradiation or neoadjuvant chemotherapy alone prior to undergoing surgical resection randomized. Patients with stage IIIA-N2 disease were randomized to neoadjuvant regimens of 3 cycles of cisplatin and docetaxel followed by radiation to 44 Gy in 22 fractions over 3 wk or chemotherapy alone. Regimens in both study groups were well tolerated, as 91% of patients completed all three cycles of neoadjuvant chemotherapy and 7% experienced radiation-induced grade 3 or higher dysphagia. The primary endpoint of event-free survival was not significantly different between both groups. Those in the neoadjuvant chemoradiation group had median PFS of 12.8 mo compared to patients in neoadjuvant chemotherapy group with a median PFS of 11.6 mo (HR = 1.1). MS for both groups were 37.1 and 26.2 mo respectively (HR = 1), and also not different from one another. The proportion of patients with pathological complete response or nodal downstaging were 61% and 44% in neoadjuvant chemoradiation and chemotherapy group respectively, which was significantly different. While preoperative chemoradiation did not improve survival, it did significantly increase the proportion of patients with mediastinal downstaging and histopathological response. Such improvement in tumor response could improve local control and even survival for carefully selected patients, and neoadjuvant chemoradiation should be further evaluated.

Given that preoperative chemotherapy improves survival for resectable stage IIIA patients, a phase III trial[36] evaluated whether adding preoperative chemoradiation in addition to induction chemotherapy could improve treatment outcomes. This trial randomized 524 stage IIIA/B (N2/3) patients to receive either induction chemotherapy and chemoradiation (intervention) or induction chemotherapy alone (control) prior to surgical resection and PORT. The toxicity and perioperative morbidity were similar between both arms. Pneumonectomies were performed at a rate of 35% in both arms. Hematological toxicities (10% *vs* 0.5%, $P < 0.0001$) and Grade 3 or higher esophagitis (19% *vs* 4%, $P < 0.0001$) were more frequent in the intervention group, whereas Grade 3 or higher pneumonitis was more common in the control group (1% *vs* 7%, $P = 0.0006$). A significantly higher proportion of patients receiving neoadjuvant chemoradiation (46%) experienced mediastinal downstaging compared to those receiving induction chemotherapy alone (29%) ($P = 0.02$). Sixty percent of patients receiving neoadjuvant radiation achieved > 90% tumor regression compared to 20% of patients among the induction chemotherapy group ($P < 0.0001$). While response rates were significantly improved by chemoradiation, neoadjuvant chemoradiation did not improve the primary endpoint for PFS for the entire cohort. Secondary endpoints for OS, rate of disease progression, or site of first progression were also similar for all patients. Five-year PFS between intervention and control groups were 16% and 14%, respectively (HR = 0.99), and 5-year OS were 21% and 18% (HR = 1) respectively. However, subset analysis did demonstrate improved PFS (HR = 1.58, $P = 0.043$) and OS (HR = 2.07, $P = 0.03$) in patients undergoing a complete resection with successful downstaging of the mediastinum from N2-3 to N0-1 following induction radiation compared to patients with incomplete resections. These data suggest that survival outcomes may improve with mediastinal clearance and downstaging prior to surgery, and neoadjuvant chemoradiation should be considered as a treatment option for patients with potentially resectable stage III disease.

Randomized phase III trials have not yet successfully demonstrated a survival advantage of induction chemotherapy or chemoradiation prior to surgery over

definitive chemoradiation. EORTC 08941[37] reported comparable MS and 5-year OS for stage IIIA-N2 initially unresectable patients receiving induction platinum-based chemotherapy and randomized to either surgery or radiation therapy. Disease was considered unresectable if there was any N2 disease for non-squamous histology or lymph node spread beyond levels 4R or levels 5/6 for right or left squamous primaries, respectively. Treatment-related mortality was greater perioperatively (4%) compared to one death (0.6%) following radiation pneumonitis. This study suggested that surgical resection may not improve treatment outcomes compared to definitive radiotherapy. Within the context that radiotherapy leads to lower morbidity and mortality compared to surgery, definitive chemoradiation is a reasonable treatment option for patients with stage IIIA-N2 disease. However, several criticisms with this study have been made including that only 50% of patients randomized to the surgery arm received radical resection, and 40% of surgical arm patients received PORT. The chemoradiation regimen used is not an accepted standard, making extrapolation of this trial to current practice challenging. An intergroup trial, INT 0139[38] tested the benefits of trimodality with sequential cisplatin/etoposide with 45 Gy of radiation prior to surgical resection compared to concurrent chemoradiation alone. After a median follow-up of 22.5 mo, 5-year OS and MS were not improved with the induction chemoradiation. Five-year PFS was significantly higher under the intervention arm (22.4%) compared to chemoradiation arm (11.1%) (P = 0.017), which was not observed from EORTC 08941[37]. However, relatively high treatment-related deaths were observed in the trimodality arm (7.9%) compared to definitive chemoradiation arm (2.1%). No benefit of surgery was observed in patients who received pneumonectomies, likely due to an increased rate of death without progression. While induction chemoradiation may have improved 5-year PFS, a survival benefit was not observed. Such results could have been confounded by the higher perioperative mortality observed in the intervention arm, particularly among pneumonectomy patients. A subgroup analysis showed that median survival was significantly improved with induction chemoradiation prior to lobectomies (P = 0.002). In addition, 5-year OS rates were significantly better (P < 0.0001) among those with pathologic stage N0 (41%) and N1-3 (24%) at time of thoracotomy compared with those who did not receive surgery (8%). These subgroup analyses suggest that a survival advantage of trimodality over definitive chemoradiation may be demonstrated in carefully selected candidates.

To minimize perioperative mortality that was observed in INT 0139, surgeons in the RTOG 0229 trial[39] were required to demonstrate expertise in performing surgery following chemoradiation. RTOG 0229 was a multi-institutional phase II trial that followed 57 stage III-N2/3 patients receiving neoadjuvant chemoradiation of carboplatin, paclitaxel, and 50.4 Gy to the mediastinum with 10.8 Gy boost to gross disease followed by surgical resection. An impressive rate of 63% of patients achieved mediastinal disease clearance while residual disease remained in 16% of patients. The primary endpoint of improving mediastinal disease from 50% to 70% with a power of 80% was achieved. One-year OS and PFS were 77% and 52%. Fourteen percent of patients in RTOG 0229 experienced Grade 3 postoperative pulmonary complications. It is important to note that this was not increased compared with other trials of chemoradiation alone. The rate of pneumonectomies was much lower in this trial (5%) compared to INT 0139 (34%). Moreover, rate of perioperative morbidity was 3% (1 patient) which compared favorably to the relatively high rate of morbidity observed in INT 0139 (7.9%). The ability of neoadjuvant chemoradiation to sterilize mediastinal nodal disease was confirmed by this study, and thus should be considered as an option for multi-modality therapy for select patients. Lobectomy should be the preferred surgical management, and surgery should be performed by a thoracic surgeon skilled in this specific approach.

A recent trial[40] studied the outcomes of surgery *vs* definitive chemoradiation boost following both neoadjuvant chemotherapy and chemoradiation. This was a phase III multi-centered randomized control trial for stage IIIA-N2 and select IIIB patients receiving three cycles of cisplatin/paclitaxel as well as induction cisplatin/vinorelbine, and accelerated radiotherapy of 45 Gy in twice daily 1.5 Gy fractions. Patients were reassessed for resectability, and randomized to either receive chemoradiation boost to 65-71 Gy in arm A or surgery in arm B. Grade 3 or higher toxicities were acceptable and balanced between both groups. After median follow-up of 78 mo, 5-year OS was 40% in arm A and 44% in arm B, while 5-year PFS rates were 35% and 32% in arms A and B, respectively. No significant differences were found for either OS or PFS between the two groups, thus making either strategies acceptable for resectable stage IIIA, and select inoperable IIIA or IIIB patients.

Multi-modality management is efficacious for select stage IIIB patients as well. Because induction radiation and chemotherapy improves mediastinal downstaging and pathological response, tumor resectability has proven to increase among stage IIIB patients in several phase II trials[41-45]. 3-year OS rates have approached to 60%[44], and resectability rates increased up to 80%[43]. Table 1 summarizes trials for multi-modality therapy for stage IIIA/B patients.

MANAGEMENT OPTIONS FOR STAGE IIIB AND UNRESECTABLE/INOPERABLE STAGE IIIA NSCLC

Chemoradiation

Definitive chemoradiation remains a standard of care in the management of stage IIIB disease or IIIA patients

Table 1 Prospective trials of multi-modality therapy for resectable stage III non-small-cell lung cancer

Ref.	Phase	Study design	Chemo regimen	RT	Number of patients	Stage	Median f/u (mo)	OS	Median OS (mo)	PFS	Median PFS (mo)	Response rate
Pless *et al*[35] (2015)		Induction chemoRT + surgery *vs* induction chemo + surgery	Cisplatin/ docetaxel	44 Gy in 2 Gy fxns over 3 wk	232	IIIA (N2)	52.4		37.1 *vs* 26.2		12.8 *vs* 11.6 (P = 0.67)	ORR: 61% *vs* 44%
Thomas *et al*[36] (2008)	3	Induction chemo + induction chemoRT + surgery *vs* induction chemo + surgery	Induction: Cisplatin/ etoposide ChemoRT: Carboplatin/ vinorelbine	45 Gy in 1.5 Gy fxns (twice daily)	524	III A/B (N2/3)		5-yr, 21% *vs* 18% (P = 0.97)	15.7 mo *vs* 17.6 mo	5-yr, 16% *vs* 14% (P = 0.87)	9.5 *vs* 10	CR: 60% *vs* 20% (P < 0.0001) Mediastinal downstaging: 46% *vs* 29% (P < 0.02)
EORTC 08941 Van Meerbeeck *et al*[37] (2007)	3	Induction chemo + surgery *vs* chemoRT	Platinum-based	60-62.5 Gy in 1.95-2.05 Gy daily fxns	332	IIIA (N2)	> 72	5-yr, 15.7% *vs* 14% (P = 0.6)	16.4 *vs* 17.5 (P = 0.6)	2-yr, 27% *vs* 24% (P = 0.6)	9 *vs* 11.3 (P = 0.6)	
INT 0139 Albain *et al*[38] (2009)	3	Induction chemoRT + surgery *vs* chemoRT	Cisplatin/ etoposide	45 Gy boost to 61 Gy if definitive chemoRT	396	IIIA (N2)	22.5	5-yr, 27.2% *vs* 20.3% (P = 0.10)	23.6 *vs* 22.2 (P = 0.24)	5-yr, 22.4% *vs* 11.1% (P = 0.017)	12.8 *vs* 10.5 (P = 0.017)	
RTOG 0229 Suntharalingam *et al*[39] (2010)	2	Induction chemoRT + surgery	Carboplatin/ paclitaxel	50.4 Gy + 10.8 Gy to gross disease	60	III A/B (N2/3)		1-yr, 77%	26.6	1-yr, 52%	13.1	Improved mediastinal sterilization 50% to 70% met
ESPATUE Eberhardt *et al*[40] (2015)	3	Induction chemotherapy + induction chemoRT + RT boost *vs* Induction chemotherapy + induction chemoRT + surgery	Induction chemo: Cisplatin/ paclitaxel Induction chemoRT: Cisplatin/ vinorelbine	45 Gy in 1.5 Gy twice daily fxns Definitive chemoRT: Boost to 65-71 Gy	246	III A/B (N2/N3)	78	5-yr, 40% *vs* 44% (P = 0.34)		5-yr PFS, 35% *vs* 32% (P = 0.75)		
Eberhardt *et al*[40] (2015)	3	Induction chemo + induction chemoRT + surgery *vs* induction chemo + definitive chemoRT	Induction: Cisplatin/ paclitaxel ChemoRT: cisplatin/ vinorelbine	45 Gy in 1.5 Gy fxns (twice daily)	246	IIIA (N2), select IIIB (N3)	78	5-yr, 40% *vs* 44%		5-yr, 35% *vs* 32%		

CR: Complete response; ORR: Overall response rate; OS: Overall survival; RT: Radiotherapy; PFS: Progression free survival.

with unresectable or inoperable disease[3]. Radiation provides local therapy for inoperable tumors, and chemotherapy not only reduces or prevents micrometastatic spread of the disease, but also acts as a radiosensitizer to increase the therapeutic index of radiation therapy. Chemotherapy plays a critical role in the management for advanced NSCLC, and when given with radiation, the combination improves survival over supportive care or radiation therapy alone[46-49]. Standard radiation is typically 60-66 Gy in 2Gy daily fractions over 6 wk, as established by RTOG 7301 trial[50], and platinum-based doublet chemotherapy is typically used with standard radiation[3].

Sequential vs concurrent chemoradiation

Concurrent chemoradiation has proven to be superior to sequential chemoradiation, and is now considered standard of care. RTOG 9410[51] was a pivotal trial esta-

blishing the superiority of concurrent chemoradiation. This trial randomized 610 inoperable stage II-III NSCLC patients into one of three groups: Sequential cisplatin/vinblastine and conventionally fractionated radiation to 63 Gy (arm 1), concurrent chemotherapy and radiation to 63 Gy (arm 2), or concurrent chemotherapy with accelerated hyperfractionation of 69.6 Gy in twice daily 1.2 Gy fractions over 6 wk (arm 3). Five-year OS rates among the three groups were 10%, 16%, and 13% respectively, and was significantly higher in the standard chemoradiation arm compared to arm 3 (P = 0.046), but not against arm 1 (P = 0.46). MS was 17 mo in arm 2 while it was 14 mo in arm 1. Furthermore, the response rate in arm 2 was 70% and statistically significantly higher compared to sequential chemoradiation (P < 0.05). While acute Grade 3 or higher non-hematologic toxicity rates, particularly severe acute esophagitis, were higher with concurrent therapy, late toxic effects were ultimately similar in concurrent or sequential therapies.

Since RTOG 9410, the superiority of concurrent over sequential chemoradiation has been confirmed by several other studies, including a meta-analysis evaluating seven randomized controlled trials[52]. Concurrent chemoradiation improved OS by an absolute benefit of 4.5% after 5-years, increasing 5-year OS rate from 10.6% to 15.1% (HR = 0.84)[52]. Moreover, locoregional progression decreased by an absolute rate of 6.1% at 5 years, lowering the rate from 35% to 28.9% after concurrent chemoradiation. While concurrent chemoradiation provides better locoregional control, it does not lower distant disease progression compared to sequential chemoradiation (HR = 1.04). Concurrent chemoradiation, however, is associated with higher rates of Grade 3 or higher esophageal toxicity, and can reach up to 18%. The higher toxicity rates were thought to be clinically acceptable and manageable. Induction or consolidation chemotherapy in addition to chemoradiation was not necessary, as it has not been shown to improve 2-year OS or MS[53-56]. However, it could be considered for patients with bulkier tumors whose gross disease could not be treated with radiation without leading to radiation-induced toxicity[57]. Concurrent chemoradiation is better suitable for patients with minimal co-morbidities, favorable performance statuses, and minimal weight loss[53,58]. Patients who are unable to tolerate concurrent chemoradiation should still receive sequential regimens since it still incurs some benefit over radiotherapy alone by increasing 5-year OS from 5% to 10%[59-62].

Current and future directions with chemotherapy regimens for chemoradiation

Chemoradiation therapy is complex, and the agents needed to achieve the best disease control and survival are unknown. The most commonly used regimens are cisplatin/etoposide or carboplatin/paclitaxel. Cisplatin-based regimens have demonstrated to provide better outcomes compared to carboplatin-based regimens[63-65]. In a phase II randomized trial[63] comparing outcomes from 60 Gy thoracic radiation combined with either cisplatin/etoposide (PE) *vs* carboplatin/paclitaxel (PC), OS was significantly better in the PE arm. Three-year OS was 33.1% in the PE arm, but only 13% in the PC arm (P = 0.04). In a meta-analysis from individual patient data[65], cisplatin achieved significantly higher objective response rate of 30% compared to 24% from carboplatin (P < 0.001) among nine trials using platinum-based agents in first-line treatments. While cisplatin-based chemotherapy was more efficacious, it has also led to increased toxicity, especially Grade 3/4 neutropenia[15,63,65].

An individual patient data meta-analysis[65] also observed patients with non-squamous tumors experienced significantly higher mortality when treated with carboplatin and third-generation chemotherapy (HR = 1.12). However, a small number of studies have reported equivalent outcomes with carboplatin as with cisplatin[66,67]. An analysis of over 1842 patients from Veterans Health Administration data demonstrated PC having similar survival as PE. In fact, PE was associated with more hospitalizations, outpatient visits, acute kidney disease, and esophagitis/mucositis compared to PC[66]. However, the results from this trial should be interpreted with caution since 98% of patients were men, and approximately 50% of tumors was squamous cell histology *vs* approximately 20% adenocarcinoma. This was not representative of true population of stage III NSCLC[51,68,69]. Therefore, carboplatin may be more beneficial for men presenting with squamous NSCLC[70]. Liew *et al*[67] also found PC to have similar survival outcomes *vs* PE, with MS to be 20.7 and 13.7 mo with PC and PE, respectively. Relapse free survival was also comparable, and median PFS was 12 mo with PC *vs* 11.5 mo with PE. PC cause significantly less hematological toxicities compared to PE. Therefore, carboplatin therapy may also be more beneficial for older patients and those with multiple co-morbidities.

Third generation chemotherapy agents are increasingly being incorporated into the management of stage III NSCLC patients (Table 2). Their use has not been shown to improve treatment outcomes compared to "older" generation agents like cisplatin/etoposide. A retrospective review[5] compared PE, PC, and cisplatin/docetaxel (PD), and found that MS from PD was not significantly better compared to PE or PC. Median survivals were 27, 36, and 23 mo respectively. Median PFS were 21, 10, and 15 mo in PE, PC, and PD arms respectively, and was significantly better under PE arm (P = 0.01). PE not only has better treatment outcomes, but also had better objective response rates compared to PD or PC. Additionally, WTOG 0105 trial[71] was a phase III study directly comparing second to third generation regimens in the setting of concurrent chemoradiation for inoperable stage III NSCLC. In this study, patients were randomized to receive MVP, carboplatin/irinotecan, or PC along with 60 Gy of concurrent radiation for 6

Table 2 Chemotherapy agents for non-small-cell lung cancer by generation

Generation	Agents	Effect on survival for stages II-III
First	Methotrexate Cyclophosphamide Vincristine Doxorubicin	No effect
Second	Cisplatin, cisplatin-based combinations Ifosfamide Mitomycin Vindesine Vinblastine Etoposide	Combination with radiation superior to radiation alone Concurrent superior than sequential chemotherapy and radiation
Third	Paclitaxel, paclitaxel-based combinations Docetaxel Gemcitabine Vinorelbine Irinotecan Topotecan	Expected to be superior to second generation agents given with radiation

wk. Five-year OS rates for the three arms were 17.5%, 17.8%, and 19.8% respectively. Thus third generation agents did not significantly improve survival; however, it was also not inferior to second generation agents. While third generation agents may be non-inferior to second generation agents, more treatment interruptions were observed with patients receiving carboplatin/irinotecan compared to other chemotherapy groups. Other studies that have chosen to focus on understanding the efficacy of other single-agent third generation chemotherapy such as vinorelbine have findings that agree with prior phase III trials[72,73]. While third generation agents are equivocal to second generation agents regarding survival and response rates, these agents should still be further investigated, even though they do not add benefit to survival or response rates.

Pemetrexed is a new multi-targeted anti-folate chemotherapy agent commonly used with cisplatin in first-line, second-line, and maintenance therapies for non-squamous NSCLC[55,74,75]. Several phase II studies demonstrated that pemetrexed can be safely administered with either cisplatin or carboplatin, yielding a median survival ranging from 18.7 to 34 mo, and esophageal and pulmonary toxicities reaching no higher than 16% and 23% respectively[76-78]. Better outcomes among non-squamous tumor histologies were observed[76-78]. The PROCLAIM trial[79] was a phase III trial comparing concurrent chemoradiation using cisplatin/pemetrexed *vs* PE among non-squamous NSCLC. Although enrollment ended early due to futility, 598 patients were ultimately randomized. MS were 26.8 mo in the pemetrexed arm and 25 mo in etoposide arm (HR = 0.98), which were similar to those observed in phase II trials. PFS was also not significantly different between pemetrexed over etoposide regimens, but trended in favor of pemetrexed. Median PFS were 11.4 and 9.8 in pemetrexed and etoposide arms respectively (HR = 0.86). Moreover, pemetrexed yielded a mildly higher response rate (35.9%) compared to etoposide (33%). Pemetrexed had significantly lower Grade 3 or higher adverse effects compared to PE (P = 0.01), including neutropenia, febrile neutropenia, and thrombocytopenia.

Targeted therapy

Treatment response varies greatly among individuals, and the heterogeneity of tumor biology is expansive. Few driving mutations that may be exploited by therapy have been discovered. Incorporation of therapies targeted to these driver mutations has not yet been successful and remains under investigation. EGFR and ELM4-ALK mutations are likely candidates for targeted therapy in definitive treatment. EGFR inhibitors include monoclonal antibodies targeting the extracellular domain of EGFR, while tyrosine kinase inhibitors (TKI) target the intracellular domain of EGFR and also act as radiosensitizers.

Early studies with cetuximab have shown some promise. The FLEX trial[80] was an international open-labeled phase III trial that compared the efficacy of cetuximab plus chemotherapy with chemotherapy alone among EGFR-positive NSCLC patients. Patients who were given cetuximab in addition to chemotherapy survived significantly longer than those receiving chemotherapy alone (P = 0.04). MS was 11.3 and 10.1 mo respectively (HR = 0.871). The main toxicity associated with cetuximab was an acne-like rash, and 10% of patients on cetuximab experienced severity of grade 3. The RTOG 0324 phase II trial[81] evaluated whether cetuximab given in conjunction with chemoradiation would provide any benefit for unresectable stage III patients. Through this single-arm trial, MS was 22.7 mo and 2-year OS is 49.3%, which was higher than previous reports at the time[51,56]. With such promising results, RTOG 0617 phase III trial[82] evaluated the use of cetuximab with standard and high-dose chemoradiotherapy. MS among patients receiving cetuximab was 25 mo and 24 mo who did not receive cetuximab (HR = 1.07). Moreover, the addition of cetuximab was associated with significantly higher rate of toxicities (P < 0.0001). Grade 3 or higher toxicity rates were 86% with cetuximab and 70% without. Therefore, the addition of cetuximab to concurrent chemoradiation or consolidation treatment did not provide any survival benefit while increasing treatment-related toxicities.

In contrast, TKIs like gefitinib and erlotinib play a larger role in the management of locally advanced NSCLC. Gefitinib is reserved for patients with disease refractory to standard chemotherapy. When used as a first-line or maintenance agent, it has not shown to improve survival[83-85]. INTACT trials randomized unresectable locally advanced to metastatic, chemotherapy-naïve patients to receiving gefitinib with platinum-doublet chemotherapy or platinum-doublet therapy alone. The addition of gefitinib with chemotherapy as first line

treatment did not improve MS, time to progression, or response rates. In SWOG S0023[85], MS with gefitinib maintenance following concurrent chemoradiation with PE decreased to 23 mo compared to 35 mo from placebo (P = 0.013). The decreased survival is primarily due to disease progression rather than treatment toxicity, as toxic death rate was not different from placebo. It is important to notice that these trials enrolled patients with and without EGFR mutations. Perhaps selectively treating patients only with EGFR mutations with gefitinib may lead to different outcomes.

Erlotinib is often used for patients with locally advanced and metastatic disease. The TRIBUTE study[86] randomized 1059 stage ⅢB and Ⅳ NSCLC to either erlotinib or placebo in combination with six cycles of PC. While there was no benefit with the addition of erlotinib to OS and time to disease progression, there was a survival benefit among patients who never smoked. MS with erlotinib increased to 22 mo compared to 10 mo with just PC alone. In a secondary analysis, patients specifically with EGFR mutations were associated with better response rates (P < 0.05) and a trend toward improved time to disease progression (P = 0.092)[87]. However, OS remained similar with the addition of erlotinib among this subset of patients (P = 0.96).

The IPASS trial[88] was a phase Ⅲ trial randomizing stage ⅢB and Ⅳ pulmonary adenocarcinoma patients in East Asia and who were nonsmokers or light smokers to receive either gefitinib alone or carboplatin/paclitaxel as first line therapy. The primary endpoint for non-inferior PFS was met, and surpassed. Gefitinib resulted in 12-mo PFS rate of 24.9% compared to 6.7% achieved with carboplatin and paclitaxel. For patients specifically with EGFR mutations, PFS survival was significantly longer from gefitinib therapy (P < 0.001). A similar phase Ⅲ trial[89] for European NSCLC patients with EGFR mutations randomized patients to receiving erlotinib alone or standard chemotherapy (cisplatin with either docetaxel or gemcitabine), and demonstrated that erlotinib significantly improved median PFS. Thus, TKIs are now considered first-line therapeutic options for patients harboring EGFR mutations.

Crizotinib is an oral small-molecule tyrosine kinase inhibitor against the product of the EML4-ALK fusion gene. For patients who harbor this mutation, crizotinib can be used as a first-line treatment. As a first line therapy, PROFILE-1014 phase Ⅲ trial[90] demonstrated that locally advanced or metastatic ALK-positive NSCLC patients experience longer progression free survival (10.9 mo) compared to cisplatin/pemetrexed therapy (7 mo) (P < 0.001), and improved overall response rate of 74% *vs* 45%, respectively (P < 0.001). However, 1-year survivals between the two groups were not significantly different. Similar findings were found when crizotinib was used as a second-line agent among patients who received prior platinum-based chemotherapy treatment[91]. Unfortunately, acquired resistance to crizotinib can occur, and manifests after a median period of 7-11 mo[90,91]. In this situation, a more potent agent, ceritinib, can be used to treat ALK-positive NSCLC patients refractory to chemotherapy and crizotinib. ASCEND-2 is a single-arm phase Ⅱ trial that demonstrated a durable response for these patients[92]. The majority of patients enrolled in this study also had brain metastases. Whole body overall response rate was 38.6%, with median duration of response of 9.7 mo and median PFS of 5.7 mo. Similarly, ASCEND-4 and 5 trials are two phase Ⅲ randomized control trials designed to compare progression free survival of ceritinib with or without chemotherapy in chemo-naïve or previously treated patients with stage ⅢB and Ⅳ NSCLC. Based upon their success in patients with metastatic disease, a role for erlotinib and crizotinib are being investigated in the potentially curative setting. RTOG 1306 is a phase Ⅱ in which patients with Stage Ⅲ NSCLC with susceptible mutations are randomized to standard chemoradiation alone or with the addition of erlotinib or crizotinib.

Besides EGFR and EML4-ALK inhibitors, other molecular targets are being explored to use in conjunction with chemoradiation for unresectable stage Ⅲ patients. Bevacizumab is one such anti-angiogenic therapy that could have synergistic effects with radiation[93,94]. Phase Ⅲ trials have shown promising results with higher response rates, and longer OS and PFS. However, the high rate of grade 3 or worse esophagitis including formation of trachea-esophageal fistula makes this agent less likely to be used with chemoradiation[95]. Nivolumab, a PD-1 immune checkpoint inhibitor antibody, is garnering attention. Two recent randomized, international phase Ⅲ trials demonstrated that Nivolumab prolonged 1-year OS, 1-year PFS, and response rates compared to docetaxel for patients whose disease had progressed during or after platinum-doublet chemotherapy for both squamous and non-squamous histologies[96,97]. With such promising results, perhaps immunotherapy will play an increasing role in the management of locally advanced NSCLC patients in the future.

Current and future directions with radiation for chemoradiation

Definitive radiotherapy alone continues to yield poor outcomes for stage Ⅲ patients. MS continues to range from 10 to 26 mo[6,98,99], with a 5-year survival rate of less than 25%[98,100,101]. Such low outcomes are related to the failure to eradicate local disease as well as development of distant metastasis. Several ways to improve local control and survival include dose escalation and altered fractionation schedules.

Increasing dose intensity has been shown to improve local control and survival in early studies. A retrospective analysis[102] of 7 prospective RTOG trials demonstrated that the higher biological effective dose (BED) of radiotherapy was associated with better outcomes in locally advanced NSCLC. Phase Ⅰ and Ⅱ dose escalation studies[103-105] using conformal radiation demonstrated that conformal thoracic radiation up to 74 Gy was fea-

sible and tolerable, and led to encouraging survival and response rates with acceptable toxicity levels. A modified phase I/II trial[103] randomized 62 unresectable stage III NSCLC patients to one of four cohorts where radiation dose was escalated from 60 to 74 Gy. No dose-limiting toxicity was observed from any cohorts, making 74 Gy the maximum tolerated dose (MTD). The most common toxicity was esophagitis, and approximately 8% of patients experienced grade 3/4 esophagitis. Overall response rate was 52%, and MS of 26 mo. Three-year OS rate was 40% and 3-year PFS was 29%. RTOG 0117 trial[104] confirmed that MTD was 74 Gy with 3D-CRT, since doses beyond 74 Gy incurred high pulmonary toxicity levels. Delivering 74 Gy concurrently with PC led to encouraging response rate of 66.6% and 1-year OS of 66.7%. MS was 24.3 mo, surpassing the study's predefined MS benchmark of 18 mo which was chosen to be the best that was achieved by CALGB. Despite such encouraging early results, results from the intergroup phase III RTOG 0617 trial[82] did not recommend use of 74 Gy as OS was significantly worse than the standard dose of 60 Gy. MS was 20.3 mo after delivery of 74 Gy compared to 28.7 mo from standard dose (HR = 1.38, P = 0.004). The rate of severe esophagitis was significantly worse at 21% in high dose group *vs* 7% in standard dose group (P <0.0001). Constraints for heart dose were not mandated, and heart doses were significantly higher among patients receiving high dose radiation, and this likely contributed to a survival detriment in those patients.

Accelerated hyperfractionation (hyperFRT) is a way to deliver a higher dose of radiation over the same time period as one would with conventional fractionation schedules. To do so, a lower dose per fraction is delivered more frequently, typically twice a day. The benefits of hyperFRT schedule were evaluated by various trials, in which early reports were rather mixed. RTOG 8311[106] was a phase I trial of radiation dose escalation. Patients were randomized to receive total doses of total doses of 60.0 Gy, 64.8 Gy, 69.6 Gy, 74.4 Gy or 79.2 Gy delivered in 1.2 Gy twice daily fractions five days a week. Survival did not improve at doses beyond 69.4 Gy. At this dose, MS was 13 mo and 2-year OS was 29%, which was significantly better than lower radiation doses tested (P = 0.02). With an optimal dose set for hyperFRT, the phase III RTOG 8808 trial[107] compared outcomes of conventional fractionation plus induction cisplatin/vinblastine (arm 1), hyperFRT at 69.4 Gy in 1.2 Gy fractions (arm 2), and conventional fractionation RT alone (arm 3). While survival from arm 2 was better compared to arm 3, it was not significantly better than arm 1[107]. Five-year OS rates were 8%, 6%, and 5% respectively, with MS rates of 13.2, 12, and 11.4 mo respectively. RTOG 9410[51] study echoed similar findings as RTOG 8808. This study compared sequential cisplatin/vinblastine and conventional RT (arm 1), concurrent cisplatin/vinblastine and conventional RT (arm 2), and concurrent cisplatin/etoposide with hyperFRT (arm 3). Conventional fractionation was 63 Gy in 1.8 Gy fractions over 7 wk), and hyperFRT delivered 69.6 Gy in 1.2 Gy twice daily fractions. Five-year OS were 10%, 16% and 13% respectively, and significantly better in arm 2 (P = 0.046). MS were 14.6, 17 and 15.6 mo, respectively. Between the two concurrent chemoradiation treatments, overall response rates were similar between arms 2 (70%) and 3 (65%), respectively. Grade 3 or higher toxicities were observed in 45% of patients receiving hyperFRT, though was not significantly different from arm 2. Incorporation of hyperFRT into multi-modality therapy has also been tested. Pöttgen *et al*[108] retrospectively compared outcomes of neoadjuvant chemotherapy and hyperFRT (45 Gy in 1.5 Gy twice daily fractions) *vs* conventional RT (46 Gy in 2 Gy daily fractions). While complete response rates were higher in neoadjuvant concurrent chemotherapy and hyperFRT compared to the control group using conventional RT (P < 0.006), the use of hyperFRT was not associated with improved survival.

Continuous hyperfractionated accelerated radiotherapy (CHART) delivers less than 1.8-2 Gy per fraction in an accelerated course to allow for less normal tissue injury per fraction and inter-fraction normal tissue repair. Despite that total dose of radiation and dose per fraction delivered are lower compared to conventional fractionation schemes, it is hypothesized that delivering greater radiation dose per unit of treatment time outpaces tumor cell repopulation which could improve treatment outcomes[109-111]. Standard CHART delivers 54 Gy in 1.5 Gy fractions three times per day for 12 consecutive days. A randomized control trial[112] comparing the efficacy of CHART to conventional fractionation, which delivered 60 Gy in daily 2Gy fractions, showed that CHART significantly improved 2-year OS by 9%, increasing it from 20% to 29% (HR = 0.76, P = 0.004). This finding translated to a 22% overall reduction in relative risk of death. The largest benefit of CHART was observed within patients with squamous NSCLC, where 2-year survival improved by 14%, increasing the survival rate from 19% to 33%. Adverse effects were higher in patients receiving CHART compared to conventional fractionation schemes within the first three mo of therapy. Severe dysphagia in particular was seen in 19% and 3% of patients, respectively. Overall, acute and late toxicities were not different between groups. CHARTWEL was a modification of CHART in that treatments were not given during weekends. A phase III trial[113] randomized 460 patients to either CHARTWEL or conventional fractionation. Five-year OS were 11% and 7% from CHARTWEL and conventional RT, and were not significantly different from each other. Local control rates were found to improve after CHARTWEL among patients with higher T or N staging (P = 0.006) or after receiving neoadjuvant chemotherapy (P = 0.019). Acute dysphagia and radiation-induced pneumonitis were frequent among CHARTWEL patients. Therefore, unlike CHART, CHARTWEL did not exhibit a survival benefit. Results from CHARTWEL was a proof-of-concept that

delivering lower total dose can be compensated by shorter treatment time, and that time is an important factor for the management of unresectable locally advanced NSCLC patients. The addition of neoadjuvant chemotherapy to CHART did not significantly improve survival or response rates[114,115], but was associated with less toxicity compared to standard fractionated concurrent chemoradiation and therefore could still be an option for locally advanced patients. In a recent small phase I trial[100], escalating total delivered dose from 54 Gy to 64.8 Gy in the setting of CHART was feasible and did not exhibit dose-limiting toxicities. MS was 24 mo across all dose cohorts, and Grade 3 or worse adverse effects were found in 6 of 18 patients. Thus, CHART potentially enhances survival and response outcomes compared to conventional fractionation radiation. Table 3 summarizes key prospective trials evaluating hyperFRT fractionation schedules over conventional fractionation radiotherapy.

A meta-analysis of studies comparing hyperfractionated to conventional radiation[8] determined that hyperFRT ultimately has significant survival benefit despite mixed results from earlier trials. HyperFRT increased 5-year OS by 2.5% (P = 0.009) over conventional fractionation and decreased the risk of death by 12% (P = 0.02). However, hyperFRT did not significantly improve PFS, and was associated with higher toxicities compared to conventionally fractionated radiotherapy. While hyperFRT regimens may be superior to conventionally fractionated radiotherapy, the cost of greater toxicity, particularly severe esophagitis, and logistics of treating patients multiple times per day has prevented its wider adoption in a clinical setting.

Hypofractionation (hypoFRT) delivers a higher dose per fraction compared to conventional fractionation schedules. The overall delivered dose is lower than conventional fractionation schemes, but tumor repopulation may be outpaced with greater tumor cell kill per fraction. HypoFRT is potentially able to deliver higher biologically equivalent dose to provide better local control[102,109]. Hypofractionation also offers advantages of less total fractions and less machine time per patient. In a pilot study[116] of 59 stage IIIA/B patients treated with 75 Gy in 28 daily fractions (2.68 Gy/fraction) over 5.5 wk, patients had a MS of 10 mo, and a 3- and 5-year OS of 18% and 4%, respectively. Only three of 59 patients experienced severe late complications from therapy, suggesting that hypoFRT is an acceptable and tolerable regimen. A randomized control trial[117] compared conventional RT (60 Gy in 2 Gy fractions over 6 wk) to hypoFRT (60 Gy in 5Gy weekly fractions for 12 wk). One- and two-year OS were 49% and 23% in the conventional RT arm, and 59% and 29% in hypoFRT arm respectively. These survival rates were not statistically significantly different from each other, but agree with previous reports. Local failure and response rates from hypoFRT were similar to conventional RT as well, thus suggesting hypoFRT is as efficacious as conventional RT but not superior. The EORTC 08972-22973 trial[61] tested the efficacies of sequential gemcitabine/cisplatin *vs* hypoFRT or concurrent cisplatin and hypoFRT therapies. While the trial was underpowered to detect any significant difference, OS and toxicity rates favorably trended towards concurrent arm of the trial. Two-year OS rates for patients treated with sequential chemoradiation is 34% while those in concurrent chemoradiation arm is 39% survival rate. MS for the sequential and concurrent arms are 16.2 and 16.5 mo respectively. The SOCCAR phase II trial[101] also tested sequential *vs* concurrent cisplatin/vinorelbine with hypoFRT. The primary endpoint of this trial was treatment-related mortality, which occurred in 2.9% and 1.7% of patients on concurrent and sequential arms, respectively. The rate of Grade 3 or worse esophagitis was similar between the two arms, as were 2-year OS, median survival, 1-year PFS rates, and median PFS. This trial demonstrated that hypoFRT given with full dose chemotherapy has similar outcomes to previous trials and had a low, acceptable treatment-related mortality rate. Table 4 summarizes key prospective trials evaluating hypoFRT fractionation schedules for NSCLC treatment.

Intensity modulated radiotherapy (IMRT) delivers radiation using inverse computer planning to determine multiple intensity levels across varying beam shapes, which has allowed for improved homogenous and conformal dose distributions for complex target volumes while sparing critical adjacent structures. While there is a hypothetical advantage of reducing toxicity by reducing dose to normal tissue compared to 3D-CRT, there has been no prospective evidence to guide when to use IMRT for select NSCLC patients. There have been concerns with using IMRT which have limited its adoption. It can expose a larger volume of lungs to low-dose radiation, which is often associated with pneumonitis[118]. Additionally, there are uncertainties regarding the delivery of radiation related to multi-leaf collimator movement and respiratory-related tumor motion[119]. These concerns lack convincing evidentiary support. There have been several retrospective institutional studies reporting improvements in overall dosimetry and rates of toxicity with IMRT. Notably, a review of 151 NSCLC patients treated from MD Anderson Cancer Center compared rates of treatment-related pneumonitis among patients treated with 3D-CRT *vs* IMRT[118]. While patients treated with IMRT had more advanced disease, debilitated performance status, and larger median gross tumor volume, rates of Grade 3 or higher treatment-related pneumonitis at 1-year was 8%, compared to 32% observed for patients treated with 3D-CRT (P = 0.002). IMRT also significantly reduced V20 doses compared to 3D-CRT (P < 0.001). RTOG 0617[82] included patients treated with IMRT. Planned secondary analyses for survival outcomes, toxicities, and quality of life from RTOG 0617 trial were done. IMRT had comparable OS and PFS to 3D-CRT[120]. However, IMRT was associated with significantly higher lung V5, while having lower lung V20 (P = 0.08) and heart doses at V5,

Table 3 Prospective trials for hyperfractionated radiation schedules for non-small-cell lung cancer treatment

Ref.	Phase	Study design	Chemo regimen	RT	No. of patients	Stage	Median f/u (mo)	OS	Median OS (mo)	Response rate	Toxicity
RTOG 83-11 Cox *et al*[106] (1990)	1 and 2	Randomized 1 of 5 dose groups: 60, 64.8, 69.6, 74.4, 79.2 Gy	None	Dose delivered in 1.2 Gy twice daily fxns	848	III	N/A	2-yr, 29% (69.6 Gy arm)	13 (69.6 Gy arm)		Risk for severe/ life-threatening pneumonitis- 2.6% (60 Gy), 5.7% (64.8 Gy), 5.7% (69.6 Gy), 8.1% (74.4 Gy)
RTOG 8808/ ECOG 4588 Sause *et al*[107] (2000)	3	Conv. RT + chemo *vs* hyperFRT *vs* conv. RT	Cisplatin/ vinblastin	Conv RT: 60 Gy in 2 Gy daily fxns HyperFRT: 69.6 Gy in 1.2 Gy twice daily fxns	458	II-IIIB, unresectable	> 60	5-yr, 8%, 6%, 5%	13.2, 12, 11.4		6 G4+ RT-related toxic events-4 of them in hyperFRT arm
RTOG 9410 Curran *et al*[51] (2010)	3	Sequential chemoRT (conv., arm 1) *vs* concurrent chemoRT (conv., arm 2) *vs* concurrent chemoRT (hyperFRT, arm 3)	Cisplatin/ vinblastine (arms 1 and 2) Cisplatin/ etoposide (arm 3)	Conv: 63 Gy in 1.8 daily fxns HyperFRT: 69.6 Gy in 1.2 Gy twice daily fxns	610	II-III, inoperable	132	5-yr, 10%, 16%, 13%)	14.6, 17, 15.6	ORR- 61%, 70%, 65%	G3+ acute esophagitis- 4%, 22%, 45% No difference in G5 toxicities
Saunders *et al*[112] (1999)		CHART *vs* conv. RT	None	Conv RT: 60 Gy in 2 Gy daily fxns HyperFRT: 54 Gy in 1.5, 3 x daily fxns, for consecutive days	563	III	> 48	2-yr, 29% *vs* 20% (P = 0.004) 2-yr, 33% *vs* 19% if SCC			Severe dysphagia, 19% *vs* 3%
ARO 97-1 Baumann *et al*[113] (2011)		CHARTWEL *vs* conv. RT	None	Conv RT: 66 Gy in 2 Gy fxns for 6.5 wk CHARTWEL: 60 Gy in 1.5, 3 x daily fxns for 2.5 wk	460	I-IIIB	40.8	2-yr, 31% *vs* 32% 3-yr, 22% *vs* 18% 5-yr, 11% *vs* 7%			Higher incidence of acute dysphagia with CHARTWEL
INCH trial Hatton *et al*[114] (2011)		Induction chemo + CHART *vs* CHART alone	Cisplatin/ vinorelbine	54 Gy in 1.5 Gy fxns (3 x daily) for 12 consecutive days	46	I-III, inoperable	33		25 *vs* 17		G3/4 adverse effects 65% *vs* 57%
ECOG 2597 Belani *et al*[115] (2005)	3	Induction chemo + conv. RT *vs* induction chemo + CHART	Carboplatin/ paclitaxel	Conventional RT: 64 Gy in2 Gy fxns (daily) 57.6 Gy in 1.6 Gy fxns (3 x daily) for 15 d	141	IIIA/B, inoperable	> 36	2-yr, 24% *vs* 44% 3-yr, 14% *vs* 34%	14.9 *vs* 20.3	ORR, 22% *vs* 25%	Acute esophagitis 16% *vs* 25% G3/4 acute pulmonary toxicity observed in conventional RT arm
Hatton *et al*[100] (2016)	1	Randomized 1 of 4 dose groups: 54, 57.6, 61.2, 64.8 Gy	None	Each dose group delivered in 1.8 Gy, 2-6 fxns daily	18	IIIA/B	21	2-yr, 49% (entire cohort)	24 (entire cohort)	ORR, 61% (entire cohort) CR, 28% (entire cohort)	G3/4 adverse effects in 6 of 18 patients No dose-limiting toxicities

SCC: Squamous cell carcinoma; OS: Overall survival; RT: Radiotherapy.

V20, and V40. V20 was ultimately predictive of grade 3 pneumonitis. Rate of Grade 3 or higher pneumonitis was 2 fold lower among patients treated with IMRT (3.5%) *vs* 3D-CRT (7.9%) despite that patients with IMRT

Table 4 Prospective trials for hypofractionation radiation schedules for non-small-cell lung cancer treatment

Ref.	Phase	Study design	Chemo regimen	RT	No. of patients	Stage	Median f/u (mo)	OS	Median OS (mo)	Response rate	Toxicity
RTOG 8312 Graham *et al*[116] (1995)	Pilot	HypoFRT	None	75 Gy in 2.68 fxns daily for 5.5 wk	59	IIIA/B		1-yr, 41% 2-yr, 25% 3-yr, 18% 5-yr, 4%	10		Most common was G1/2 pulmonary fibrosis and pneumonitits
Slawson *et al*[117] (1990)		Conv. RT *vs* HypoFRT		Conv. RT: 60 Gy in 2 Gy fxns (daily) HypoFRT: 60 Gy in 5Gy fxn (weekly)	150	Locally advanced, unresectable	36	1-yr, 49% *vs* 59% 2-yr, 23% *vs* 29%		CR, 17% *vs* 26%	No difference for later reactions
EORTC 08972-22973 Belderbos *et al*[61] (2007)	3	Sequential *vs* concurrent chemo + hypoFRT	Gemcitabine/ cisplatin	66 Gy in 2.75 Gy fxns in 32 d	158	I-IIIB, Inoperable	39	2-yr, 34% *vs* 39% 3-yr, 22% *vs* 34%	16.2 *vs* 16.5		G3 hematological toxicity higher in sequential arm (30% *vs* 6%) Esophagitis more common in concurrent arm (5% *vs* 14%)
SOCCAR Maguire *et al*[101] (2014)	2	Sequential *vs* concurrent chemo + hypoFRT	Cisplatin/ vinorelbine	55 Gy in 2.75 Gy fxns over 4 wk	130	III, inoperable	N/A	2-yr, 46% *vs* 50%	18.3 *vs* 24.3		G3+ esophagitis 8.5% *vs* 8.8% Tx-related mortality, 1.7% *vs* 2.9%
Liu *et al*[126] (2013)		Concurrent chemo + HypoFRT dose escalation	Carboplatin/ vinorelbine	60-75 Gy in 3 Gy fxns for 5 wk	26	IIIA/B, unresectable	11.5	1-yr, 60.9%	13	CR, 26.9% Partial, 53.8% Stable, 19.2% ORR, 80.8%	Acute esophagitis, 88.5% (G3 = 15.4%) Pneumonitits, 42.3% (G3 = 77%)
Lin *et al*[127] (2013)	1	Concurrent chemo + hypoFRT dose escalation	Carboplatin/ vinorelbine	60-72 Gy in 3Gy fxns for 5 wk	13	IIIA/B, unresectable	10			CR, 23.1% Partial, 15.4% Stable, 15.4% ORR, 84.6%	4 instances dose-limiting toxicities, all occurring in 72 Gy arm
Kim *et al*[128] (2013)		Concurrent chemo + hypoFRT IMRT dose escalation	Cisplatin/ vinorelbine	48 Gy in 2.4 Gy fxns with boosts of 16.8 Gy/7, 20 Gy/7, or 22.7 Gy/7	12	II-IIIB, unresectable	22	1-yr, 58.3%	12.7	CR, 75% Partial, 33% Stable, 25%	No G3 acute or late radiation-toxicities

HypoFRT: Hypofractionation; IMRT: Intensity-modulated radiotherapy; CR: Complete response.

had more advanced stage disease and larger PTV to lung ratios compared to those treated with 3D-CRT[120]. Quality of life at 12 mo was significantly higher for patients treated with IMRT than those with 3D-CRT[121]. In an attempt to identify patients who may derive a survival benefit from IMRT over 3D-CRT, Jegadeesh *et al*[119] used the National Cancer Data Base to analyze stage III NSCLC treated with chemoradiation for curative intent. This analysis suggested that patients with T3 and T4 disease are associated with improved median survival (17.2 and 14.6 mo respectively) and 5-year OS (19.9% *vs* 13.4% respectively). T stage and treatment time was significantly associated on multivariate and propensity-matched cohort analysis. With such promising results,

a prospective randomized trial comparing IMRT and 3D-CRT for NSCLC is needed.

Proton therapy for the treatment of NSCLC is under active research. Protons have characteristic energy "Bragg peaks", which limit exit dose into adjacent tissues[122]. This unique feature could reduce the irradiated volume of normal tissues, such as the heart, normal lungs, esophagus, and spinal cord, relative to photon dose distributions. This may limit toxicity to allow improved tolerance of relatively higher doses than photon radiation. Proton therapy from single-institution reports have delivered 74 cobalt gray equivalent (CGE) with concurrent chemotherapy for locally advanced NSCLC. In various small trials and single-institution reports, MS typically ranged from 26.7 to 30.4 mo[99,123,124], which was longer compared to that achieved in RTOG 0117 trial which delivered 74 CGE with conventional photon RT. Local recurrences range from 5.5% to as high as 40%[99,124,125], and development of distant metastases is still difficult to control as up to 45% of patients experience distant progression[123,124]. Toxicity rates were expectedly lower compared to those experienced at 74 Gy with conventional photon RT from RTOG 0117 trial[124]. Results of RTOG 1308, a phase III randomized trial comparing overall survival outcomes after photon *vs* proton chemoradiation for inoperable stage II-IIIB NSCLC patients, is anticipated.

CONCLUSION

Locally advanced stage III NSCLC continues to be a deadly disease, and consists of a heterogeneous patient population. Generally, treatment requires combined modalities that address local disease control, with surgery or radiation, and control of systemic spread with chemotherapy. Several combinations and various sequences of systemic and local therapies have been investigated with similar or conflicting outcomes making determination of the optimal management for these patients challenging. Multiple strategies have been developed in order to maximize survival through improved disease control through treatment intensification; however, disease progression treatment-related toxicities continue to limit survival. For patients with resectable disease, surgery offers highest rates of local control. With new awareness of chemotherapy and radiation, the role of surgery as well as disease staging are being evaluated. Multi-modality therapy is playing an increasingly important role for both resectable and unresectable stage III patients. Concurrent chemoradiation remains the standard of care in the management of inoperable or unresectable patients. In an effort to maintain or improve outcomes with less toxic effects, 3rd generation chemotherapy agents have been studied and incorporated into treatment. Targeted therapy, immunotherapy, and other non-cytotoxic drug therapies are also being investigated, and may play a greater role in the future. While dose escalation with conventional RT has not proven to improve treatment outcomes, alternative fractionation, particularly hypofractionation, may play a larger role in future management. IMRT and proton radiotherapy provides an opportunity to provide higher radiation doses with less toxicity. Future work will be needed to exploit biological tumor differences and integrate advancements in radiation technology.

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REVIEW

Three-dimensional bio-printing: A new frontier in oncology research

Nitin Charbe, Paul A McCarron, Murtaza M Tambuwala

Nitin Charbe, Unit of Clinical Pharmacology, L. Sacco University Hospital, University of Milan, 20157 Milan, Italy

Paul A McCarron, Murtaza M Tambuwala, School of Pharmacy and Pharmaceutical Science, University of Ulster, Coleraine, County Londonderry, Northern Ireland BT52 1SA, United Kingdom

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Correspondence to: Dr. Murtaza M Tambuwala, PhD, School of Pharmacy and Pharmaceutical Science, University of Ulster, Coleraine, County Londonderry, Coleraine, Northern Ireland BT52 1SA, United Kingdom. m.tambuwala@ulster.ac.uk
Telephone: +44-28-70124016
Fax: +44-28-70123518

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Abstract

Current research in oncology deploys methods that rely principally on two-dimensional (2D) mono-cell cultures and animal models. Although these methodologies have led to significant advancement in the development of novel experimental therapeutic agents with promising anticancer activity in the laboratory, clinicians still struggle to manage cancer in the clinical setting. The disappointing translational success is attributable mainly to poor representation and recreation of the cancer microenvironment present in human neoplasia. Three-dimensional (3D) bio-printed models could help to simulate this micro-environment, with recent bio-printing of live human cells demonstrating that effective *in vitro* replication is achievable. This literature review outlines up-to-date advancements and developments in the use of 3D bio-printed models currently being used in oncology research. These innovative advancements in 3D bio-printing open up a new frontier for oncology research and could herald an era of progressive clinical cancer therapeutics.

Key words: Cancer; Three-dimensional bio-printing; *In vitro*; *In vivo*; Biomaterials

Core tip: This review highlights the recent advancements in three-dimensional (3D) bio-printing in the field of oncology research and how the use of 3D bio-printed models can revolutionise and accelerate the development of new cancer therapeutics for human use.

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INTRODUCTION

Cell culture and animal models are the accepted evaluative methodology in all types of preclinical studies, including oncology research. These models have contributed a lot to the overall understanding of the pathological mechanisms of several diseases including different types of cancers, however, their value in predicting the effectiveness of treatment options and strategies in clinical trials have remained doubtful[1,2]. Apart from the ethical controversies; lead by the animal activist, the main problems with animal models lays in the species differences when compared with human. These species differences often causes misleading interpretation[3]. In fact, clinical trials are mandatory because preclinical studies on cell culture and animal models do not envisage with sufficient confidence the likely outcomes in human studies.

In oncology research, due to the ethical concerns associated with human experimentation, animal models and cell culture studies have become an important source of information. However, the average rate of successful clinical translation from animal models to clinical trials are not very encouraging; at present not more than 8%[4]. Animal models have the restricted ability to mimic the complex process of human cell proliferation and pathophysiology conditions. In oncology research, studies on cell culture and animal models are critical instruments in determining the efficacy, pharmacodynamics and mechanism of action of novel anti-cancer drugs. It should be remembered that heterogeneity of the tumour cells leads to the huge diversity with a high degree of genetic instability and phenotypic variation.

Prior to plunge into the trial of a promising anticancer drug, pharmaceutical companies and institutional investigators conduct wide pre-clinical experimental studies. *In vitro* and *in vivo* studies preliminary covers safety, efficacy, toxicity and pharmacokinetic profiles of the candidate molecules. Early *in vivo* testing aims specifically to provide initial safety and efficacy data to supports investigators claims about compound under investigation. To justify further development, preclinical experiments add sufficient confidence to the research data. This is important because as per the Food and Drug Administration guidelines, successful animal need/preclinical testing have to be completed before humans are exposed to the potential therapeutic entity[5].

Apart from possible misleading *in vitro* results, relating to inaccuracies in potency, efficacy, toxicity, genotoxicity and carcinogenicity, the financial cost of clinical research also plays a decisive role in the development and establishment of the successful therapeutics. Given that three-dimensional (3D) bio-printed structures could produce better models of the *in vivo* microenvironment, there is the significant potential for cost reductions in pre-clinical research. The 3D bio-printed tissues and organs have the capacity to provide viable substitutes to cell cultures and animal models. The 3D printing of solid objects is already guiding major innovations in diverse areas, such as education, manufacturing, engineering, art, pharmaceuticals and medicine[6]. Recent innovation in 3D printing and material science have enabled construction of complex 3D functional living constructs (tissues and organs)[6]. Without worrying about the rejection, 3D bio-printing has already been used for the generation and transplantation of several important tissues including, bones, skin, heart tissue, *etc*. Other lucrative applications include developing more reliable 3D bio-printed tissue models for pharmaceutical and drug discovery research. Accurate reproduction of the tissue or an organ is a significant feature of the 3D bio-printing which ultimately could lead to the standardisation of therapeutic testing[7]. This is possible to achieve by reproducing all the functional components of the tissues and organs, such as mimicking the exact branching patterns of the tinniest capillary in a complex organ like the heart, kidney, liver and lungs, or manufacturing the biomaterials to take care of the natural physiology.

PRECLINICAL *IN VITRO* MODELS AND THERAPEUTIC DEVELOPMENT

New drug development programmes generally take about 12 years to get an experimental lead compound to the patient bedside. The average cost involved in this process can be as high as exceeding $1.2 billion dollars[8,9]. The drug development process is highly risky in terms of economic gain; evident by an overall average attrition rate of approximately 90%, which means that only 10% of clinical trial compounds could finally reach to the market[10]. Consequently, scientists are now putting greater efforts in reducing the cost of the drug development process. Computer aided drug design[11], *in silico* pharmacokinetics[12] and toxicity testing[13] are few of the newer methodologies available, which could reduce the initial cost of the drug development process.

Accurate preclinical determination of efficacy and toxicity would lower the failure rate of new molecules during the important stage of clinical evaluation. Drug testing on 3D bio-printed human organs could eliminate the possibility of drawing uncertain conclusions from preclinical animal and cell culture studies. Conflicting conclusions from preclinical animal models and human experiments usually surface during the final stage of the clinical trials, when most of the resources have already been invested in the research and development process. Several promising lead candidates have faced failures in clinical trials after successful animal testing[14-19]. Preventing these problems in the first place would improve the cost and time involved in bringing a new drug to the market. To accurately predict the unwanted parameters of the drug candidates in clinical trials, various classical, existing and emerging technologies (models) are available. This comprehensive list includes

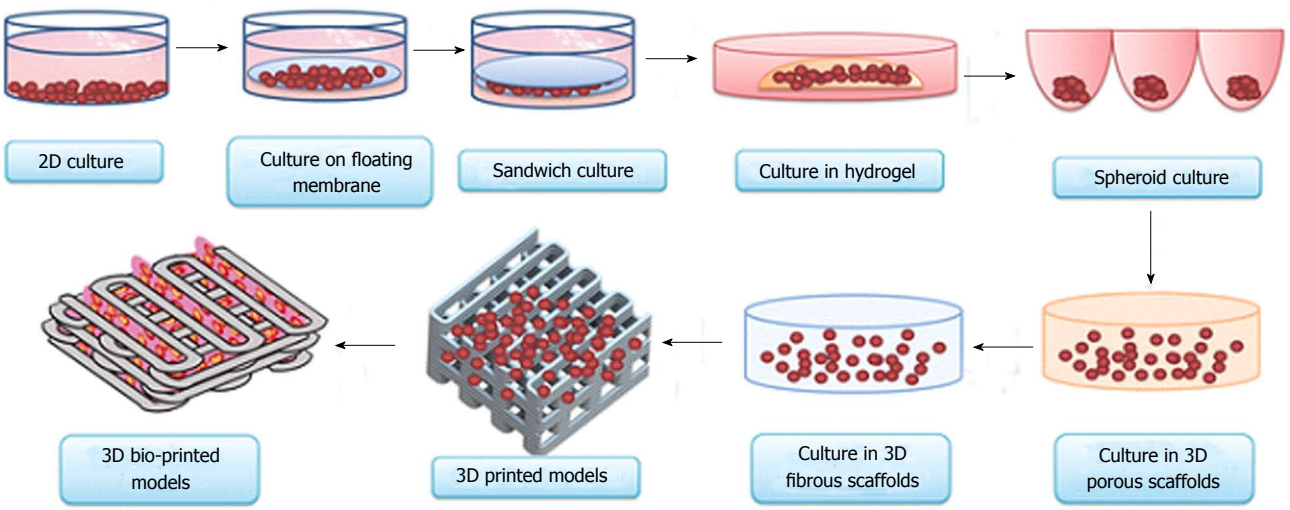

Figure 1 Evolution of cell-culture models from simple two-dimensional to complex three-dimensional bio-printed models. Currently, 3D bio-printing is the most sophisticated technique used to make tissue/organ constructs[65]. 3D: Three-dimensional.

traditional 2D tissue culture[20], classical whole rodent models[21], humanised mouse models[22], 3D culture models[23], co-culture systems[24] and 3D tissue models[25] (Figure 1).

Traditional 2D cell culture systems which employ cell lines in a single layer, themselves contain abundant genetic mutations. 2D cell culture systems also lack the important natural microenvironment present in the tissues and organ from which they were originally seeded[26]. Traditional culture performed with primary cells do not offer 3D microenvironmental characters similar to that of its origin[27]. Classical cell culture systems not only lack the influential tissue microenvironment and gradient but may also include the rapid loss of important proteins and its functions and gene expression profiles. To get a better representation of tissue complexity, microenvironment and whole-body physiological impact, studies on the animal model have become the backbone of preclinical studies. However, as discussed earlier, basic molecular, physiological and pathophysiological differences between the species lead to the likelihood of erroneous conclusions being drawn about an under trial candidate. Erroneous conclusions are the leading cause of failures in clinical trials.

Co-culture systems, 3D culture models, 3D tissue models and humanised mouse models which could mimic the host microenvironment are available for preclinical studies. To some extent, these methodologies allow drug testing in human-like systems, eliminating the species differences and, thereby, increasing acceptability in clinical trials. Developing pharmacological assays based on configuring multiple cells into a 3D-orientated structure could provide more realistic data. The 3D cell culture and models could mimics native tissue architecture more closely and hence could address drug development concerns in a more actual ambience than traditional 2D culture models.

Humanised mice model is another approach to testing drugs in more human-like conditions. This type of the animal models include mice bearing tumours derived from humans, known as xenografts or mice in which the endogenous liver has been compromised and repopulated with human liver cells[22]. Xenografts are important and proving useful in anticancer drug development. Xenografts often enable the assessment of drug efficacy, safety and toxicity in the context of tumour phenotypic and genotypic heterogeneity. Similarly, mice with humanised liver offer the ability to assess drug pharmacokinetics and metabolism preclinically *in vivo*. Humanised liver is an important tool to understand drug excretion and toxicity[28]. One important thing to remember about all humanised models is their chimeric nature. They are a single human tissue or cell type planted within the animal body, which may lead them to behave differently from their native environment. This may propagate false interpretation due to inter-species variations. For example, the stromal and vascular components of xenograft models largely come from an animal in origin[29]. Similarly, mice with humanised livers contain human hepatocytes, however, the other cell types found in the liver and all of the interrelated organ systems are of mouse[30] which ultimately could affect the liver functions. Hence, such models cannot be considered as the perfect model for human systems modelling. However, as stated earlier, humanised mouse models are a popular model in the study of human cancer. They provide an understanding of factors involved in pathology, physiology, metastasis and invasion.

In xenograft models, human tumour cells are transplanted into a different species, either into the organ type in which the tumour originated or under the skin. Human tumour cells are commonly transplanted into the mice which are severely immunocompromised. The weak immune system of such mice accepts foreign human cells readily. For example, the xenograft (foreign

cells or organ) will be readily accepted by athymic nude mice (lacking T cells producing thymus), severe combined immunodeficiency mice strains, or other immunocompromised mice[31,32]. Therapeutic agents can be studied in these immunocompromised mice as it readily allows the growth of human tumour within itself. The size of the tumour is generally depends on upon the number of cells originally transplanted, however, growth occurs over 1 to 8 wk to give more natural humanised environment. Genetically engineered mouse (GEM) model is another type of widely used animal model used for studying human cancer.

GEM mouse model allows the investigator to study the genes which are speculated to be the reason of the malignancy. Such genes are deleted, silenced or sometimes overexpressed and the animal is observed for the molecular and phenotypical changes over the period of time to study the therapeutic response. GEM provides an opportunity to study the therapeutic response *in vivo.* Xenograft models and immunocompromised athymic nude mice have been in used for several decades to increase our understanding of pathophysiological and genetic factors involved in uncontrolled cell proliferation and metastasis. Recent information about the role of the microenvironment on the tumour progression, growth and resistance towards the drugs has made GEM and primary human xenografts in humanised mouse models a primary choice for the experiments. However, because of the species difference, xenografts of human cell lines in mice to test drug responses do not always necessarily correlate with the actual pathophysiological condition in patients[29].

The importance of the tumour microenvironment on tumour growth not only leads to the general acceptance of the humanised mouse models and GEM for the development of the cancer therapeutics but also paved ways for the development of 3D printed tissues and organs in oncology research. The 3D culture and co-culture systems already exist and recent refinement increases their availability for therapeutic research. Certain drawbacks, such as low cell density, and use of artificial matrices and scaffolds add a non-human or non-native aspect to the system, which could affect the final outcome. However, more recent approaches that generate 3D culture systems, such as 3D bio-printing, could help nullify the non-human aspect.

3D BIO-PRINTING

The 3D bio-printed tissues and organs could be designed to mimic the exact cellular density of target tissues and organs, with proper consideration given for cellular component, extracellular matrix and three-dimensional spatial components. Since complex tissues are not constructed exclusively from a single cell type, 2D mono-cell culture models are of debatable value[33]. However, 3D bio-printers deposit more than one cell type, co-culturing them in one single spatial arrangement making them a closer match for the natural architectural arrangement. With the recent advancement in bio-printing, it is now feasible to combine the most important elements of spatial patterning to generate 3D *in vitro* tissue/organ systems that could mimic the key cellular and extracellular functional machinery, including innervation[33]. The 3D printers use various types of cells in the form of bio-inks, which technically have enhanced the speed of 3D printing of organs and tissues. The 3D organ scaffold generated with the help of computed tomography or another imaging technology and the solid surface made up of the biocompatible materials is used as the substrate to generate the 3D tissues and organs. Bio-inks are made up of cells suspended in a biocompatible gel-like material then deposited on the substrate using 3D printers which work on the principal like mechanical extrusion[33]. During and after deposition on the substrate the bio-ink is gelled by polymeric inter-linking with the help of photo or thermal activation. Because of the involvement of the high energy, care is always taken to leave the cells intact and functional. Hydrogels not only play an important role in physically restraining the suspended cells and in the maintenance of the cell viability but also can be personalised and tailored according to the biocompatible material or dimensions[33].

The development of aqueous-based systems enabled direct printing of bio-inks into 3D scaffolds[34]. Sequential deposition of the living cells, biocompatible extracellular and materials with spatial control over the 3D architectural parameters is the heart of the 3D bio-printing and 3D bio-printed organs. The 3D bio-printing works on the several established principals based on bio-mimicry, autonomous self-assembly and mini-tissue building blocks[6].

Technological advancement in imaging and digital design technology has positively impacted the 3D bio-printing by reproducing and visualising the very complex, heterogeneous architecture of complex tissues and organs. Non-invasive imaging techniques, like computed tomography, magnetic resonance imaging, computer-aided design and computer-aided manufacturing tools and mathematical modelling, are used to collect and digitise the complex tomographic and architectural information of the tissues/organs. The 3D digital images of complex organs are then used to print tissues and organs using techniques like inkjet[35-38], micro-extrusion[39-41] and laser-assisted printing (Figure 2)[42-44].

The 3D printing technologies first became prominent in non-biological applications, such as the deposition of ceramics, metals and thermoplastic polymers in heavy and light industries. Organic solvents, high temperatures and cross-linking agents (*e.g.,* photo-activation) used in 3D printing poses immediate compatibility problems for delicate living cells, thermal liable biological (*e.g.,* proteins) and biocompatible materials[6,45,46]. Among several, one of the main and dare challenges in the

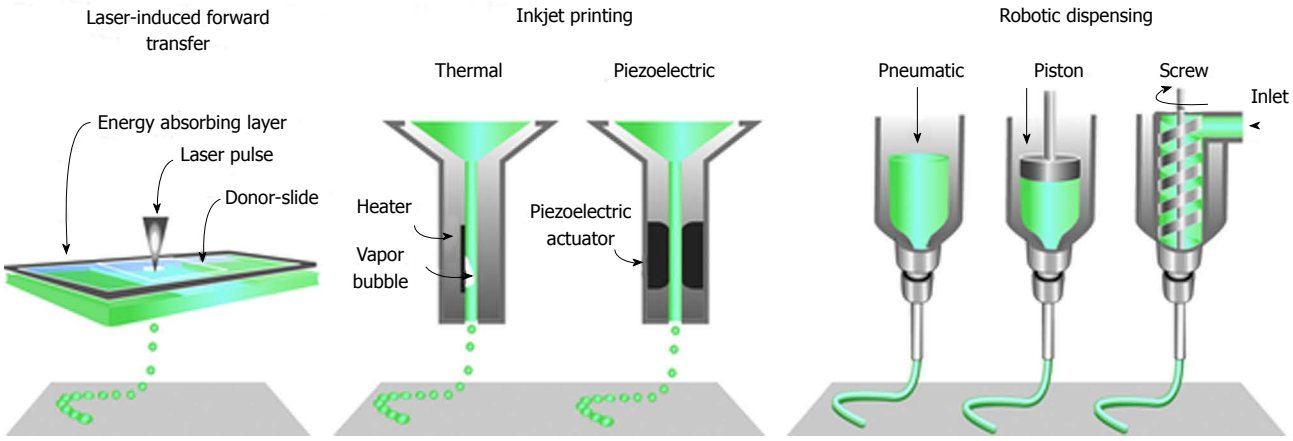

Figure 2 The common approaches currently used to bio-print tissue, are laser-assisted, inkjet-based and extrusion-based robotic dispensing techniques[110].

3D bio-printing of tissues and organs is to develop the compatible materials that not only should go well with the several other biological materials and the harsh printing process but should also provide the required mechanical and functional properties to the 3D bio-printed constructs. Materials currently used in the field of regenerative medicine are based on either natural polymers (*e.g.,* alginate, gelatin, collagen, chitosan, fibrin and hyaluronic acid *etc.*) or synthetic molecules (*e.g.,* polyethylene glycol). Some of the major advantages of the natural polymers in 3D bio-printing are its similarity to the human extracellular matrix, non-toxic nature and inherent bioactivity. Whereas the typical advantage of the synthetic polymers is that they can be personalised and tailored to the specific application and can also be obtained in the most purified form. But like other synthetic molecules, synthetic polymers not only possess the risk of the poor bio-acceptability but could also lead to the toxicity because of the toxic degradation. Other challenges could be the loss of the mechanical strength over the period of time and immunogenicity. Despite this, synthetic hydrogels polymers owing to its hydrophilic, absorbent and manageable physical and chemical properties are an attractive alternative in 3D bio-printing. The correct functioning of the 3D fabricated tissue or organ does not only depend on upon the accurate deposition of the cells but the choice of the cells is also crucial. Other criteria need to be satisfied is that the cell chosen for 3D bio-printing should have the capability to proliferate of its own. Precise control of cell proliferation (*in vitro* and *in vivo*) ensures the functionality of the construct. In addition to the primary cell of interest (*e.g.,* hepatocytes in liver construct), most tissues also contain other cell types that are involved in supportive, structural or barrier functions (selective transport) (*e.g.,* liver also contains sinusoidal endothelial cells and phagocytic Kupffer cells) and may also be involved in vascularization or may play role in stem cell maintenance and differentiation.

Presently, 3D bio-printing involves the deposition of multiple primary cell types into patterns that accurately represent the native tissue. In the case of the auto-rearrangement and self-assembly to the 3D construct, printing involves the bio-ink of the stem cells that can proliferate and differentiate into the required cell types. Maintenance and exact mimicking of the physiological function of cells in 3D construct are important and hence the criteria applied for selecting the cells plays the decisive role in proper functioning[47].

Rejection by the host immune system is the challenge in the tissue and organ transplant. This issue can be sort out by using the autologous cells for 3D bio-printing of organs and tissues. Autologous cell source involves biopsies, generation and differentiation of autologous stem cells or induced pluripotent stem cells. Although autologous cells are the very reliable source, it's of no use in case if the patient is already ill, cells are infected or have metabolic or hereditary disorders. In such cases, especially in the case of genetic disorder, 3D construct is not useful for the transplant but could be useful in case of therapeutic development (*e.g.,* genetic mutation in cancer cells will be useful to construct 3D bio-printed tumour model). In the case of the metabolic disorder, autologous cells may not be able to produce the normally desired function in bio-printed organs.

Prolong functionality of any 3D bio-printed tissues and organs are the key to the success. However, cells types like heart, liver and immune cells are not only difficult to isolate from the source but is also difficult to culture in a lab because of their limited lifespan[48]. Self-renovating, ability to differentiate into any cell type and capability to generate multi-functional tissue-specific cell phenotypes is the solution for such problems. Embryonic stem cells and induced pluripotent stem cells have all these characters and hence are the promising cell types for 3D bio-printed organs and tissues[49]. The 3D bio-printed organs require the self-renovating or self-replenishing character to maintain the functionality, in this regard pluripotent stem cells ability to multiply several times highlight its potential in 3D bio-fabricated

construct. Other types of stem cells, such as stem cells from bone marrow[50-52] and fat[53] or perinatal stem cells from amniotic fluid[54] or placenta[55], have limited multi-potent differentiation ability. These cell types but are considered safer for 3D bio-printed construct. These cells also satisfy the criteria of the autologous cell types and hence have the potential application in regenerative medicine. Mesenchymal stromal cells (MSC) are also a good cell source but its Isolation is difficult. However, the establishment of the new protocols for isolation, expansion and differentiation now make them the reliable and promising source for bio-fabricated constructs. Clinically required amount of MSC has been effectively generated *in vitro* and have found application in clinical trials and regenerative medicine[50-52]. Future development in biotechnology and cell-culture techniques is likely to be useful to exploit other stem cell populations for bio-printing and regenerative medicine; this is not just a hypothesis but a potential possibility.

3D PRINTING IN PRE-CLINICAL TESTING AND THERAPEUTIC DEVELOPMENT OF ANTI-CANCER DRUGS

Therapeutic drug development and therapy optimisation experiments in genetically modified mouse, 2D cell culture, 3D co-culture and xenografts of human tumour cells into nude mice are the important tool and have immensely contributed in the oncology research[31,56,57]. Physiologically, tumour microenvironment is extremely complex in which genetically mutant and phenotypically proliferative cancerous cells not only interact with each other but also reciprocally interact with the stromal and immune system microenvironment[58]. Modelling the heterogeneous complexity of a typical tumour using 3D bio-printed tissues and organs for preclinical testing could be an innovative and novel approach for the pre-clinical testing and therapeutic development of anti-cancer drugs.

Determination of the efficacy, toxicity, pharmacodynamics, pharmacokinetics and mechanism of action are the critical studies towards the development of efficient anti-cancer therapeutics. Cell culture and animal studies have played important roles in this process. Tumour cells and host microenvironment interaction leads to the recruitment of the components essential for the inflammatory and immune signalling. This recruitment of the signalling components is preceded by the fibroblasts and endothelial cells activation. The microenvironment of the host tumour is modified to select and adapts the genetic and phenotypic characters of the tumour cells. In fact, the modified microenvironment of the host organ in cancer pathology ultimately helps in the growth of the tumour cells. This reciprocal interaction between tumour cells and the microenvironment is actually essential for tricking the immune system, proliferation and metastasis[59]. Host microenvironment not only subjected to the different environmental stimuli but if looked from the population perspective it is genetically and phenotypically so diverse that the same tumour will grow and behave differently in different physiological condition (different patient). Simulation of such huge diversity (thousands of genes) in 2D cell culture and in animal models to test the toxicity and efficacy of drug candidate is the mammoth task. Essentially, it is impossible to extrapolate the results obtained from single or two test models to the numerous tumour variants in a broad genetically heterogeneous population.

Cell cultures derived from the human tumour cell line only offers the advantage of the biology to the primary tumour but it cannot simulate or mimic the complexity involved in the interaction between the proliferating tumour cells and microenvironment. Xenografts in immunocompromised mice interact with the surrounding cell types which are different from the native cell types and hence grafted tumour cells could behave differently in mice. Overall, the xenografts mice models have added limited value to the 2D cell culture. Similarly, lack of working the immune system and insufficient interactions between the human tumour cells and human stromal cells do not essentially represent the human tumour microenvironment.

Organovo is now an early-stage but established medical research company, which designs and develops functional 3D human tissues and organs for medical and pharmaceutical research and therapeutic development. The main focus of this innovative company is to speed up the preclinical and clinical drug testing by bio-printing human tissues and organs which mimics the human organ *in vitro*. The 3D bio-printed constructs enable the researcher to develop treatments and therapeutics faster, at very low cost and without risk to the living subjects. To assist the drug development process, Organovo now associated itself with biopharmaceutical and pharmaceutical companies and renounced academic medical research centres to design, build, standardised and validate more human-like *in vitro* tissues for disease simulation and drug, efficacy and toxicity testing.

The 3D bio-printed tissues and organs printed form human/autologous cells theoretically provides similar microenvironment as that of tissues and organs inside the body. Individual cells of the 3D construct experience the similar microenvironment as that of the tissues of the body. This provides an opportunity to the researcher to carry out the drug testing experiments *in vitro* in living tissues and organs. This also eliminates the possibility of the testing of drugs in living human subject; thereby bridging the gap between preclinical experiments and clinical trials.

Organovo's bio-printed tissues are created from human cells. Bio-printed construct recreates various biological aspects *in vitro*, *e.g.,* microenvironment and biology, reciprocal interactions between cells and micro-environmental factors and simulation of original tissue extracellular matrix including extracellular electrolytes. Organovo's exVive3D™ bio-printed human tissues may

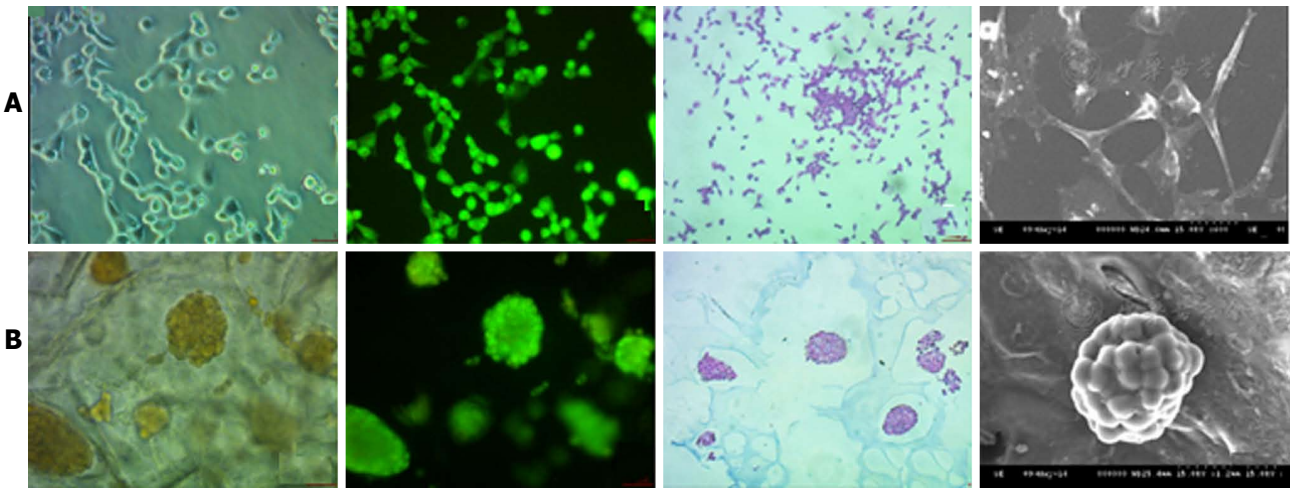

Figure 3 Nonsmall-cell lung cancer 95D cell morphology under two-dimensional and three-dimensional culture conditions. The 2D cultured cells (A) are tiled, polygonal, of long spindle shape and display more pseudopodia. In contrast, 3D morphology culture groups (B) are a combination of round and oval shapes, display intercellular tight aggregation and adhesion. Furthermore, there is evidence of multiple sizes of cells distributed in different scaffold pores[60]. 2D: Two-dimensional; 3D: Three-dimensional.

reduce the failure risks and costs involved in the drug and therapeutic development process. Drug testing experiments *in vitro* 3D printed human tissues enable to secure human tissue-specific data prior to initiating the clinical trials in humans.

The liver is the primary site for the metabolism of many endogenous (*e.g.,* hormones) and exogenous (*e.g.,* xenobiotics) substances. Organovo's exVive3D liver is a bio-printed human liver model composed primary of hepatocytes, hepatic stellate cells and endothelial cells. Organovo's exVive3D liver tissue secretes important proteins like fibrinogen, albumin and transferrin proportional to levels in whole liver. Levels of ATP and lactate dehydrogenase secreted are also in the normal range when compared with the whole liver. This liver model could be a very important tool to study the route of metabolism of various exogenous and endogenous substances.

The realistic implications of 3D printing technology in drug discovery and development process involves the optimisation of the preclinical and clinical research methodologies. The research gap present between the lead molecule optimisation, preclinical studies and clinical research could be filled by the 3D construct of human tissues. Moreover, 3D constructs can reduce the failure risk and cost associated with the final stages of the drug discovery and development process. The 3D bio-printed models, unlike traditional cell culture models, could be standardised and validate for answering the complex questions related to the human cancer biology at molecular and tissue levels.

Today's 3D bio-printed human research data is not sufficient enough to replace the classical cell culture and animal models. However, the recent pragmatic shift towards the 3D bio-printed tissues and organs may be sufficient enough to generate enough evidence to prove its usefulness in drug discovery process. Sooner or later the researcher will be confident enough to make a call with a high level of confidence. The Early conclusion at the preclinical stage could be possible with the advancement in the 3D bio-printed technology; thereby reducing the risk associated with final-stage clinical trials.

Early prediction of the risk associated with the drug discovery process could be reduced with the help of 3D printed tissues, *e.g.,* Mou *et al*[60] used non-small cell lung cancer 95D cells to co-culture with a 3D bio-printed scaffold to construct a lung cancer model *in vitro*. This study of Mou *et al*[60] was focused on the relative comparison of the biological functions of lung cancer cells under the 2D and 3D environmental conditions. The 3D scaffold was constructed using the natural products like agarose and alginate and 3D printing technique was utilised to deposit the cell cultures on the scaffold. 95D cells types were used to co-cultured with this scaffold. The most important observation of this research tells us about the spindle and polygonal morphology of the cell cultured in 2D wells, whereas those cells which were grown in the 3D culture aggregated into spheroids and was able to migrate and invade the surrounding area of the scaffold (Figure 3).

Cell metabolic activity assay showed that the multiplication rates of the 3D-cultured cells for 2-6 d were significantly lower when compared with the 2D-cultured cells. On the other hand, those cells which were cultured for a longer time (8-9 d) were significantly higher than that of the 2D-cultured cells, demonstrating the proliferative activity of the cancer cells grown in 2D cultures for 8-9 d was inhibited. It is also observed that the cells grown on 3D scaffolds maintained a high rate of proliferation over the longer period of time. At the end, it was concluded that not only the cell mor-

phology and proliferation rate was different but also the associated protein expression was different. The growth of the lung cancer cells in 3D culture was also found to be different from the 2D cultured cells. We can also conclude that the agarose-alginate 3D scaffold can better simulate the microenvironment of lung cancer *in vivo* and in future this 3D construct may be established as a promising model for research in lung cancer.

Bone were constructed using human mesenchymal stem cells which were co-printing with acrylated peptides and acrylated poly (ethylene glycol). Inkjet bio-printing technique was used to make this construct[61]. Bone marrow stem cells with hydrogels like alginate, agarose, Matrigel®, and Lutrol® F127 were dispensed together using 3D bio-printer[62]. The printed bone marrow stem cells in combination with hydrogels were found to be functional and viable in the 3D construct. A mechanically stronger 3D bio-printed construct containing two different cell types has also been fabricated for osteochondral tissue regeneration[63].

Adipose-derived stem cells have the versatile ability to differentiate along with multiple lineage pathways. These cells could be isolated from human adipose tissue and could play the crucial role in regenerative medicine. Yao *et al*[64] used adipose-derived stem cells along with hydrogel (gelatin-alginate) to bio-print 3D construct in cubical shape. This work has significantly contributed to the idea of 3D construct of adipose tissue with functional vessels for efficient blood flow. Development of blood vessels inside the 3D printed adipose tissue means the better simulation to study complex biological phenomenon's *in vitro*, *e.g.,* differentiation of stem cell, cell signalling and interaction *etc*. One important finding of this study is that adipose stem cells not only proliferated of its own but were also found to differentiate within the 3D construct. When basic fibroblast growth factor was added, cells present in the 3D scaffold converted into endothelial cells and the cells rooted in the hydrogel separated into adipose-like cells. The constructs were found to remained intact for around 60 d[65].

Lee *et al*[66] used cells like keratinocytes, fibroblasts and collagen to develop the skin construct *in vitro*. Keratinocytes represented and converted to epidermis layer, fibroblasts into dermis layer and collagen epitomised the extracellular matrix of the skin (Figure 4). Histological, biochemical, light and fluorescence microscopic examinations have proved that the 3D printed skin was not only morphologically but was also found to be biologically similar to the natural skin[66,67]. Koch *et al*[68] on the other hand utilised laser-induced forward transfer (LIFT) for the development of 3D skin. Koch *et al*[68] used skin cells like fibroblasts and keratinocytes to represent the cells of dermis and epidermis layers of skin respectively and also used human mesenchymal stem cells for differentiation into other useful cells. All these cells were used in the form of bio-ink and were then deposited using laser-induced forward transfer method.

Vascular system transports oxygen, nutrients and toxic residue to-and-fro from the cell and hence considered as the very important component of the complex organ system. In regenerative medicine, development of the *in vitro* vascular structures could help us to bio-print the bigger and hugely complex organ[69]. Skardal *et al*[70] was the first to cross-linked tetrahedral polyethylene glycol tetracrylates with hyaluronan hydrogels to generate the 3D bio-constructed vascular system. Skardal *et al*[70] utilised bio-printers which work in the principle of extrusion (Figure 5). Recently Kolesky *et al*[71] also developed the complex vascular scaffold using gel-based cellular suspensions, sacrificial and fugitive gel and casting cavity filled with a GelMA gel.

Miller *et al*[72] first time used bio-printed complex vascular structure using carbohydrate glass. Carbohydrate glass was used as a sacrificial substrate/template for the cell adhesion. The sacrifice of the carbohydrate glass after cell deposition lead to the formation of the cylindrical vessels. Carbohydrate glass wall was lined with endothelial cells and the blood was forced through it under high pulsated pressure. After sacrifice of the carbohydrate glass wall, the hollow channel network left behind was populated with human umbilical vein endothelial cells to attach themselves to the wall of hollow channels. As compared with the other methods discussed earlier, Miller *et al*[72] approach is not only simple and gives greater control over the network geometry but is also well-suited with the different types of natural and synthetic extracellular materials, different variety of cells and various cross-linking methods. Miller *et al*[72] also proved that the vascular system was able to tolerate the metabolic function of rat hepatocytes in 3D engineered constructs[72]. Norotte *et al*[73] on the other hand, developed a method for preparation of the scaffold-free vascular tissue l. Norotte *et al*[73] utilised fibroblasts and smooth muscle cells with agarose as the supporting gel.

To study the inflammation in the intestinal mucosa Leonard *et al*[74] developed a complex *in vitro* model. Leonard *et al*[74] have utilised enterocyte cell line, immunocompetent macrophages and dendritic cells to construct 3D-fabricated intestinal mucosa model. This 3D printed intestinal mucosa model was then stimulated with the help of lipopolysaccharides from *Escherichia coli* and *Salmonella typhimurium*, interleukin-1β, and interferon-γ. Stimulation helped to develop the natural pathophysiological changes which occur in the intestine during inflammation. Different cell lines like Caco-2, HT-29 and T84, were used to develop the 3D constructs and were stimulated with the same pro-inflammatory molecules. It was observed that the Caco-2 cells were highly responsive towards the pro-inflammatory interleukin-1β molecules (Figure 6).

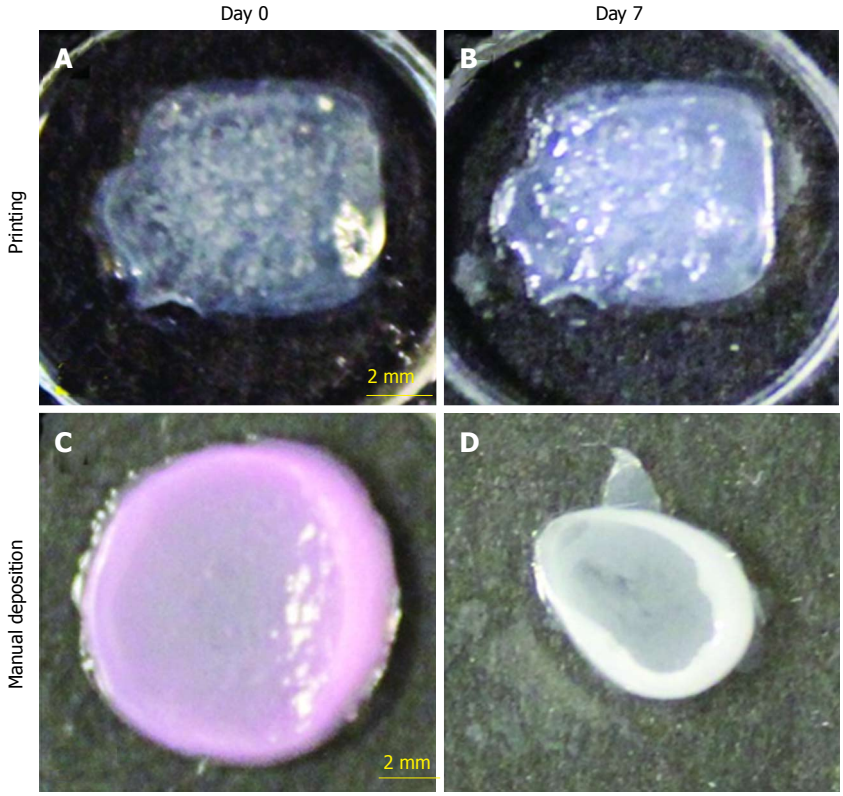

Figure 4 Shape and form of printed skin tissue. A comparison of skin tissues fabricated *via* 3D bio-printing and manual deposition indicates that printed skin samples (A, B) retain their form (dimensions) and shape, whereas manually deposited structures (C, D) shrink and form concave shapes (buckle) under submerged culture condition after 7 d.

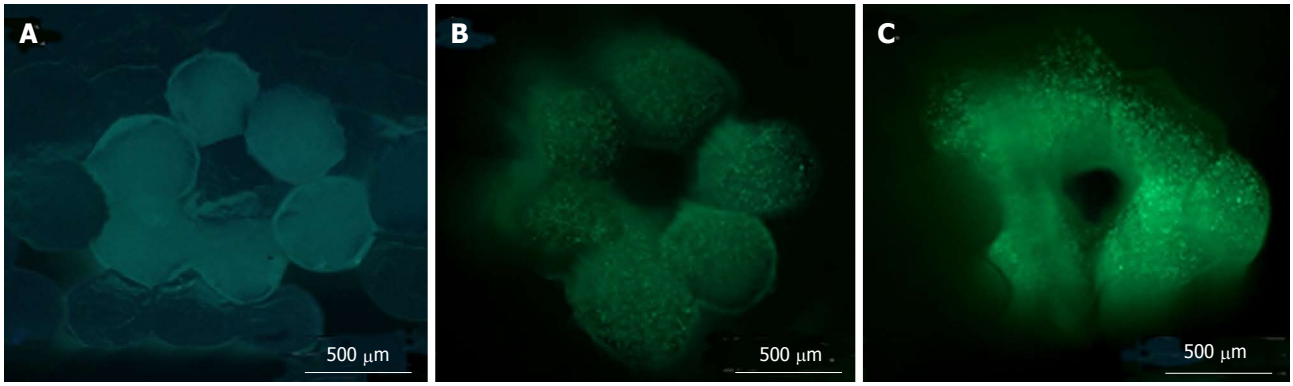

Figure 5 Cross-sectional images of three-dimensional bio-printed tissue (NIH 3T3 cells) containing an encapsulated fluorescent HA-BODIPY tracer for increased visualisation. Cross-sectional views of the bio-printed vascular constructs were taken (A) immediately after printing; (B) at 14 d; and (C) at 28 d of culture using LIVE/DEAD staining to highlight viable and dead cells. Green fluorescence indicates calcein AM-stained live cells[70].

The above-mentioned examples of 3D bio-printed tissues and organs could fasten the therapeutics development process and would facilitate the *in vitro* study of cancer pathophysiology. Recent advancement in the stem cell technology (Induced pluripotent stem cell) will hugely supplement the research in 3D bio-printing. Induced pluripotent stem cell has the unique character of dedifferentiated and then redifferentiated into tissues of choice[75]. Induced pluripotent stem cell technology has the very important role to play in 3D bioprinting and in solid organ transplantation. In the future, patient specific 3D tumour model also has the ability to revolutionised the field of personalised treatment.

ADVANTAGES OF 3D PRINTED TUMOUR MODELS - A COMPARISON WITH 2D PLANAR MONO-CULTURE AND 3D CO-CULTURE MODELS

The most efficient way of learning about the tumour progression and anticancer drug evaluation is by regulated and structured clinical trials on humans. However, direct evaluation of pathophysiological process in cancer

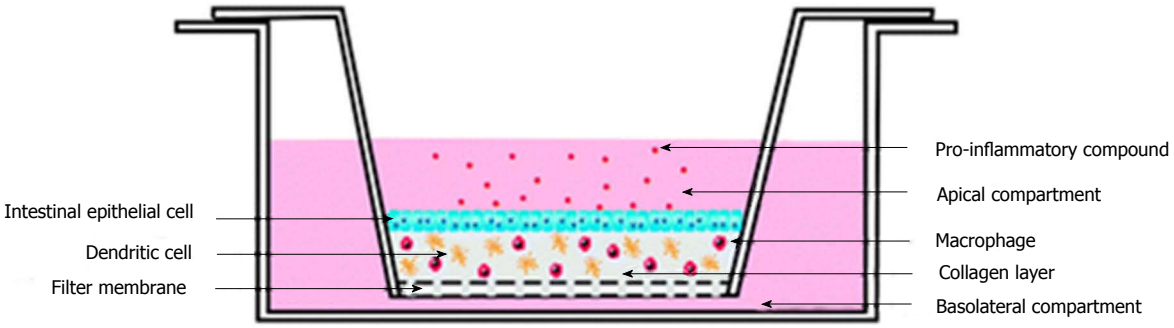

Figure 6 Experimental setup of three-dimensional co-culture comprising of intestinal epithelial cells, macrophages and dendritic cells[74].

development and anticancer activity of drugs is highly unethical because of the safety concerns. To overcome ethical challenges, preclinical studies on tumour models are highly appreciated. Several preclinical tumour models like cell culture, xenograft, mouse model and 3D tissue culture are developed which are thought to resemble with the natural tumours in terms of pathophysiological processes involved[76-78]. Evidence are now available which proves that the tumour microenvironment is the key regulator of the several stages involved in the pathophysiology of cancer progression. Tumour microenvironment is particularly very important in terms of the development of resistance, inventions of the distance organs and escape from the immune surveillance

This recent development not only challenged the past concept which mostly focused on the tumour cells but also impacted the research strategies of future. In future, the medical interventions in clinical oncology will also involve the therapeutics targeting the microenvironments. A systematic and methodological study of the tumour microenvironment, with the help of 3D bio-printed tumour models, would promote evaluation and selection of candidate agents from preclinical trials[79]. This would not only fasten the drug development process but would also save the resources.

A factor that plays an important role in the advanced malignancies is inappropriate activation of the supportive tissue called stroma. In most of the malignancy cases, stroma loses its connective and structural role. The various types of stromal cells are pericytes, smooth muscle cells, adipocytes immune cells, endothelial cells, fibroblasts, *etc.* Tumour microenvironment also found to contain various growth factors, many hormones, several structural and functional proteins, enzymes, cytokines and small cytokines of which most works as a primary and secondary signalling molecules and ligands for the receptors. The presence of all these functionalities in microenvironment could widely affect not only the pharmacokinetics but also the pharmacodynamics of the anticancer drugs. Thus the therapeutic outcome is widely regulated by the normal or abnormal expression of these extracellular proteins. It is now well recognised that protein and gene function varies strangely when studied them *in vivo* and *in vitro*. Studying the effect of these genetic alterations on drug response in either original or damaged neoplastic microenvironment is very critical for the fruitful drug development, translational anticancer regimes, and optimisation of therapies. These and several other factors are vital for the development of malignancies and are very difficult to re-orchestrate in 2D and co-culture models[80,81].

The genetically activated stroma of sarcomas and carcinomas is not only composed of cancer associated fibroblasts and myofibroblasts but can be identified due to altered matrix components, change in the proteins synthesis associated with repair machinery and reprogrammed breakdown process[80,81]. Except for the supportive function, stromal cells also play the important role in the physical and biological protection of microenvironment protection. This functionality actually limits the effective delivery of the therapeutic drugs to the cancer cells. Altered components of the tumour microenvironment, including the synthesis of the proteins involved in the repair mechanism, allows the unrestricted growth of the tumour cells. Tumour cells in favourable environment successfully evade the apoptosis signals triggered by cytotoxicity and develop various resistance strategies to select the malignant phenotypes.

Correlation of the survival rate and capability of stroma to overpower the carcinogenesis is already established[82]. However, once distorted to a tumour-associated neighbour because of the stimuli like inflammation, infection, mutation, *etc.,* the stromal protective function can be altered to stimulate the proliferation[83-85]. Under the altered condition, stromal cells start to evolve with the cancer cells and begin synthesis of growth factors, cytokines, chemokines, *etc.,* which fast-track the disease progression[86]. In addition to this, many *in vitro* studies have proved the complex role of the tumour microenvironment in cancer development. Experiments with genetically modified stroma proved the importance of the tumour microenvironment in disease progression[87,88].

Infection, immune-associated signalling and inflammation have been found to be associated with several cancer types. For example, liver carcinoma which is the leading cause of death in patients with liver cirrhosis and

increased the risk of colorectal cancer in the patients with increased inflammation is credited to unresolved inflammatory signalling[89]. Similarly viruses, bacteria and parasites are also the leading cause of the variety of cancers. A higher incident of multiple cancers like gastrointestinal tract, lung, reproductive and skin cancers has been found in female immunosuppressed organ transplant recipients[90]. Retrospective analysis revealed a higher incidence of AIDS-associated cancers (*e.g.,* Kaposi's sarcoma, Cervical cancer, Non-Hodgkin lymphoma), and non-AIDS-related cancers (*e.g.,* tongue, skin, lung, CNS and multiple myelomas) in HIV-infected patients[91]. Various enzymatic proteins, like matrix metalloproteinase, in particular, matrix metalloproteinase-2 and matrix metalloproteinase-9 have a role in the tumour progression. For example, matrix metalloproteinase-2 and matrix metalloproteinase-9 allow cancer cells to breach through the extracellular matrix of the tumour microenvironment and are closely related to cancer metastasis. The activity of the various matrix metalloproteinase is found to increase with the development of cervical cancer[92] and can be studied efficiently in 3D bio-printed tumour models[93].

Development of the resistance towards the therapeutic intervention is the foremost challenge in clinical oncology. In addition to fuelling the tumour growth, the altered tumour microenvironment modifies treatment responses by affecting cell sensitivity towards anticancer agents. Decreased cell sensitivity towards anticancer drugs gives rise to the drug resistance. The drug resistance facilitated by the alteration tumour microenvironment is not limited to classical agents like chemotherapies. Instead, it covers various therapeutic materials, including targeted agents and targeted drug delivery systems[94]. The role of tumour microenvironment in the protection of acute myeloid leukaemia or chronic lymphocytic leukaemia cells from pharmaceutical agents like anthracyclines, alkylating agents, imatinib and nucleoside analogues has been recently evaluated. The defending role of tumour microenvironment is detected in the protection of the mutant Janus kinase 2 cells from Janus kinase inhibitors. Tumour microenvironment role is also observed in protecting solid tumours from erlotinib and cetuximab. Similarly, recent findings described the protection of melanoma against RAF inhibitors, like vemurafenib[95-97]. Tumour microenvironment assisted resistance is found to be directed through several cell lineages and alteration in the stromal components (*e.g.,* fibroblasts, endothelial cells, *etc.*)[94,98].

Tumour microenvironment assisted protection of tumour cells applies to multiple therapeutic strategies and varies with the inter-individual differences. For example, in the treatment of melanoma by mitogen-activated protein kinase pathway inhibitors, tumour-associated macrophages multiplies and release cytokine-like tumour necrosis factor-α as a crucial growth factor that provides resistance to the targeted therapy through the microphthalmia transcription factor[99]. Similarly, certain cancer endothelial cells secrets interleukin-6 and tissue inhibitor of metalloproteinases-1 as the survival factors. Both of the factors were found to be significantly involved in the resistance of lymphoma when the Eμ-Myc mice model of Burkitt's lymphoma treated with anticancer antibiotic doxorubicin. This could be reversed or good chemotherapeutic efficacy could be achieved by the inhibition of these survival factors or by stimulating the p38 mitogen-activated protein kinase pathway[100]. Another noted example of tumour microenvironment-exerted protection of cancer cells is the chemoresistance caused by the amplification of the CXCL1/2-S100A8/9 loop by antineoplastic agents used in breast cancer treatment[101].

The examples illustrated above demonstrate various pathways by which therapies or targeted agents can be affected by the changes in the tumour microenvironment. Tumour microenvironment not only contains the tumour cells but also contains the several other cells, *e.g.,* immune cells, lymphatics cells fibroblasts, pericytes, *etc*. This composition of microenvironment essentially affects the therapeutic outcome[102]. The 2D monolayered and 3D coculture cellular models lack illustrated characteristics of natural 3D tissues *in vivo*[103]. 2D monolayered culture has the increased drug diffusion properties which do not match with the natural tumour character. A lot of drugs have their site of action inside the cells and hence their penetration is very important for effectiveness. This character of cell culture models explains the importance of three-dimensional arrangements for the proper success of the therapy.

To overcome the drawback of the cell culture models various alternative animal models were developed, *e.g.,* genetically altered and immunocompromised mice models. Animal models have contributed enormously to the present understanding of cancer, however, they could not reflect the actual pathophysiology involve in disease progression because of the species differences[104].

To overcome the hurdles of simulating the exact complex tumour microenvironment in cell culture, 3D printing technology was adapted to produce the 3D bio-printed tissues and organs. Similarly, 3D printing technology could be easily utilised to produce the 3D tumour models which subsequently could be utilised to study the cancer biology and anticancer drugs[105,106]. Various techniques, such as cell-seeding 3D scaffolds, hydrogel embedding, multicellular spheroids, cell patterning and microfluidic chips have been explored for the construction of 3D tumour models *in vitro*[76].

Several advances in 3D printing technology and stem cell research offers unique opportunity for the construction of complex organs and tumours. The 3D printed organs and tumour models essentially simulate the exact physiological and pathophysiological microenvironments. The exact recreation of the tumour microenvironment facilitates the better understanding

of the disease[107,108].

Till date, very few reports have been published describing the 3D printed tumour models. Zhao *et al*[93] demonstrated the use of HeLa cells in gelatin/alginate/fibrinogen hydrogels to bio-print the 3D *in vitro* models of cervical tumours. When compared with 2D cell culture model, 3D printed tumour model have shown 90% proliferation rate. Zhao *et al*[93] also observed the increased expression of matrix metalloprotease protein and chemoresistance in 3D printed tumour models when compared with 2D cell culture model. Work of Zhao *et al*[93] is just one example of the advancement of 3D bio-printed tumour model, with further advancement in 3D printing technology, *a revolution* in the field of cancer research is on the corner.

CONCLUSION

The 3D bio-printing of tissue and organ models is a developing field in which several ground-breaking results have been obtained over the past few years. The 3D-bioprinted tissue constructs are being prepared not only for the solid organ transplantation but also for use in drug discovery process. Fabrication of the realistic tissues, organs and tumour models with the help of the various 3D bio-printing techniques is now possible. Extrapolation of the results obtained from the cell culture and animal models are not trustworthy because of the species differences. This challenge of species difference could be overcome by printing the 3D tissues and organs from the human cells. The 3D printed tumour model fabricated from the human tumour cell lines will definitely revolutionise the oncology research. The 3D printing is the very precise which could be demonstrated by its (inkjet printer) use in transfecting genes into cells[109,110]. In coming days, 3D bio-printed tissues and organs will find its way in the pharmacological and toxicological testing of the molecules under drug development process. Bio-printing has the potential to change the way the drug enters the clinical trials after preclinical studies. The 3D printing not only has the capability to improve the attrition rate of the clinical trials but will also reduce the cost and time required in the drug discovery process. This is possible because of the speedy identification of the efficient candidate molecule. Use of 3D bio-printed models will eliminate the need of animal models and hence the data obtained in the preclinical studies will be more trustworthy.

Most published results are the early prediction and only a few studies methodologically explored the developmental method parameters. Standardisation and optimisation of the printing process parameters are essential for the successful adaption of the 3D printed tissues and organs to use them in drug development process. This is possible to achieve to by establishing the relationship between structural and functional parameters. Moreover, modern fabrication schemes rely on mathematical modelling and computer simulations for optimising the process design and making predictions[107,109]. Therefore the performance of the tissue constructs could be predicted virtually using computer simulations before actually printing the construct.

Stem cells already have revolutionised the field of regenerative medicine and have very important role to play in the construction of 3D tissue, organs and tumour models. Stem cells (*e.g.,* induced pluripotent stem cells) offer greater possibility for fabricating complex constructs because of their ability to differentiate in various another kind of cells, as highlighted by various research groups[107,109,111,112]. However, some issues need to be fixed before stem cells can be used for 3D bio-printing. This issue includes optimisation of the cellular microenvironment to combine the advantages of cell attachment, cell stimulation and mechanical stability to mimic the *in vivo* environment to the highest degree.

Printed 3D models match closely with the natural organs and when compared with the cell culture models. Novel 3D cell printing technology may help to develop the tumour models *in vitro* which will be more useful in studying cancer cell biology. Although, 3D bio-printing techniques are still in their infancy, they offer potential to overcome many challenges associated with the production of complex tissues and organs. This technique is a promising tool for replacing current and often misleading results obtained from cell culture and animal based screening of pharmaceuticals. Interdisciplinary research and collaboration of the researcher from the various field are required to overcome the hurdles before 3D bio-printed concept accepted by the institutional and pharmaceutical researchers. To be successful, we will have to sort-out the progressive challenges of 3D bio-printing, including cell sources and biocompatible material requirements, proper vascularization and autonomous maturation and continuous functionality of the construct.

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REVIEW

From targeting the tumor to targeting the immune system: Transversal challenges in oncology with the inhibition of the PD-1/PD-L1 axis

Melissa Bersanelli, Sebastiano Buti

Melissa Bersanelli, Sebastiano Buti, Medical Oncology Unit, University Hospital of Parma, 43126 Parma, Italy

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Correspondence to: Sebastiano Buti, MD, PhD, Medical Oncology Unit, University Hospital of Parma, Via Gramsci 14, 43126 Parma, Italy. sebabuti@libero.it
Telephone: +39-05-21702316
Fax: +39-05-21995448

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Abstract

After that the era of chemotherapy in the treatment of solid tumors have been overcome by the "translational era", with the innovation introduced by targeted therapies, medical oncology is currently looking at the dawn of a new "immunotherapy era" with the advent of immune checkpoint inhibitors (CKI) antibodies. The onset of PD-1/PD-L1 targeted therapy has demonstrated the importance of this axis in the immune escape across almost all human cancers. The new CKI allowed to significantly prolong survival and to generate durable response, demonstrating remarkable efficacy in a wide range of cancer types. The aim of this article is to review the most up to date literature about the clinical effectiveness of CKI antibodies targeting PD-1/PD-L1 axis for the treatment of advanced solid tumors and to explore transversal challenges in the immune checkpoint blockade.

Key words: Immune checkpoint inhibitors; PD-1; PD-L1; Checkpoint inhibitors; Cancer treatment; Immune checkpoint blockade; Anti-PD-1 antibodies; Anti-PD-L1 antibodies

Core tip: The onset of PD-1/PD-L1 targeted therapy in oncology has demonstrated the importance of this axis in the immune escape across almost all human cancers. A sort of revolution has been happening with the investigation of the new immune checkpoint inhibitors in the field of anticancer therapy. The aim of this article is to review the most up to date literature about the clinical effectiveness of the antibodies targeting PD-1/PD-L1 axis for the treatment of advanced solid tumors and to explore transversal challenges in the immune checkpoint blockade.

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INTRODUCTION

After that the era of chemotherapy in the treatment of solid tumors have been overcome by the "translational era", with the innovation introduced by targeted therapies, medical oncology is currently looking at the dawn of a new "immunotherapy era" with the advent of immune checkpoint inhibitors (CKI) antibodies.

The strategy to maintain physiologic self-tolerance and to restore latent anti-tumor immunity is currently going through the whole oncology, gradually revolutionizing the standard of treatment of the most chemo-resistant tumors such as melanoma, lung and renal cancer. From the first class of antibodies against cytotoxic T-lymphocyte-associated antigen 4 (CTLA-4), like ipilimumab and tremelimumab, burdened by significant autoimmune toxicity, the scenario is currently evolving in favor of the antibodies against programmed cell death protein 1 (PD-1) and its ligand PD-L1, in both cases inhibiting the PD-1/PD-L1 axis[1].

The monoclonal antibodies nivolumab and pembrolizumab (anti-PD-1), atezolizumab, durvalumab and avelumab (anti-PD-L1), have been tested against multiple cancer types in the last years and are currently under investigation in several phase Ⅱ and phase Ⅲ clinical trials. Further similar antibodies are currently undergoing phase Ⅰ experiences, in order to compete with the first arrivals on the clinical scenario[2-4]. All the antibodies cited in the text are reported in Table 1.

In all cases, the mechanism targets the inhibitory signal that contributes to the balance between co-stimulatory and inhibitory pathways in the regulation of T-cell response, starting from the antigen recognition by T-cell receptor. In fact, in contrast to other antibodies currently used for cancer therapy, CKI do not target tumor cells directly, but instead they target lymphocyte receptors or their ligands, with the aim to enhance endogenous antitumor response[5].

PD-1 belongs to the inhibitory B7-family molecules; it is upregulated and expressed by activated T-cells (but also B-cells, T regulatory and natural killer cells) and engaged through its ligands PD-L1 and PD-L2, expressed by the antigen presenting cells (APC) and by non-hematopoietic stem cells, aside from tumor cells. The role of PD-1 consists in the inhibition of the effector T-cells activity in peripheral tissues during the inflammatory response to infection and in the regulation and limitation of autoimmunity[6]. Within the tumor microenvironment, this endogenous mechanism favors immune resistance[7]. The major PD-1 ligand expressed on solid tumors cells is PD-L1, whose most important signal for induction is interferon-γ (IFN-γ), produced by T helper 1 (Th1) cells[8]. Most types of solid tumors have been demonstrated to express high levels of PD-L1 (melanoma, ovarian, lung cancer and genitourinary tumors among others), and more recently the importance of PD-L1 expression on the immune cells infiltrating the tumor also emerged, in particular on tumor-infiltrating lymphocytes (TILs). Nevertheless, the evidence about the prognostic and predictive role of these elements have not yet been clarified and it seems to be different basing on tumor type[5].

Table 1 Immune-checkpoint inhibitors antibodies with their targets

CKI	Mechanism of action
Nivolumab	Anti-PD-1
Pembrolizumab	Anti-PD-1
Atezolizumab	Anti-PD-L1
Durvalumab	Anti-PD-L1
Avelumab	Anti-PD-L1
BMS936559	Anti-PD-L1
Pidilizumab	Anti-PD-1

CKI: Checkpoint inhibitors.

Despite these unresolved issues, the findings described above provided the rationale for the capacity of the blockade of PD-1/PD-L1 axis to enhance intratumoral immune responses in a transversal way across different tumor types, firstly encouraged by preclinical evidence and then largely satisfied by the early results of several recent clinical studies.

RESEARCH

The aim of this article is to review the most up to date literature about CKI antibodies targeting PD-1/PD-L1 axis for the treatment of advanced solid tumors, particularly considering phase Ⅲ randomized trials, starting from the first performed trials on the issue. Published papers were obtained from the Medline database. The search was implemented by reviewing the most important international scientific meetings abstract databases. In addition, indirect data on the topic were achieved by reading the most recent publications related to the use of CKI in different types of solid tumors.

The ongoing trials were reached on the official website www.clincaltrials.gov, considering only randomized phase Ⅲ studies.

RESEARCH RESULTS

Melanoma

Treatment of advanced melanoma has been radically changed by the advent of CKI. After that the anti-CTLA4 antibody ipilimumab in the last years had become the backbone of this malignant tumor treatment, where traditional chemotherapy harvested very little success, the introduction of the anti-PD-1 antibodies nivolumab

Table 2 Phase Ⅲ randomized clinical trials currently ongoing with PD-1/PD-L1 axis blockade in adjuvant setting for solid tumors

Trial name/NCT	Cancer type	Immune checkpoint inhibitor	Arms	Primary endpoint	Expected primary completion date	No. of patients
KEYNOTE-054[20]	Melanoma	Pembrolizumab	Pembrolizumab *vs* placebo	RFS	2018	900
NCT02506153[21]	Melanoma	Pembrolizumab	Pembrolizumab *vs* high dose recombinant interferon-α-2B or ipilimumab	OS	2020	1378
KEYNOTE-091 (PEARLS)[22]	NSCLC	Pembrolizumab	Pembrolizumab *vs* placebo	DFS	2021	1380
IMvigor010[23]	Bladder cancer	Atezolizumab	Atezolizumab *vs* observation	DFS	2021	440
IMpower010[24]	NSCLC	Atezolizumab	Atezolizumab *vs* BSC after adjuvant CT[1]	DFS	2020	1127
NCT02768558[25]	NSCLC (locally advanced)	Nivolumab	Nivolumab *vs* placebo (after CT[1]-RT)	OS	2022	660
ANVIL[26]	NSCLC	Nivolumab	Nivolumab *vs* observation	DFS	2018	714
CheckMate 238[27]	Melanoma	Nivolumab	Nivolumab + placebo *vs* ipilimumab + placebo	RFS	2018	800
CheckMate 274[28]	Urothelial cancers	Nivolumab	Nivolumab *vs* placebo	DFS	2020	640
CheckMate 577[29]	Esophageal or gastroesophageal junction cancer (locally advanced)	Nivolumab	Nivolumab *vs* placebo (after CT[1]-RT and surgery)	DFS	2019	760
PACIFIC[30]	NSCLC (locally advanced)	Durvalumab	Durvalumab *vs* placebo (after CT[1]-RT)	OS	2017	702
NCT02273375[31]	NSCLC	Durvalumab	Durvalumab *vs* placebo	DFS	2025	1100

[1]According to the standard of care and basing on the choice of the investigator. RFS: Recurrence free survival; NSCLC: Non-small cell lung cancer; DFS: Disease free survival; CT: Chemotherapy; OS: Overall survival; RT: Radiotherapy.

and pembrolizumab further improved the therapeutic armamentarium for melanoma.

The first published phase Ⅲ randomized study about PD-1/PD-L1 axis inhibition in this disease demonstrated, at the beginning of 2015, the advantage of nivolumab over chemotherapy with dacarbazine both in terms of overall survival (OS) and of progression free survival (PFS) among previously untreated patients with metastatic melanoma without *BRAF* mutation. Median PFS of 5.1 mo in the nivolumab group was more than doubled when compared to dacarbazine treated patients, with 2.2 mo [hazard ratio (HR) = 0.43, 95%CI: 0.34-0.56, $P < 0.001$]. OS was not reached in the nivolumab group, instead being 10.8 mo in the group treated with chemotherapy (HR = 0.42, 99%CI: 0.25-0.73, $P < 0.001$)[9].

An analogous comparison was made in patients who progressed after anti-CTLA4 treatment in the phase Ⅲ randomized study CheckMate 037, reporting a response rate (RR) of 32% for nivolumab *vs* 11% with chemotherapy according to investigator's choice. These findings have resulted in the inclusion of nivolumab in the new treatment options for a cancer with high unmet need[10].

In parallel, pembrolizumab was compared with ipilimumab as the new standard of care for first line treatment of advanced melanoma in a phase Ⅲ randomized trial, demonstrating to prolong PFS and OS with less toxicity respect to the CTLA4 inhibitor[11].

Nevertheless, the new frontier for untreated melanoma is currently represented by the combination of anti-CTLA4 and anti-PD-L1 antibodies: Larkin *et al*[12] demonstrated that the association of nivolumab and ipilimumab resulted in a significantly longer PFS than ipilimumab alone, despite 55% of treatment-related adverse events (AEs) of grade 3 or 4 (G3-4) *vs* 16% in the nivolumab group and 27% in the ipilimumab group. This three arms phase Ⅲ randomized trial closed the matter of first line ipilimumab alone, otherwise confirming good effectiveness for nivolumab monotherapy in this setting[12].

Further phase Ⅲ-Ⅳ trials are currently ongoing to test different dosing schedules of CKI[13], others to verify their efficacy in particular subgroups of patients like those with brain metastases[14], or to establish the correct duration of anti-PD-1 therapy in metastatic melanoma, especially in the case of long responders[15]. Again, more others are investigating alternative combinations[16,17] or treatment sequences, like ipilimumab plus nivolumab followed or preceded by dabrafenib and trametinib in *BRAF* mutated patients[18].

Moreover, after the Food and Drug Administration approval of ipilimumab for the adjuvant setting for melanoma[19], as discussed below, the PD-1 and PD-L1 inhibitors are currently under investigation for the adjuvant and neoadjuvant setting also in different tumor types in several clinical trials, which results are eagerly awaited, given the lower toxicity expected from this "second generation" of CKI (Table 2)[20-31].

Lung cancer

Lung cancer immunotherapy have an historical back-

ground, but it has not shown significant survival benefit until the recent advent of CKI.

Conversely to anti-CTLA4 antibodies, which demonstrated a certain efficacy only when combined with chemotherapy, the inhibition of PD-1/PD-L1 axis clearly works as single strategy in non-small cell lung cancer (NSCLC)[32].

The first step through immunotherapy for lung cancer in clinical practice was the approval of CKI monotherapy with nivolumab (and more recently with atezolizumab) for NSCLC patients pretreated with first line chemotherapy, on the basis of the first published randomized trials[33-35].

Anti-PD1 antibodies are going to radically revolutionize lung cancer treatment regardless of the histology, especially after the recently published results of KEYNOTE 024 trial[36], providing the outstanding evidence of pembrolizumab superiority compared to chemotherapy as first line treatment for NSCLC, in terms of PFS (10.3 mo *vs* 6 mo, $P < 0.001$), OS (80% *vs* 72% at 6 mo, $P = 0.005$), RR (45% *vs* 28%) and safety among patients bearing strong PD-L1 expression on tumor cells (at least 50% was required for enrollment). This latter evidence, despite concerned to the 30% of overall NSCLC population, will provide the rationale to radically change the therapeutic paradigm for NSCLC, shifting CKI treatment option to first line in a great subgroup of patients. The selection of patients basing on a single biomarker, despite potentially harmful, has been demonstrated to be effective in this case, as proven by the recently announced failure of the analogue phase Ⅲ trial with nivolumab, whose patients were enrolled independently from PD-L1 status[37].

Several phase Ⅲ studies are currently still ongoing in order to investigate further CKI antibodies in all treatment lines, in different treatment regimens and with alternative combinations targeting PD-1/PD-L1 axis in advanced NSCLC (Table 3)[37-96].

Also adjuvant paradigm has been pursued in lung cancer: Table 2 summarizes all the ongoing phase Ⅲ studies in this field.

Squamous cell lung cancer: Squamous cell histology had the first indication for CKI therapy, basing on the outstanding results of CheckMate 017 trial comparing nivolumab *vs* docetaxel in advanced squamous NSCLC (SqNSCLC) progressive to previous chemotherapy[33]. With a median OS of 9.2 mo *vs* 6 mo, nivolumab reduced the risk of death of 41%, with an HR of 0.59 (95%CI: 0.44-0.79), $P < 0.001$. The advantage was confirmed also for RR, PFS and safety profile, finally providing an unprecedented treatment option also in terms of tolerability.

Non-squamous cell lung cancer: With a slight delay and with not as brilliant but positive results, nivolumab was also approved for non-squamous NSCLC (non-SqNSCLC) treatment after failure of chemotherapy, on the basis of an analogous phase Ⅲ randomized trial demonstrating an improvement of median OS from 9.4 mo with docetaxel to 12.2 mo (HR = 0.73, 95%CI: 0.59-0.89, $P = 0.002$)[34]. In this study, nivolumab was associated with better OS and RR but not with longer PFS compared to chemotherapy. A crossing of the PFS curves suggested a delay of the benefit with nivolumab, consistent with the results of previous immune system modulating agents, probably reflecting a pattern of response typical of immunotherapy and the use of inadequate response assessment measurements for this type of drug[97].

Other thoracic malignancies: Among other thoracic tumors, small cell lung cancer (SCLC), malignant pleural mesothelioma (MPM) and thymic epithelial tumors (TETs), under the thrust of true unmet medical needs, came across immunotherapy with CKI.

Preliminary data for PD-1/PD-L1 blockade in SCLC were encouraging and currently ongoing phase Ⅲ studies are investigating CKI both in pretreated and untreated advanced SCLC patients[72,93] or as maintenance treatment after standard treatment either in extensive or in limited disease[91].

Great expectations have been made for MPM, because of the known relationship between neoplastic and inflammatory counterpart in this tumor, recognized to have a T-cell inflamed phenotype. At the moment, only preliminary data have been published and CKI are currently under proposal for further investigations in this disease. Finally, early phases studies are ongoing to test CKI immunotherapy also in TETs[98].

Renal cancer

After the pivotal trial Checkmate 025, nivolumab has vowed to became the cornerstone of previously treated metastatic renal cell carcinoma (mRCC) therapy, finally offering an OS improvement in a setting where targeted therapies have fallen short of expectation[99]. The median OS was 25 mo (95%CI: 21.8-not estimable) with nivolumab and 19.6 mo (95%CI: 17.6-23.1) with everolimus, with a HR of 0.73 and a RR of 25% *vs* 5% ($P < 0.001$). Also in terms of toxicity, nivolumab was superior to the standard treatment everolimus, with 19% *vs* 37% of AEs.

In the light of these results, nivolumab currently represents a new standard of treatment for mRCC after disease progression to first line antiangiogenic therapy. On this auriferous vein other phase Ⅲ randomized trials have been planned and their results are eagerly awaited. Worthy of note, a phase Ⅲ randomized trial with an innovative design is comparing the combination of lenvatinib and everolimus (which recently achieved great results in phase Ⅱ[100]) with the combination of lenvatinib and pembrolizumab *vs* the standard sunitinib. Such ambitious trials will probably provide the cornerstone of the future clinical practice in RCC[41,101].

After reaching the indication for second line treat-

Table 3 Phase III randomized clinical trials currently ongoing with PD-1/PD-L1 axis blockade in advanced setting for solid tumors

Trial name/NCT	Cancer type	Immune checkpoint inhibitor	Arms	Treatment line	Primary endpoint	Expected primary completion date	No. of patients
STOP-GAP[15]	Melanoma	PD-1 inhibitor (any)	Intermittent *vs* continuous therapy	Any	OS	2025	550
NCT02752074[16]	Melanoma	Pembrolizumab	Pembrolizumab + epacadostat *vs* pembrolizumab + placebo	I line	PFS	2018	600
MASTERKEY-265[17]	Melanoma	Pembrolizumab	Pembrolizumab + talimogene laherparepvec *vs* pembrolizumab + placebo	I line	PFS	2018	660
KEYNOTE-048[82]	HNSCC	Pembrolizumab	Pembrolizumab *vs* CT[1] + pembrolizumab *vs* CT[1]	I line	PFS	2018	780
KEYNOTE-040[38]	HNSCC	Pembrolizumab	Pembrolizumab *vs* methotrexate or docetaxel or cetuximab	From II line	OS	2017	466
KEYNOTE-204[39]	Hodgkin lymphoma	Pembrolizumab	Pembrolizumab *vs* brentuximab	From II line	PFS	2019	300
KEYNOTE-045[40]	Urothelial cancers	Pembrolizumab	Pembrolizumab *vs* paclitaxel, docetaxel or vinflunine	From II line	OS	2017[2]	470
NCT02811861[41]	Renal cell carcinoma	Pembrolizumab	Pembrolizumab + lenvatinib *vs* lenvatinib + everolimus *vs* sunitinib	I line	PFS	2020	735
KEYNOTE-426[102]	Renal cell carcinoma	Pembrolizumab	Pembrolizumab + axitinib *vs* sunitinib	I line	PFS, OS	2019	840
KEYNOTE-240[42]	HCC	Pembrolizumab	Pembrolizumab *vs* BSC	II line	PFS	2019	408
KEYNOTE-189[43]	NSqNSCLC	Pembrolizumab	Platinum and pemetrexed ± pembrolizumab	I line	PFS	2017	570
KEYNOTE-407[44]	SqNSCLC	Pembrolizumab	CT[1] ± pembrolizumab	I line	PFS	2018	560
KEYNOTE-042[45]	NSCLC PD-L1-positive	Pembrolizumab	Pembrolizumab *vs* platinum based CT[1]	I line	OS	2018	1240
KEYNOTE-010[46]	NSCLC	Pembrolizumab	Pembrolizumab *vs* docetaxel	From II line	OS	2019	1034
KEYNOTE-119[47]	Triple negative breast cancer	Pembrolizumab	Pembrolizumab *vs* monochemotherapy	II-III line	PFS	2017	600
KEYNOTE-355[48]	Triple negative breast cancer	Pembrolizumab	CT[1] + pembrolizumab *vs* CT[1] + placebo	I line	PFS	2019	858
KEYNOTE-177[49]	MSI-H or dMMR colorectal carcinoma	Pembrolizumab	Pembrolizumab *vs* CT[1]	I line	PFS	2019	270
KEYNOTE-181[50]	Esophageal/ esophago-gastric junction carcinoma	Pembrolizumab	Pembrolizumab *vs* monochemotherapy[1]	II line	PFS	2018	600
KEYNOTE-061[51]	Esophageal/ esophago-gastric junction adenocarcinoma	Pembrolizumab	Pembrolizumab *vs* paclitaxel	II line	PFS	2017	720
KEYNOTE-062[52]	Esophageal/ esophago-gastric junction carcinoma	Pembrolizumab	Pembrolizumab *vs* CT[1] + pembrolizumab *vs* CT[1]	I line	PFS	2019	750
JAVELIN Ovarian 200[53]	Ovarian cancer (platinum resistant)	Avelumab	Avelumab *vs* avelumab plus PLD *vs* PLD	From II line	OS	2018	550
JAVELIN Ovarian 100[54]	Ovarian cancer	Avelumab	CT[1] *vs* CT[1] followed by avelumab maintenance *vs* CT[1] + avelumab followed by avelumab maintenance	I line	PFS	2019	951
JAVELIN Renal 101[55]	Renal cell cancer	Avelumab	Avelumab with axitinib *vs* sunitinib	I line	PFS	2018	583
JAVELIN Bladder 100[56]	Urothelial cancer	Avelumab	Avelumab *vs* BSC (maintenance after CT[1])	I line maintenance	OS	2019	668

JAVELIN Gastric 100[57]	Adenocarcinoma of the stomach or of the gastro-esophageal junction	Avelumab	CT[1] continuation *vs* avelumab in maintenance after CT[1]	I line	OS	2018	666
JAVELIN Gastric 300[58]	Adenocarcinoma of the stomach or of the gastro-esophageal junction	Avelumab	Avelumab + BSC *vs* CT[1] + BSC *vs* BSC	III line	OS	2017	330
JAVELIN Lung 100[59]	NSCLC (PD-L1 positive)	Avelumab	Avelumab *vs* platinum based CT[1]	I line	PFS	2017	420
JAVELIN Lung 200[60]	NSCLC (PD-L1 positive)	Avelumab	Avelumab *vs* docetaxel	From II line	OS	2017	650
OAK[61]	NSqNSCLC	Atezolizumab	Atezolizumab *vs* docetaxel	From II line	OS	2017	1225
IMvigor211[62]	Bladder cancer	Atezolizumab	Atezolizumab *vs* monochemotherapy	II line	OS	2017	932
IMvigor130[63]	Urothelial carcinoma (ineligible for cisplatin)	Atezolizumab	Atezolizumab + CT[1] *vs* placebo + CT[1]	I line	PFS	2019	435
IMpower110[64]	NSqNSCLC	Atezolizumab	Atezolizumab *vs* platin + pemetrexed	I line	PFS	2019	570
IMpower111[65]	SqNSCLC	Atezolizumab	Atezolizumab *vs* gemcitabine + platin	I line	PFS	2017	ND
IMpower131[66]	SqNSCLC	Atezolizumab	Atezolizumab + nab-paclitaxel + carboplatin *vs* atezolizumab + paclitaxel + carboplatin *vs* nab-paclitaxel + carboplatin	I line	PFS	2023	1200
IMpower210[67]	NSCLC	Atezolizumab	Atezolizumab *vs* docetaxel	II line	OS	2019	563
IMpower130[68]	NSqNSCLC	Atezolizumab	Atezolizumab + nab-paclitaxel + carboplatin *vs* nab-paclitaxel + carboplatin	I line	PFS	2019	550
IMpower150[69]	NSqNSCLC	Atezolizumab	Atezolizumab + carboplatin + paclitaxel ± bevacizumab *vs* carboplatin + paclitaxel + bevacizumab	I line	PFS	2017	1200
IMpassion130[70]	Triple negative breast cancer	Atezolizumab	Atezolizumab + nab-paclitaxel *vs* placebo + nab paclitaxel	I line	PFS	2020	900
IMmotion151[71]	Renal cell carcinoma	Atezolizumab	Atezolizumab + bevacizumab *vs* sunitinib	I line	PFS	2020	900
IMpower133[72]	SCLC	Atezolizumab	Carboplatin and etoposide ± atezolizumab	I line	OS	2019	400
NCT02788279[73]	Colorectal carcinoma	Atezolizumab	Atezolizumab + cobimetinib *vs* atezolizumab *vs* regorafenib	From III line	OS	2019	360
KESTREL[74]	HNSCC	Durvalumab	Durvalumab *vs* durvalumab + tremelimumab *vs* SOC	I line	PFS	2017	628
MYSTIC[75]	NSCLC	Durvalumab	Durvalumab *vs* durvalumab + tremelimumab *vs* SOC	I line	PFS	2017	1092
Danube[76]	Bladder cancer	Durvalumab	Durvalumab *vs* durvalumab + tremelimumab *vs* SOC1	I line	PFS	2017	525
Lung-MAP[77]	SqNSCLC (biomarker-targeted)	Durvalumab, nivolumab	Docetaxel *vs* durvalumab vs erlotinib *vs* AZD4547 *vs* ipilimumab *vs* palbociclib *vs* rilotumumab *vs* taselisib	Any	PFS	2022	10000

CAURAL[78]	NSCLC T790M mutation positive	Durvalumab	AZD9291 + durvalumab *vs* AZD9291	II-III line	PFS	2018	350
NCT02369874[79]	HNSCC	Durvalumab	Durvalumab *vs* durvalumab + tremelimumab *vs* SOC[1]	II line	OS	2018	720
NEPTUNE[80]	NSCLC	Durvalumab	Durvalumab + tremelimumab *vs* SOC[1]	I line	OS	2018	800
ARCTIC[81]	NSCLC	Durvalumab	Durvalumab *vs* durvalumab + tremelimumab *vs* SOC[1]	II-III line	OS	2016	730
NCT02224781[18]	Melanoma BRAFV600 mutated	Nivolumab	Dabrafenib + trametinib followed by ipilimumab + nivolumab *vs* ipilimumab + nivolumab followed by dabrafenib + trametinib	I line	OS	2019	300
NIBIT-M2[14]	Melanoma brain metastases	Nivolumab	Fotemustine *vs* ipilimumab + fotemustine *vs* ipilimumab + nivolumab	Any	OS	2018	168
CheckMate 026[37]	NSCLC PD-L1 positive (all)	Nivolumab	Nivolumab *vs* CT[1]	I line	PFS	2016[2]	535
CheckMate 651[83]	H&N SCC	Nivolumab	Nivolumab + ipilimumab *vs* platinum + fluorouracil + cetuximab	I line	OS	2020	490
CheckMate 459[84]	HCC	Nivolumab	Nivolumab *vs* sorafenib	I line	TTP	2017	726
NCT02267343[85]	Gastric cancer	Nivolumab	Nivolumab *vs* placebo	From II line	OS	2017	480
NCT02569242[86]	Esophageal cancer	Nivolumab	Nivolumab *vs* docetaxel/paclitaxel	From II line	OS	2019	390
CheckMate 214[87]	Renal cell carcinoma	Nivolumab	Nivolumab + ipilimumab *vs* sunitinib	I line	PFS	2019	1070
CheckMate 143[88]	Glioblastoma	Nivolumab	Nivolumab *vs* bevacizumab	II line	OS	2017	440
CheckMate 141[89]	H&N SCC	Nivolumab	Nivolumab *vs* cetuximab/ methotrexate/docetaxel monotherapy	Any	OS	2018	360
CheckMate 227[90]	NSCLC	Nivolumab	Nivolumab *vs* nivolumab + ipilimumab *vs* nivolumab + platinum doublet CT[1]	I line	OS	2018	1980
CheckMate 451[91]	SCLC	Nivolumab	Nivolumab *vs* nivolumab + ipilimumab *vs* placebo after platinum based CT[1]	Maintenance after I line	OS	2018	810
CheckMate 498[92]	Glioblastoma (unmethylated MGMT)	Nivolumab	Nivolumab + RT *vs* temozolomide + RT	I line	PFS	2019	550
CheckMate 331[93]	SCLC	Nivolumab	Nivolumab *vs* topotecan/amrubicin	II line	OS	2018	480
CheckMate 078[94]	NSCLC	Nivolumab	Nivolumab *vs* docetaxel	From II line	OS	2018	500
NCT02339571[95]	Melanoma	Nivolumab	Nivolumab + ipilimumab ± sargramostim	I line	OS	2021	400
CheckMate 401[96]	Melanoma	Nivolumab	Nivolumab + ipilimumab *vs* nivolumab	I line	OS	2021	615

[1]According to the standard of care and basing on the choice of the investigator; [2]The trial has results but it is still unpublished. OS: Overall survival; PFS: Progression free survival; HNSCC: Head and neck squamous cell carcinoma; HCC: Hepatocarcinoma; NSqNSCLC: Non-squamous non-small cell lung cancer; SqNSCLC: Squamous non-small cell lung cancer; CT: Chemotherapy; NSCLC: Non-small cell lung cancer; MSI-H: High microsatellite instability; dMMR: Deficient mismatch repair; PLD: Pegylated liposomal doxorubicin; SCLC: Small cell lung cancer; TTP: Time to progression; ORR: Objective response rate.

ment, also first line setting has been investigated, with the planning of interesting trials currently still ongoing. In previously untreated RCC patients, atezolizumab in combination with bevacizumab is being compared to sunitinib[71]; the same standard of treatment is in turn compared to pembrolizumab combined with axitinib[102] and then to nivolumab plus ipilimumab[87]. Eventually, also avelumab plus axitinib is being investigated *vs*

sunitinib[55]. In all cases, the control arm is represented by such a big standard of therapy (sunitinib) that, in case of positive results, the clinical practice for RCC will completely change, switching from angiogenesis inhibition to immune-checkpoint blockade.

Urothelial cancers

Since no significant improvements have been achieved in metastatic bladder cancer for long time, the impressive results of recent trials with CKI, in particular with the anti-PD-L1 atezolizumab, have given new hope to finally cure urothelial cancer[103,104].

Atezolizumab is currently been approved for treatment of urothelial cancer on the basis of a randomized phase Ⅱ trial comparing this anti-PD-L1 with standard treatment, demonstrating its advantage over chemotherapy in both platinum pretreated ineligible patients and in chemotherapy pretreated patients[105]. At the same time, phase Ⅲ studies in second line setting are ongoing and both atezolizumab and pembrolizumab have been compared to different second line chemotherapeutic regimens in all urothelial cancers: The trial with pembrolizumab has been recently early stopped due to the meeting of the primary endpoint (OS)[40,62]. Also avelumab and durvalumab reached phase Ⅲ investigation in bladder cancer, but in the first line setting; the latter combined with the anti-CTLA4 tremelimumab *vs* standard first line chemotherapy[56,76]. A further interesting study in metastatic urothelial cancer is recruiting naive patients ineligible to cisplatin to receive atezolizumab in combination with chemotherapy (gemcitabine and carboplatin) as first line treatment[63].

Not less significant the promising evidence about the role of CKI in the adjuvant setting of urothelial cancer: Atezolizumab is under investigation *vs* only observation after cystectomy in PD-L1 positive high risk muscle-invasive bladder cancer[23] and also nivolumab is being tested in this setting[28].

Head and neck cancer

Head and neck squamous cell carcinoma (HNSCC) undoubtedly a promising candidate for CKI because of the profound immune suppression from which is characterized. As the matter of fact, a phase Ⅲ randomized study comparing nivolumab to the standard of treatment in pretreated HNSCC patients was early stopped after the clear demonstration of an improvement in terms of OS for nivolumab[89]. This trial provided very promising results in platinum refractory disease, encouraging the planning of further phase Ⅲ studies, currently ongoing, also for pembrolizumab[38,82] and early phases trials with durvalumab and avelumab[106].

Despite an apparently not so favorable toxicity profile, also anti-CTLA4 antibodies are being tested in combination with anti-PD-1 or anti-PD-L1 agents in HNSCC. Phase Ⅲ studies with this therapeutic strategy are currently ongoing both in pretreated patients and in first line setting[74,79].

Other tumors

The PD-1/D-L1 axis has been targeted in other tumor types than those cited above, with an interesting rationale and supported by phase Ⅰ-Ⅱ experiences, despite still remaining in shadow waiting for phase Ⅲ results.

In ovarian cancer, despite several early phase studies currently ongoing with nivolumab, pembrolizumab, BMS936559 (an anti-PD-L1) and avelumab, the emerged response rates are relatively low, in front of a manageable safety profile[53,54,107].

Pembrolizumab, aside from early investigations in soft tissue and bone sarcomas[108], is currently under phase Ⅲ investigation in hepatocellular carcinoma[42], in esophageal and gastric carcinoma[50-52], in Hodgkin and non-Hodgkin lymphoma[39].

In these latter malignancies also nivolumab and pidilizumab, anti-PD-1 antibodies, besides from atezolizumab and durvalumab, anti-PD-L1 antibodies, are being evaluated in early phases[109]. Furthermore, different treatment lines of advanced gastric cancer are being tested with avelumab[57,58].

Some initial encouraging data are emerging from ongoing studies in favor of the employment of CKI also in central nervous system (CNS) malignancies, such as glioblastoma, where unmet clinical needs are leading to new investigations[88,92]. Disappointing results were instead obtained for pancreatic cancer, despite a certain evidence for durvalumab[110].

About colorectal cancer, despite the initial evidence to be not responsive to nivolumab, a subset of patients has been identified as potentially best responders to pembrolizumab, revealing that the mismatch repair (MMR) status can predict clinical benefit with enhanced responsiveness in tumors with microsatellite instability (MSI)[111]. With this rationale, phase Ⅲ randomized studies have been initiated in order to compare standard therapy with pembrolizumab in MSI colorectal cancer patients[49]. Furthermore, atezolizumab is currently under investigation alone or in combination with cobimetinib (mitogen activate protein kinase-inhibitor) *vs* regorafenib (antiangiogenic multi-kinase inhibitor) in all advanced colorectal tumors[73].

Eventually, a great interest for PD-1/PD-L1 blockade is represented by triple negative breast cancer: Phase Ⅲ trials are currently ongoing with pembrolizumab compared to chemotherapy and with atezolizumab combined with nab-paclitaxel both in neo-adjuvant and advanced setting[47,48,70,112].

Transversal challenges

Immune-related toxicity: The management of the "new toxicities" of CKI is transversal to all malignancies and to all cited antibodies, unavoidably involving other specialists beyond the oncologist, such as the endocrinologist and the immunologist in first line.

These immune-related adverse events (irAEs) are due to the infiltration of tissues by activated T-lymphocytes responsible of autoimmunity. As a consequence, the block of the immune-checkpoint can amplify any immune response in all organs: Skin, gastrointestinal tract, endocrine glands, lung, CNS, liver, kidney, hematological cells, muscular-articular system, heart and eyes can all be affected. Nevertheless, most of these irAEs are rare and only fatigue, rash, pruritus, diarrhea, nausea and arthralgia occurs in > 10% of cases. On the other hand, despite being rare, interstitial pneumonitis is the main life-threatening toxicity for anti PD-1/PD-L1 agents[113].

Potentially predisposing conditions for irAEs development could be represented by personal or family history of autoimmune disease (genetic determinants), by underlying silent autoimmunity, chronic viral infections or other personal ecological factors (such as the microbiome in the case of enterocolitis)[114].

The prevention, the anticipation, the detection and then the treatment (with multidisciplinary approach) and monitoring of irAEs are the principles of their correct clinical management. Depending on their severity, irAEs require temporary or permanent discontinuation of CKI therapy, use of high doses corticosteroids or, in severe cases, of anti-TNF treatment with infliximab. The current management guidelines are based on recent expert consensus recommendations published about the issue[115].

Response assessment: RECIST *vs* immune-related criteria: Based on survival analysis, traditional response evaluation criteria in solid tumors (RECIST) might underestimate the benefit of CKI[116].

The pattern of response of immunotherapy, radically different from those of standard chemotherapy and also of antiangiogenic agents, is frequently not captured by the conventional RECIST[117]. This led to the development of the immune-related response criteria (irRC)[118], assessing tumor burden as a continuous variable and evaluating percentage changes in several target lesions overtime. In this system, the appearance of new lesions does not mean progressive disease but it is considered and reassessed in the context of a dynamic evaluation. Moreover, the thresholds to determine progression or response (25% increase and 50% decrease) are higher than those of RECIST (20% increase and 30% decrease)[119]. Given the reported evidence, modified criteria are undoubtedly mandatory in the response assessment to the new immunotherapy, in order to prevent premature discontinuation of treatment.

PD-L1 expression as response predictor: In the context of solid tumors treated with PD-1/PD-L1 inhibitors, the predictive role of PD-L1 expression on tumor cells and, as more recently discovered, on immune infiltrating cells, represents an actual issue of great interest and constitutes a significant cue of discussion for clinical researchers[120].

Currently, on the basis of the state of art, the predictive value of PD-L1 on tumor cells is limited to NSCLC and melanoma, especially for anti-PD-1 antibodies, whilst a more predictive significance of PD-L1 expression on the immune cells infiltrating the tumor seems to emerge for urothelial cancers in the case of anti-PD-L1 antibodies[121,122]. Nevertheless, a great limit of such speculations is represented by the scarce reliance and reproducibility of the different methods used for the biomarker's detection, with controversial results depending on the staining technique, on the different anti-PD-L1 antibodies and finally on the sample used for immune-histochemical assay (primary tumor *vs* metastases samples, with the challenge of heterogeneity). Moreover, confusing data emerged from the use (and the lack of validation) of different cut-off for PD-L1 expression, from 1%, to 5%, to 50% threshold in different trials[120].

Aside from PD-L1 expression, further multiple factors have been explored and are currently undergoing investigations as predictive elements for response to CKI: Among these, an increasing interest is being acquired by the micro-environmental features of the tumor, such as the infiltrating immune cells sub-populations and their biomarkers expression[123].

Microsatellite instability and hyper-mutational status: The MSI phenotype, as a consequence of a defective DNA-MMR system, characterizes a subgroup of tumors harboring a large number of somatic mutations (high mutational load). Since these mutations have the potential to encode a great number of immunogenic neoantigens, a particular susceptibility of MSI-hypermutated cancers to PD-1/PD-L1 axis blockade have been hypothesized and more recently proven[124]. As the matter of fact, MSI tumors have a microenvironment characterized by abundant T-cell infiltrate, with activated $CD8^+$ cytotoxic T lymphocyte (CTL) and activated Th1 producing IFN-γ, high expression of PD-L1 (in particular by TILs and myeloid cells infiltrating the tumor) and great overexpression of immune-checkpoint related proteins[125]. All these elements configure the elective candidate cancer for immune-checkpoint inhibition and suggest to investigate CKI in all cancer types with MMR defects.

Additionally, tumors with polymerase E (POLE) mutations, despite stable microsatellites, have been demonstrated to contain a high mutational load. Also these POLE-ultra-mutated cancers are characterized by an active Th1/CTL microenvironment and upregulated immune checkpoints, constituting an ideal target for CKI therapy as well as MSI tumors[126].

In conclusion, among apparently resistant cancer types (such as colon cancer), CKI have been proven to exert an effect in case of MMR defects and trials on this selected population are currently ongoing to investigate

the efficacy of anti-PD-1 antibodies[49].

Immune system modulation with sequential or association strategies: Given the great benefit in terms of OS and the long lasting impact of CKI therapy on patients' survival in the responding cases, probably due to immunological memory, two major issues remain to be addressed: The sensitization of non-responders and the disease control in patients initially pseudo-progressive. With these aims, combination strategies have been planned and investigated in the last years, either combining immunotherapy with chemotherapy, radiotherapy and targeted agents or associating different CKI[127].

The strategy to increase the immunogenicity of tumors can be pursued through the enhancement of antigen presentation (boosting antigens release or stimulating APC function), the stimulation of major histocompatibility complex (MHC) class Ⅰ expression, the down-regulation of the T-reg cells and the stimulation of the T-cells infiltration. Some of these mechanisms can be achieved with promising combination strategies.

Chemotherapeutic agents are capable to induce immunogenic cancer death, generating a strong immune stimulation. Among these, cyclophosphamide have additionally been shown to reduce the number of circulating T-reg cells, removing a key element of immunosuppression, and moreover to sensitize tumor cells to T-cell mediated apoptosis, potentially boosting the effect of the immune checkpoint blockade[128-130]. Considering the criticism of a combination between CKI and chemotherapy, given expected short term immunosuppressive effect of the latter, in our opinion a sequential strategy could represent a good opportunity to take advantage of cell death and antigen release caused by an induction chemotherapy, in order to prepare a more immunogenic environment for the subsequent CKI[131].

A great interest for the potential stimulation of the immune-response through radiotherapy has been suggested by the evidence about the immune-mediated abscopal effect[132]. Aside from interesting case reports, clinical trials in this field are currently in early phases and eagerly awaited[133].

Targeted therapy combinations with immunotherapy are currently under investigation, in early phases, with interesting results[127]. The rationale of such strategies could be represented by the aim to obtain a more rapid RR and to boost PFS with the targeted agent, in expectation of the long-term effect on survival of the CKI.

Finally, the combination of anti-PD-1 and anti-CTLA4 antibodies, despite the increased immune-related toxicity, has been shown to improve the outcomes in a phase Ⅲ randomized trial in metastatic melanoma, early changing the standard of treatment a few years after the onset of the new immunotherapy with ipilimumab[134]. Several trials investigating such association of CKI are currently ongoing: The management of irAEs will probably represent the main criticism of such strategies[127].

Targeting PD-1/PD-L1 axis in adjuvant setting: The rationale for the PD-1/PD-L1 axis inhibition for adjuvant purposes is in the concept of "immunological memory", generated by the cancer-immunity cycle, starting from the release of cancer cell antigens also in the early phases of tumorigenesis. After the APC migration in the lymph nodes and the presentation of antigens in the context of MHC-I molecules to $CD8^+$ T cells, aside from effector T-lymphocytes capable of activation against cancer neo-antigens, memory T-cells are also generated. These quiescent lymphocytes are appointed to the subsequent immune-response and could contribute to avoid disease relapse[135].

Considering the widely acceptable toxicity profile of CKI, the proposal of using them as adjuvant therapy, to prevent relapses after surgery of early disease while maintaining a good quality of life, appears very favorable. In support of this, we have the approval of the CTLA4 inhibitor ipilimumab for adjuvant treatment in melanoma, on the basis of a recent pivotal trial[136]. For PD-1/PD-L1 axis inhibitors, nevertheless, the investigation in adjuvant setting is quite early, in spite of a more favorable safety management. A noteworthy issue about immune-adjuvant treatment with these compounds (unlike the case of ipilimumab) is the correct duration of therapy, ranging from one to more years in different planned trials. The currently ongoing studies are reported in Table 2.

PERSPECTIVES

Considering the wide range of settings and combinations covered by the ongoing clinical trials with CKI treatment, we think that the future directions for immunotherapy are still to be written and they are probably different basing on cancer types. The reason of this latter statement, not so obvious as it may seem, is likely due to the other different therapies to whom immune-checkpoint blockade needs to be sequenced and alternated in each tumor, more than to a real difference in the target, which is always represented by the immune system and by its relationship with the tumor rather than by the tumor itself.

From this point of view, a key issue could be represented by the immunomodulating potential of the current standard of treatment in each case, sometimes widely unknown and rarely explored before the "immunotherapy era"[137].

The great advantage of anti-PD-1/PD-L1 agents is undoubtedly represented by their very favorable safety profile, with large tolerability in almost all patients. Combinations of CKI with standard chemotherapy or targeted therapies, despite possibly more effective, have the risk of became unsustainable both in terms of costs and of toxicity, significantly impacting on the final outcome. Nevertheless, alternating targeted and

immunotherapy might permit to modulate tumor metabolism, inflammation and immune infiltration, allowing to modify the relationship between cancer and immune system.

Thus, in order to fully take advantage of its potential, the winning strategy with immune-checkpoint blockade could be represented by an ingenious sequence, exploiting the immunomodulating properties of previous and subsequent drugs with the aim of boosting immune system activation against the tumor.

CONCLUSION

The onset of PD-1/PD-L1 targeted therapy has demonstrated the importance of this axis in the immune escape across almost all human cancers. Despite being burdened by some issues not still addressed, such as the correct duration of therapy in the responsive patients, the new CKI allowed to significantly prolong survival and to generate durable response, demonstrating remarkable efficacy in a wide range of cancer types. However, such benefit is not extended to all patients, and some of them experienced immune escape despite therapy. The investigation about mechanisms leading to the development of primary or secondary immune escape must represent the key element of future studies in the whole immuno-oncology, with the aim of resensitize these patients to the immune checkpoint blockade. The future approach to the problem may be represented by a personalized cancer immunotherapy, allowed only by multiparameter biomarkers approaches, as interestingly suggested by Kim *et al*[138] in a recent review about the "step to success (or failure)" to PD-1/PD-L1 blockade. In their proposal, a hypothetical algorithm could provide the assessment of specific immune-related biomarkers in each patient's tumor, allowing to create a personal mapping according to which characteristics the oncologist could chose (or exclude) the optimal immunotherapy or immunotherapeutic combination for each single case.

Waiting for the possible realization of such sophistication of therapy, the immune checkpoint blockade in oncology is currently experiencing promising huge advances, shifting the classical paradigm of anticancer treatment from targeting the tumor to targeting the immune system and increasing our hopes to gain the immune control of oncological disease.

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ORIGINAL ARTICLE

Basic Study

Nanoparticle-linked antagonist for leptin signaling inhibition in breast cancer

Tia Harmon, Adriana Harbuzariu, Viola Lanier, Crystal C Lipsey, Ward Kirlin, Lily Yang, Ruben R Gonzalez-Perez

Tia Harmon, Adriana Harbuzariu, Viola Lanier, Crystal C Lipsey, Ward Kirlin, Ruben R Gonzalez-Perez, Department of Microbiology, Biochemistry, and Immunology, Morehouse School of Medicine, Atlanta, GA 30310, United States

Lily Yang, Department of Pathology and Laboratory Medicine, Emory University School of Medicine, Atlanta, GA 30322, United States

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Correspondence to: Ruben R Gonzalez-Perez, PhD, Department of Microbiology, Biochemistry, and Immunology, Morehouse School of Medicine, 720 Westview Drive, Hugh Gloster Suite 329, Atlanta, GA 30310, United States. rgonzalez@msm.edu
Telephone: +1-404-7521581

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Abstract

AIM

To develop a leptin peptide receptor antagonist linked to nanoparticles and determine its effect on viability of breast cancer cells.

METHODS

The leptin antagonist, LPrA2, was coupled *via* EDAC [1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide] to iron oxide nanoparticles (IONP-LPrA2) to increase its efficacy. IONP-LPrA2 conjugation was confirmed by Western blot and nanoparticle tracking analysis. Human triple negative breast cancer (TNBC) MDA-MB-231, HCC1806 and estrogen receptor positive (ER^+) MCF-7 cells were analyzed for the expression of the leptin receptor, Ob-R. The effects of leptin and antagonist on levels of leptin-induced STAT3 phosphorylation and cyclin D1, cell cycle progression, cell proliferation, and tumorsphere formation in breast cancer cells were determined. Doses of the chemotherapeutics [cisplatin

(Cis), cyclophosphamide (CTX), doxorubicin (Dox) and paclitaxel (PTX)] to effectively reduce cell viability were calculated. The effects of combination treatments of IONP-LPrA2 and chemotherapeutics on cell viability were determined.

RESULTS

Western blot analysis of coupling reaction products identified IONP-LPrA2 at approximately 100 kD. IONP-LPrA2 significantly decreased leptin-induced pSTAT3 levels in HCC1806 cells and drastically decreased cyclin D1 levels in all cell lines. IONP-LPrA2 significantly reduced leptin-induced S phase progression and cell proliferation in all breast cancer cell lines and the formation of tumorspheres in MDA-MB-231 cells. Also, IONP-LPrA2 showed an additive effect on the reduction of breast cancer cell survival with chemotherapeutics. Cis plus IONP-LPrA2 produced a significant reduction in the survival of MDA-MB-231 and HCC1806 cells. CTX plus IONP-LPrA2 caused a significant decrease in the survival of MDA-MB-231 cells. Dox plus IONP-LPrA2 caused a marked reduction in the survival of HCC1806 cells. Although, PTX plus IONP-LPrA2 did not have a major effect on the viability of the breast cancer cells when compared to PTX alone.

CONCLUSION

Present data indicate that IONP-LPrA2 may be a useful adjuvant for chemotherapeutic treatment of breast cancer, particularly for TNBC which lacks targeted therapeutic options.

Key words: Triple negative breast cancer; Obesity; Leptin; Leptin peptide receptor antagonist 2; Iron oxide nanoparticles; Chemotherapy adjuvant

Core tip: Breast cancer is the second leading cause of cancer deaths in women. Triple negative breast cancer is an aggressive subtype that lacks targeted therapy. Obesity is a risk factor for breast cancer and is associated with high leptin levels. Leptin induces the expression of cell cycle associated proteins advancing cell cycle progression. Leptin also increases breast cancer stem cell growth, which promotes chemotherapeutic resistance. We have developed a leptin antagonist linked to iron oxide nanoparticles (IONP-LPrA2) which significantly inhibits leptin-induced cell proliferation and survival of breast cancer cells treated with chemotherapeutics. IONP-LPrA2 can increase chemotherapeutic efficacy in breast cancer.

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INTRODUCTION

The American Cancer Society estimates that there will be nearly 300000 new breast cancer cases diagnosed worldwide and approximately 50000 women will die from breast cancer in 2016[1]. Triple negative breast cancer (TNBC) accounts for 15% of all breast cancer diagnoses. TNBC is a subtype of breast cancer characterized by the lack of expression of the estrogen receptor (ER), progesterone receptor (PgR), and human epidermal growth factor receptor-2 (HER2)[1,2]. Due to the absence of receptor expression; this form of breast cancer, which predominantly affects younger, African American and Hispanic patients lacks targeted therapeutic options[3]. TNBC patients are commonly treated with chemotherapy; however these patients make up approximately 30% of breast cancer-related deaths annually[4]. This necessitates the development of targeted therapies for this more aggressive form of the disease.

There are many factors that increase the risk of developing TNBC including environment, genetic susceptibility, and obesity[5]. Obesity has a negative impact on breast cancer patient survival and, like TNBC, is associated with an increased risk of recurrence[6]. Obesity is correlated to high levels of leptin, a cytokine produced by adipose tissue which regulates satiety. The leptin signaling pathway occurs in approximately 80% of breast cancers[7]. The binding of leptin to its receptor, Ob-R, leads to activation of pathways involved in cell proliferation, migration, and survival[8]. Leptin is a survival factor in breast cancer and may have the ability to limit the effectiveness of chemotherapy drugs by activating the JAK2/STAT3, MAPK/ERK, and PI3/Akt signaling pathways[9,10]. Therefore leptin signaling inhibition has become a promising therapeutic area for breast cancer, particularly in the case of TNBC for which there is no targeted therapy[11].

The binding of leptin to Ob-R upregulates Notch, interleukin 1 (IL-1), vascular endothelial growth factor (VEGF), and its receptor VEGFR2; which promote breast cancer cell survival and angiogenesis[12]. The harmful effects of leptin signaling on breast cancer onset and progression have been shown to be diminished by the leptin peptide receptor antagonist 2 (LPrA2)[13]. LPrA2 and the pegylated form (PEG-LPrA2) have been shown to cause a delay in cancer onset and progression as well as a reduction in 4T1-tumor growth in BALB/C mice[14]. Additionally, PEG-LPrA2 has been shown to decrease MDA-MB-231 and MCF-7-tumor growth in SCID mice[15]. In another study, diet-induced obese (DIO) C57BL/6J mice treated with the carcinogen 7, 12-Dimethylbenz (a) anthracene (DMBA) along with PEG-LPrA2 did not develop tumors[16]. The anti-tumor activity of LPrA2 provides mounting evidence for its usefulness in cancer therapy.

The leptin signaling pathway plays a major role in breast cancer cell growth, angiogenesis, as well as metastasis and invasion[8]. Although the leptin

antagonist LPrA2 attenuates leptin signaling, it is limited by its insolubility in water and short half-life of 1-2 h[14,17,18]. Here we describe the coupling of LPrA2 to a nanoparticle delivery system which uses iron oxide nanoparticles (IONPs) to capture multiple LPrA2 peptides. We assessed the conjugation of LPrA2 to IONPs (IONP-LPrA2) to determine the inhibitory effect on breast cancer cell growth and survival. Because LPrA2 decreases breast cancer tumor growth and chemotherapy is widely used in the treatment of breast cancer, we sought to assess if combining IONP-LPrA2 and chemotherapeutic drugs would allow for reduction of the effective dose. Thus, we evaluated the survival of human breast cancer cell lines with IONP-LPrA2 and a panel of anti-cancer drugs.

MATERIALS AND METHODS

Reagents and antibodies

IONPs were obtained from Ocean Nanotech San Diego, CA. 1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDAC), Sulfo-NHS, and other chemicals were purchased from Sigma Aldrich St. Louis, MO. Ob-R (sc-8325), Cyclin D1 (sc-246), pSTAT3 (sc-8059), STAT3 (sc-8019) antibodies were purchased from Santa Cruz Biotechnology Santa Cruz, CA. Anti-rabbit and anti-mouse conjugated to horseradish peroxidase were obtained from Bio-Rad Laboratories Hercules, CA. Dulbecco's Modified Eagles Medium (DMEM), Iscove's Modified Dulbecco's Medium (IMEM), Protease and Phosphatase Inhibitor cocktails, Penicillin/Streptomycin, Slide-a-lyzer dialysis cassette, and Western blotting chemiluminescence substrate were purchased from Thermo Fisher Scientific Rockford, IL. Mammocult complete medium was obtained from Stem Cell Technologies Vancouver, BC. Fetal bovine serum was obtained from Med Supply Partners Atlanta, GA. Leptin was purchased from R and D Systems Minneapolis, MN. 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) kit was purchased from Molecular Probes Eugene, OR. Annexin V/fluorescein Isothiocyanate (FITC) and propidium iodide (PI) were obtained from Nexcelom Bioscience Boston, MA. Cisplatin (Cis) was purchased from Millipore Billerica, MA. Cyclophosphamide (CTX), paclitaxel (PTX), and doxorubicin (Dox) were obtained from SelleckChem Houston, TX.

Nanoparticle conjugation

LPrA2 was synthesized as described[8,19]. LPrA2 was desalted using the slide-a-lyzer dialysis cassette (Thermo Fisher). LPrA2 was conjugated to IONPs (Ocean Nanotech) by the outlined method[20].

Western blot analysis

IONP-LPrA2 was separated by SDS-PAGE. LPrA2 and LPrA2-Scramble (Sc) were used as positive and negative controls, respectively. The peptides were transferred onto nitrocellulose membranes (Bio-Rad). The membranes were probed with an LPrA2 antibody, purified from antigen injected rabbit bleeds. Anti-rabbit IgG conjugated to horseradish peroxidase (Bio-Rad) was used for further detection of the peptides. Chemiluminescent detection of the bands was displayed by Western blotting substrate (Thermo Fisher).

Nanoparticle tracking analysis

Dilutions of 1:10000 of the bound and unbound particles were sonicated for 30 min. The size and distribution of the conjugated and unconjugated IONPs were determined by the NanoSight (Malvern Instruments Ltd., Worcestershire, United Kingdom).

Cell culture

Human ER$^+$ MCF-7 cells in addition to TNBC MDA-MB-231 and HCC1806 cells (American Type Culture Collection, ATCC, Manassas, VA) were cultured in DMEM (Thermo Fisher) with 10% FBS and 1% penicillin/streptomycin (Thermo Fisher) and maintained in an incubator at 37 ℃ with 5% CO_2.

Cell lysis and immunoblotting analysis

Cells were seeded at 2 × 10^5 in 6 well cell culture plates and grown to 70%-80% confluence. The cells were treated with leptin (1.2 nmol/L) (R and D Systems), or IONP-LPrA2 (0.0036 pmol/L) plus leptin (1.2 nmol/L) for 24-48 h. Basal cells served as untreated controls. The cells were lysed with RIPA buffer (Sigma) containing protease/phosphatase inhibitors (Thermo Fisher). Proteins were pulled down by Immunoprecipitation. Immunoblotting analysis was performed as described[21]. The membranes were incubated with Ob-R, cyclin D1, pSTAT3, and STAT3 (Santa Cruz Biotechnology) antibodies overnight at 4 ℃. GAPDH (Sigma) was used as a protein loading control. Relative protein levels were determined by Image J software (National Institute of Health, NIH).

Cell cycle analysis

Cells were seeded at 2 × 10^5 in 6 well cell culture plates and grown to 70%-80% confluence. They were treated with IONP-LPrA2 (0.0036 pmol/L) plus leptin (1.2 nmol/L) at indicated concentrations for 24-48 h. Leptin and unconjugated LPrA2 served as controls. The cells were trypsinized, washed with 1 × PBS, and resuspended in cold 100% methanol (Sigma). The were stored at -20 ℃ prior to analysis (< 1 wk). Afterward, the cells were centrifuged to remove the methanol. They were resuspended in 50 μL PI (Nexcelom) and incubated at 37 ℃ for 40 min. The cells were centrifuged to remove the PI, resuspended in 1 × PBS, and analyzed by the Nexcelom Cellometer Vision® image based cytometer to determine the percentage of cells in the S phase of the cell cycle.

MTT assay

Cells were seeded at 5 × 10^3 in 96 well cell culture plates and grown to 70%-80% confluence. The cells were treated with leptin (1.2 nmol/L), or IONP-LPrA2

(0.0036 pmol/L) plus leptin (1.2 nmol/L) for 24-48 h. Basal cells served as untreated controls. The media was removed from the cells, the wells were washed with 1 × PBS, and 200 μL of IMEM (Thermo Fisher) together with 10 μL of sterile 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), 5 mg/mL in PBS, (Molecular Probes) were added. The plates were incubated for 4 h at 37 ℃. Following incubation the media was removed, 50 μL of Dimethyl sulfoxide was added to the wells, and the plates were incubated at 37 ℃ for 30 min. The absorbance was read at 540 nm using a microplate reader (Molecular Devices) to measure cell proliferation.

Tumorsphere formation

MDA-MB-231 cells were seeded at 5 × 10^3-2 × 10^4 cells/mL in low attachment plates and grown for 1-2 wk in Mammocult complete medium (Stem Cell Technologies) supplemented with heparin and hydrocortisone and treated with leptin (1.2 nmol/L), or IONP-LPrA2 (0.0036 pmol/L) plus leptin (1.2 nmol/L). Basal tumorspheres served as untreated controls. The tumorspheres were visually assessed by light microscopy. The size of the tumorspheres were determined and the number of tumorspheres were counted manually in triplicate.

Apoptosis assay

Cells were seeded at 2 × 10^5 in 6 well cell culture plates and grown to 70%-80% confluence. They were treated with the chemotherapeutic drugs: Cis (Millipore), CTX, PTX, and Dox (SelleckChem) in 5% FBS with or without IONP-LPrA2 for time periods ranging from 1-6 d. Before trypsinizing, the supernatants were transferred into microfuge tubes for subsequent analysis. The trypsinized cells were added to the supernatants and centrifuged. The pellets were washed with 1 × PBS and resuspended in Annexin V binding buffer (Nexcelom). Annexin V/FITC, and PI, 5 μL each (Nexcelom) were added with mixing. The samples were incubated in the dark at room temperature for 15 min. The cells were washed with 1 × PBS, centrifuged, and resuspended in Annexin V binding buffer to a concentration of 3 × 10^4 cells per 20 μL. The samples were analyzed by the Cellometer Vision. The viability was determined by multiplying the percentage of live cells by the total cell count.

Statistical analysis

All experiments were performed in triplicate. One-way ANOVA (SigmaPlot) was used to determine statistical significance among treatment groups and controls. Data presented as the average ± standard deviation (SD). *P* values of $P < 0.05$ were considered statistically significant.

Biostatistics statement

The statistical review was performed by Ward Kirlin, PhD. The appropriate ANOVA of variance was performed on the data presented in this paper, and levels of statistical significance are based on the F-values and Tukey's multiple comparisons between group means as determined using SigmaPlot (Systat Software, Inc.). Mean + SDs are indicated in the graphical analysis, based on replicates of densitometry analysis of Western blots, the percentage of cells in S-phase of the cell cycle, or percentage of proliferating cells as indicated in the figures.

RESULTS

Generation and characterization of IONP-LPrA2

The leptin antagonist, LPrA2, has been shown to inhibit breast cancer growth and progression *in vitro* as well as *in vivo*[2,22,23]. To increase its efficacy, LPrA2 was conjugated to IONPs. IONPs are amphiphilic and have a 10 nm core[20]. The binding of LPrA2 to IONPs was facilitated by EDAC, which activates the carboxyl group on the IONP surface and allows the formation of an amide bond with the amino group of LPrA2 (Figure 1A). To confirm the binding of the LPrA2 peptides to the nanoparticles, the conjugates were analyzed by SDS-PAGE and Western blot. With LPrA2 antibody incubation, bands were detected at approximately 100 kD, indicating conjugated LPrA2, and approximately 3 kD indicating unbound LPrA2. Unconjugated LPrA2 and LPrA2-Sc were used as positive and negative controls, respectively (Figure 1B). To further characterize IONP-LPrA2, 1:10000 dilutions of the conjugated and unconjugated IONPs were measured by NanoSight nanoparticle tracking analysis (Malvern); in which the left and right Y-axes show particle number and percent distribution, and the X-axis displays particle size. The size of the unconjugated IONP was found to be 14 nm while conjugated IONP-LPrA2 measured 20 nm. This data suggests that the conjugation of LPrA2 to IONPs was successful.

Ob-R expression and effect of IONP-LPrA2 on leptin-induced pSTAT3 and cyclin D1 levels in human breast cancer cells

In order to determine the effects of IONP-LPrA2 on leptin signaling inhibition, we first had to confirm expression of the leptin receptor, Ob-R, in the human breast cancer cell lines. Immunoprecipitation and subsequent Western blot analysis showed Ob-R expression in MDA-MB-231, HCC1806, and MCF-7 cells (Figure 2A). Leptin signaling activates the JAK2/STAT3, MAPK/ERK, and PI3/Akt signaling pathways, which are implicated in its anti-apoptotic activity[9]. For this reason, we aimed to determine the effect of IONP-LPrA2 treatment on active/phosphorylated, pSTAT3. Leptin significantly increased the level of pSTAT3 in MDA-MB-231 and HCC1806 cells. IONP-LPrA2 significantly inhibited the effect of leptin on pSTAT3 levels in HCC1806 cells. No significant changes occurred in pSTAT3 levels in MCF-7 cells treated with

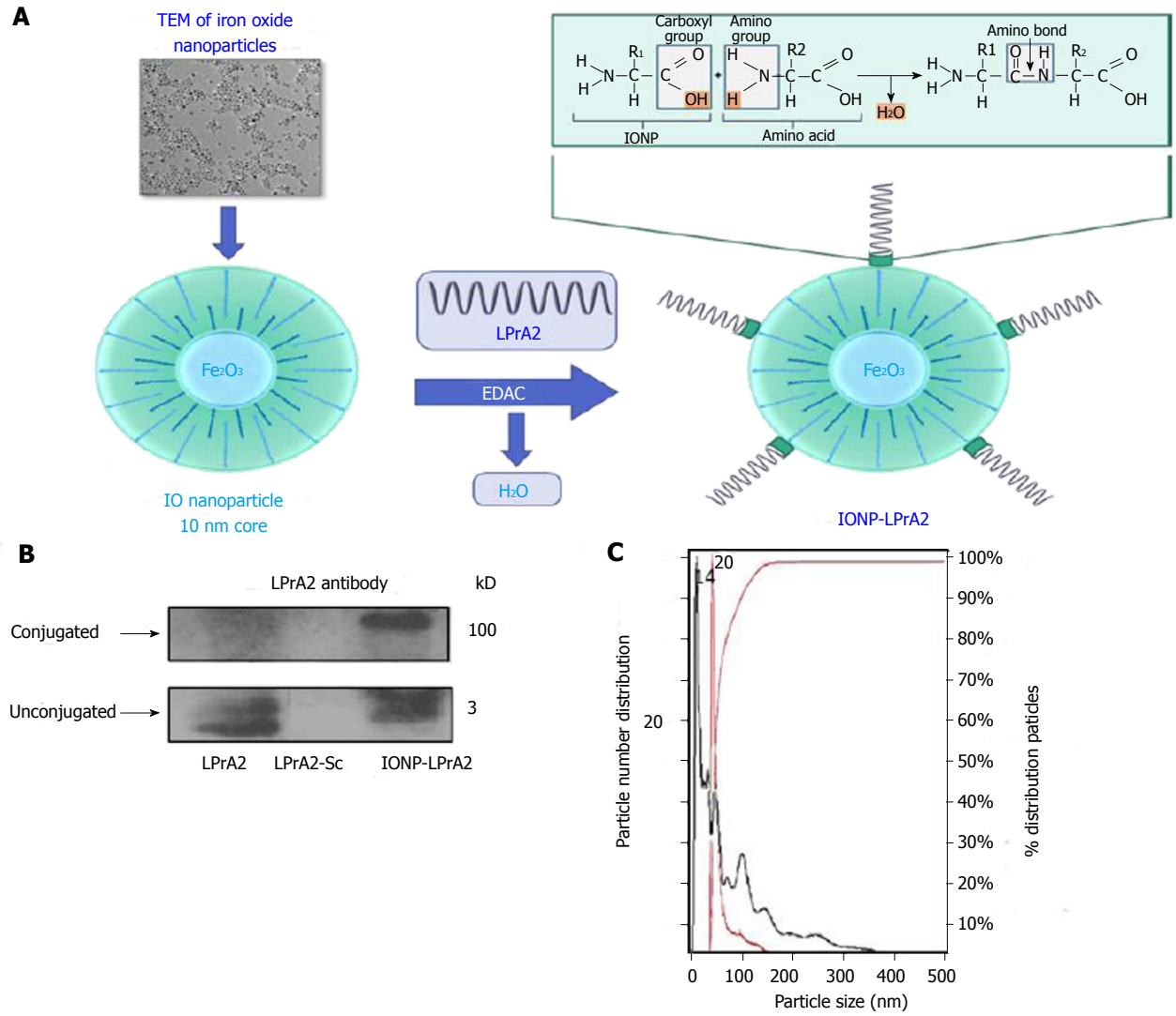

Figure 1 Generation and characterization of iron oxide nanoparticles-LPrA2. A: Conjugation of iron oxide nanoparticles (IONP)-LPrA2. LPrA2 was conjugated to IONPs *via* 1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDAC), which activates the carboxyl group on the IONP surface allowing it to form a covalent bond with the amino group of LPrA2 (displayed by TEM, transmission electron microscopy, Ocean Nanotech); B: Western blot confirmation of IONP-LPrA2 conjugation. Conjugated IONP-LPrA2 (100 kD) was detected by Western blot with an LPrA2 antibody, purified from antigen injected rabbit bleeds. Unconjugated LPrA2 (3 kD) and the scrambled peptide LPrA2-Sc (3 kD) served as positive and negative controls, respectively; C: NanoSight analysis of unconjugated and conjugated IONPs. The particle size of unconjugated IONP (14 nm) shown in black and the conjugated IONP-LPrA2 (20 nm) shown in red were determined by nanoparticle tracking analysis. The hyperbolic curve shows that the particles are 100% homogeneous.

leptin and IONP-LPrA2 (Figure 2B and C). Because leptin has been shown to increase cyclin D1 levels in breast cancer cells[14,15], we sought to determine the effect of IONP-LPrA2 treatment on cyclin D1 expression in MDA-MB-231, HCC1806, and MCF-7 breast cancer cells. Leptin significantly induced cyclin D1 expression in all cell lines (Figure 2B and C). The addition of IONP-LPrA2 significantly inhibited the effect of leptin on cyclin D1 expression in all cell lines (Figure 2B and C). These results suggest that IONP-LPrA2 abrogates the effect of leptin on leptin-induced signaling pathways.

IONP-LPrA2 inhibits leptin-induced cell cycle progression of human breast cancer cell lines

Leptin has been shown to increase expression of the cell cycle associated protein, cyclin D1[14,15]. To illustrate the effect of leptin on cell cycle progression, the number of cells in the S phase was determined by cell cycle analysis with the Cellometer Vision (Nexcelom). MDA-MB-231, HCC1806, and MCF-7 human breast cancer cells lines were treated with leptin (1.2 nmol/L) and IONP-LPrA2 plus leptin in order to determine its antagonistic effect. The cells were treated with IONP-LPrA2 concentrations ranging from 0.0018-0.036 pmol/L. MDA-MB-231 and HCC1806 TNBC cell lines were treated for 24 h while the ER^+ MCF-7 cells were treated for 48 h to produce an effect. Treatment with leptin caused a significant increase in cell cycle progression in HCC1806 (Figure 3B) and MCF-7 (Figure 3C), but had no significant effect on MDA-MB-231 cells (Figure 3A). Treatment with IONP-LPrA2 plus leptin abrogated leptin-induced cell cycle progression at 0.0018-0.0036 pmol/L

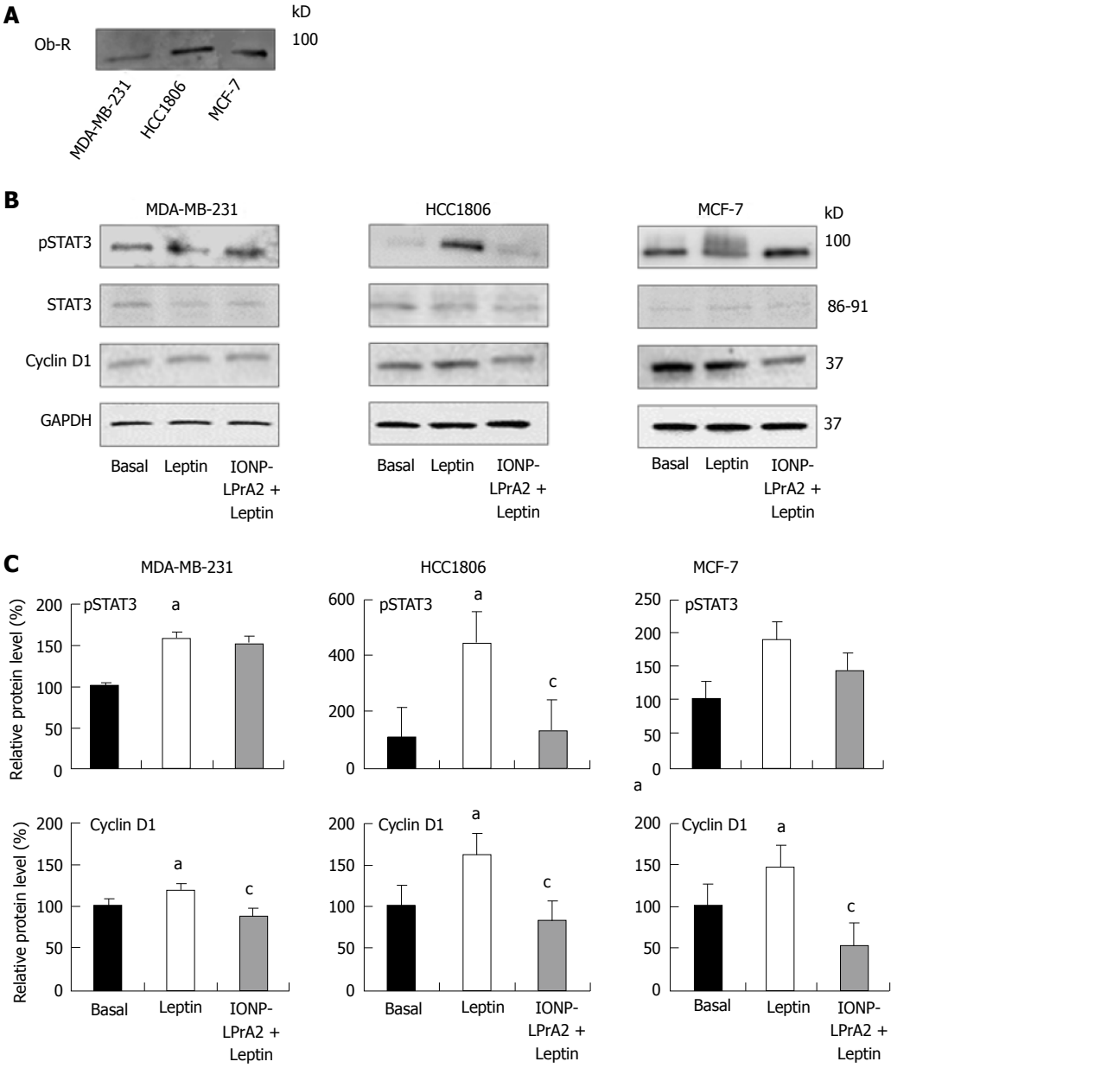

Figure 2 Ob-R expression and effect of iron oxide nanoparticles-LPrA2 on leptin-induced pSTAT3 and cyclin D1 levels in human breast cancer cells. A: Detection of Ob-R expression. The expression of Ob-R was detected by Western blot in MDA-MB-231, HCC1806, and MCF-7 cells; B: Iron oxide nanoparticles (IONP)-LPrA2 inhibition of leptin-induced pSTAT3 and cyclin D1 levels. Lysates were obtained from MDA-MB-231, HCC1806, and MCF-7 cells treated with leptin (1.2 nmol/L) or IONP-LPrA2 (0.0036 pmol/L) plus leptin (1.2 nmol/L) for 24-48 h. pSTAT3 and cyclin D1 levels were detected by Western blot. STAT3 served as a loading control for pSTAT3. GAPDH served as a loading control for cyclin D1; C: Densitometric analysis of pSTAT3 and cyclin D1 levels. Graphs represent quantitative analysis of pSTAT3 and cyclin D1 levels in MDA-MB-231, HCC1806, and MCF-7 cells with Image J software. Relative protein level was significantly increased in leptin treated cell lines compared to basal (untreated) cells, $^{a}P < 0.05$. Relative protein level in cells pretreated with IONP-LPrA2 and then leptin differed significantly from those treated with leptin alone, $^{c}P < 0.05$.

in MDA-MB-231, at 0.0018-0.036 pmol/L in HCC1806, and at 0.0018-0.0072 in MCF-7 cells (Figure 3). This data elucidated the effective dilution of IONP-LPrA2 for abrogation of leptin-induced cell cycle progression in each of the cell lines.

IONP-LPrA2 inhibits leptin-induced cell proliferation in human breast cancer cells

Leptin signaling stimulates breast cancer cell survival and proliferation[8]. To ascertain the manner in which IONP-LPrA2 affects cell proliferation, an MTT assay was performed. MDA-MB-231, HCC1806, and MCF-7 cell were treated with leptin (1.2 nmol/L) and IONP-LPrA2 (0.0036) plus leptin (1.2 nmol/L). Leptin treatment significantly increased cell proliferation and IONP-LPrA2 significantly diminished the effect of leptin in all of the cell lines (Figure 4). This data indicates that IONP-LPrA2 prevents leptin induction of cell proliferation.

IONP-LPrA2 decreases MDA-MB-231 tumorsphere formation

Self-renewal is a hallmark of cancer. Leptin has been

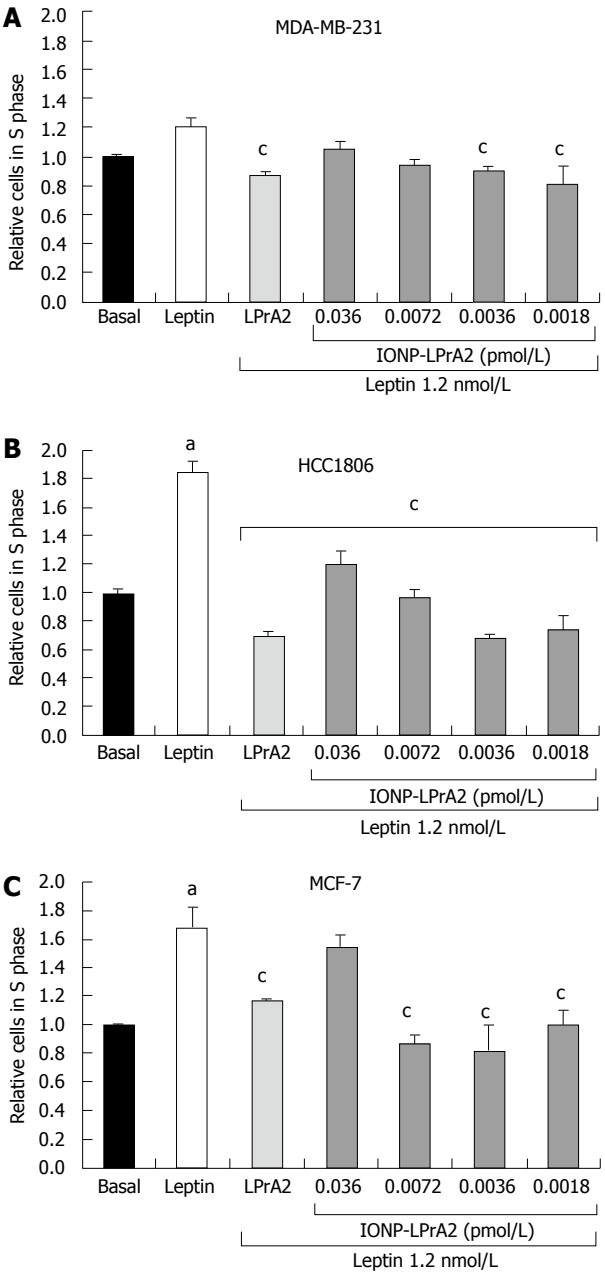

Figure 3 Iron oxide nanoparticles-LPrA2 inhibits leptin-induced cell cycle progression of human breast cancer cell lines. Iron oxide nanoparticles-LPrA2 inhibits S phase progression in breast cancer cells. A: MDA-MB-231; B: HCC1806; C: MCF-7. The cells were seeded in 6 well plates and treated with leptin (1.2 nmol/L), LPrA2 (1.2 nmol/L) plus leptin (1.2 nmol/L), or IONP-LPrA2 at indicated concentrations plus leptin (1.2 nmol/L) for 24-48 h. The percentage of cells in S phase was determined by cell cycle analysis, a measure of propidium iodide (PI) fluorescence. Relative percentage of cells in S phase was significantly increased in leptin treated cell lines compared to basal (untreated) cells, $^{a}P < 0.05$. Relative percentage of cells in S phase pretreated with leptin antagonists and then leptin differed significantly from those treated with leptin alone, $^{c}P < 0.05$.

shown to increase self-renewal and breast cancer stem cell (BCSC) growth[24]. To learn how IONP-LPrA2 affects BCSC growth, tumorsphere formation was assessed. MDA-MB-231 TNBC cells were treated with leptin (1.2 nmol/L) and IONP-LPrA2 (0.0036 pmol/L) plus leptin (1.2 nmol/L). Untreated, basal, MDA-MB-231 cells developed few small and medium tumorspheres

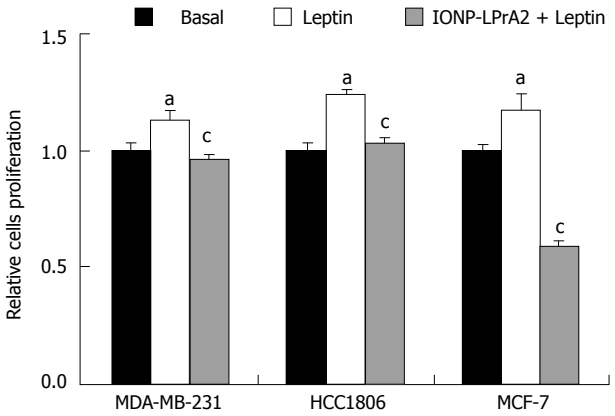

Figure 4 Iron oxide nanoparticles-LPrA2 inhibits leptin-induced cell proliferation in human breast cancer cells. MDA-MB-231, HCC1806, and MCF-7 cells were seeded in 96 well plates and treated with leptin (1.2 nmol/L) and iron oxide nanoparticles (IONP)-LPrA2 (0.0036 pmol/L) plus leptin (1.2 nmol/L) for 24-48 h. Cell proliferation was determined by MTT assay. Relative percentage of proliferating cells was significantly increased in leptin treated cell lines compared to basal (untreated) cells, $^{a}P < 0.05$. Relative percentage of proliferating cells pretreated with IONP-LPrA2 and then leptin differed significantly from those treated with leptin alone, $^{c}P < 0.05$.

(100-200 μm), cells treated with leptin showed a significant increase in the development of medium (200 μm) and large tumorspheres (> 200 μm) in comparison to basal. Cells treated with IONP-LPrA2 plus leptin displayed a significant decrease in medium tumorsphere growth relative to the leptin treated (Figure 5). This data shows that IONP-LPrA2 treatment may decrease BCSC growth.

The effect of chemotherapeutics on survival of breast cancer cell lines

Chemotherapy is among the most common treatments for breast cancer in addition to radiation and surgery[25]. To determine the effective dose of chemotherapeutics, cells were treated with a panel of anti-cancer drugs and viability was tested by the Annexin V FITC/PI Assay (Nexcelom). MDA-MB-231, HCC1806, and MCF-7 cells were treated with Cis (0.001-1.1 μmol/L), CTX (0.01-100 μmol/L), Dox (0.01-50 μmol/L), and PTX (0.05-1.0 μmol/L) for time periods ranging from 1-6 d to determine an effective dose to reduce cell viability (Figure 6). Cis and Dox reduced cell viability in 24 h while CTX and PTX treated cells required up to 6 d to produce an effect. All cell lines displayed a similar response to Cis and PTX (Figure 6A and D). MDA-MB-231 cells appeared to be more sensitive to CTX and Dox (Figure 6B and C).

Determination of the effect of IONP-LPrA2 on survival of breast cancer cells treated with chemotherapeutics

Chemotherapy has many detrimental side effects; because of this it is advantageous to utilize adjuvant therapies in order to reduce the effective dose. To determine the adjuvant potential of IONP-LPrA2, cells were treated with chemotherapeutics combined with IONP-LPrA2 and analyzed for viability by the Annexin

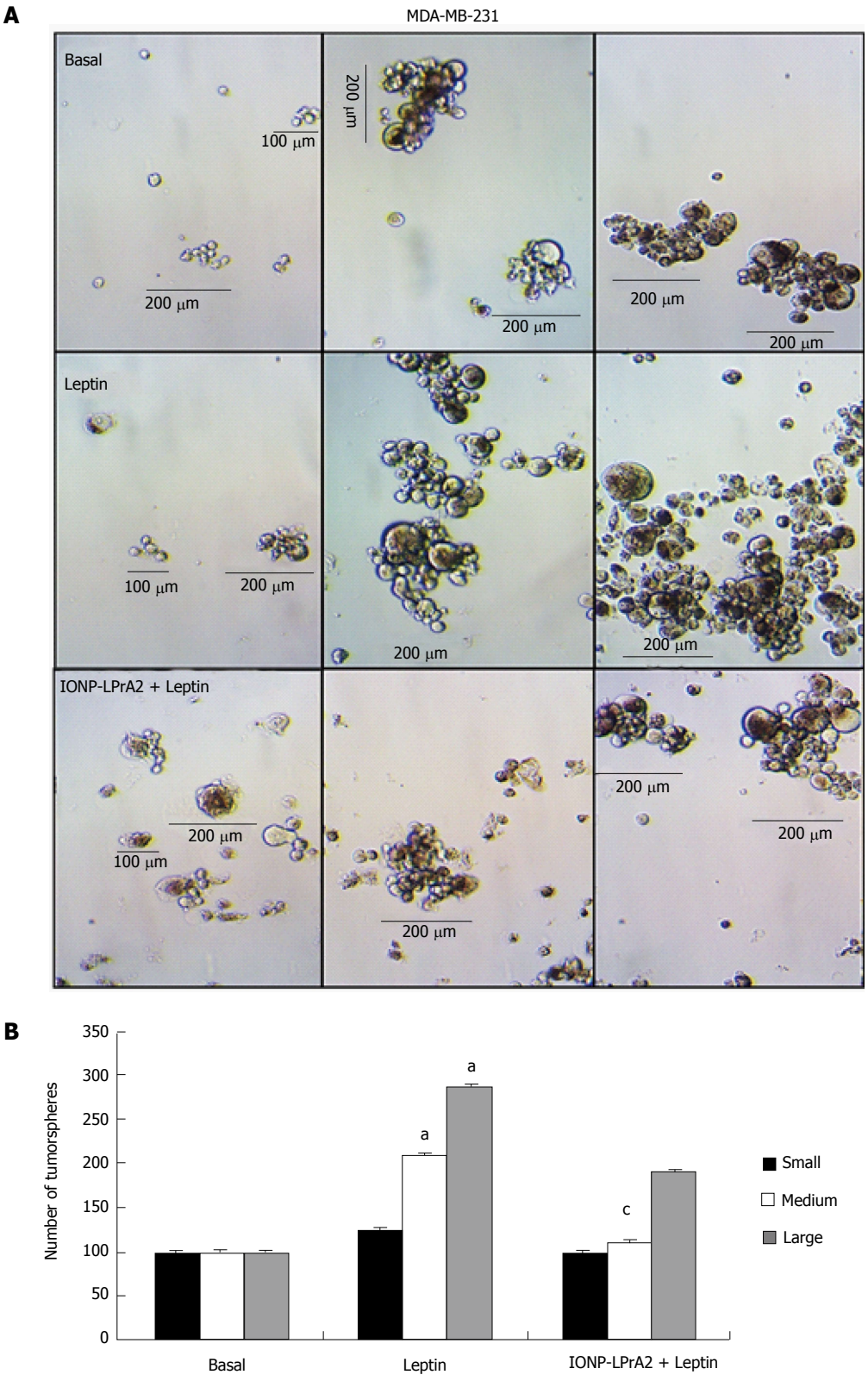

Figure 5 Iron oxide nanoparticles-LPrA2 decreases MDA-MB-231 tumorsphere formation. A: Iron oxide nanoparticles (IONP)-LPrA2 attenuation of leptin-induced tumorsphere formation. MDA-MB-231 cells were grown in low attachment plates with mammocult medium for 1-2 wk, under treatment with leptin (1.2 nmol/L) and IONP-LPrA2 (0.0036 pmol/L) plus leptin (1.2 nmol/L). Tumorspheres were counted. Tumorspheres were grouped according to size: Small (< 100 μm), medium (100-200 μm) and large (> 200 μm); B: Effect of leptin and IONP-LPrA2 on number and size of tumorspheres. Graph represents quantitative analysis of small, medium, and large tumorspheres in response to leptin and IONP-LPrA2 treatment. The number of colonies was significantly increased in leptin treated cells compared to basal (untreated) cells, $^{a}P < 0.05$. The number of colonies pretreated with IONP-LPrA2 and then leptin differed significantly from those treated with leptin alone, $^{c}P < 0.05$.

V FITC/PI Assay (Nexcelom). MDA-MB-231, HCC1806, and MCF-7 cells were treated with chemotherapeutics

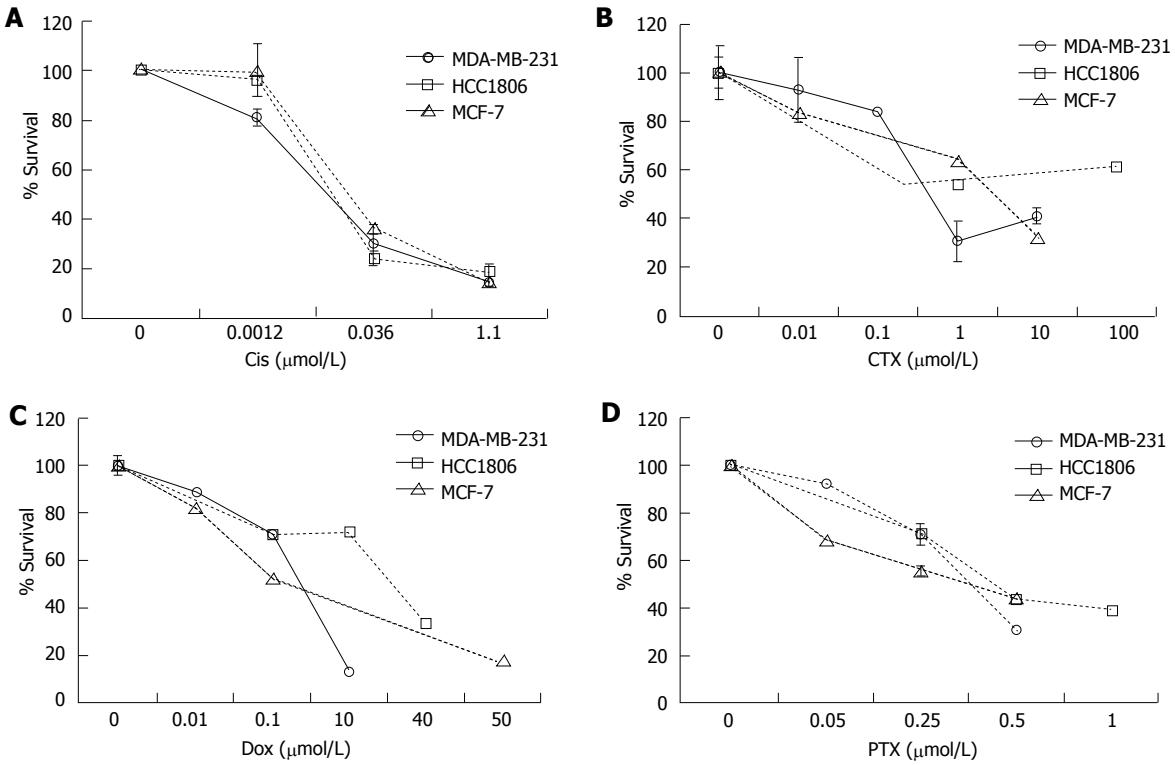

Figure 6 The effect of chemotherapeutics on survival of breast cancer cell lines. The effective dose of the chemotherapeutics. A: Cisplatin (Cis); B: Cyclophosphamide (CTX); C: Doxorubicin (Dox); D: Paclitaxel (PTX) were determined in MDA-MB-231, HCC1806, and MCF-7 cells. The cells were seeded in 6 well plates and treated with Cis (0.001-1.1 μmol/L), CTX (0.01-100 μmol/L), Dox (0.01-50 μmol/L), and PTX (0.05-1.0 μmol/L) for 1-6 d. Percent survival was determined by the Annexin V/FITC and PI assay. The relative survival was determined by multiplying the percentage of live cells by the total cell count.

at concentrations determined in Figure 6 in media containing 5% FBS to mimic physiological leptin levels, in addition to IONP-LPrA2 (0.0036 pmol/L) for time periods ranging from 1-6 d. The treatment concentrations were MDA-MB-231 (Cis 0.001 μmol/L, CTX 0.5 μmol/L, Dox 0.4 μmol/L, PTX 0.5 μmol/L); HCC1806 (Cis 0.036 μmol/L, CTX 1 μmol/L, Dox 10 μmol/L, PTX 0.5 μmol/L); and MCF-7 (Cis 0.036 μmol/L, CTX 5 μmol/L, Dox 0.01 μmol/L, PTX 1 μmol/L). MDA-MB-231 TNBC cells treated with IONP-LPrA2 displayed a significant decrease in viable cells when dosed with Cis and CTX (Figure 7A and B). HCC1806 TNBC cells treated with IONP-LPrA2 showed a significant reduction in viable cells when dosed with Cis and Dox (Figure 7A and C). ER+ MCF-7 cells treated with IONP-LPrA2 did not show a significant decrease in viable cells when treated with chemotherapeutics (Figure 7). Although cells were treated with PTX for up to 6 d to reduce cell viability, IONP-LPrA2 showed no additional decrease in viability when combined with PTX (Figure 7D). PTX is an anti-microtubule agent which acts on the M phase of the cell cycle while the other chemotherapeutics act on DNA which affects the S phase[2]. This data suggests that IONP-LPrA2 increases the potency of chemotherapeutics on TNBC cells, particularly anti-cancer drugs which target DNA.

DISCUSSION

In spite of methods for early detection of breast cancer, it remains the second leading cause of cancer deaths in women in the United States[1]. TNBC is a subtype of breast cancer characterized by the lack of hormone receptor expression. The absence of hormone receptors makes this more aggressive form of breast cancer even more difficult to treat. Obesity is often associated with poorer outcomes in individuals with breast cancer, particularly those with TNBC[25]. Obesity is characterized by an excess of the inflammatory cytokine, leptin. Elevated leptin levels display a significant correlation with metastasis and lower breast cancer patient survival[26]. The leptin antagonist, LPrA2 has been shown to inhibit leptin signaling in breast and other cancer types, but the actions of LPrA2 are restricted by its low MW of < 3 kD, short half-life, and insolubility in water[8,27]. IONP-LPrA2 was developed to circumvent these limitations. IONPs conjugated to other peptides, such as the amino terminal fragment of urokinase type plasminogen activator (ATF-uPA) are stable for more than 48 h in *in vivo* imaging experiments[20]. IONPs are amphiphilic, small (10 nm core size), and uniformly sized to facilitate delivery which prevents phagocytosis[28]. The characteristics of IONPs make them an ideal delivery system for LPrA2 to target and treat breast cancer. In the present study, IONP-LPrA2 was used to evaluate its ability to inhibit leptin signaling in human breast cancer cells. The data indicates that IONP-LPrA2 abrogates cell cycle progression and acts as an adjuvant when administered with chemotherapeutics.

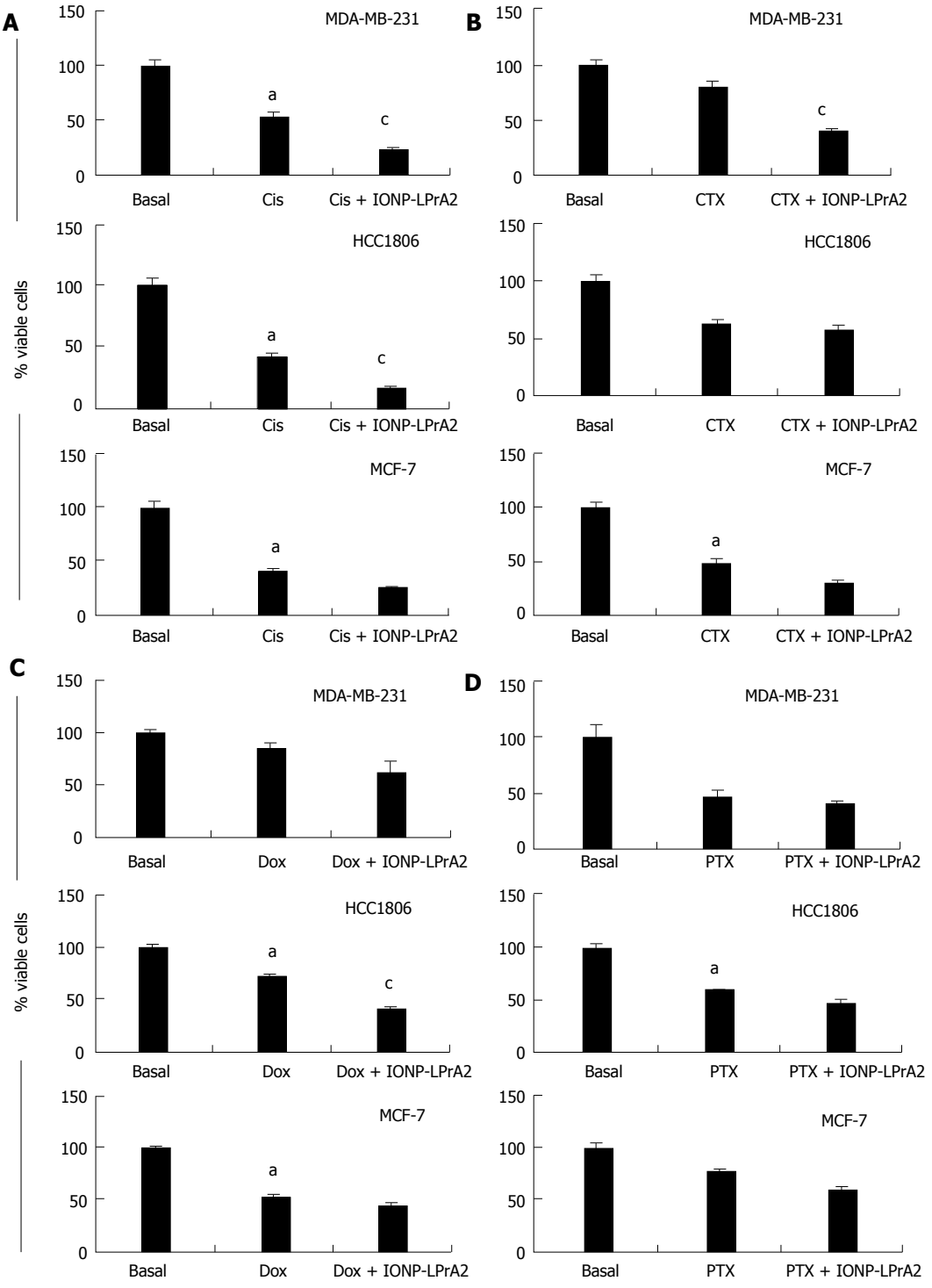

Figure 7 Determination of the effect of iron oxide nanoparticles-LPrA2 on survival of breast cancer cells treated with chemotherapeutics. MDA-MB-231, HCC1806, and MCF-7 cells were treated with an effective dose of the chemotherapeutics. A: Cisplatin (Cis); B: Cyclophosphamide (CTX); C: Doxorubicin (Dox); D: Paclitaxel (PTX) plus IONP-LPrA2 (0.0036 pmol/L). The cells were seeded in 6 well plates and treated with chemotherapeutics at effective concentrations determined in Figure 6 for 1-6 d. The treatment concentrations were MDA-MB-231 (Cis 0.001 μmol/L, CTX 0.5 μmol/L, Dox 0.4 μmol/L, PTX 0.5 μmol/L); HCC1806 (Cis 0.036 μmol/L, CTX 1 μmol/L, Dox 10 μmol/L, PTX 0.5 μmol/L); and MCF-7 (Cis 0.036 μmol/L, CTX 5 μmol/L, Dox 0.01 μmol/L, PTX 1 μmol/L). Percent of survival was determined by the Annexin V/FITC and PI assay. The relative survival was determined by multiplying the percentage of live cells by the total cell count. Perecent viability was significantly decreased in cells treated with chemotherapeutic compared to basal (untreated) cells, $^{a}P < 0.05$. Cells treated with chemotherapeutic and IONP-LPrA2 differed significantly from those treated with chemotherapeutic alone, $^{c}P < 0.05$.

Decreased levels of pSTAT3 and cyclin D1 with IONP-LPrA2 treatment were shown by Western blot. Cyclin D1 is a cell cycle regulatory gene. STAT3 is a transcription factor responsible for the regulation of cyclin D1[10]. Decreased levels of pSTAT3 with IONP-LPrA2 treatment were seen at time points as early as 5-15 min post

treatment. Previous studies have shown that leptin is mitogenic and increases cyclin D1 in ER^+ MCF-7 breast cancer cells[14,15]. Because leptin increases cyclin D1 and IONP-LPrA2 inhibits the effect of leptin, utilizing agents that target cyclin D1 may be a plausible method to treat breast cancer. In this study, we have shown that IONP-LPrA2 decreases pSTAT3 and cyclin D1. The decreased levels of these leptin-induced targets may inhibit cell cycle progression in ER^+ MCF-7 cells as well as MDA-MB-231 and HCC1806 TNBC cells.

Inhibition of cell cycle progression by IONP-LPrA2 was displayed by image based cytometry. Leptin has been shown to increase levels of cyclin D1[14,15]. In this study, we show that IONP-LPrA2 decreases cyclin D1 expression, but the effect on cell cycle progression was yet to be determined. Here we show that IONP-LPrA2 treatment decreases the percentage of cells in the S phase of the cell cycle, where DNA is synthesized, as or more effectively than LPrA2 alone. Interestingly, the greatest decrease in the percentage of cells in S phase with IONP-LPrA2 treatment was seen in HCC1806 TNBC cells derived from a non-metastatic squamous cell carcinoma in contrast to MCF-7 and MDA-MB-231 cells derived from metastatic adenocarcinomas. This data suggests that IONP-LPrA2 inhibition of cell cycle progression may reduce the advancement of breast cancer, and may be particularly beneficial in the treatment of non-metastatic and squamous cell carcinomas.

Chemotherapy is the first line of treatment for TNBC. Although TNBC is generally more responsive to chemotherapy than other forms of breast cancer, there is an increased risk of developing drug resistance[29]. BCSC growth and self-renewal play an important role in breast cancer drug resistance and leptin increases the risk[24]. These cells express molecular markers for breast cancer, $CD44^+CD24^-/ALDH^+$[10]. We have demonstrated that leptin induces *in vitro* BCSC, tumorsphere, formation and treatment with IONP-LPrA2 attenuates the effect of leptin in MDA-MB-231 TNBC cells. These results indicate that IONP-LPrA2 prevents BCSC formation and may decrease chemoresistance in TNBC.

Chemotherapeutic treatment of breast cancer is plagued with high toxicity. Toxic side effects and the development of drug resistance are cause for the development of adjuvant therapies. The need for adjuvant therapies is exacerbated in TNBC patients who often experience relapse and develop resistance to chemotherapy[29]. TNBC is commonly treated with combination chemotherapy[25]. Here, we treated breast cancer cells with a panel of commonly used chemotherapeutics (Cis, CTX, Dox and PTX) in addition to IONP-LPrA2 to test its ability to decrease cell viability more than the drugs alone. We demonstrated that TNBC cells, MDA-MB-231 displayed a significant decrease in viability with Cis and CTX plus IONP-LPrA2; and HCC1806 showed a significant reduction in live cells when treated with Cis and Dox plus IONP-LPrA2. ER^+ MCF-7 cells treated with chemotherapeutics plus IONP-LPrA2 did not show a significant decrease in viable cells. Also, there was no significant decrease in viability in the cells treated with PTX plus IONP-LPrA2. This may be due, in part, to PTX's anti-microtubule action, which affects the M phase of the cell cycle[25]. Cis, CTX, and Dox act on DNA which affects the S phase[25]. These drugs may work synergistically with IONP-LPrA2, which also appears to act on the S phase. These data indicate that IONP-LPrA2 may act as a chemotherapeutic adjuvant by decreasing viability, thereby decreasing the effective dose in TNBC.

In conclusion, IONP-LPrA2 was found to decrease the level of leptin-induced targets pSTAT3 and cyclin D1. IONP-LPrA2 decreased DNA synthesis during the S phase of the cell cycle and reduced proliferation in both ER^+ and TNBC cells. When combined with chemotherapeutics, particularly drugs targeting the S phase, IONP-LPrA2 showed an additive effect on the reduction of live breast cancer cells. These findings indicate that IONP-LPrA2 may be useful in the prevention of tumor cell growth and proliferation in breast cancer. Further, treatment with IONP-LPrA2 may allow for lower chemotherapeutic dosing. These results are potentially beneficial for obese patients with elevated leptin levels, whom have a higher incidence and thus poorer outcome of TNBC. Taken together, the present data provides confirmation of our hypothesis that IONP-LPrA2 treatment may be useful in impairing tumor growth and when given in combination with the indicated chemotherapeutics has the potential to increase drug effectiveness. These data indicate that there is a synergistic effect with IONP-LPrA2 and chemotherapeutics which affect the S phase of the cell cycle *in vitro*.

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COMMENTS

Background

Obesity and high leptin levels are strongly associated with breast cancer relapse, drug resistance, and poorer patient outcomes. Overexpression of leptin and its receptor, Ob-R, induce breast cancer cell growth and proliferation. Triple negative breast cancer (TNBC) is a subtype of breast cancer which comprises approximately 15% of cases and is an aggressive form of the disease with no targeted therapy. TNBC chemotherapeutic treatment often leads to chemoresistance and shows several undesirable side effects. Leptin is proliferative and is a survival factor for breast cancer treated with chemotherapeutics. Therefore, the authors have developed a leptin peptide receptor antagonist coupled to iron oxide nanoparticles (IONP-LPrA2), which successfully inhibits leptin signaling as well as increases chemotherapeutic effectiveness in breast cancer and is particularly promising for TNBC treatment.

Research frontiers

IONP-LPrA2 could be a new and effective biological for blocking pro-oncogenic and drug resistance effects of leptin in breast cancer, especially in obese patients suffering from TNBC that are treated with chemotherapeutics.

Innovations and breakthroughs

This study describes for the first time the production and characterization of a new biological bound to nanoparticles that can effectively block leptin signaling inducing proliferation and survival in breast cancer cells treated with chemotherapeutics.

Applications

In recent years, IONPs have become an important tool for biomedical applications. The use IONPs has been employed in vaccinations, drug delivery, MRI, and molecular imaging. The authors' data suggests combining IONPs with the leptin antagonist, LPrA2, prevents the growth of breast cancer cells and acts as a chemotherapeutic adjuvant by reducing the effective dose.

Terminology

Leptin signaling occurs when the hormone is secreted by the adipose tissue and binds to its receptor, Ob-R. Breast cancer, particularly in obese individuals, is associated with high levels of leptin. Leptin signaling leads to increased breast cancer cell growth, proliferation and drug resistance. The inhibition of leptin signaling with the nanoparticle-linked leptin antagonist, IONP-LPrA2, provides a promising new way to improve breast cancer chemotherapy.

Peer-review

This manuscript provides useful information to the medical students, clinicians, and researchers in this field, therefore, is acceptable for publication.

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ORIGINAL ARTICLE

Basic Study

NDRG2 gene copy number is not altered in colorectal carcinoma

Anders Lorentzen, Cathy Mitchelmore

Anders Lorentzen, Cathy Mitchelmore, Eucaryotic Cell Biology, Department of Science and Environment, Roskilde University, 4000 Roskilde, Denmark

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Correspondence to: Cathy Mitchelmore, PhD, Associate Professor, Department of Science and Environment, Roskilde University, Universitetsvej 1, Postbox 260, 4000 Roskilde, Denmark. mitch@ruc.dk
Telephone: +45-46743201
Fax:+45-46743011

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Abstract

AIM

To investigate if the down-regulation of *N-myc Downstream Regulated Gene 2* (*NDRG2*) expression in colorectal carcinoma (CRC) is due to loss of the *NDRG2* allele(s).

METHODS

The following were investigated in the human colorectal cancer cell lines DLD-1, LoVo and SW-480: NDRG2 mRNA expression levels using quantitative reverse transcription-polymerase chain reaction (qRT-PCR); interaction of the *MYC* gene-regulatory protein with the *NDRG2* promoter using chromatin immunoprecipitation; and *NDRG2* promoter methylation using bisulfite sequencing. Furthermore, we performed qPCR to analyse the copy numbers of *NDRG2* and *MYC* genes in the above three cell lines, 8 normal colorectal tissue samples and 40 CRC tissue samples.

RESULTS

As expected, NDRG2 mRNA levels were low in the three colorectal cancer cell lines, compared to normal colon. Endogenous MYC protein interacted with the *NDRG2* core promoter in all three cell lines. In addition, the *NDRG2* promoter was heavily methylated in these cell lines, suggesting an epigenetic regulatory mechanism. Unaltered gene copy numbers of *NDRG2* were observed in the three cell lines. In the colorectal tissues, one normal and three CRC samples showed partial or complete loss of one *NDRG2* allele. In contrast, the *MYC* gene was amplified in one cell line and in more than 40% of the CRC cases.

CONCLUSION
Our study suggests that the reduction in *NDRG2* expression observed in CRC is due to transcriptional repression by MYC and promoter methylation, and is not due to allelic loss.

Key words: N-myc downstream-regulated gene 2; Colorectal carcinoma; MYC; Tumor suppressor; Allelic loss; Gene amplification; Copy number

Core tip: *NDRG2* is a putative tumor suppressor gene whose expression is reduced in many cancer forms, including colorectal carcinoma (CRC). We set out therefore to investigate if down-regulation of *NDRG2* expression was due to loss of one or both alleles and/or to other mechanisms. In our paper, we show that allelic loss of *NDRG2* is a rare event in CRC. To our knowledge, this is the first study that has specifically investigated gene copy number of *NDRG2* in CRC. Furthermore, our results suggest that *MYC* is amplified in more than 40% of CRC cases. MYC is known to repress transcription of *NDRG2*. Our results lead us to suggest that it is the transcriptional control of *NDRG2* expression, including repression by MYC and epigenetic regulation, that results in decreased *NDRG2* mRNA levels in CRC, rather than allelic loss of *NDRG2*.

Lorentzen A, Mitchelmore C. *NDRG2* gene copy number is not altered in colorectal carcinoma. *World J Clin Oncol* 2017; 8(1): 67-74 Available from: URL: http://www.wjgnet.com/2218-4333/full/v8/i1/67.htm DOI: http://dx.doi.org/10.5306/wjco.v8.i1.67

INTRODUCTION

N-myc downstream regulated gene 2 (*NDRG2*) is one of four genes belonging to the *NDRG* gene family. Common for these genes is an NDR domain, a protein motif covering almost the entire protein, but the cellular functions of these genes are currently unclear[1,2]. *NDRG2* expression has been found to be down-regulated in several human cancers including colorectal carcinoma (CRC), hepatocellular carcinoma, glioblastoma and thyroid cancer[3-7]. *NDRG2* is a candidate tumor suppressor gene, with a better overall survival for CRC, hepatocellular carcinoma and glioma patients displaying expression of the gene compared to low or no expression[8-12]. Further evidence of the tumor suppressor function of NDRG2 comes from the observation that NDRG2-lacking mice develop various types of tumors, and from xenograft studies showing that NDRG2-expressing tumor cells implanted in nude mice form smaller tumors and fewer metastases than control cells[13-15]. NDRG2 has a number of downstream targets, including activation of phosphatase and tensin homolog, a known tumor suppressor in the PI3K-AKT pathway[13,16].

Several mechanisms have been suggested as possible regulators of *NDRG2* expression, of which epigenetic silencing, due to promoter hypermethylation, is the most widely observed[4,8,9,13,14,17]. However, other regulatory mechanisms may also play a role. One example could be the transcription factor *MYC*, which is characterised as a proto-oncogene often altered in human cancers[18]. The biological function of MYC seems to be to either activate or repress the transcription of target genes[19,20]. Zhang *et al*[21] have previously shown that ectopically expressed MYC is able, *via* Miz-1, to interact with and to repress transcription from the *NDRG2* promoter. Moreover, correlation of high *MYC* with reduced *NDRG2* expression has been observed in different cancers and cancer cell lines[15,22-24]. However, an inverse relation between *MYC* levels and *NDRG2* expression seems not to apply to all cancer types[25].

CRC is, like most other cancers, a malignant disease with a combination of both genetic and epigenetic changes. One of these changes is chromosome instability, which affects one or several chromosomal regions. Many groups have analysed changes in gene copy numbers in CRC by different approaches and found numerous chromosomal gains and losses[26-29]. In the study by Lagerstedt *et al*[29], the status of CRC samples classified as Dukes stages A-D was analysed, showing an increasing frequency of allelic losses at more severe stages (Dukes C and D). According to their data, allelic deletions in chromosome 14, containing the *NDRG2* gene, is already found at earlier stages (Dukes A and B) and becomes more frequent at the later stages. Although chromosome 14 is not considered one of the deletion hot spot regions, such as chromosome 8p or 18q[27,28,30,31], we hypothesised that deletions in chromosome 14 could lead to loss of one or both of the *NDRG2* alleles. On the other hand, the *MYC* gene is found on chromosome 8q, and gains of this large chromosome arm are frequently found in CRC[26,28,32]. Analysing the gene copy number of *MYC* is therefore of interest with regards to its possible regulatory effect on *NDRG2*.

In this study, we demonstrate a frequent increase in the gene copy number of *MYC* in CRC. In contrast, we find that changes in the copy number of the *NDRG2* locus are rare in CRC, and we suggest that reduced expression of NDRG2 in CRC is due to epigenetic and MYC-related transcriptional repression.

MATERIALS AND METHODS

Cell lines and genomic DNA

The DLD-1, LoVo and SW-480 colorectal cancer cell lines were a gift from Associate Professor Ole Vang, Roskilde University. Cells lines were incubated and maintained at 37 ℃ in an environment of humidified air with 5% CO_2 in McCoy's 5A + GlutaMaxTM-1 media with 10% Fetal Bovine Serum and 1% Penicillin-Streptomycin (Invitrogen). RNA from cell lines was purified with

the SV total RNA isolation kit (Promega) and genomic DNA was purified by ethanol precipitation after an overnight Proteinase K treatment. Reference human genomic DNA, purified from blood lymphocytes, was obtained from Roche Diagnostics, United States (Cat. No.11691112001). As a normal colonic control we used commercially available DNA (BioChain Institute Inc., D4234090). Human colon genomic DNA from tissue classified as either normal or tumorigenic was obtained from BioChain Inc, United States (Cat. no. D8235090-1; Supplementary Table S1). The commercial supplier confirms that tissue and data collection were ethically approved by their Institutional Regulatory Board and that informed consent was obtained from all human subjects.

Chromatin immunoprecipitation

The chromatin immunoprecipitation (ChIP) kit from Abcam (Ab500) was used according to the instructions, with inclusion of a final ethanol precipitation to increase the DNA concentration. Antibody against MYC (Abcam, ab56-100) was used at a concentration of 5 μg per reaction. The primers used in the PCR step were designed to cover the core promoter region in *NDRG2* (-80 to +93, Figure 1A) and their sequences were (5'-3'): CTTGAGGCATTGACCCCAGAG and CTCTTTGCTGCGTCCCGAC.

Bisulfite treatment and sequencing

Bisulfite treatment of genomic DNA was performed as previously described[33], using glycogen as carrier, and the precipitated DNA was redissolved in TE buffer, amplified by PCR and sequenced directly. The primers were designed to cover 16 CpG sites in the promoter region in *NDRG2* (Figure 1A) and their sequences were (5'-3'): TTTTCGAGGGGTATAAGGAGAGTTTATTTT and CCAAAAACTCTAACTCCTAAATAAACA[34]. A positive control with *in vitro* methylated (IVM) DNA was prepared by mixing 2 μL NEB2 buffer, 1 μL 20 x S-adenosylmethionine (New England Biolabs, B9003S), 200 ng reference human genomic DNA and 1 μL SssI methyltransferase (New England BioLabs, M0226S) in a total of 20 μL. Samples were incubated at 37 ℃ overnight with occasional addition of 2 μL 20 x S-adenosylmethionine to ensure sufficient methyl-donor substrate. The following description was used for each CpG site: Unmethylated (no methylation signal); weakly methylated (methylation signal was less than or approximately equal to unmethylated signal); and strongly methylated (methylation signal was greater than unmethylated signal).

Quantitative real-time PCR

Determination of gene copy number was based on the LightCycler technology using SYBR Green. The sequences of the primers were (5'-3'): *NDRG2* (5' end): CCCCTTGCCTTCTAACTTCCCA and ACAGCCCCTCCTCCCACCTT; *NDRG2* (3' end): GGGGTGAACGAAGAACAAAACAAAG and CGAGGGAGACGGTGAGATGAGG; *MYC*: CCAGAGGAGGAACGAGCTAA and TTGGACGGACAGGATGTATG; *GFAP*: TGACCCTCTCCACCCCATAGTGAC and CAGCAGCAGTGCCCTGAAGATTAG; and *MECP2*: TCAGAGGGTGTGCAGGTGAA and TTGAAAAGGCATCTTGACAAGGA. In a validation experiment using a control sample, a dilution series was produced and assayed for *NDRG2, MYC*, *GFAP* and *MECP2*. When C_t values were plotted against log dilution it was shown that the assays are quantitative over a range of 625-fold dilution for *NDRG2* (5' end), *NDRG2* (3' end), *MYC*, *MECP2* and 125 for *GFAP*. All samples were quantified in triplicates and mean C_t values were normalised to *GFAP* and used to calculate delta delta C_t (ddCt) relative to the reference human genomic DNA[35]. Copy number was defined as a loss for ddCt < 0.75 and as a gain for ddCt > 1.25. Quantification of *NDRG2* mRNA expression levels in colorectal cancer cell lines, using qRT-PCR and normalisation to β-actin, was carried out as previously described[25].

Statistical analysis

All statistical tests were carried out using GraphPad Prism 4 software and *P* values of < 0.05 were considered significant. An unpaired two-tailed *t*-test was used to compare the means of normal-distributed data for the two groups (normal *vs* tumor). The null hypothesis is that there is no difference between the two groups. When data of the two groups did not have equal variance, by *F* test analysis, we used a Mann-Whitney test.

RESULTS

NDRG2 expression is down-regulated in colorectal cancer cell lines

In order to examine how *NDRG2* expression is regulated in colorectal cancer, we chose to work with three cell lines. First of all, we quantified *NDRG2* mRNA levels in the three colorectal cancer cell lines DLD-1, LoVo and SW-480 and observed no or very low expression of *NDRG2*, when normalised to β-actin and compared to human colon mRNA from healthy controls (Table 1).

MYC binds to the NDRG2 gene promoter in colorectal cancer cell lines

We were interested in seeing whether endogenous MYC was bound to the *NDRG2* promoter in these cell lines, since ectopically expressed MYC is a transcriptional repressor of *NDRG2*[21]. A ChIP experiment did indeed show binding of endogenous MYC protein to the core promoter region of *NDRG2* in all three colorectal cancer cell lines (Figure 1B).

The NDRG2 promoter is heavily methylated in colorectal cancer cell lines

In silico analysis of the NDRG2 promoter predicted a CpG island between -380 and +1471 relative to the transcriptional start site (%GC = 66.3, observed/expected CpG = 0.673, cpgislands.usc.edu/cpg.aspx).

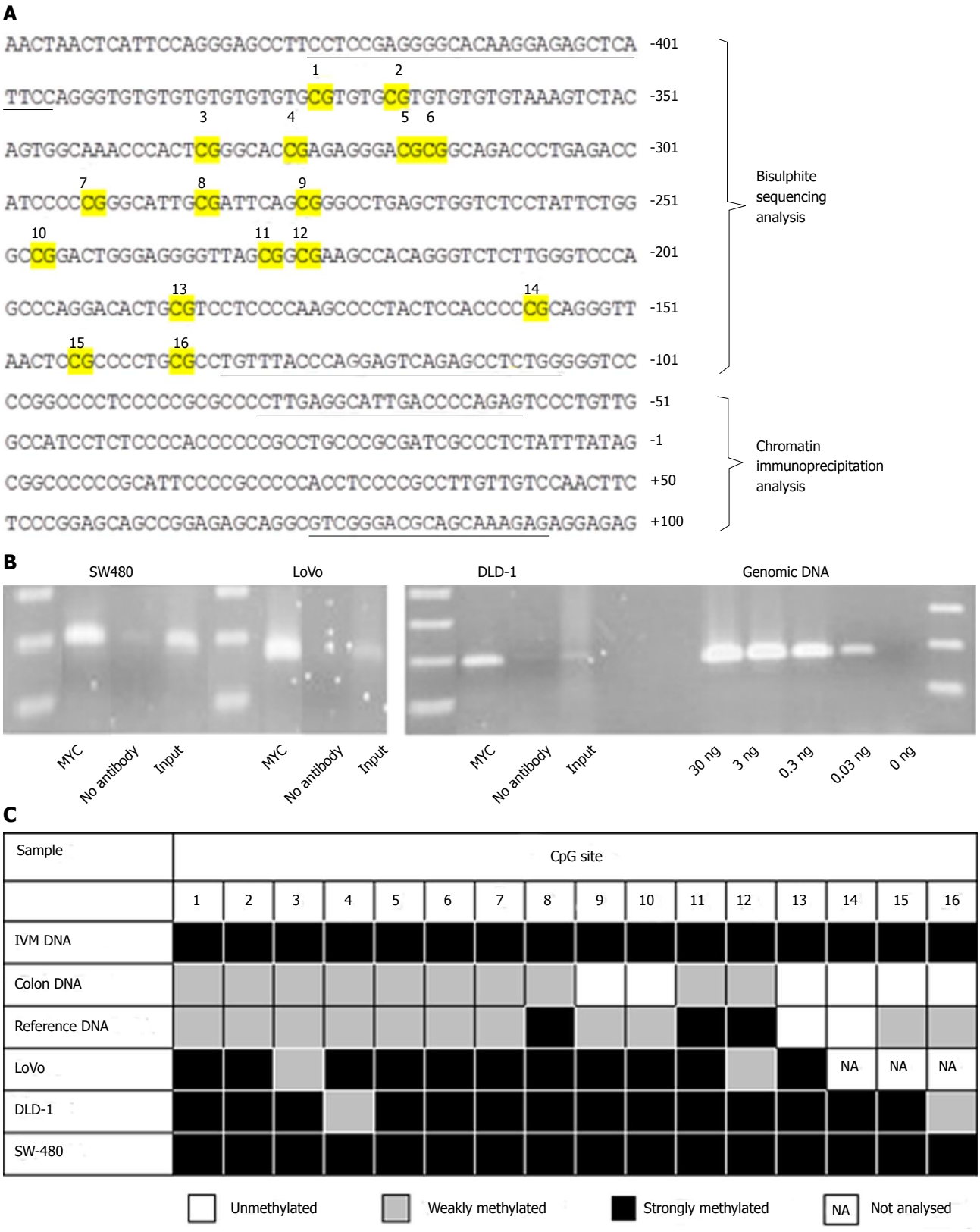

Figure 1 Epigenetic and chromatin immunoprecipitation analysis of the *NDRG2* promoter in three colorectal cancer cell lines. A: The *NDRG2* gene sequence around the transcriptional start site at +1. Primer-binding regions for PCR are underlined and CpG sites subjected to methylation analysis are numbered 1 to 16; B: Endogenous MYC interacts with the *NDRG2* core promoter. ChIP analysis was carried out on SW-480, LoVo and DLD-1 cell extracts using antibody against the transcription factor MYC. "No antibody" was without antibody and "input" served as a positive control. Genomic DNA was used as positive control for the PCR reaction; C: The NDRG2 promoter is hypermethylated in three colorectal cancer cell lines. Bisulfite sequencing was carried out on human genomic DNA from LoVo, DLD-1 and SW-480 cell lines, normal colonic DNA, reference DNA and *in vitro* SssI-methylated (IVM) DNA. Each CpG site was rated as unmethylated, weakly methylated (≤ 50% methylated), or strongly methylated (> 50% methylated).

Table 1 Mean values of normalised levels of *NDRG2* mRNA in colorectal cancer cell lines and healthy colonic tissue

Sample	mRNA level
DLD-1 cell line	0
LoVo cell line	0.005
SW-480 cell line	0.001
Control human colon[a]	0.034 ± 0.009

All samples were analysed in technical triplicates and normalised to β-actin mRNA levels. [a]Previously published data for the mean ± standard deviation for 15 individuals[3].

To establish the methylation status of the *NDRG2* proximal promoter in all three cell lines, we carried out bisulfite treatment and sequencing of the region from -426 to -107, which contains 16 CpG sequences. Bisulfite treatment converts all unmethylated cytosines into uracils, while cytosines with a methyl group attached remain unaltered. As controls, we compared our results with healthy colon genomic DNA, reference genomic DNA from normal blood lymphocytes, and IVM genomic DNA. As presented in Figure 1C, the normal colon genomic DNA and reference genomic DNA sample were predominantly weakly methylated, whereas the *in vitro* methylated control was completely methylated at all cytosines. The three colorectal cancer cell lines, LoVo, DLD-1 and SW-480, displayed strong methylation at the majority of CpG sites (Figure 1C).

NDRG2 gene copy number is not altered in colorectal cancer

We wished to determine the allelic copy numbers of both *NDRG2* and *MYC* in human colorectal carcinoma. By combining qPCR with the mathematical delta delta C_t equation (ddCt), we were able to quantify both losses and gains of these genes. Our experimental setup was validated by analysing the copy numbers of the X-chromosome linked *MECP2* gene in males and females - with the expected one and two X-chromosomes, respectively. As visualised in Figure 2, DNA from 3 females were scored with a ddCt value close to 1.00, which means that the same gene copy ratio between *MeCP2* and *GFAP* was present in both the analysed samples and the reference female genomic sample. A ddCt value of 1.00 therefore represents the normal two alleles. On the contrary, males displayed a ddCt value of approximately 0.50, which represents one allele. Finally, we tested our setup on an unknown sample clearly showing the pattern for male DNA. The conclusion was, therefore, that our setup clearly could differentiate between females and male, *i.e.*, one and two alleles, and has the potential to analyse the copy numbers of *NDRG2* and *MYC*.

We have previously published data showing a statistically significant down-regulation of *NDRG2* mRNA in CRC[3], and the main aim in the present study has therefore been to analyse if allelic loss of *NDRG2* could explain cases of decreased *NDRG2* mRNA levels. For a thorough investigation of *NDRG2,* we selected two regions of the genomic sequence of *NDRG2*, one lying in the 5′ part of the sequence and the other lying in the 3′ end. We first analysed the three colorectal cancer cell lines for both *NDRG2* and *MYC* and found no changes in the copy number of *NDRG2*, in contrast to *MYC*, for which we observed copy number loss in the LoVo cell line, the normal two alleles in DLD-1 cells and a clear copy number gain in SW-480 (Table 2). This latter result is in agreement with a previous study showing a 5 to 10-fold genomic amplification of *MYC* in SW-480 cells[36].

We next analysed 8 normal and 40 CRC tissue samples. In one case out of the eight normal samples, our data indicated copy number loss at the 5′ end of the *NDRG2* gene; otherwise, none of the samples showed any copy number alterations for *NDRG2* (Table 3). As summarised in Table 3, 29 out of the 40 CRC samples (72%) had an unaltered copy number, 2 samples showed loss at either the 5′ or the 3′ end of *NDRG2*, and only in one case did we observe loss at both ends of the gene. In contrast, we found complete copy number gain of *NDRG2* in 3 cases and partial gain in 9 cases (Supplementary Table S2).

Finally, we determined the copy numbers of *MYC* in the same 8 normal and 40 CRC samples, and observed one case of genomic amplification in the normal samples. Otherwise, we did not find any allelic changes in the normal samples (Table 3). For the 40 CRC samples, we observed copy number loss in 4 cases, the normal two copies in nearly half the cases (19 out of 40), and copy number gains of the *MYC* gene in the remaining 17 samples (42.5%) (Supplementary Table S1). However, the observed differences in copy number between normal and CRC tissue did not reach statistical significance (Mann-Whitney test, Table 3).

DISCUSSION

We and others have previously published data showing a statistically significant reduction in *NDRG2* mRNA levels in CRC compared to normal colorectal tissue samples[3,12,23]. Similar findings have been observed in other cancers including gliomas, hepatocellular carcinoma, breast cancer, thyroid cancer and meningioma[5-7,25,37]. Exactly how and why *NDRG2* expression is reduced is not fully understood, but repression by the MYC transcription factor is likely to be involved in some cases, just as promoter hypermethylation seems to play an important role[4,14,21,34]. Here, we show that 16 potential methylation sites in the proximal promoter of *NDRG2* are heavily methylated in all three colorectal cancer cell lines tested. Methylation of the analysed region from -426 to -107 could reduce accessibility to the transcription factors WT1 and HIF1α, which have binding sites in this region[38,39] and/or result in transcriptional silencing. In support of this, previous studies have shown that reversal of methylation by 5-aza-2′-deoxycytidine treatment leads to increased NDRG2 mRNA levels in the colorectal cancer cell lines CaCo2, HCT116 and SW480[34]. Furthermore,

Table 2 ddCt values and corresponding copy numbers for the *NDRG2* and *MYC* genes in colorectal cancer cell lines

Cell line	*NDRG2* - 5′ end		*NDRG2* - 3′ end		MYC	
	ddCt ± SD	Copy number	ddCt ± SD	Copy number	ddCt ± SD	Copy number
LoVo	1.23 ± 0.47	2	1.12 ± 0.51	2	0.91 ± 0.31	2
DLD-1	1.04 ± 0.23	2	1.08 ± 0.50	2	0.74 ± 0.22	Loss
SW-480	1.04 ± 0.26	2	0.94 ± 0.44	2	4.88 ± 0.30	Gain

Copy number loss is defined as ddCt < 0.75 and a gain is defined as ddCt > 1.25. ddCt: Delta delta Ct; SD: Standard deviation.

Table 3 Alteration in copy numbers for the *NDRG2* and *MYC* genes in colorectal tissue

Colorectal tissue	Number of samples	Loss ddCt < 0.75	Unaltered ddCt 0.75-1.25	Gain ddCt > 1.25	Normal *vs* CRC
NDRG2 - 5′ end					
Normal	8	1	7	0	
CRC	40	2	29	9	$P = 0.194^a$
NDRG2 - 3′ end					
Normal	8	0	8	0	
CRC	40	2	32	6	$P = 0.470^a$
MYC					
Normal	8	0	7	1	
CRC	40	4	19	17	$P = 0.135^b$

[a]*P* value for comparison of ddCt values (supplementary table S2) in normal and CRC samples using an unpaired two-tailed *t* test; [b]*P* value for comparison of ddCt values (supplementary table S2) in normal and CRC samples using a Mann-Whitney test. ddCt: Delta delta Ct.

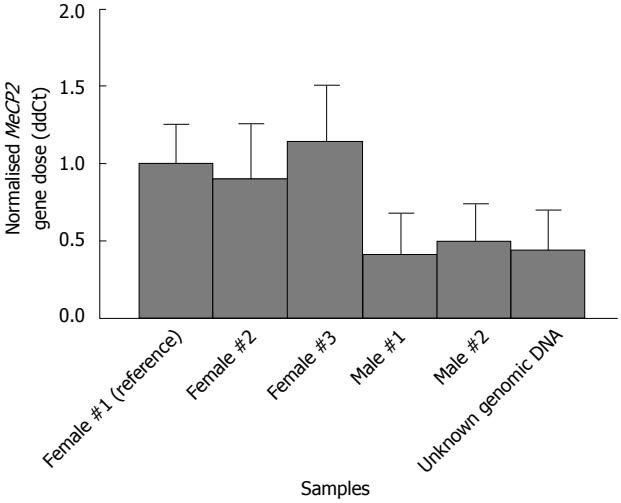

Figure 2 Validation of the gene copy number experimental setup. Bar diagram showing the calculated delta delta Ct values (ddCt) of the X-linked *MeCP2* gene normalised to *GFAP*, giving the expected result (one copy in males and two copies in females). A ddCt value of 1.00 in the reference female genomic sample represents the normal two alleles. Data are presented as mean (filled bars) and SD (whiskers).

DNA methylation at the *NDRG2* promoter was shown to be significantly higher in CRC tissue compared to normal colonic tissue from the same patients[14,34].

Our ChIP experiments on three colorectal cancer cell lines showed that endogenous MYC interacts with the *NDRG2* core promoter. Although MYC is considered a classical transcription factor, it is also involved in the maintenance of chromatin structure[40,41]. For example, MYC has been shown to recruit DNA methyltransferase 3a to the promoter region of a gene to exert its repressive activity[42]. Thus, we suggest that MYC could be involved in the regulation of *NDRG2* by recruitment of other proteins to produce an epigenetic silencing of *NDRG2*.

However, the suggested regulatory mechanisms cannot explain all cases of down-regulation of *NDRG2* expression, and we were therefore interested in looking at allelic loss to see if this genetic event could contribute to the decreased *NDRG2* mRNA levels observed in CRC. To investigate this question, we designed an experimental setup making it possible to quantify the copy numbers of any gene. In a validation experiment, we could easily differentiate between one or two copies of the X-chromosome linked gene *MECP2*. Our data indicate that allelic loss at the *NDRG2* locus is not very frequent in CRC. On the contrary, a subset of CRC cases showed gains of one or both ends of the *NDRG2* gene, which might lead to elevated levels of *NDRG2* mRNA. These findings were unexpected, since allelic losses in chromosome 14 are more frequently observed than gains[27,28]. Although we have only looked at copy number changes in CRC, our results might be applied to other cancers and could explain why we observed an increase in *NDRG2* levels in approximately 8% of 154 paired normal and tumor samples analysed from 19 different tumor types[25].

The proto-oncogene *MYC* is located on chromosome 8 at the q24.12 region, and several groups have shown amplification of chromosome 8q[27,28,43]. Indeed, we observed an increase in *MYC* gene copy numbers in nearly every second CRC sample, confirming a frequent gain at this particular gene locus. However, we did not detect the same high percentage of *MYC* amplification as a previous study focusing on the 8q24 region, which revealed that nearly 80% of the cases analysed had some kind of gene amplification[32]. Since MYC has the potential to repress *NDRG2* transcription[21], increased copy numbers of the *MYC* gene could lead to higher levels of MYC protein and thereby a reduced level of *NDRG2* mRNA.

Finally, copy number loss of the 5′ end of *NDRG2* and a gain of *MYC* were observed in separate normal samples and might indicate a rare, but real, genomic alteration in healthy tissue. An alternative explanation is that since all normal samples were obtained from patients diagnosed with CRC and classified as normal, the tissue might be at an early pre-malignant stage with

no visual changes, but where genetic abnormalities had already occurred.

In conclusion, we observed *NDRG2* promoter hypermethylation and interaction of endogenous MYC with the core promoter in three colorectal cancer cell lines, together with absent or low *NDRG2* mRNA expression. Frequent allelic loss was not found at the *NDRG2* locus in the colorectal cancer cell lines and tissue samples from either normal or tumor tissues. In contrast, we observed partial or complete NDRG2 copy number gains in more than 25% of the CRC cases, compared to none in the normal samples. We also found that more than 40% of CRC cases displayed *MYC* amplification, which indicates that the level of *MYC* mRNA is elevated in CRC. We conclude that epigenetic silencing and transcriptional repression by MYC are likely to be more important than copy number loss for the reduced levels of *NDRG2* mRNA observed in CRC.

COMMENTS

Background

A frequent change observed in colorectal carcinoma (CRC) is chromosomal instability, in which gain or loss of chromosomal regions affects levels of gene expression. Thus, loss of one or both alleles could explain the reduced expression of tumor suppressor genes, such as *NDRG2*, that is observed in CRC. Alternatively, *NDRG2* down-regulation could be due to transcriptional and epigenetic mechanisms.

Research frontiers

In order to understand the origin of CRC, it is important to investigate changes at the epigenetic, genetic and transcriptional level. This study investigated regulation of *NDRG2* gene expression using bisulfite-sequencing to study gene methylation, quantitative polymerase chain reaction to study gene copy number as well as chromatin immunoprecipitation to study DNA-binding of the endogenous gene-regulatory protein MYC.

Innovations and breakthroughs

This study shows for the first time that gene copy number for *NDRG2* is unaltered in CRC cell lines and clinical samples.

Applications

The authors describe a validated approach to determine gene copy number, relative to a control gene, using the comparative (ddCt) approach. Future approaches could focus on re-activating expression of *NDRG2* in CRC.

Terminology

NDRG2 is a newly described tumor suppressor gene that is down-regulated in a large range of cancers, including CRC. Interest in *NDRG2* as a therapeutic target is supported by studies showing a better prognosis in patients having higher *NDRG2* expression in tumor tissues.

Peer-review

The paper is very good.

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ORIGINAL ARTICLE

Prospective Study

Salient concerns in using analgesia for cancer pain among outpatients: A cluster analysis study

Salimah H Meghani, George J Knafl

Salimah H Meghani, Department of Biobehavioral Health Sciences, NewCourtland Center for Transitions and Health, Leonard Davis Institute of Health Economics, University of Pennsylvania, Philadelphia, PA 19104-4217, United States

George J Knafl, University of North Carolina at Chapel Hill School of Nursing, Chapel Hill, NC 27599, United States

Author contributions: Meghani SH designed the study, participated in the acquisition of the data, drafted the manuscript and interpreted the data; Knafl GJ conducted the data analysis, drafted the manuscript, and participated in the interpretation and presentation of the data; both authors revised the article critically for important intellectual content.

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Correspondence to: Salimah H Meghani, PhD, MBE, RN, FAAN, Associate Professor, Department of Biobehavioral Health Sciences, NewCourtland Center for Transitions and Health, Leonard Davis Institute of Health Economics, University of Pennsylvania, Claire M. Fagin Hall 418 Curie Boulevard, Room 337, Philadelphia, PA 19104-4217, United States. meghanis@nursing.upenn.edu
Telephone: +1-215-5737128
Fax: +1-215-5737507

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Abstract

AIM

To identify unique clusters of patients based on their concerns in using analgesia for cancer pain and predictors of the cluster membership.

METHODS

This was a 3-mo prospective observational study (n = 207). Patients were included if they were adults ($\geqslant$ 18 years), diagnosed with solid tumors or multiple myelomas, and had at least one prescription of around the clock pain medication for cancer or cancer-treatment-related pain. Patients were recruited from two outpatient medical oncology clinics within a large health system in Philadelphia. A choice-based conjoint (CBC) analysis experiment was used to elicit analgesic treatment preferences (utilities). Patients employed trade-offs based on five analgesic attributes (percent relief from analgesics, type of analgesic, type of side-effects, severity of side-effects, out of pocket cost). Patients were clustered based on CBC utilities using novel adaptive statistical methods. Multiple logistic regression was used to identify predictors of cluster

membership.

RESULTS

The analyses found 4 unique clusters: Most patients made trade-offs based on the expectation of pain relief (cluster 1, 41%). For a subset, the main underlying concern was type of analgesic prescribed, *i.e.*, opioid *vs* non-opioid (cluster 2, 11%) and type of analgesic side effects (cluster 4, 21%), respectively. About one in four made trade-offs based on multiple concerns simultaneously including pain relief, type of side effects, and severity of side effects (cluster 3, 27.5%). In multivariable analysis, to identify predictors of cluster membership, clinical and socioeconomic factors (education, health literacy, income, social support) rather than analgesic attitudes and beliefs were found important; only the belief, *i.e.*, pain medications can mask changes in health or keep you from knowing what is going on in your body was found significant in predicting two of the four clusters [cluster 1 (-); cluster 4 (+)].

CONCLUSION

Most patients appear to be driven by a single salient concern in using analgesia for cancer pain. Addressing these concerns, perhaps through real time clinical assessments, may improve patients' analgesic adherence patterns and cancer pain outcomes.

Key words: Cancer pain; Analgesia; Opioids; Preferences; Conjoint analysis; Side-effects

Core tip: Lack of adherence to analgesia for cancer pain is a prevalent clinical problem. The 2016 Centers for Disease Control and Prevention guidelines provide recommendations to clinicians for opioid prescription. However, this focus will be incomplete without understanding what concerns anchor patients' decisions to use analgesia for cancer pain. We used a trade-off analysis technique and novel adaptive methods to first show that unique clusters of patients exist based on the main concerns that anchor their preferences for analgesia for cancer pain. We then identified factors that predict membership in each preference cluster. We found that socioeconomic factors, including education, health literacy, income (rather than attitudes and beliefs about analgesics) played a role in predicting three out of four clusters. Most analgesic beliefs and concerns, including the widely indicated addiction concerns, did not predict cluster membership.

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INTRODUCTION

In the early part of 2016, the Centers for Disease Control and Prevention (CDC) released guidelines for prescribing opioids in chronic pain, including cancer pain beyond active cancer treatment[1]. While the guidelines are shaping a conversation and debate among professionals and policy makers on opioid prescription[2-4], little is known about the other side of the coin-patients' preferences that shape their analgesic taking behaviors. Cancer pain in the United States is mainly managed using analgesics[5]. Non-pharmacological pain treatment approaches are either not consistently offered to patients by their clinicians/covered by health insurance or lack data on clinical effectiveness[6-10]. For the treatments that have demonstrated clinical effectiveness, the cost burden for the patients may be excessive[11,12]. Thus, clinicians and oncologists rely on analgesics as well as opioid medications to help patients whose daily lives and function are affected by significant pain[11]. Unfortunately, patients with unrelieved chronic pain have some of the lowest quality of life observed for any medical condition[13].

Despite widespread use of analgesics in managing cancer pain, there is serious paucity of literature to understand the heuristics cancer patients may employ in making decisions to use analgesics. The few extant studies had methodological aims, that is to investigate the predictive validity of a trade-off analysis technique in eliciting analgesic preferences with diverse subgroups of patients with cancer pain[14]. Others investigating analgesic trade-offs included patients with cancer as part of the broader category of chronic pain sufferers[15]. Also, to our knowledge, no studies have investigated the sociodemographic and clinical predictors of patients' analgesic preferences. Thus, the purpose of this study was to investigate if unique clusters exist with regard to cancer patients' preference to use analgesics for cancer pain and factors predicting cluster membership.

MATERIALS AND METHODS

This was a prospective study conducted with a cohort of adult (18 years or older) patients who were diagnosed with solid tumors or multiple myelomas and had at least one prescription of around-the-clock pain medication for cancer or cancer-treatment-related pain. Patients were self-identified African-Americans and Whites and were recruited from two outpatient medical oncology clinics within a large health system in Philadelphia, United States. Data were collected at baseline and at 3-mo. This study was approved by the Institutional Review Board of the University of Pennsylvania. All patients provided written informed consent.

Measures

Analgesic concern: Analgesic preferences (utilities) for cancer pain was derived from a choice-based con-

joint (CBC) analysis experiment, which is a valuation technique based on the Random Utility Theory[16] and mathematical psychology[17]. The goal of CBC is to elicit what people value and what really drives them to choose one set of alternatives over another when facing competing choices[18]. CBC proposes that the overall utility or desirability of any good can be described based on the value of its separate, but, conjoined parts[19], which are termed "attributes". Each attribute may have multiple levels. Individuals are asked to make trade-offs between attributes and attribute levels generating a unique set of values called part-worth utilities. A higher part-worth utility represents a higher level of value or importance individuals assign to that attribute. The design of CBC experiments is tailored based on the needs of an individual study.

We used a systematic approach to designing the CBC study to elicit analgesic utilities reported in the present study. The procedures are detailed in a previously published manuscript[14]. Trade-offs were elicited on five analgesic attributes: (1) type of analgesic, (2) percentage pain relief with analgesics; (3) type of side-effects; (4) severity of side-effects; and (5) out-of-pocket cost of analgesics. In addition to the design components, we also investigated the internal, external predictive validity and temporal stability of the CBC experiment over the study period[14].

Analgesic attitudes and barriers: Barriers Questionnaire-II[20,21] was used to assess patients' attitudes and beliefs about the management of cancer pain. It is a 27-item measure which elicits patients' pain management concerns in eight domains: (1) fear of addiction; (2) fear of tolerance; (3) fear of side effects; (4) fatalism about cancer pain; (5) desire to be a good patient; (6) fear of distracting health provider from treating cancer; (7) fear that the analgesics impair the immune system; and (8) concern that analgesics may mask ability to monitor illness symptoms. The response range is from 0 (do not agree) to 5 (agree very much). The scores are based on sums for items for the total scale and four subscales (physiological, fatalism, communication, and harmful effects). The internal consistency reliability of the scale is excellent at 0.89[20].

Analgesic side-effects: Side-effects resulting from taking analgesics were assessed using the Medication Side-effects Checklist (MSEC). MSEC elicits information on the presence and severity of eight common analgesic side-effects (*i.e.*, constipation, drowsiness, nausea, vomiting, confusion, dry mouth, stomach irritation, itching) on a scale of 0-10 (no severity-extreme severity). The internal consistency reliability is 0.80[21].

Pain severity and pain-related function: The Brief Pain Inventory (BPI) was used to assess pain severity. The BPI has two subscales; pain intensity (4-items) and pain-related functional interference (7-items: General activity, mood, walking ability, normal work, relationships, sleep and enjoyment of life)[22]. Each item is scored on a 0-10 scale (0 = no pain and 10 = pain as bad as you can imagine; and 0 = no interference and 10). The psychometric properties of the BPI are well-established with cancer patients with a Cronbach's alpha that ranges from 0.77 to 0.91[23,24].

Pain management index: Pain management index (PMI) is a measure of adequacy of pain treatment based on the World Health Organization's (WHO) guidelines for managing cancer-related pain[25,26]. The measure takes into account the most potent analgesic prescribed to patients relative to the level of their reported pain. PMI is calculated by subtracting patient's "pain worst" score (from BPI coded as mild, moderate, or severe) from the most potent analgesia prescribed based on the 3-step WHO analgesic ladder. A negative PMI means inadequate analgesic prescription relative to the pain level.

Social support questionnaire: A 6-item instrument was used to measure participants' perceptions of social support and satisfaction with social support[27]. The first part of the question asks participants to list individuals who provide social support and the second part asks them to indicate the level of satisfaction with this support. This questionnaire is an abridged version of the original 27-item Social Support Questionnaire[27].

Prescribed analgesics: Prescribed analgesics were coded according to the WHO analgesic ladder[25,26]. This included step 1 (non-opioid analgesics); step 2 (weak opioid analgesics such as codeine); and step 3 (strong opioids such as morphine, oxycodone, methadone).

Sociodemographic and clinical variables: Sociodemographic data were gathered on age, gender, self-identified race, marital status, education, health insurance, household income, job status and health literacy. Health literacy was assessed using three brief screening questions that were previously validated[28] and performs well against the widely used Test of Functional Health Literacy in Adults[28]. The brief questions were also found to be effective in identifying inadequate health literacy (areas under the receiver operating characteristic curve of 0.87, 0.80 and 0.76, respectively for the three questions).

Clinical variables (collected from patients' medical records) included stage of cancer, time since cancer diagnosis, past history of drug or substance abuse, comorbidities to compute the Charlson Comorbidity Index[29], presence of chronic kidney disease, and presence of depression. Pain and treatment related variables included total number and types of analgesics and co-analgesics, most potent analgesic prescribed, hours pain medications are effective, and pain relief with analgesics.

Statistical analysis

Descriptive statistics were generated for available baseline variables. A wide variety of variables were considered within the four categories of sociodemographic; illness; pain, function and pain treatment; and analgesic attitudes and barriers. Patients were clustered on their responses to the five analgesic attributes determined by the CBC analysis using the adaptive statistical methods of Knafl *et al*[30]. A variety of clustering procedures and numbers of clusters were considered, but restricted to alternatives with each cluster containing at least 10% of the patients, thereby avoiding sparse clusters. A clustering alternative was selected using likelihood cross-validation (LCV) scores with likelihoods based on mixtures of multivariate normal distributions as commonly used in cluster analysis.

Models were evaluated and compared using 10-fold LCV scores. These were computed by first randomly partitioning the data into 10 disjoint subsets, called folds. Likelihoods were then computed for the data in each fold using parameter estimates computed from the data in the other folds. These deleted fold likelihoods were combined over all the folds into a LCV score.

A larger LCV score indicates a better model for the data but not necessarily a distinctly better model. This issue was addressed using LCV ratio tests, based on the χ^2 distribution (and so analogous to standard likelihood ratio tests). These tests were expressed in terms of a threshold for a distinct (or substantial or significant) percent change in the LCV scores. A percent decrease larger than the threshold indicates that the model with the larger LCV score provides a distinct improvement over the model with the smaller score. Otherwise, the model with the smaller score is a competitive alternative, and if also simpler then preferable as a parsimonious, competitive alternative. The threshold changes with the sample size.

The indicators for being in each of the CBC clusters were modeled separately using logistic regression. This approach allows for identification of a different set of predictors for each cluster and so was considered preferable to multinomial regression modeling of membership in all four clusters combined since that would use the same predictors for all clusters. Each available baseline variable was used to adaptively identify an associated binary characteristic for predicting being in a CBC cluster by dichotomizing the associated variable's values and choosing the dichotomization that maximized the LCV score (with likelihoods based on the Bernouilli distribution as appropriate for logistic regression). Only dichotomizations with both sets of values having at least 10% of the data were considered to avoid sparse cases. The binary characteristic was defined using the indicator variable with value 1 for the set of values generating an odds ratio (OR) > 1. This indicator was conservatively set to 0 for missing variable values if there were any. The total BQ-Ⅱ along with each of its subscales and items were considered as predictors to provide a broad assessment of the impact of analgesic attributes and barriers on the analgesic preferences (CBC types or clusters).

Dichotomization can sometimes result in loss of predictive capability compared to using the associated variable as an unadjusted predictor. This can be assessed for ordinal and continuous variables by comparing LCV scores for models based on those variables to the models based on the associate binary characteristics, but only when there are no missing values. LCV ratio tests can be used to assess whether binary characteristics provide a distinct improvement or not by comparing their LCV scores to the score for the constant model (*i.e*., with only an intercept).

An adaptive multiple binary characteristics model was generated for each CBC-cluster indicator based on the binary characteristics that were individually significantly ($P < 0.05$) related to it in bivariate models using standard Wald χ^2 tests. The adaptive modeling process[31] is based on a heuristic search guided by LCV scores through alternative models. First, the model is systematically expanded adding in predictors, in this case binary characteristics, to the model. The expanded model is then contracted to remove extraneous predictors. LCV ratio tests are used to decide when to stop the contraction, leaving the adaptively generated model. This modeling process is implemented in a SAS® (SAS Institute Inc., Cary, NC) macro available upon request from G. Knafl. All results were computed in SAS Version 9.4.

Biostatistics statement

The statistical methods of this study were reviewed by Dr. George Knafl, Biostatistician and Professor in the School of Nursing at the University of North Carolina at Chapel Hill.

RESULTS

Complete data were available for 207 patients (Figure 1). The baseline demographic and illness related data are presented in Tables 1 and 2, respectively. The mean age of the respondents was 54 years (SD = 11). More than half were married (53%) and had college or more than college education (64%). About one-third (35%) reported a household income of less than $30000 year. None of the patients had any missing CBC analgesic attribute values. Only three of all these variables had any missing values. The threshold for a distinct percent change in LCV score for data with 207 observations is 0.92% (in contrast, the percent decrease is 2.00% for 95 observations and 1.00% for 190 observations).

Unique analgesic preference clusters

Using methods described (see data analysis), a 4-cluster solution was chosen. Figure 2 contains plots of the four cluster centroids, that is, the vectors with entries equal to averages of the five CBC analgesic attributes for patients in the clusters. Based on these plots, the clusters were characterized in terms of the more

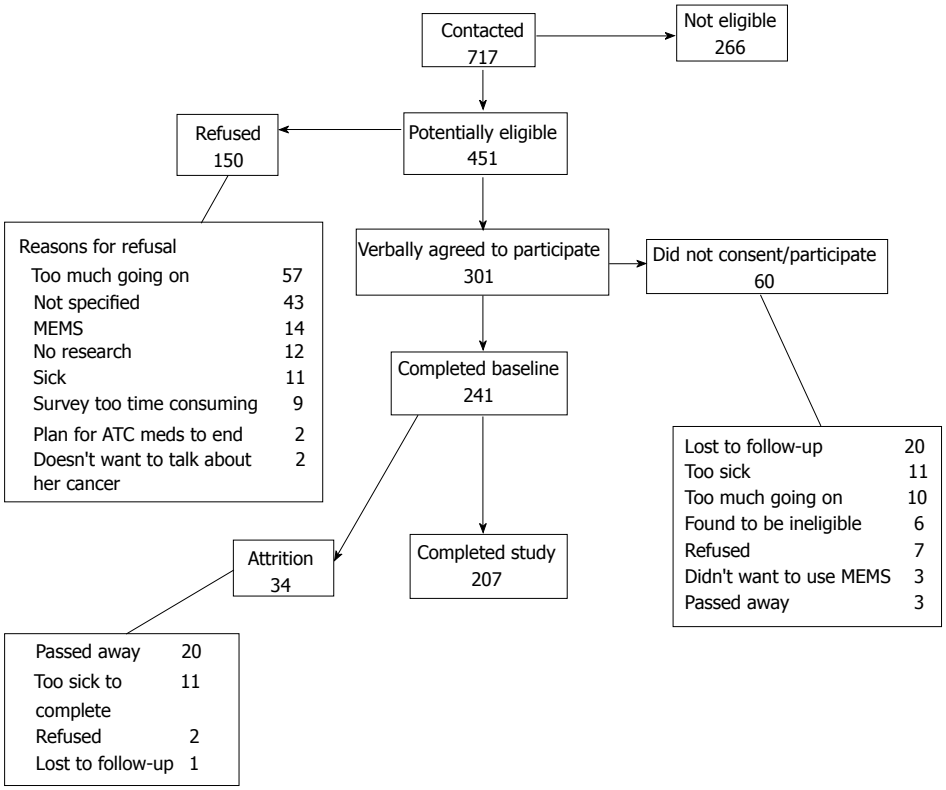

Figure 1 Participant recruitment flow diagram. MEMS: Medication Event Monitoring; ATC: Around-the-clock.

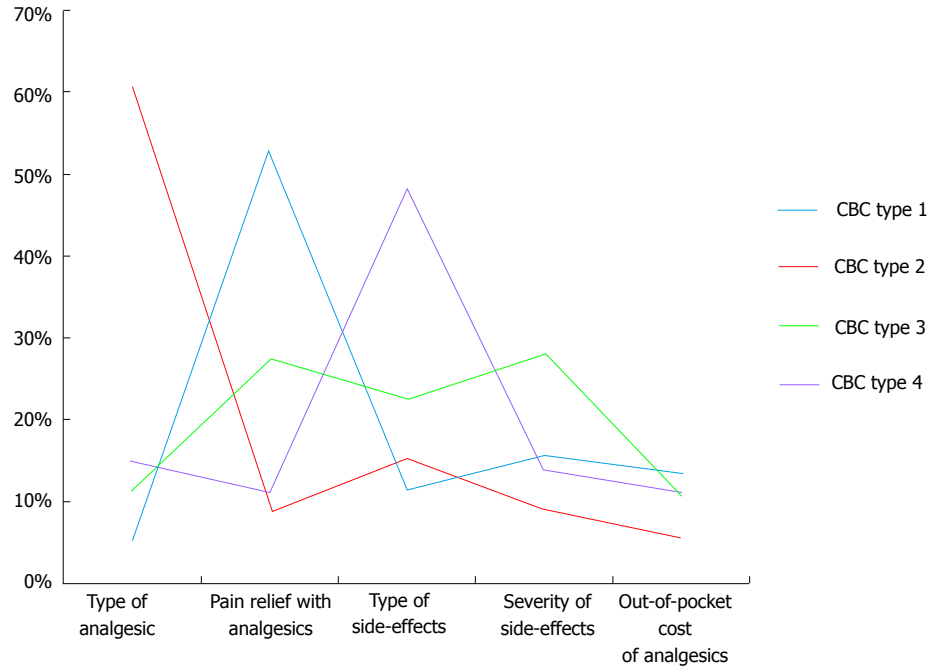

Figure 2 Choice-based conjoint analgesic attribute types. CBC: Choice-based conjoint.

strongly rated analgesic attributes (Table 3).

Cluster 1 (pain relief)

For less than half the patients (41%) in this study, expectation of pain relief was the main anchor in making analgesic related trade-offs for cancer pain. A total of 16 individually significant binary characteristics were identified for patients in this cluster (Supplemental Table 1). Patients in cluster 1 were more likely be White/Caucasians, carried a private health insurance, had higher education and health literacy, and reported less analgesic-related barriers in general. The strongest of

Table 1 Baseline sociodemographic variables (*n* = 207)

Variable	Range	*n* (%)[1]	Mean (SD)
Age	23-75		53.8 (11.1)
Education	Elementary	3 (1.5)	
	High school	70 (33.8)	
	College/Trade school	101 (48.8)	
	More than college	33 (15.9)	
Employment status	Employed outside home (full-time)	43 (20.8)	
	Employed outside home (part-time)	12 (5.8)	
	Employed at home (full-time)	4 (1.9)	
	Employed at home (part-time)	4 (1.8)	
	Retired	44 (21.3)	
	Unemployed	25 (12.1)	
	Other	75 (36.2)	
Health literacy	3-15		13.1 (2.6)
Income	< $10000	28 (13.5)	
	$10000-$20000	26 (12.6)	
	$20000-$30000	19 (9.2)	
	$30000-$50000	36 (17.4)	
	$50000-$70000	37 (17.9)	
	$70000-$90000	24 (11.6)	
	> $90000	37 (17.9)	
Primary insurance (1 missing)	Private	107 (51.9)	
	Medicare	41 (19.9)	
	Medicaid	27 (13.1)	
	Multiple	25 (12.1)	
	VA/other	6 (2.9)	
Marital status	Married	110 (53.1)	
	Separated/Divorced	48 (23.2)	
	Widowed	8 (3.9)	
	Never married	41 (19.8)	
Race	Black/African American	86 (41.5)	
	White/Caucasian	121 (58.5)	
Social support	0.17-9.00		3.7 (2.1)

[1]No missing values unless otherwise indicated. SD: Standard deviation; VA: Veterans Administration.

Table 2 Baseline illness and pain variables (*n* = 207)

Variable	Range	*n* (%)[1]	Mean (SD)
Cancer stage	I	20 (9.7)	
	II	33 (15.9)	
	III	37 (17.9)	
	IV	64 (30.9)	
	Unknown or unsure	53 (25.6)	
Time since cancer diagnosis	1-120 mo		36.7 (35.5)
Charlson comorbidity index	0-13		4.3 (2.6)
General health	Excellent	9 (4.3)	
	Very good	23 (11.1)	
	Good	63 (30.4)	
	Fair	77 (37.2)	
	Poor	35 (16.9)	
Physical health not good (number of days within last 30 d)	0-30		14.7 (10.7)
Mental health not good (number of days within last 30 d)	0-30		9.5 (10.7)
Past history of substance abuse	No	172 (83.1)	
	Yes	35 (16.9)	
Presence of depression	No	120 (58.0)	
	Yes	87 (42.0)	
Worst pain (last week)	0-10 (no pain - pain as bad as you can imagine)		6.9 (2.4)
Average pain (last week)	0-10 (no pain - pain as bad as you can imagine)		4.9 (2.1)
Least pain (last week)	0-10 (no pain - pain as bad as you can imagine)		3.4 (2.0)
Pain-related functional interference score	7-70 (does not interfere-completely interferes)		35.2 (15.9)
Pain relief with medications (last week)	1-10 (10%-100%)		7.2 (2.1)
Pain management index	-2	5 (2.4)	
	-1	13 (6.3)	
	0	92 (44.4)	
	1	63 (30.4)	
	2	31 (15.0)	
	3	3 (1.4)	
Number of analgesic side effects (MSEC)	0-8		3.8 (2.4)
Severity of analgesic side effects (MSEC)	8-80 (not severe-extremely severe)		25.2 (15.0)
BQ-II analgesic barriers (total)	0-96		39.8 (20.1)
No. of complementary alternative modalities used	0-8		2.1 (1.7)

[1]No missing values unless otherwise indicated. BQ-II: Barriers questionnaire; MSEC: Medication Side-effects Checklist; SD: Standard deviation.

these predictors, that is, the one generating the best (largest) LCV score, was lower endorsement of the belief that pain medicine can mask changes in your health with LCV score 0.51908 (LCV scores not reported).

The individually significant binary characteristics were adaptively combined into a multiple logistic regression model (Table 4). The three factors that remained in the multiple risk factor model and predicted membership in cluster 1 included, higher education, poor physical health and a lower endorsement of the belief that pain medications can mask changes in health. The most important of these (*i.e.*, the one whose removal generated the lowest LCV score) was BQ-II item, pain medicine can mask changes in your health. The LCV score was 0.53503, and so this model provided a distinct improvement over the best individual binary characteristic model with percent decrease 2.98% (since this was larger than the threshold of 0.92%).

Cluster 2 (type of analgesic)

For only 11% of patients in this study, the main anchor for analgesic trade-offs was "type of analgesic". A total of 15 individually significant binary characteristics were identified for patients in cluster type 2 (Supplemental Table 2). Patients in cluster 2 were more likely to have lower income, lower social support, greater burden of comorbidities and pain, and lower relief from taking pain medications. Patients in this cluster were more likely to hold beliefs such as pain medications can harm immune system, or make you addicted. However, the strongest

Table 3 Description of analgesic preference clusters (n = 207)

Cluster	n (%)	Salient concern(s)
1	84 (40.6)	Pain relief
2	23 (11.1)	Type of analgesic
3	57 (27.5)	Pain relief, type of side-effects and severity of side-effects
4	43 (20.8)	Type of side-effects

of these predictors was lower (≤ $50000) income with LCV score 0.71212 (LCV scores not reported).

In the multiple logistic regression model, lower social support, health literacy and income levels were predictive of membership in this cluster (Table 5). The most important of these was health literacy (LCV score was 0.72894), and so this model provided a distinct improvement over the best individual binary characteristic model with percent decrease 2.31%.

Cluster 3 (pain relief, type of side-effects and severity of side-effects)

More than one in four patients (28%) made trade-offs based on multiple factors including expectation of pain relief, type of side-effects, and severity of side-effects. A total of 18 individually significant binary characteristics were identified for patients in cluster 3 (Supplemental Table 3). Patients in this cluster were more likely to be married, had greater social support, reported lower pain and pain related functional impairment, and greater pain relief with analgesics. They were less likely to report analgesic side-effects and had lower endorsement for BQ items indicating lower attitudinal barriers. The strongest of these predictors was lower average pain (≤ 6) in the last week with LCV score 0.56530 (LCV scores not reported). In the multiple logistic regression model, being married, having greater social support, having lower average pain, lower side-effects predicted membership in cluster 3 (Table 6).

Cluster 4 (type of side-effects)

For one in five patients (21%), type of side-effects experienced was the main factor driving analgesic trade-offs. A total of 21 individually significant binary characteristics were identified for patients in cluster type 4 (Supplemental Table 4). Patients in this cluster had lower education and health literacy, were more likely to be Blacks/African Americans, reported lower relief with medications and reported shorter duration of relief with pain medications. Patients in this cluster were more likely to report greater severity of analgesic side-effects and past history of substance abuse but fewer number of days when mental health was not good. Patients in this cluster had the highest number of BQ barriers than any other cluster.

In the multiple logistic regression model, four factors including, lower health literacy, mental health, more analgesic side effects, and belief that pain medications keep you from knowing what is going on in your body predicted membership in this cluster (Table 7).

DISCUSSION

This is the first study to identify the sociodemographic and clinical predictors of unique clusters based on what may drive patients' preference for analgesic treatment for cancer pain. Lack of adherence to analgesia for cancer pain is a prevalent clinical problem[32-35]. Studies in cancer[35] and non-cancer[36-43] pain settings suggest that patterns of analgesic adherence are consequential in explaining clinical and health services outcomes. The 2016 CDC guidelines provide recommendations to clinicians for opioid prescription[1]. However, this focus will be incomplete without an understanding of how patients take prescribed analgesics and what salient concerns anchor their decisions. Previous studies have documented correlates of non-adherence to analgesia for cancer pain[44-47]. These studies do not allow discerning how risk factors and predictors may be distributed dissimilarly across subgroups of cancer patients. Using a well-established trade-off analysis technique (CBC) and more novel adaptive methods, we first showed that unique clusters of patients exist based on the main concern(s) anchoring their preferences for analgesia for cancer pain. We then identified sociodemographic and clinical factors that predict membership in each preference cluster.

Importantly, for an overwhelming majority in this study, analgesic preference for cancer pain was driven by a single salient underlying concern (see cluster 1, 2 and 4). In multivariable analysis to identify predictors of these clusters, "clinical" and "socioeconomic factors" (rather than attitudes and beliefs) were found important. Of note, at least one socioeconomic factor (including education, health literacy, income) played a role in predicting three out of four preference clusters. Furthermore, most analgesic beliefs and concerns, including the widely implicated addiction concerns, did not play a role as predictors of cluster membership. Only the belief that pain medications can mask changes in health or keep you from knowing what is going on in your body was found significant in predicting two of the four clusters. This is a common clinical concern among cancer patients and relates to the fear of disease progression[48-50].

An interesting finding was the contrast between cluster 1 and 4. Unlike cluster 1 (pain relief), those in the side-effects cluster (cluster 4) had lower health literacy and greater analgesic barriers using BQ-II questionnaire. Patients in this cluster were more likely to report greater burden of analgesic side-effects. Of note, there is a stark difference in the identified correlates of these two clusters. The correlates of cluster 1 included being white/Caucasian and having higher education, income and health literacy and lower analgesic barriers. Cluster 4, however was predicted by being African Americans and having lower education, literacy, and

Table 4 Multiple binary characteristics model for cluster 1 (pain relief)

Variable domain	Variable	Characteristic	*n* (% out of 207)	*P* value	OR	95%CI
Sociodemographic	Education	College/trade school or more than college *vs* Elementary or High school	134 (64.7)	0.001	3.88	1.75-8.59
Illness	Physical health not good (number of days within last 30 d)	⩾ 22 *vs* < 22	59 (28.5)	0.002	2.81	1.47-5.38
Pain, function and pain treatment	NS					
Analgesic attitudes and barriers	BQ-II item - pain medicine can mask changes in your health	⩽ 3 *vs* > 3	158 (76.3)	0.016	2.26	1.17-4.36

BQ-II: Barriers questionnaire II; CI: Confidence interval; OR: Odds ratio; NS: None significant.

Table 5 Multiple binary characteristics model for cluster 2 (type of analgesic)

Variable domain	Variable	Characteristic	*n* (% out of 207)	*P* value	OR	95%CI
Sociodemographic	Health literacy	= 15 *vs* < 15	93 (44.9)	0.006	3.86	1.46-10.2
	Income	⩽ $50000 *vs* < $50000	109 (52.7)	0.017	3.64	1.26-10.5
	Social support	⩽ 4.17 *vs* > 4.17	137 (66.2)	0.027	4.25	1.18-15.4
Illness	NS					
Pain, function and pain treatment	NS					
Analgesic attitudes and barriers	NS					

CI: Confidence interval; OR: Odds ratio; NS: None significant.

Table 6 Multiple binary characteristics model for cluster 3 (pain relief, type of side-effects and severity of side-effects)

Variable domain	Variable	Characteristic	*n* (% out of 207)	*P* value	OR	95%CI
Sociodemographic	Marital status	Married *vs* Separated, Divorced, Widowed or Never married	110 (53.1)	0.023	2.26	1.12-4.56
	Social support	⩾ 1.83 *vs* < 1.83	177 (85.5)	0.022	4.55	1.24-16.7
Illness	Mental health not good (number of days within last 30 d)	⩾ 2 *vs* < 2	140 (67.6)	0.002	3.46	1.55-772
Pain, function and pain treatment	Average pain (last week)	⩽ 6 *vs* > 6	163 (78.7)	0.01	4.41	1.42-6.86
	Severity of analgesic side effects (MSEC)	⩽ 28 *vs* > 28	133 (64.3)	0.005	3.11	1.41-6.86
Analgesic attitudes and barriers	NS					

CI: Confidence interval; OR: Odds ratio; MSEC: Medication Side-effects Checklist; NS: None significant.

Table 7 Multiple binary characteristics model for cluster 4 (type of side-effects)

Variable domain	Variable	Characteristic	*n* (% out of 207)	*P* value	OR	95%CI
Sociodemographic	Health literacy	⩽ 13 *vs* > 13	84 (40.6)	0.004	3.11	1.43-6.76
Illness	Mental health not good (number of days within last 30 d)	⩽ 12 *vs* > 12	144 (69.6)	0.001	6.18	2.06-18.5
Pain, function and pain treatment	Severity of analgesic side effects (MSEC)	⩾ 40 *vs* < 40	37 (17.9)	0.002	4.19	1.68-10.5
Analgesic attitudes and barriers	BQ-II item - pain medicine can keep you from knowing what's going on in your body	⩾ 4 *vs* < 4	42 (20.3)	< 0.001	5.25	2.32-11.9

BQ-II: Barriers questionnaire; MSEC: Medication Side-effects Checklist; CI: Confidence interval; OR: Odds ratio.

more analgesic barriers. Another interesting noteworthy contrast between the two clusters (1 and 4) was that in the multiple logistic regression models, individuals in cluster 1 (pain relief) were less likely to believe that pain medications can mask changes in your health whereas patients in cluster 4 were more likely to endorse pain can keep you from knowing what is going on in your body. Thus, literacy and analgesic beliefs appear to be

at play in different ways in the two clusters.

Previous studies have investigated and found racial and socioeconomic disparities in pain management in general, including cancer pain management[51-54]. Our findings indicate that analgesic side-effects are also poorly treated in cancer patients with lower health literacy. These patients will benefit from meticulous assessment of pain and symptoms and accessible interventions that promote self-advocacy and negotiation of pain and side-effects management with their clinicians and oncologists.

In the last few decades, significant resources have been devoted towards psychoeducational interventions that have a major focus on dismantling analgesic beliefs and barriers[20,55,56]. Unfortunately, a number of systematic reviews show that these interventions do not improve adherence to analgesia for cancer pain or cancer pain outcomes[57,58]. Our findings imply that meticulous assessment of clinical factors such as pain levels, analgesic side-effects, and addressing SES factors (such as health literacy) may play a role in improving cancer pain outcomes. Also, the finding that decision-making for most patients was driven by single salient underlying factor raises an exciting possibility of designing two-part interventions focused on eliciting real-time trade-offs and linking real-time preferences sequentially to brief, tailored, and patient-centered clinical interventions.

Study limitations

The clusters identified in this study are based on the CBC design. While CBC is a well-established method and we previously tested the validity of the CBC utilities used in this study, there is a notable consideration. About 1 in 3 patients used lexicographic decision rules (*i.e.*, unwillingness to trade more or less of one attribute in favor or detriment of the other)[14]. These processes may represent patients' actual preferences or mental shortcuts to get through the CBC exercise, potentially compromising the clinical validity of the data. Our confidence that the clusters represent actual preferences is enhanced by the study findings. For instance, patients in cluster 4 (side effects) were more likely to report greater burden of analgesic side-effects, which remained significant in the multivariable model. Similarly, patients in cluster 3 weighed multiple factors similarly (pain relief, type and severity of side-effects) possibly because of their experience of lower pain severity and lower burden of side-effects (*e.g.*, MSEC < 28 in cluster 3 *vs* > 40 in cluster 4). These findings increase confidence that the clusters identified in this study represent actual preferences rather than mental shortcuts. Also, we restricted our analysis to those patients who completed the study to avoid having missing data that may have affected the conclusions of the study. Excluded patients were with advanced illness who died or were too sick to complete the study (Figure 1), thus we caution against generalizing the findings to those with advanced illness. Nevertheless, our findings inform a scarce body of literature on what anchors cancer patients' preferences in using analgesia for cancer pain and a potential new path to brief, tailored, and accessible interventions to improve pain and functional outcomes among cancer patients.

COMMENTS

Background

The purpose of this study was to investigate if unique clusters exist with regard to patients' concerns in using analgesics for cancer pain and factors predicting cluster membership.

Research frontiers

The new Centers for Disease Control and Prevention opioid guidelines are shaping a national conversation among professionals and policy makers on opioid prescription. Little is known about the other side of the coin, *i.e.*, cancer patients' concerns in using analgesia and factors shaping these concerns and preferences that may relate to their analgesic taking patterns. This study fills this important gap.

Innovations and breakthroughs

The authors employed novel statistical methods to understand unique subgroups of patients based on their concerns in using analgesics for cancer pain and identified sociodemographic and clinical correlates of these unique clusters. In recent decades, significant resources have been committed to psychoeducational interventions that have a major focus on dismantling analgesic beliefs and barriers. However, recent systematic reviews show that psychoeducational interventions do not consistently improve adherence to analgesia for cancer pain or cancer pain outcomes. The authors' findings suggest that careful assessment of clinical factors such as analgesic side-effects and addressing social determinants, such as patients' health literacy, may play a role in improving cancer pain outcomes.

Applications

The authors' study finding that decision-making for most patients was driven by single salient underlying factor raise an exciting possibility of designing two-part interventions focused on eliciting real-time trade-offs and linking real-time preferences sequentially to brief, tailored, and patient-centered clinical interventions.

Terminology

Analgesic concerns and preferences in this study were elicited using choice-based conjoint (CBC) analysis, which is a trade-off analysis technique. Individuals are asked to make trade-offs between attributes (*e.g.*, pain relief, side-effects) and attribute levels (*e.g.*, percent pain relief, severity of side-effects) generating a unique set of values called part-worth utilities. A higher part-worth utility represents a higher level of value or importance an individual assign to that attribute.

Peer-review

The paper contributes important information.

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CASE REPORT

Intermittent facial spasms as the presenting sign of a recurrent pleomorphic adenoma

Rosalie A Machado, Sami P Moubayed, Azita Khorsandi, Juan C Hernandez-Prera, Mark L Urken

Rosalie A Machado, Sami P Moubayed, Mark L Urken, Thyroid, Head and Neck Cancer Foundation, New York, NY 10003, United States

Rosalie A Machado, Sami P Moubayed, Mark L Urken, Department of Otolaryngology-Head and Neck Surgery, Mount Sinai Beth Israel, New York, NY 10003, United States

Azita Khorsandi, Department of Radiology, Mount Sinai Beth Israel, New York, NY 10003, United States

Juan C Hernandez-Prera, Department of Pathology, Mount Sinai Beth Israel, New York, NY 10003, United States

Author contributions: Urken ML designed the report; Machado RA, Moubayed SP, Khorsandi A and Hernandez-Prera JC wrote the manuscript; Urken ML made final edits to the manuscript.

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Correspondence to: Rosalie A Machado, Research Associate, Thyroid Head and Neck Cancer Foundation, 10 Union Square E, New York, NY 10003,
United States. rmachado@thancfoundation.org

Telephone: +1-212-8446441
Fax: +1-212-8448465

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Abstract

The intimate anatomical relationship of the facial nerve to the parotid parenchyma has a significant influence on the presenting signs and symptoms, diagnosis and treatment of parotid neoplasms. However, to our knowledge, hyperactivity of this nerve, presenting as facial spasm, has never been described as the presenting sign or symptom of a parotid malignancy. We report a case of carcinoma arising in a recurrent pleomorphic adenoma of the left parotid gland (*i.e.*, carcinoma *ex pleomorphic* adenoma) that presented with hemifacial spasms. We outline the differential diagnosis of hemifacial spasm as well as a proposed pathophysiology. Facial paralysis, lymph node enlargement, skin involvement, and pain have all been associated with parotid malignancies. To date the development of facial spasm has not been reported with parotid malignancies. The most common etiologies for hemifacial spasm are vascular compression of the ipsilateral facial nerve at the cerebellopontine angle (termed primary or idiopathic) (62%), hereditary (2%), secondary to Bell's palsy or facial nerve injury (17%), and hemifacial spasm mimickers (psychogenic, tics, dystonia, myoclonus, myokymia, myorthythmia, and hemimasticatory spasm) (17%). Hemifacial spasm has not been reported in association with a malignant parotid tumor but must be considered in the differential diagnosis of

this presenting symptom.

Key words: Facial spasm; Pleomorphic adenoma; Benign mixed parotid tumor; Reconstructive surgery; Salivary glands

Core tip: This report represents the first case of hemifacial spasm associated with transformation of a recurrent pleomorphic adenoma into a carcinoma *ex pleomorphic* adenoma. The causation of hemifacial spasms is discussed.

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INTRODUCTION

The intimate anatomical relationship of the facial nerve to the parotid gland has a significant influence on the symptoms/signs, diagnosis, and treatment of parotid neoplasms[1]. Involvement of the facial nerve by parotid malignancies usually results in partial or total hemifacial paralysis[2]. However, to our knowledge, hyperactivity of this nerve presenting as facial spasm has not been reported as the presenting feature of a malignant parotid tumor. Facial spasm has nonetheless been reported twice in the literature as a presenting feature of a benign parotid tumor[3]. We report a case of carcinoma arising in recurrent pleomorphic adenoma (*i.e.,* carcinoma *ex pleomorphic* adenoma) that presented with hemifacial spasms. We outline the differential diagnosis of hemifacial spasm as well as a proposed pathophysiology.

This is a single institutional case report in a tertiary referral hospital. Institutional Review Board was not required to report one case at our institution.

CASE REPORT

A 56-year-old female smoker had a history of a pleomorphic adenoma in the left parotid gland treated with a superficial parotidectomy at the age of 18. Nineteen years following that surgery, the patient presented with multifocal recurrence. Surgical exploration was undertaken and the tumor was found inseparable from the facial nerve. At that time, the resection was abandoned and the facial nerve was not sacrificed and gross disease was left in the parotid bed. The patient underwent external beam radiation therapy and the size of the tumor remained stable for 10 years on serial computed tomography (CT) and magnetic resonance imaging (MRI) monitoring. The patient had been clinically asymptomatic until she started developing intermittent ipsilateral hemifacial spasms occurring spontaneously and involving all portions of the left facial musculature, which prompted her to return for evaluation.

Repeat CT scan showed enlargement of avidly and uniformly enhancing solid tumor without areas of necrosis or extracapsular extension with extension into the left stylomastoid foramen, along with suspicious changes in enlarged (15 mm) left level IV lymph node (Figure 1A). Fine-needle aspiration biopsy of the tumor was suspicious for carcinoma *ex pleomorphic* adenoma. After a negative systemic metastatic work-up, the patient was brought to the operating room for a radical parotidectomy with facial nerve sacrifice, ipsilateral selective neck dissection (levels I-IV), and a de-epithelialized anterolateral thigh free flap for volume restoration and to enhance wound healing. The vertical segment of the facial nerve in the mastoid was exposed. Primary facial nerve repair was performed using sural nerve grafting from the main trunk to the temporal branch of the facial nerve, nerve to masseter grafting to the dominant midfacial branches of the facial nerve, together with construction of an oral commissure suspension with a fascia lata sling.

Final surgical pathology confirmed a 5.2 cm pleomorphic adenoma with a multinodular growth pattern. Well-circumscribed neoplastic nodules of variable sizes were embedded in densely fibrotic connective tissue (Figure 2). Nerve bundles were also entrapped in the scar tissue in-between the nodules, but no true perineural invasion was detected. Within the nodules, two foci of early non-invasive carcinoma were noted. Within one nodule a 4 mm focus of malignant cells surrounded by benign epithelial elements was identified. In a separate nodule, an intraductal malignant neoplastic proliferation with an intact benign myoepithelial cell rim was also noted. None of the malignant neoplastic foci showed invasion into adjacent fibroadipose tissue and nerves. Thirteen level II-V lymph nodes were negative for tumor involvement. The primary tumor was staged as rT4N0M0.

The hemifacial spasms subsided after surgery, and the patient remains disease free at 6 mo of follow-up. The patient has recovered facial tone but has yet to develop dynamic muscular activity.

DISCUSSION

Zbären *et al*[4] reported that pleomorphic adenomas comprised 60% of all of their benign and malignant parotid neoplasms**.** When left untreated, pleomorphic adenoma has a malignant transformation risk of 5% to 25% over a span of 15-20 years[5]. The risk of recurrence after primary superficial parotidectomy is 2%-5%[4], and malignant change in recurrent pleomorphic adenomas has an incidence of 2%-24%[6].

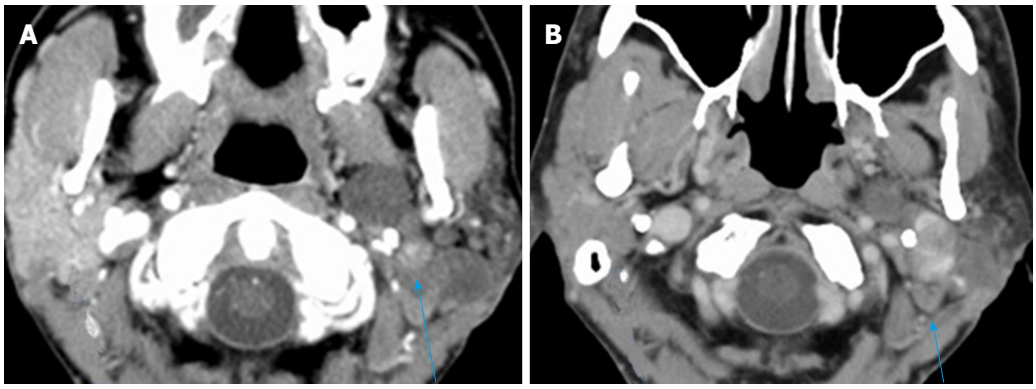

Figure 1 Axial computed tomography of the neck with contrast demonstrates oval shaped enhancing lesion of the left parotid gland deep to the left ramus of the mandible, centered at the left stylomandibular tunnel. A: The lesion measured 9 mm × 7 mm × 8 mm in 2007; B: The lesion measured 3.1 cm × 2.8 cm × 4.5 cm in 2015.

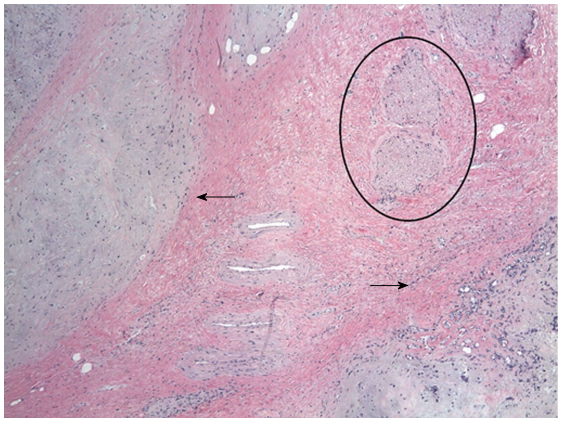

Figure 2 A 5.2 cm pleomorphic adenoma (circle) with a multinodular growth pattern and well-circumscribed neoplastic nodules with variable sizes were embedded in fibroadipose tissue (arrows).

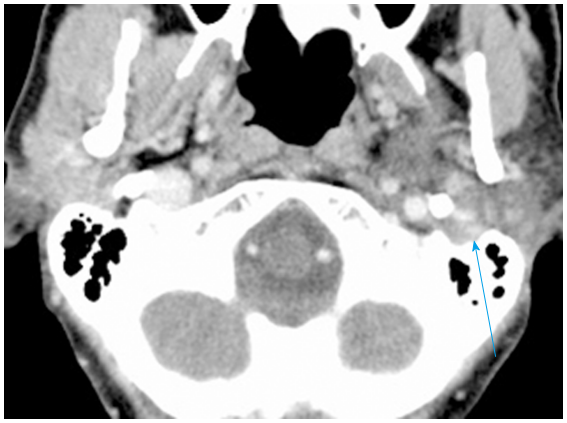

Figure 3 2015: Showing new extension into the left stylomastoid foramen not present on the examination of 2007.

Zbären *et al*[4] postulates that the risk of de novo malignant change increases with time from first presentation and the number of recurrent episodes of the tumor.

Treatment of recurrent pleomorphic adenoma involves primary surgery that can either be a superficial or total parotidectomy based on the site of the recurrence and the extent of previous facial nerve exploration[6]. Adjuvant radiotherapy is another treatment option that is suitable for patients whose tumor is not completely excised[6]. According to Witt et al, retrospective analysis provides evidence that radiotherapy improves local control of this tumor[6]. The risk of malignant change in salivary glands following radiation therapy to the neck in 11047 patients with Hodgkins Lymphoma was investigated by Boukheris *et al*[7]. They reported that 21 patients developed salivary gland carcinoma with an observed-to-expected ratio of 16.9 and a confidence interval of 95%[7]. The risk was highest in patients under 20 years of age and those who survived more than 10 years[7].

In a review of the literature, Gnepp reported that carcinoma *ex pleomorphic* adenoma was present in 3.6% of all salivary gland neoplasms, 6.2% of all mixed tumors, and 11.6% of all malignant salivary gland neoplasms[2]. The malignant tumor is mainly found between the sixth to eighth decades of life[2]. Carcinoma *ex pleomorphic* adenoma represents a malignant change in a primary or recurrent pleomorphic adenoma[2]. Nouraei *et al*[8] and Zbären *et al*[4] reported that 25% of their 28 patients and 21% of their 24 patients, respectively, had a previously treated parotid adenoma. Carcinoma *ex pleomorphic* adenoma predominantly affects the major salivary glands with a majority of cases noted in the parotid and submandibular glands[2]. Nouraei *et al*[8] and Olsen *et al*[9] reported that the carcinoma *ex pleomorphic* adenoma was located in the parotid gland in of 96% and 86% of their cases, respectively. The most common clinical presentation of carcinoma *ex pleomorphic* adenoma is as a firm mass in the parotid gland[2]. This tumor though typically non-invasive, confined to the capsule of the parotid adenoma and asymptomatic, has been reported to become invasive and involve local structures[2].

Carcinoma *ex pleomorphic* adenoma may present with pain when it is associated with invasion of local tissues[2]. Involvement of the facial nerve causes facial paresis or palsy[2]. Olsen *et al*[2] reported that

32% of the patients in their series had facial nerve involvement manifesting as partial or complete facial muscle weakness. Rarely, patients presented with skin ulceration, tumor fungation, skin fixation, palpable lymphadenopathy and dysphagia[2].

No case of hemifacial spasms or twitching associated with carcinoma *ex pleomorphic* adenoma or any other parotid or submandibular gland malignancies has been reported in the literature. The only malignant neoplasm presenting with facial spasm that we identified in the literature was a malignant astrocytoma located at the cerebellopontine angle[10]. Following resection of that tumor, the facial spasms resolved[10]. The two cases of hemifacial spasm have been reported with benign parotid tumors. Behbehani *et al*[11] reported the case of a 47-year-old man who presented with a right parotid mass and hemifacial spasm. The hemifacial spasms did not abate following surgery, but responded 8 mo later to botulinum toxin-A injections[11]. Destee *et al*[3] also reported a case of a pleomorphic adenoma in a 70 year-old man who presented with hemifacial spasms. During total parotidectomy, it was noted that the facial nerve was pale and appeared ischemic[3]. The hemifacial spasms reduced 8 days post operatively and had almost completely subsided within 6 mo[3].

The most common causes of hemifacial spasm are vascular compression of the ipsilateral facial nerve at the cerebellopontine angle (termed primary or idiopathic) (62%), hereditary (2%), secondary to Bell's palsy or facial nerve injury (17%), and hemifacial spasm mimickers (psychogenic, tics, dystonia, myoclonus, myokymia, myorthythmia, and hemimasticatory spasm) (17%)[12]. In addition to a thorough history and a complete neurological examination, some authors recommend magnetic resonance imaging and angiography of the cerebellopontine angle[12]. However, such imaging may not be cost-effective in all patients[13], as the presence of an ectatic artery on magnetic resonance imaging may not be specific for hemifacial spasms[12]. Therefore, this may be reserved for patients with atypical features such as numbness and weakness[13].

The authors postulate that in this patient the hemifacial spasm commenced with the onset of the malignant transformation in the recurrent pleomorphic adenoma in the parotid gland. In the absence of any evidence of perineural invasion, we believe that peri-tumoral inflammatory responses caused the neural stimulation that resulted in hemifacial spasm. This patient did not have any prior ear surgery or any other known etiology to account for this symptom. An alternative explanation to the patient's neurological symptoms is external compression to the facial nerve. This could be related to the dense fibrotic tissue surrounding both tumor nodules and nerves or to direct tumor extension into the left stylomastoid foramen[14] (Figure 3). The latter mechanism has been previously proposed by Blevins *et al*[14].

In conclusion, we present the first case of hemifacial spasm in conjunction with transformation of a recurrent pleomorphic adenoma into a carcinoma *ex pleomorphic* adenoma. The pathophysiology of hemifacial spasms is discussed.

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COMMENTS

Case characteristics

A 56-year-old female with a history of recurrent pleomorphic adenoma of the left parotid gland treated with surgery and external beam radiation therapy presented with ipsilateral hemifacial spasm.

Clinical diagnosis

The clinical diagnosis is a malignant change in a parotid pleomorphic adenoma with involvement of the facial nerve.

Differential diagnosis

The differential diagnosis is the stimulation of facial nerve by perineural invasion or an inflammatory reaction caused by malignant parotid tumor.

Imaging diagnosis

Repeat CT scan showed enlargement of avidly and uniformly enhancing solid tumor without areas of necrosis or extracapsular extension with extension into the left stylomastoid foramen, along with suspicious changes in enlarged (15 mm) left level IV lymph node (Figure 1A).

Pathological diagnosis

Fine-needle aspiration biopsy of the tumor was suspicious for carcinoma *ex pleomorphic* adenoma. Final surgical pathology confirmed a 5.2 cm pleomorphic adenoma with a multinodular growth pattern with two foci of early non-invasive carcinoma and no malignant spread to adjacent fibroadipose tissue, nerves or thirteen level II-V lymph nodes.

Treatment

A radical parotidectomy with facial nerve sacrifice, ipsilateral selective neck dissection (levels I-IV), and a de-epithelialized anterolateral thigh free flap was performed. A sural nerve grafting from the main trunk of the facial nerve to its branches and an oral commissure suspension with a fascia lata sling was done.

Experiences and lessons

The authors postulate that the hemifacial spasm commenced with the onset of the malignant transformation in the recurrent pleomorphic adenoma in the ipsilateral parotid gland. In the absence of any evidence of perineural invasion, they believe that peri-tumoral inflammatory responses caused the neural stimulation that resulted in hemifacial spasm.

Peer-review

This is the first reported case of malignant transformation of a recurrent pleomorphic adenoma in a parotid gland presenting with ipsilateral hemifacial spasm. In the absence of evidence of perineural invasion of the ipsilateral facial nerve, it is postulated that peri-tumoral inflammatory responses were responsible for the excitation of this nerve and the resultant hemifacial spasm.

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CASE REPORT

Difficult endoscopic diagnosis of a pancreatic plasmacytoma: Case report and review of literature

Nicolas Williet, Radwan Kassir, Muriel Cuilleron, Olivier Dumas, Leslie Rinaldi, Karine Augeul-Meunier, Michèle Cottier, Xavier Roblin, Jean-Marc Phelip

Nicolas Williet, Olivier Dumas, Leslie Rinaldi, Xavier Roblin, Jean-Marc Phelip, Department of Gastroenterology, University Hospital of Saint Etienne, 42270 Saint-Priest en Jarez, France

Radwan Kassir, Department of General Surgery, University Hospital of Saint Etienne, 42270 Saint-Priest en Jarez, France

Muriel Cuilleron, Department of Radiology, University Hospital of Saint Etienne, 42270 Saint-Priest en Jarez, France

Karine Augeul-Meunier, Department of Hematology and Oncology, Cancérologie Lucien Neuwirth, 42271 Saint-Priest en Jarez, France

Michèle Cottier, Department of Cytology, University Hospital of Saint Etienne, 42270 Saint-Priest en Jarez, France

Author contributions: Williet N, Kassir R, Roblin X and Phelip JM designed this work; Williet N wrote the paper; Williet N performed the systematic review; Cuilleron M, Dumas O and Rinaldi L contributed equally to this work; Augeul-Meunier K edited the first version text and provided her onco-hematologic regard on this case; Cottier M wrote cytology part of the text and provided cytological pictures.

Institutional review board statement: This case report was exempt from the internal Review Board standards of the Hepato-gastroenterology department managed by Pr Jean-Marc Phelip, at University of Saint-Etienne in Saint-Priest en Jarez.

Informed consent statement: The patient who is involved in the present case report gave his verbal informed consent before his death, authorizing use and disclosure of his protected health information.

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Correspondence to: Dr. Nicolas Williet, MD, Department of Gastroenterology, University Hospital of Saint Etienne, Avenue Albert Raimond, 42270 Saint-Priest en Jarez, France. nwilliet@yahoo.fr
Telephone: +33-4-77829029

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Abstract

A 71-year-old man, with history of plasmacytoma in relapse since one year, was hospitalized for a initial presentation of acute pancreatitis and hepatitis. Although there was a heterogeneous infiltration around the pancreas head, the diagnosis of an extramedullary localization of his plasmacytoma was not made until later. This delayed diagnosis was due to the lack of specific radiologic features and the lack of dilatation of biliary ducts at the admission. A diagnosis was made with a simple ultrasound guided paracentesis of the low abundance ascites after a transjugular hepatic biopsy, an endoscopic ultrasound-guided fine needle aspiration of the pancreatic mass, and a failed attempt of biliary drainage through endoscopic retrograde cholangiopancreatography. In order to document the difficulty of this diagnosis, characteristics of 63 patients suffering from this condition and diagnosis were

identified and discussed through a systematic literature search.

Key words: Plasmacytoma; Pancreas; Diagnosis; Ultrasound endoscopy; Review

Core tip: We wrote an interesting case report about a pancreatic plasmacytoma for which diagnosis, including endoscopic diagnosis, was a challenge. In a second part, a systematic pubmed search was performed from 1950 to June 2016, reporting characteristics and route to diagnosis of 63 similar cases reports! Strengths of our paper are the original route to diagnosis (by a simple ultrasound guided paracentesis, after failed of the endoscopic route) and our literature search which is particularly exhaustive: we are first to identify more 20 case similar reports (63!!) and their characteristics.

Williet N, Kassir R, Cuilleron M, Dumas O, Rinaldi L, Augeul-Meunier K, Cottier M, Roblin X, Phelip JM. Difficult endoscopic diagnosis of a pancreatic plasmacytoma: Case report and review of literature. *World J Clin Oncol* 2017; 8(1): 91-95 Available from: URL: http://www.wjgnet.com/2218-4333/full/v8/i1/91.htm DOI: http://dx.doi.org/10.5306/wjco.v8.i1.91

INTRODUCTION

Here we describe the case of a pancreatic plasmacytoma and difficulties to establish the diagnosis. Characteristics of patients and routes to diagnosis in this condition will be identified through a systematic literature search, in a second part.

CASE REPORT

A 71-year-old man was hospitalized for a clinical and biological presentation of acute pancreatitis. Pain occurred suddenly and was associated with an increased level of lipase above 2000 UI/L, a cholestatic icterus (bilirubin: 103 μmol/L) and a hepatic cytolysis (ALT: 154 UI/L; AST: 131 UI/L). An initial computerized tomography (CT) scan showed a significant but unspecific infiltration around the pancreas head, without dilatation of biliary ducts. A first endoscopic ultrasound (EUS) (Pentax, EG 3670 URK, France) showed similar data. The hypoechoic infiltration of the pancreas head was heterogeneous and extended to the hepatic hilum, in contact with portal vein. There was no biliary lithiasis, nor context of alcohol consumption during the last days before the admission. However, the patient was treated with Lenalidomide plus dexamethasone for a Immunoglobulin A (IgA) plasmacytoma diagnosed 3 years ago [t(4;14) positive, del(17p) negative; at baseline: LDH: 173 UI/L, monoclonal immunoglobulin peak: 40.5 g/L, Kappa and Lambda serum free light chain: 11.7 and 18.6 mg/L, respectively], without hypercalcemia nor kidney failure. He relapsed dramatically one year ago, with an extramedullar localization (L4 lumbar spine). Based on hematotoxicity (platelets: 41000 G/mm^3) and lake of specific radiologic features, the initial diagnosis suspected was a dual hepatic and pancreatic toxicity of Lenalidomide. Indeed, acute pancreatitis and hepatitis had been occasionally reported as a side effect of Lenalidomide[1,2]. Common hepatitis viral serologies were tested before carrying out a trans-jugular hepatic biopsy which showed a histological aspect compatible with the diagnosis of drug hepatitis or hepatitis related to a biliary obstruction (centrilobular and portal infiltrate of polymorphs inflammatory cells including eosinophils). Although an empirical treatment with 500 mg intravenous methylprednisolone daily was started, bilirubin level increased at 345.8 μmol/L within the following ten days. Hence, a new CT-scan was performed and showed the occurrence of a mild to moderate dilatation of biliary ducts and a low abundance ascites. At the moment of admission, the infiltration of the pancreas head significantly resembled a tumor (Figure 1) and the diagnosis of a pancreatic localization of the plasmacytoma was suspected. After platelets support, EUS (Pentax, EG 3670UTK, France) guided fine needle aspiration (FNA) was carried out with a 22-gauge needle. Tumor infiltration appeared to be growing due to portal vein invasion. Linear EUS passage through the pylorus was drastically limited, so that FNA was performed from the gastric antrum. Then, an endoscopic retrograde cholangiopancreatography was attempted to place a biliary stent for palliative treatment, but the cannulation of the bile duct had failed due to a major parietal oedema of the duodenum which was easily bleeding due to the contact of the sphincterotome. A percutaneous biliary drainage was considered, but an ultrasound-guided paracentesis was preferred, taking into account technical difficulties of the biliary drainage. Cytology of the FNA was not contributory (epithelial cells of pancreas without malignity signs) while the analysis of ascites showed plasmacytosis with severe atypia enabling the diagnosis of pancreatic plasmacytoma (Figure 2). Bone marrow was exempted from dystrophic plasma cells, proving an extramedullar relapse. The increase of the monoclonal spike (from 2.3 g/L to 8.1 g/L within 4 mo) and LDH (259 UI/L) was compatible with this diagnosis. Kappa and Lambda free light chain, at this time of the disease, were 0.4 mg/L and 24.8 mg/L, respectively, without hypercalcemia, Bence Jones proteinuria, nor kidney failure. Hence, after contacting the referral hematologist of the patient, a cure of 40 mg dexamethasone daily was started inciting a drastic decrease of bilirubin level within the next three days (183.1 μmol/L). Then, a second line of chemotherapy (Bortezomib + Cyclophosphamide) was started with a good short-term safety. Although

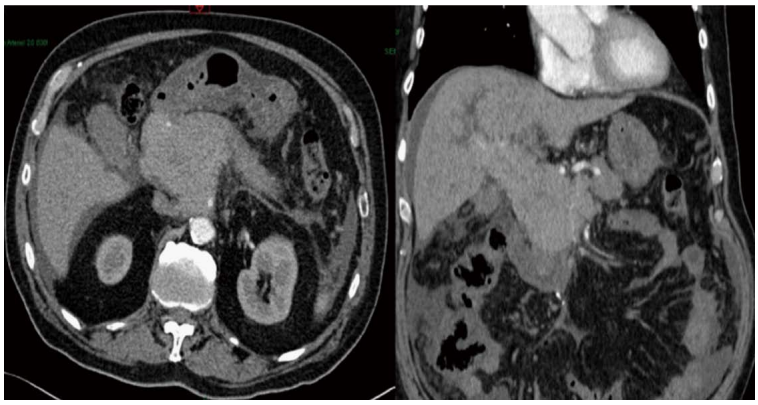

Figure 1 Abdominal computerized tomography scan showing a head pancreas mass extended to the hepatic hilum with mild to moderate dilatation of biliary ducts and a low abundance ascites.

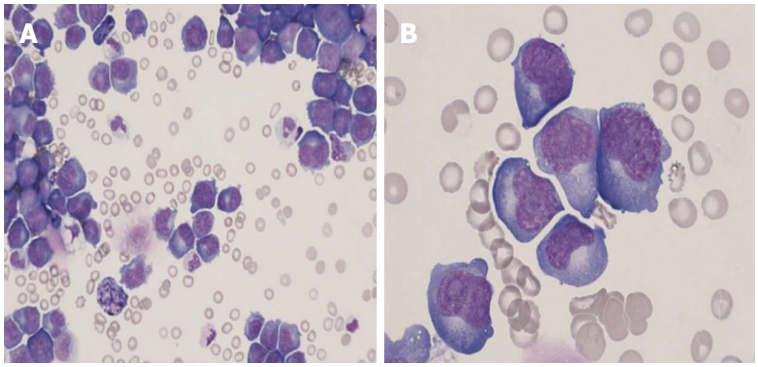

Figure 2 Peritoneal fluid Cytology, May-Grünwald-Giemsa stain. A: An almost pure population of myeloma cells (× 40); B: Malignant plasma cells exhibiting severe atypia (× 100).

a biological response, especially for monoclonal peak (2.1 g/L), at one month, the patient died 4 mo after the diagnosis of pancreatic plasmacytoma.

DISCUSSION

Extramedullary plasmacytoma involvement is not an uncommon presentation, occurring in 10%-15% of patients[3]. They are commonly identified after the diagnosis of multiple myeloma. The most commonly involved organs are those located around skeletal lesions, and less frequently, skin, liver, kidney, or central nervous system. Regarding the digestive system, liver and spleen are classically the organs which could be damaged by disease through deposits of amyloid proteins[4]. Extramedullary plasmacytomas involving the pancreas is a very rare condition with a prevalence rate estimated at 2.3%, based on autopsy studies[5].

After conducting a systematic Pubmed search, we identified 63 case reports of pancreatic plasmacytoma and collected a set of clinical and diagnostic data which were reported in Table 1. About half of them were male, with a median age of 58.5 years old, and presented jaundice in 70.0% with (36%) or without pain. About 2/3 of patients (68.4%) had a known history of plasmacytoma since 1 year (0-13) (median, interquartile ranges 25%-75%), before the involvement of the pancreas head. Only two cases involved the body or the tail of the pancreas[6,7]. Only 1/3 of patients (32.6%) were diagnosed by EUS-guided FNA *vs* 1/5 (20.9%) by CT-guided percutaneous FNA. About ¼ of patients (25.6%) have needed for a surgical biopsy, including situation involving bowel obstruction. A direct biopsy of the mass was possible in 16.3% during an upper gastrointestinal endoscopy. Most of patients were treated with chemotherapy (56.0%) and/or radiotherapy (52.0%), providing a 100% tumor response rate. A biliary stent was placed in half of patients with jaundice (46.7%).

Hence, to the best our knowledge, this is the first case report of a pancreatic plasmacytoma which was diagnosed by ascites analysis. Diagnosis by noninvasive procedures and rapid response to conservative therapy were important in this patient's care. It is very difficult to radiologically differentiate extramedullary plasmacytoma of the pancreas from other pancreatic tumors. EUS guided FNA provides the easiest and most safe route to diagnosis of pancreatic plasmacytoma. Studies have shown that the overall accuracy of EUS-guided FNA ranges between 71% and 90% in case of pancreatic tumor[8]. However, there is no corresponding data in case of pancreatic plasmacytoma.

In our case, the missed diagnosis of pancreas plasmacytoma through EUS-guided FNA may be due to a sampling bias. Furthermore, we made only one diagnostic EUS attempt while in few cases reported, authors specified the need for repeating EUS-guided FNA[9-13].

This case highlights that a pancreatic mass in patients with plasmacytoma should be systematically considered as an extramedullary extension of the disease until proven otherwise. Ascites analysis could

Table 1 Main characteristics of the 63 patients who had been reported to date with a pancreas plasmacytoma: Results of a PubMed search from 1950 to June 2016

Demographic characteristics	*n* (%)
Male	22 (56.4)
Age (years, median, IQR)	58.5 [51.2-82]
Symptom(s) at diagnosis	
Jaundice	35 (70.0)
Pain	18 (36.0)
Myeloma	
Known history of myeloma	26 (41.3)
Disease duration at diagnosis of pancreas plasmacytoma (years, median, IQR)	1 [0-13]
Type Kappa	13 (71.4)
Immunoglobulin	A (36%), G (52%), M (12%)
Diagnosis process of the pancreas plasmacytoma	
Endoscopic ultrasound FNA	14 (32.6)
Percutaneous FNA	9 (20.9)
Endoscopic biopsy	7 (16.3)
Surgical biopsy	11 (25.6)
Paracentesis	0 (0.0)
Postmortem biopsy	3 (7.0)
Management of the pancreas plasmacytoma	
Chemotherapy	14 (56.0)
Radiotherapy	13 (52.0)
Biliary stent in patients with jaundice	10 (40.0)
Surgery	8 (32.0)
Biliodigestive derivation	3 (37.5)
Duodenopancreatectomy cephalic	2 (25.0)

FNA: Fine needle aspiration; IQR: Interquartile range.

be a simple route to diagnosis, even in low abundance. Finally, in case of jaundice, excluding angiocholitis, potential risks of biliary stenting should be taken into account, regarding safety and the drastic efficacy of radiotherapy or medical treatment (dexamethasone and chemotherapy).

COMMENTS

Case characteristics

A 71-year-old man with history of plasmacytoma in relapse since one year, and treated with Lenalidomide.

Clinical diagnosis

The initial diagnosis suspected was a dual hepatic and pancreatic toxicity of Lenalidomide.

Differential diagnosis

An adenocarcinoma of the pancreas, or other less frequent pancreatic tumor such as a non Hodgkin's lymphoma, or endocrine tumor.

Laboratory diagnosis

An increased level of lipase above 2000 UI/L, a cholestatic icterus and a hepatic cytolysis.

Imaging diagnosis

Computerized tomography showed a significant but unspecific infiltration around the pancreas head, without dilatation of biliary ducts, extended to the hepatic hilum, and evolving as a pseudotumor within few days.

Cytological diagnosis

A (pancreatic) plasmacytoma.

Treatment

An empirical corticotherapy followed by a second line of chemotherapy (Bortezomib + Cyclophosphamide).

Related reports

Cytology of the mass was not contributory in contrast with the very low abundance ascites located around the liver.

Terms explanation

Extramedullary plasmacytoma involvement is not an uncommon presentation, and occurres preferentially in located around skeletal lesions, or less frequently in, skin, liver, kidney, or central nervous system.

Experiences and lessons

A pancreatic mass occurring in a patient with history of plasmacytoma and with an uncommon presentation should make suspecting an extramedullar site of the disease. No diagnostic way should be forgot, even a simple analysis of an ascites sample.

Peer-review

This is an interesting case about pancreas involvement in a case with relapsed myeloma.

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EDITORIAL

Watch and wait policy in advanced neuroendocrine tumors: What does it mean?

Nicola Fazio

Nicola Fazio, Unit of Gastrointestinal Medical Oncology and Neuroendocrine Tumors, European Institute of Oncology, 20141 Milan, Italy

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Correspondence to: Nicola Fazio, MD, PhD, Unit of Gastrointestinal Medical Oncology and Neuroendocrine Tumors, European Institute of Oncology, Via Ripamonti 435, 20141 Milan, Italy. nicola.fazio@ieo.it
Telephone: +39-02-57489258
Fax: +39-02-94379224

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Abstract

Neuroendocrine neoplasms (NENs) are a group of rare and heterogeneous malignancies, which can develop in various organs. The clinical course of NENs is quite heterogeneous, with different spontaneous growth rates after diagnosis, and different degrees of sensitivity to the same therapy even when they have similar characteristics. *Watch and wait* (W and W), is a term coined to indicate observation being conducted to assess the evolution of the tumor without administering any anti-tumor therapy. It has been applied to NENs since in extremely rare cases they tend to remain stable for a long time. Although W and W has been reported in several guidelines and recommendations it has never been validated, nor has it been specifically investigated. Furthermore it is not standardized. Therefore its application in clinical practice can differ in terms of tumor status assessment, type and timing of imaging or other exams utilized. In conclusion, while undertaking W and W to delay the first-line therapy by some weeks may be justified in good performance asymptomatic patients with low-grade NENs in order to usefully characterize the disease and patient and thereby choose the best therapy and therapeutic strategy, it seems to be far more difficult to justify W and W with the intent of avoiding an anti-tumor treatment. It should be considered that not only do NENs tend to grow even when they have very favorable biological characteristics but also that the alternative to W and W is most commonly a low toxic and effective treatment with somatostatin analogs.

Key words: Observation; Wait and see; *Watch and wait*; Surveillance; Neuroendocrine tumors

Core tip: *Watch and wait* (W and W) is a term coined to indicate observation without therapy assessing the evolution of the tumor. Given that neuroendocrine tumors sometimes are radiologically stable over months since they tend to grow slowly observation has been reported as an option to be considered in several guidelines and recommendations. However it has neither validated nor specifically investigated so far. Therefore its application

in clinical practice is arbitrary and it differs in terms of tumor status assessment, type and timing of imaging or other exams utilized. While undertaking W and W to delay the first-line therapy by some weeks may be justified in good performance asymptomatic patients with low-grade neuroendocrine neoplasms (NENs) in order to usefully characterize the disease and patient and thereby choose the best therapy and therapeutic strategy, it seems to be far more difficult to justify W and W with the intent of avoiding an anti-tumor treatment. It should be considered that not only do NENs tend to grow even when they have very favorable biological characteristics but also that the alternative to W and W is most commonly a low toxic and effective treatment with somatostatin analogs.

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INTRODUCTION

Neuroendocrine neoplasms (NENs) represent a group of rare and heterogeneous malignancies, which can develop in various organ. They are classified on the basis of their level of aggressiveness into low, intermediate and high grades of malignancy.

Neuroendocrine neoplasms from the digestive tract, are classified on the basis of proliferation index as G1 (⩽ 2% Ki-67), G2 (3%-20% Ki-67) and G3 (> 20% Ki-67). Furthermore, based on their morphology they are named "tumors" (NETs) when they are well differentiated, whereas "carcinomas" (NECs) when they are poorly differentiated"[1]. Neuroendocrine neoplasms from the thoracic region are classified into typical carcinoid, TC (< 2 mitoses/2 mm^2 with absence of necrosis), atypical carcinoid, AC (2-10 mitoses/2 mm^2 with necrosis), large cell neuroendocrine carcinoma, LCNEC (> 10 mitoses with extensive necrosis) and small cell lung cancer, SCLC (> 10 mitoses with extensive necrosis)[2].

While high-grade NENs are treated with chemotherapy in the vast majority of cases when they are in advanced stage of disease, the therapeutic approach to advanced low-intermediate grade NENs varies. Somatostatin analogs (SSA), interferon (IFN), molecular targeted agents (MTAs), chemotherapy, peptide receptor radionuclide therapy (PRRT), and liver-directed treatments (LDTs), are all potentially effective therapies to propose, often in the same clinical setting. Although some of these therapies have been approved on the basis of positive regulatory phase Ⅲ trials[3-7] in specific settings and several guidelines about NENs do exist[8,9], no sequencing or priority criteria about the different therapies have been validated. Furthermore, the clinical course of NETs is quite heterogeneous, with different spontaneous growth rates after diagnosis, and different degrees of sensitivity to the same therapy even when they have similar characteristics.

"*Watch and wait* (W and W)", "watchful waiting", "wait and see", "observation" and "active surveillance" are all terms which are used to describe assessing the evolution of the tumor without an anti-tumor therapy. These terms have been applied synonymously to NETs as in rare cases they have a spontaneous very indolent clinical course. Sometimes they are also applied to a localized disease, as in the case of so-called pancreatic "incidentaloma", namely a < 2 cm isolated nodule in the pancreas. European Neuroendocrine Tumor Society (ENETS) 2016 guidelines recommend W and W for a < 2 cm pancreatic NET, "G1 or low G2, asymptomatic, mainly in the head, with no radiological signs suspicious for malignancy", and suggest that one also consider the patient's attitude, age and comorbidity. It is specified that the follow-up should be performed with endoscopic ultrasound (EUS), magnetic resonance imaging (MRI) (or computed tomography, CT) "every 6 to 12 mo". However, the length of follow-up is not specified[10].

In the ENETS guidelines W and W is also recommended for advanced disease, for instance in NETs from the midgut when they are "non-functional, G1, low tumor burden, no symptoms, stable disease". This policy is advised even for pancreatic NETs, when they are "non-functional, G1, ⩽ 10% Ki-67, low tumor burden, stable disease or initial diagnosis, no symptoms"[11].

In both midgut and pancreatic NETs the W and W policy is a possible alternative to SSA. However, SSA compared with placebo resulted effective in two phase III randomised controlled trials, with octreotide long-acting repeatable (LAR) producing a longer time to progression (TTP) in midgut NETs in the *PROMID* trial and lanreotide autogel significantly prolonging progression free survival (PFS) in enteropancreatic NETs in the *CLARINET* trial, respectively[5,6]. Notably, time to progression (TTP) was quite short in the placebo arm of the *PROMID* trial demonstrating that also NETs with < 3% Ki-67, as were the vast majority of the tumors included in the PROMID, will progress eventually. Interestingly, NETs included in the *CLARINET* trial, which resulted as having a stable disease in 96% of cases in accordance with RECIST criteria, in fact were progressing at baseline, as showed with the so-called tumor growth rate (TGR)[12].

Another report indicating that NETs tend to grow early spontaneously, is a retrospective analysis of more than 200 patients with advanced pancreatic NETs showing that those patients who did not receive antitumor treatment during follow-up had a significantly shorter PFS compared to treated patients, thus confirming that anti-tumor therapy can favorably impact on the clinical course of the disease[13].

In the ENETS 2016 guidelines it is not specified whether radiological or functional imaging or both are recommended to monitor the tumor status of a low-grade NET; it is not clear whether some biochemical tests, such as chromogranin-A, should be performed periodically;

timing of follow-up imaging is not specified.

Furthermore no data exist about the impact of the W and W on the patient's quality of life and costs.

The W and W policy is debated also in other fields of oncology. For instance in renal cancer it was investigated in a phase Ⅱ trial including medical anti-tumor treatment-naive patients with advanced disease[14]. The decision to choose W and W over immediate systemic therapy was made jointly by the patient and treating physician. Therefore patients underwent homogeneous radiological and clinical follow-up and also filled in quality of life questionnaires. Median time to radiological progression, RECIST-based, was 9.4 mo (95%CI: 7.4-13.4); at progression, patients received a first-line systemic therapy; no observed adverse effects on quality of life, anxiety and depression, were recorded during the observation period. Although this study seems to indicate that in some selected patients with metastatic renal carcinoma, active surveillance might be a good approach, homogeneous criteria for selection of patients to undergo W and W, type of follow-up and timing of first-line therapy remain debatable.

Further while in renal cancer one of the reasons for performing W and W instead of administering treatment to patients is to avoid therapies which may well be highly toxic, in NETs the choice is almost always between W and W and SSAs, which are a very low-toxic therapy.

Finally, in good-performance status asymptomatic patients with advanced NETs, the diagnostic work-up, morphological and functional staging and characterization of the disease require some weeks. Luckily in most cases this time without therapy is not detrimental for the patient and it allows an assessment to be made of clinical behavior and tumor growth, a thorough understanding of tumor and patient characteristics, and the discussion of the global therapeutic strategy within a dedicated multidisciplinary team. All of this may be very helpful to patients when compared with starting a single first-line therapy right from the time of diagnosis of an advanced NET. Proposing a W and W policy after completing this initial period of observation to a patient with a metastatic NET means waiting for a tumor growth or a clinical progression. On the one hand it is arbitrary to define whether morphological (radiological), functional (receptorial? metabolic?) or biochemical progression should be considered and with which threshold; on the other hand it could be detrimental to start therapy only when tumor-related symptoms arise. Nonetheless patients should be informed that no study has specifically investigated this topic comparing W and W and anti-tumor therapy, and therefore we have no evidence either for or against. Patients will need to understand that follow-up will be life-long even with stable disease, that there are data showing that the vast majority of advanced NETs tends to grow and that SSAs can be active even when the tumor is very indolent.

In conclusion, W and W policy in advanced NENs is yet to be well-defined. First of all it should be clarified whether W and W means delaying or avoiding an anti-tumor treatment. Delaying may be justified in an asymptomatic good performance status patient with a low-grade NETs over some weeks in order to thoroughly characterize both disease and patient and so make a well-informed choice as to the best therapy and therapeutic strategy to pursue. This is a quite common clinical scenario in the field of NETs. By contrast it is hard to justify W and W with the intent to avoid treatment considering that low-grade advanced NETs tend to grow even when they have very favorable biological characteristics. Therefore, also in that case, rather than avoiding, it would mean once again delaying the first-line therapy. Of course the first-line therapy and the therapeutic strategy depend on the specific clinical context and on the goal of treatment. In other words in a patient who is a good candidate for a future absolute debulking, then the first-line treatment even more than an SSA should be applied even with a stable disease without any delay. On the other hand, in a patient with a metastatic low grade, really stable NET, when absolute debulking is not possible and the goal of the treatment is the tumor growth control over time with a systemic medical therapy, then a thoughtful analysis needs to be made. It is important to bear in mind the cost- and risk-benefit of SSA, which is the most commonly proposed therapy in such a context, and also the cost, invasiveness, impact on quality of life and possible detrimental effect of W and W.

I would argue that given the absence of evidence and of clinical trials designed to specifically investigate this topic, as is currently the case, clinicians should consider administering treatment to all patients, whether their NETs are advanced.

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EDITORIAL

Translating new data to the daily practice in second line treatment of renal cell carcinoma: The role of tumor growth rate

Enrique Grande, Olga Martínez-Sáez, Pablo Gajate-Borau, Teresa Alonso-Gordoa

Enrique Grande, Olga Martínez-Sáez, Pablo Gajate-Borau, Teresa Alonso-Gordoa, Medical Oncology Department, Ramón y Cajal University Hospital, 28034 Madrid, Spain

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Correspondence to: Enrique Grande, MD, PhD, Medical Oncology Department, Ramón y Cajal University Hospital, Ctra. Colmenar Viejo km 9100, 28034 Madrid, Spain. egrande@oncologiahrc.com
Telephone: +34-91-3368263
Fax: +34-91-3369181

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Abstract

The therapeutic options for patients with metastatic renal cell carcinoma (mRCC) have completely changed during the last ten years. With the sequential use of targeted therapies, median overall survival has increased in daily practice and now it is not uncommon to see patients surviving kidney cancer for more than four to five years. Once treatment fails with the first line targeted therapy, head to head comparisons have shown that cabozantinib, nivolumab and the combination of lenvatinib plus everolimus are more effective than everolimus alone and that axitinib is more active than sorafenib. Unfortunately, it is very unlikely that we will ever have prospective data comparing the activity of axitinib, cabozantinib, lenvatinib or nivolumab. It is frustrating to observe the lack of biomarkers that we have in this field, thus there is no firm recommendation about the optimal sequence of treatment in the second line. In the absence of reliable biomarkers, there are several clinical endpoints that can help physicians to make decisions for an individual patient, such as the tumor burden, the expected response rate and the time to achieve the response to each agent, the prior response to the agent administered, the toxicity profile of the different compounds and patient preference. Here, we propose the introduction of the tumor-growth rate (TGR) during first-line treatment as a new tool to be used to select the second line strategy in mRCC. The rapidness of TGR before the onset of the treatment reflects the variability between patients in terms of tumor growth kinetics and it could be a surrogate marker of tumor aggressiveness that may guide treatment decisions.

Key words: Axitinib; Everolimus; Cabozantinib; Kidney cancer; Nivolumab; Renal cell; Sequence; Second line; Sorafenib; Tumor-growth rate

Core tip: The landscape of renal cell carcinoma has dramatically changed in the last decade. Today, at least 6 agents are approved after failure with cytokines,

sunitinib or pazopanib in first line treatment. Lack of reliable biomarkers to select the best treatment in daily practice is somewhat frustrating. Therefore, our decisions in real practice are based on safety profiles, patient' co-morbidities and physician experience or preference. Here we debate the pros and cons of the tumor-growth rate as a tool to select second line systemic treatment after failure to a prior tyrosine kinase-inhibitor in patients with advanced renal cell carcinoma.

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INTRODUCTION

The increased knowledge about the underlying pathogenesis of the metastatic renal cell carcinoma (mRCC) has led to the development of new therapeutic drugs that have completely changed patient prognosis. These drugs are targeting the vascular endothelial growth factor receptor (VEGFR) axis, the mammalian target of rapamycin (mTOR) pathway or the immune system and tumor cell interactions (PD1/PDL1). The number of patients that are candidates for a second line therapy after progressing on a first line varies from 43% to 79%[1]. The second line treatment is determinant in mRCC as patients can also benefit from an improvement in overall survival (OS) already achieved with first line choice and expand their chances for a longer therapeutic sequence. In this regard, a large registry-based experience in the United Kingdom has shown that those patients who received a second line treatment lived longer (33 mo; ranging from 30.8-35.2) than those who did not receive further treatment after first line (20.9 mo; ranging from 16.4-25.3)[2]. Fortunately, options for second line therapy have multiplied with the recent approval of nivolumab, cabozantinib and the combination of everolimus with lenvatinib[3-6]. However, there are no head-to-head comparisons between them and no predictive biomarker has been validated for the second line treatment decision making[7]. Besides, the uncertainty regarding the optimal therapeutic sequence, there is an urgent need for developing prognostic and predictive variables, in order to select patients who will benefit from a specific second line treatment[8].

There are some clinical and economic-derived factors coming from the pivotal trials of each agent that could be considered at the time of second line treatment decisions (Table 1). The patient's tumor burden has been suggested from retrospective data as being strongly correlated with the progression free survival (PFS) and OS in patients with mRCC[9-12]. The expected response rate from the approved drugs has been reported to be different between cabozantinib, nivolumab and axitinib that achieve an overall response rate (ORR) of 17% to 22%, unlike the combination of everolimus with lenvatinib that has been reported to be of 35% in the phase II pivotal trial[3-6]. Moreover, the time required to achieve a tumor response is a major concern for heavily symptomatic patients that need an early tumor control. Prior tolerance and duration of response to first line treatment may identify those patients harboring a kidney tumor that greatly benefits from the angiogenic blockade (angiogenesis addiction), but may limit the decision in primary refractory patients[13,14]. Finally, we also propose the assessment of the tumor-growth rate (TGR), as a novel outcome measure that could help in the therapeutic sequence decision in the mRCC setting.

$$TGR = 100 \times [\exp(TG) - 1]$$

$$TG = \frac{3 \times \log (D2/D1)}{\text{Time (months)}}$$

D1 = tumor size at date 1; D2 = tumor size at date 2; and time (months) = (date2 - date1 + 1)/30.44

Figure 1 Tumor growth rate calculation formula. TGR: Tumor-growth rate.

Several authors have discussed that the Response Evaluation Criteria in Solid Tumors (RECIST) may be inadequate to completely evaluate the response of targeted therapies in mRCC as often induce long-lasting stable disease rather than tumor shrinkage[15-18]. In addition, these criteria do not take into account tumor growth kinetics, and might not be relevant in slow-growing diseases[19,20]. Therefore, alternate modalities to assess the drug response have been proposed to overcome the limitations of the RECIST criteria, such as Choi, SACT, MASS, ETPIC or iRECIST. These approaches include the tumor perfusion evaluation, *via* the use of CT response assessment combining reduction in both, size and arterial phase density, changes in tumor CT texture or metabolism or the immune component evaluation. However, none of them appear to be an adequate surrogate of response or clinical outcome for its application in routine clinical practice[16,18,21,22].

TGR provides a dynamic and quantitative evaluation of tumor kinetics; it estimates the percentage of change in the tumor volume over one month. TGR is usually defined as the ratio between the slope of tumor growth before the initiation of treatment and the slope of tumor growth during treatment, and between the nadir and disease progression[9,23]. We can calculate TGR according to the formula shown in Figure 1[24]. The tumor size is defined using the sum of the longest diameters (SLD) of target lesions only, without considering non-target and new lesions. However, the assessment of the TGR in clinical practice is easier as there are internet tools available (http://ec2-54-218-32-173.us-west-2.compute.amazonaws.com:3838/tgrShiny/ or http://www.gustaveroussy.fr/doc/tgr_calculator/index_en.html).

Table 1 Phase Ⅲ clinical trials evaluating approved drugs in second and subsequent treatment lines for metastatic renal cell carcinoma

	Axitinib	Cabozantinib	Lenvatinib + Everolimus	Nivolumab
Trial design	Phase Ⅲ	Phase Ⅲ	Phase Ⅱ	Phase Ⅲ
Size	361	330	51	410
Patient population	2nd Line (100%)	2L- 71% 3L- 29%	2nd Line (100%)	2L- 72% 3L- 28%
MSKCC risk % (Good/int/poor)	28/37/33	45/42/12	24/37/39	35/49/16
Comparator	Sorafenib	Everolimus	Everolimus	Everolimus
ORR% (ICR)	19%	17%	35%	22%
Progression disease (%)	22%	12%	4%	35%
PFS (m)	6.7 (HR 0.66)	7.4 (HR 0.51)	12.8 (HR 0.40)	4.6 (HR 0.88)
PFS (m) in pts with bone mets	NR	7.4 (HR 0.33)	NR	NR
OS (m)	20.1 (HR 0.96)	21.4 (HR 0.66)	25.5 (HR 0.59)	25.0 (HR 0.73)
Dose reductions	30%	60%	71%	N/A
Discontinuations due to AEs	7%	9%	25%	8%
Toxicity G3/4 (%)	56%	68%	71%	19%
Average monthly cost (US basis)	9580$	10229$	22461$	12435$

MSKCC: Memorial Sloan Kettering Cancer Center Criteria; ORR: Overall response rate; OS: Overall survival; PFS: Progression free survival; AE: Adverse events.

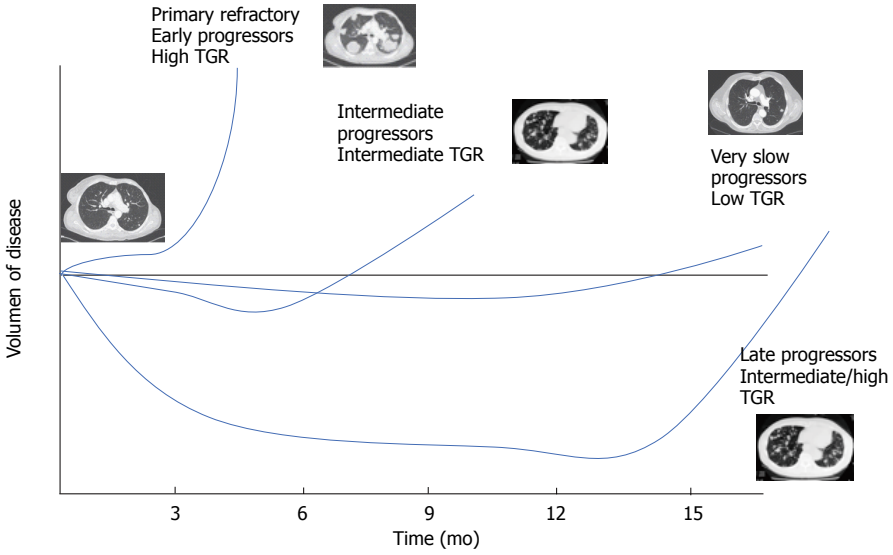

Figure 2 Hypothetical representation of different groups of patients and their patterns of response to first line treatment: Primary refractory patients with early progression and high tumor growth rate, intermediate progressors with intermediate tumor growth rate, very slow progressors with low tumor growth rate and late progressors with high tumor growth rate. TGR: Tumor-growth rate.

Current evidence from phase Ⅰ studies in solid tumors and from phase Ⅲ studies in mRCC (TARGET and RECORD trials) and metastatic neuroendocrine tumors (NETs) (CLARINET trial), although retrospective, show a significant association between prior TGR before the onset of the second line approach with the expected PFS and OS with the later systemic treatment administered[9,24-28]. Moreover, TGR could be an important tool in the evaluation of prognosis during treatment and after the discontinuation of VEGFR targeted agents. Iacovelli *et al*[29] showed that those patients with a higher than median TGR during treatment had a significantly shorter OS and, indeed, those patients with lower than the median TGR after discontinuation had longer OS, as compared to TGR after discontinuation greater than or equal to the median. Therefore, it would be possible to use TGR as a possible surrogate for tumor aggressiveness and survival in mRCC patients while on VEGFR-directed TKI in the first line. In the post hoc analysis from the CLARINET trial, TGR seemed to provide more precise information to predict pretreatment progression regarding actively growing tumors, but considered as stable disease by RECIST criteria, and more sensitive to detect early antitumor activity from treatment compared with RECIST criteria[28]. We consider that the addition of TGR in the assessment of individual patients undergoing targeted therapies may help clinicians to know if a given agent is modifying or not the course of the disease and guide the decision of which agent would be preferred in the subsequent line. However, for the use of TGR in the clinical setting, a prospective clinical trial for its validation would be needed[23].

Considering all aspects previously discussed, patients with mRCC that are candidate for a second line treatment could be differentiated into four main subgroups (Figure 2). Patients with florid symptoms, high tumor burden, short time to response to the first line (PFS less than 6 mo, so called, early progressors) and high TGR, in which we would need an early and high response, the

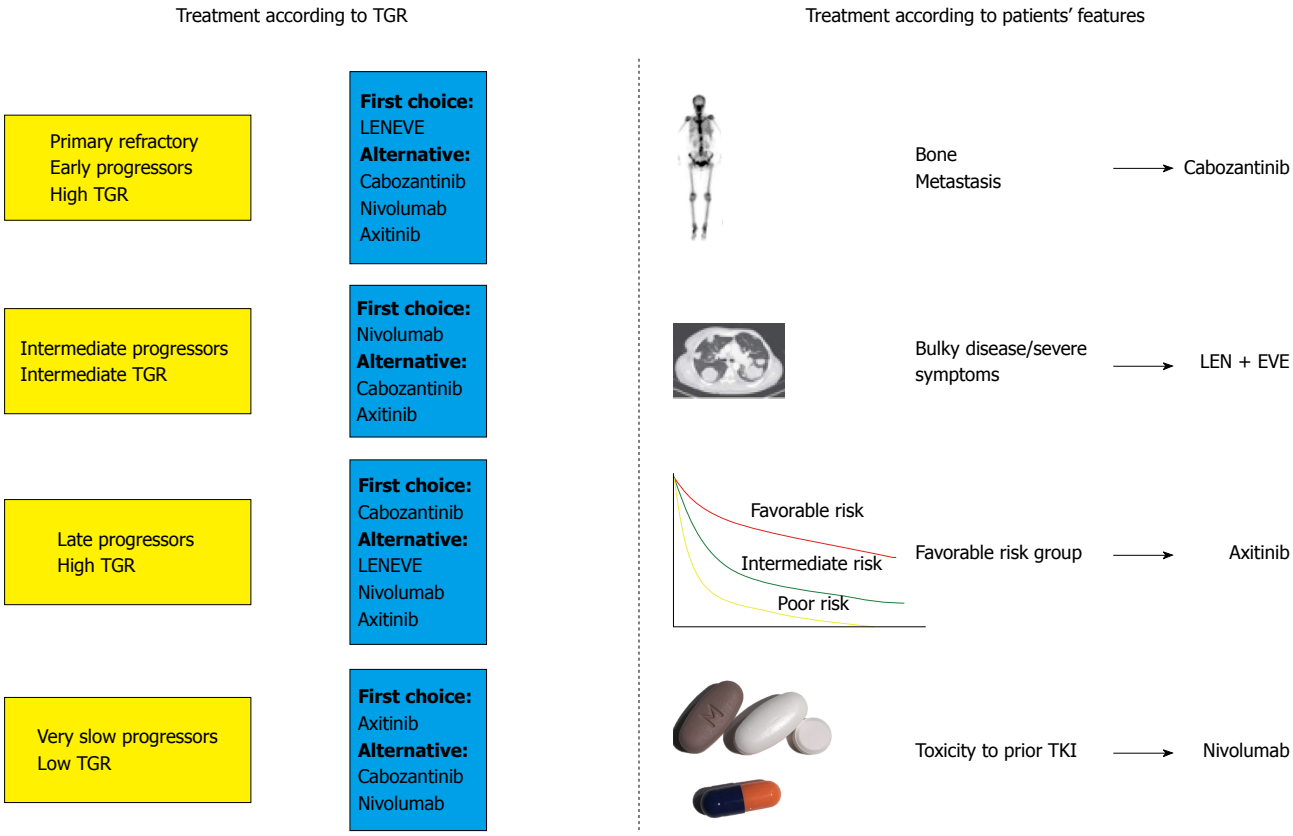

Figure 3 Adapting the study data to our clinic. A proposed algorithm to treat second line metastatic renal cell carcinoma patients according to tumor growth rate and patients' characteristics. TGR: Tumor-growth rate; TKI: Tyrosine kinase inhibitor; LEN: Lenvatinib; EVE: Everolimus.

combination of everolimus with lenvatinib should be considered, as we will target several mechanisms of action (VEGFR, fibroblast growing factor receptor, FGFR, and m-TOR pathways). In such patients, the expected benefit outweighs the increased toxicity of the combination therapy. In those patients with a long response to first antiangiogenic drug (PFS more than 18 mo, so called angiogenesis addicts) and low or intermediate TGR, the use of cabozantinib may be considered. Regarding those patients that are not responding radiographically but are stable for the advanced disease for a long period with a very low TGR (increase of less than 4% in the sum of the longest diameters per month) and have an adequate tolerability, we propose that axitinib could be a reliable option to prolong the clinical benefit. Finally, for patients with an interval free of progression with first line treatment between 6 and 18 mo, as considered intermediate-progressors, nivolumab may be the treatment of choice as an inhibitor of an actionable immune target by introducing a different mechanism of action against tumor growth.

Lastly, we highlight the upcoming availability of novel immune agents such as ipilimumab, atezolizumab, pembrolizumab either as single agent or in combination that might impact in the first line setting of patients with advanced RCC. Therefore, it is very likely that second line landscape of metastatic RCC may change shortly. Adaptation to the clinic of the amount of new data that are expected in a short term promises to be challenge.

In conclusion, patients with mRCC receiving a second line treatment achieve a median OS of more than 2 years with novel agents. Thus, the optimal treatment selection in this setting allows us to provide the maximal clinical benefit to our patients, but with no definitive biomarker to guide our decision. In this setting, we have considered some relevant clinical parameters before choosing a certain agent such as the patient's tumor burden, the expected response rate to the different drugs and the time to achieve this response, the prior response to previous VEGFR-TKIs, the toxicity profile of each agent and the patient preference. Thus, we propose the employment of the TGR as a new tool that could provide useful information in the management of mRCC patients in addition to clinical features that could better fit with one of the therapeutic alternatives (Figure 3). TGR may represent a surrogate of tumor aggressiveness, a relevant parameter before choosing a treatment and an early biomarker for treatment response and evaluation of the ability to interfere in the natural history of the tumor growth. TGR could be a valuable endpoint for clinical use in treatment decision-making favoring patients with mRCC, with more reliable information about prognosis and evaluation of response to molecular targeted agents.

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FRONTIER

Leptin signaling and cancer chemoresistance: Perspectives

Pierre V Candelaria, Antonio Rampoldi, Adriana Harbuzariu, Ruben R Gonzalez-Perez

Pierre V Candelaria, Antonio Rampoldi, Adriana Harbuzariu, Ruben R Gonzalez-Perez, Department of Microbiology, Biochemistry and Immunology, Morehouse School of Medicine, Atlanta, GA 30310, United States

Author contributions: Candelaria PV, Rampoldi A and Harbuzariu A have been involved equally, researched the literature, wrote the paper and have read and approved the final manuscript; Gonzalez-Perez RR researched the literature, analyzed data, wrote and edited the paper.

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Correspondence to: Ruben R Gonzalez-Perez, PhD, Professor, Department of Microbiology, Biochemistry and Immunology, Morehouse School of Medicine, 720 Westview Drive SW, Hugh Gloster Bldg., Room 329, Atlanta, GA 30310, United States. rgonzalez@msm.edu
Telephone: +1-404-7521581
Fax: +1-404-7521179

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Abstract

Obesity is a major health problem and currently is endemic around the world. Obesity is a risk factor for several different types of cancer, significantly promoting cancer incidence, progression, poor prognosis and resistance to anti-cancer therapies. The study of this resistance is critical as development of chemoresistance is a serious drawback for the successful and effective drug-based treatments of cancer. There is increasing evidence that augmented adiposity can impact on chemotherapeutic treatment of cancer and the development of resistance to these treatments, particularly through one of its signature mediators, the adipokine leptin. Leptin is a pro-inflammatory, pro-angiogenic and pro-tumorigenic adipokine that has been implicated in many cancers promoting processes such as angiogenesis, metastasis, tumorigenesis and survival/resistance to apoptosis. Several possible mechanisms that could potentially be developed by cancer cells to elicit drug resistance have been suggested in the literature. Here, we summarize and discuss the current state of the literature on the role of obesity and leptin on chemoresistance, particularly as it relates to breast and pancreatic cancers. We focus on the role of leptin and its significance in possibly driving these proposed chemoresistance mechanisms, and examine its effects on cancer cell survival signals and expansion of the cancer stem cell sub-populations.

Key words: Obesity-related cancer; Cancer stem cells; Leptin; Chemoresistance; Breast cancer; Pancreatic cancer

Core tip: Obesity and its main mediator leptin, are implicated in many protumorigenic processes, with

emerging evidence from both the literature and our work pointing to a significant role in the development of resistance to chemotherapies. Chemoresistance is a major concern in the field of cancer therapy as some cancers have no targeted therapies available. As obesity reaches epidemic proportions around the world, its impact on diseases like cancer and its treatment becomes more relevant. In this paper, we will discuss the current state of the literature regarding the influence of obesity and leptin on cancer treatment and the development of chemoresistance.

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INTRODUCTION

Obesity is the state of having excessive adipose tissue reserves, commonly defined as having a body mass index (BMI) of 30 or more. The global prevalence of obesity is high, with 37% of men and 38% of women being either overweight or obese[1]. There are significant health consequences for being overweight or obese. Obesity is closely associated to high rates of morbidity and mortality. It is considered responsible for an estimated 3.4 million deaths and 4% of years spent with a disability. There is a well-documented increased risk in obese and overweight people for numerous cancers, including thyroid, esophageal, kidney, colon, rectal, melanoma, leukemia, endometrial, gallbladder, pancreas and breast cancer[2-6]. In addition, weight gain before 50 has been associated with greater risk of breast cancer, especially estrogen negative breast cancer[7-9]. A contributing factor could be complications related to therapy, as obesity is correlated with breast cancer recurrence, with increasing BMI being correlated with increased risk of breast cancer relapse. Obesity impacts on life expectancy, with premenopausal and postmenopausal obese women being 1.75 and 1.34 times, respectively, at increased risk of death from breast cancer[10].

A distinctive characteristic of obesity and overweight conditions is the high serum level of the main adipokine, leptin secreted by adipose tissue. Leptin, from the Greek work "leptos", thin, is a 16 kDa protein, composed of 167 aminoacids, its gene, *Ob*, is in humans on chromosome 7q32. *Ob* gene is composed by three exons and 2 introns, spanning 20 kb. Leptin is the first discovered adipokine, a cytokine secreted by adipocytes, both from the white adipose tissue and brown adipose tissue. Placenta, ovaries, skeletal muscle, bone marrow, stomach, pituitary gland, and mammary epithelial cells have been shown to express leptin[11]. Several cancer cell types and tumor stroma also express leptin[12].

OBESITY, LEPTIN/OB-R AND CANCER

The main role of leptin is to regulate energy balance by inhibiting hunger. Leptin levels correlate to adiposity. Under physiological conditions leptin binds and activates receptors in the arcuate nucleus of the hypothalamus, which regulate appetite[13]. In obese people a decreased sensitivity to leptin was observed resulting in a decreased capacity to feel satiety[14]. A result of this resistance is overeating that results in obesity and the concomitant high serum levels of leptin. In obese individuals serum leptin levels are 10 times higher (*i.e.*, 40 ng/mL) than normal weight people (*i.e.*, 4 ng/mL)[15]. The upregulation of leptin has an important role in carcinogenesis[16].

Leptin receptor (Ob-R) is predominantly found in the hypothalamus[17], but is expressed at lower level in the whole body, including pancreas[18] and mammary epithelial cells[19]. Remarkably, cancer cells overexpress Ob-R, which enable them to respond to leptin that is more prominent in obese individuals showing high levels of the adipokine. Ob-R belongs to Class Ⅰ superfamily cytokine receptors. It is a transmembrane protein composed by an extra-cellular domain, responsible for binding leptin, a transmembrane domain and a cytoplasmic domain for signaling[20]. Currently six different isoforms of the leptin receptor have been identified, Ob-Ra-f, generated by mRNA splicing or proteolytic processing, Ob-R isoforms are divided in three classes, short and long (which are bound to the cellular membrane) and secreted (a soluble protein that binds leptin in blood). The long isoform Ob-Rb (or l) is the predominant one, expressed at high levels in different cell types. Ob-Rb has full signaling capabilities in contrast to short Ob-R isoforms. It is generally accepted that leptin binding to Ob-R provokes the formation of a homodimer that is responsible for leptin-mediated signals. Leptin and Ob-R have absolute specificity for binding. Once leptin binds to Ob-Rb, it activates several signaling pathways. Because Class Ⅰ cytokine receptors lack auto phosphorylation function they need auxiliary kinases to initiate signaling upon ligand binding. The first signaling event after leptin binding to Ob-Rb is the activation of janus kinase/signaling transducer and activation of transcription factor pathway (JAK/STAT)[21]. JAK2 recruitment to Ob-R intracytoplasmatic tail leads to the phosphorylation of the kinase, subsequent phosphorylation of Ob-R in several intracytoplasmatic sites and recruitment and phosphorylation of tyrosine residue on STATs. Phosphorylated STATs, then form hetero or homodimers and translocate to the nucleus to induce the transcription of specific genes[22].

Leptin plays roles in other physiological functions, as indicated by the presence of its receptor in different organs and tissues types besides the hypothalamus[23]. Leptin is involved in immunity, proliferation, differentiation, apoptosis, angiogenesis, inflammation, fertility and oncogenesis[12,16,22]. Leptin is known to inhibit bone formation[24]. It can also regulate the ovulatory cycle by

stimulating GnRH from the hypothalamus[25,26] and is an important factor in embryo implantation[27-29]. Leptin is involved in the onset of puberty[30], regulates glucose homeostasis[31], hematopoiesis[32], and modulate immunity like T cell activity in response to atherosclerosis[33]. Leptin has been speculated to be an inflammatory marker that responds specifically to adipose-derived inflammatory cytokines[34].

Obesity is a significant risk factor for cancer incidence and mortality. The effects of obesity on cancer could be due in part to leptin's elevated levels and Ob-R over expression in cancer cells, which enable leptin-deregulated pleiotropic signals in cancer. Leptin has been shown to have several pro-tumorigenic effects, such as increasing cancer cell proliferation, anti-apoptosis, angiogenesis, self-renewal and possibly resistance to chemotherapeutic treatment[12,16].

Several studies linked the effects of leptin on the proliferation of cancer both *in vivo* and *in vitro* experimental models, and from patient data. Leptin signaling has been consistently linked to the development of breast, endometrial, pancreatic, colon, prostatic, hepatic, skin, brain, oesophagus, stomach, thyroid gland, and ovarian cancers, and leukemia and chondrosarcoma[35-43].

Leptin induces breast cancer cell growth *in vitro* and *in vivo*. Several leptin-induced signaling pathways and factors have been linked to the proliferation of breast, endometrial and pancreatic cancer cells[12,16,36,37]. Leptin induced tumor cell growth and inhibited apoptosis in papillary thyroid cancer (PTC) cells. Serum levels of leptin were shown to be higher in patients with PTC than in negative controls[42]. An increase in the expression of leptin receptor Ob-R was observed in PTC specimens[44]. Leptin can induce the development of non-alcoholic fatty liver disease (NAFLD), one of the major cause of hepatocellular carcinoma, by promoting insulin resistance, steatosis and hepatic inflammation by increasing transforming growth factor beta (TGF-β) expression[43]. Leptin is overexpressed in colon cancer, Ob-R mRNA was found in cancer cell lines and colon tumors[45] and Ob-R protein expression was confirmed by western blot[46]. Serum leptin levels were significantly high in patients with lung cancer, compared to healthy individuals. Lung cancer tissues showed higher expression of leptin compared to normal lung tissue[47]. Leptin was shown to stimulate the proliferation of human myeloid leukemia cell lines[48], and it might play a role in the development of prostate cancer, it can increase growth and survival of prostate cancer cells and Ob-R mRNAs has been found in prostate cancer cells through RT-PCR[49]. Epithelial ovarian cancer (EOC) is one of the principal cause of death in gynecological malignancies, but the role of leptin in this disease still needs further investigation as Ob-R mRNA was found in several immortalized EOC cell lines[50]. Limited data suggested also a link between leptin and adrenal cancer[51].

Leptin induced pleiotropic effects in cancer cells. Leptin increased breast cancer cell proliferation, which was linked to the up regulation of cyclin D[52] and increased expression of anti-apoptotic proteins like Bcl-2 in breast cancer[53]. Additionally, leptin can down regulate pro-apoptotic Bax[54]. Leptin induces tumor angiogenesis that has a pivotal role in solid tumor growth and metastasis. Leptin not only promotes the expression of angiogenic factors like vascular endothelial growth factor (VEGF)[55], VEGFR-2[52,56], and fibroblast growth factor 2 (FGF-2), but also itself induces vascular endothelial cell proliferation *in vitro* with similar effects than VEGF[57]. Moreover, in the absence of VEGF, leptin induced Notch signaling pathway in endothelial cells that was linked to leptin-induced transphosphorylation of VEGFR-1 and VEGFR-2[58]. Leptin induces two angiogenic factors: Interleukin (IL)-1[59] and Notch[60] that can increase VEGF expression. Moreover, leptin induces the secretion and synthesis of proteases and adhesion molecules needed for the development of angiogenesis. Leptin induces expression of metalloproteinases 2 and 9 (MMP-2 and MMP-9) that are involved in tissue remodeling, specifically the breakdown of extracellular matrix proteins[61,62]. Additionally, leptin induces the expression of avB3 integrin that is also involved in angiogenesis[37,63]. Leptin induces production of inflammatory cytokines like IL-1, IL-6 and tumor necrosis factor (TNF)-α, which like leptin can induce the expression of metalloproteinases, promoting tumor invasion and metastasis. TNF-α acts on adipocytes increasing leptin expression[34].

LEPTIN-INDUCED NOTCH AND RBP-JK AFFECT CANCER PROGRESSION

Gonzalez-Perez's lab earlier reported that leptin signaling crosstalk to Notch in breast cancer[60]. Notch signaling is an embryonic conserved pathway involved in proliferation, angiogenesis, cell fate and development. Notch system is composed by transmembrane proteins: Receptors (Notch1-4) and ligands expressed in adjacent cells (Delta-like, Dll1-3, and Jagged-like, JAG1-2), and molecular targets hairy enhancer of split (Hes1-7), hairy/enhancer-of-split related with YRPW motif subfamilies (Hey1, Hey2, HeyL, HesL/HelT, Dec1/BHLHB2, Dec2/BHLHB3) and survivin. Notch receptors are all composed of an extracellular domain (NECD) where ligands bind, a transmembrane domain (TM) and an intracellular domain (NICD). Notch is activated upon binding to a ligand that triggers a proteolytic cascade producing activated NICD, which is transported to the nucleus where it binds to a tumor repressor, DNA-binding protein, recombination signal binding protein for immunoglobulin kappa J (RBP-Jk) or CBF1/Su(H)/Lag-1 (CSL) family of transcription factors[64].

RBP-Jk is a DNA binding factor, which mediate either transcriptional repression or transcriptional activation. RBP-Jk binds to the ubiquitous corepressor proteins (Co-R: Silencing mediator of retinoid and thyroid hormone receptors, SMRT and Ski-interacting protein, SKIP)[65], histone deacetylases (HDACs), CBF1 interacting corepressors (CIR), and SAP30 (a linker between CBF1 and the HDAC complex)[66], which repress transcription of some genes. Thus, RBP-Jk is a transcription factor

that acts as a repressor in complex with SMRT and SKIP when it is not associated with Notch. In contrast, activated NICD-RBP-Jk complex displaces co-repressors and recruits coactivator (Co-A). When RBP-Jk is associated with NICD it acts as a transcriptional activator in complex with mastermind-like proteins, MAML[67]. This process is required for Notch-induced canonical signals that increase the transcription of target genes such as Hes, Hey, nuclear factor-kappa B (NF-κB), cyclin D, c-Myc and others[64]. Additionally, Notch signaling is linked to expansion of cancer stem cell populations (CSC), which show self-renewal capabilities and can recapitulate tumor heterogeneity and are believed to be responsible for recurrence and drug resistance[68,69].

Notch signaling is deregulated in many cancers. Indeed, deregulation of Notch signaling is a hallmark of breast cancer[64]. In breast and pancreatic cancer cells leptin upregulates Notch receptors, ligands and targets[16,60]. Moreover, latest reports show a positive correlation between leptin, Ob-R and Notch components in endometrial cancer tissues from obese patients[70]. Leptin induces RBP-Jk and Notch that impacts on CSC and self-renewal[16,60,71]. Moreover, a novel crosstalk between Notch, IL-1 and leptin (NILCO) was found in breast cancer[53,60,72]. NILCO induces proliferation/migration and upregulation of VEGF/VEGFR-2, and could represent the integration of developmental, pro-inflammatory and pro-angiogenic signals critical for leptin effects in breast cancer[60]. Paradoxically, low expression of RBP-Jk has been reported in several solid tumors that was associated with increase aggressiveness[73]. Our preliminary data indicate that knockdown of RBP-Jk in breast cancer cells induces a dramatic increase of Notch 3 and Notch 4 expression, CSC population ($CD24^-/CD44^+$) and N-cadherin (epithelial-mesenchymal-transformation marker)[74]. These data may suggest that tumor repressor activities of RBP-Jk could overcome the oncogenic actions of NICD-RBP-Jk complex upon activation of Notch, thus, cancer cells downregulate RBP-Jk expression in order to proliferate and develop tumors. However, this topic deserves follow up and more deep mechanistic investigation.

LEPTIN SIGNALING INDUCES BREAST CANCER PROGRESSION

Leptin and Ob-R are low expressed in human mammary glands, yet they play a role in the normal development[75]. In contrast, leptin and Ob-R expression is upregulated in breast cancer[76]. Obese patients with breast cancer show tumoral leptin overexpression that correlated to larger and more advanced tumors[77]. The molecular mechanisms involved in obesity-related breast carcinogenesis are not very clear. The binding of leptin to its receptor on breast cancer cells induces the activation of multiple oncogenic pathways, including Jak/STAT3, ERK1/2, and phosphoinositide 3-kinase (PI-3K) pathways, cyclin D1 expression and retinoblastoma protein hyperphosphorylation[78]. Triple negative breast cancer (TNBC) showed high level of molecules correlated with metastasis and lower survival of patients of leptin (*i.e.*, IL-1, Notch and VEGF/VEGFR2). Notch, IL-1 and leptin crosstalk outcome (NILCO) seems to play essential roles in the regulation of leptin-mediated induction of proliferation/migration and expression of pro-angiogenic molecules in breast cancer[64].

Breast adipose tissue is a source of estrogen, which is involved in tumorigenesis. Estrogens promote cell proliferation by inhibiting apoptosis and inducing angiogenesis[79]. Therefore, these molecules are breast cancer markers and therapeutic targets. A functional crosstalk between estrogen and leptin exists and may act to promote tumorigenesis[80]. The aromatization of androstenedione in adipose tissue is the main source of estrogen[81], a reaction catalysed by the enzyme aromatase, whose expression is increased by leptin in ER positive breast cancer cells[82]. Leptin has been shown to induce resistance in ER positive cancer cells to Faslodex[83] and Tamoxifen[84]. Leptin binding to ObR was also shown to transactivate HER2/neu[85], which is an important oncogenic protein involved in breast cancer growth. All these data indicate that leptin is involved in the development of breast cancer. Therefore, the use of leptin-signaling targeting drugs could be a novel strategy in breast cancer management.

LEPTIN SIGNALING PROMOTES THE EXPANSION OF CANCER STEM CELLS

Breast cancer stem cells

The cancer stem cell (CSC) theory postulates the existence of a sub-population of cancer cells with the ability to undergo self-renewal and also tumor differentiation[86]. The presence of these cells is a risk factor for carcinogenesis. CSC can recreate the bulk of the tumor, and are believed to be responsible for tumor initiation, cancer recurrence and metastatic progression[87]. CSC in breast cancer (BCSC) initiate and drive carcinogenesis and tumor differentiation[88]. BCSC can be identified by several molecular phenotypic markers. Networks of cytokines and growth factors, including leptin, have been implicated in BCSC interaction with the tumor micro-environment[89]. BCSC exhibit a high sensitized responses to leptin. It was reported that leptin mediates microenvironment effects on BCSC activity that establishes a self-reinforcing signaling circuit. Leptin upregulates several factors considered BCSC markers in several breast cancer cell lines like, including CD44, ALDH1[60], HER2[90], Oct-4 and Sox2[91]. Leptin is also involved in activation of transcriptional factors associated with BCSC, like STAT3[92] and NF-κB[93]. BCSC markers are shown in Table 1[60,90,91,94-105].

PANCREATIC CANCER STEM CELLS

Characterization of pancreatic cancer stem cells

Pancreatic cancer stem cells (PCSC) are characterized by the expression of cell markers, including $CD24^+CD44^+$, $CD133^+$, $CD24^+CD44^+$ and epithelial specific antigen (ESA^+ or $EpCAM^+$) and aldehyde dehydrogenase ($ALDH^+$)[106-108]. PCSC represent a rare cell population of 0.5%-1% of

Table 1 Breast cancer stem cells markers

Markers	Localization	Ref.	Markers	Localization	Ref.
CD44	Cell surface	Guo *et al*[60], 2011	MET	Cell surface	Baccelli *et al*[100], 2013
CD24	Cell surface	Kakarala *et al*[94], 2008	CD133	Cell surface	Tume *et al*[101], 2016
Epcam	Cell surface	Chiotaki *et al*[95], 2015	CD338	Cell surface	Leccia *et al*[102], 2014
CD49f	Cell surface	Chiotaki *et al*[95], 2015	ALDH1	Cytoplasm	Guo *et al*[60], 2011
MUC1	Cell surface	Nigam[96], 2013	Bmi I	Cytoplasm	Kim *et al*[103], 2015
CD29	Cell surface	Yeo *et al*[97], 2016	GLI I	Cytoplasm	Fernandez-Zapico[104], 2013
CD61	Cell surface	Yeo *et al*[97], 2016	Sox2	Cytoplasm	Feldman *et al*[91], 2012
CD47	Cell surface	Zhang *et al*[98], 2015	4-Oct	Cytoplasm	Feldman *et al*[91], 2012
HER2	Cell surface	Korkaya *et al*[90], 2008	NANOG	Cytoplasm	McClements *et al*[105], 2013
eHSP90	Cell surface	Stivarou *et al*[99], 2016			

total PC cells (Table 2). Remarkably, when isolated and inoculated into nude mice PCSC generate tumors, whereas implantation of PC cells negative for these markers could not. Rasheed *et al*[109] showed that a subpopulation of PCSC, $CD133^+CXCR4^+$ was found in patients with PC metastatic disease. Additionally, PC $ALDH^+$ cells showed enhanced clonogenic growth, migratory potential and affected negatively the overall survival of PC patients. In 2011, Li *et al*[106] described a new population of PCSC c-Met^+ involved in PC growth and metastasis. Recent preclinical data suggest PC c-Met^+ cells are involved in drug resistance. Indeed, the use of a c-Met inhibitor (Cabozantinib) in PC patient overcomes Gemcitabine resistance[110]. PCSC could also be identified by flow cytometry using Hoechst 33342 dye. PC side population that can exclude Hoechst 33342 dye correlated with chemoresistance and poor survival[111]. Wang *et al*[112] described a similar PC side population (Hoechst 33342 negative) showing high expression for $CD133^+$, $ABCG2^+$ and $Notch1^+$, which were more chemoresistant compared to non-side population cells. A PCSC population marked by the expression of Doublecortin and Ca/Calmodulin- Dependent Kinase-Like 1 (Dclk1) was described by Bailey *et al*[113] in 2014. PCSC $Dclk1^+$ were found in PanIN (pancreatic intraepithelial neoplasia) lesions, as well as in invasive stages of PC. These findings suggest that PCSC populations can be identified at the early stages of pancreatic tumorigenesis and may serve as a biomarker for early detection of this deadly disease.

PCSC show self-renewal and multipotency, and can initiate and propagate the parental tumor while serial passage into immunocompromised mice[114]. CSC including PCSC have retained the expression of at least three of the transcription factors that are characteristic to embryonic stem cells (ESC) (Oct-4, Sox-2 and Nanog). Increased levels of Oct-4 and Nanog are correlated with early stages of carcinogenesis and worse prognosis. Oct-4 and Nanog play important roles in embryonic development, and also in maintaining the stemness of PCSC. In contrast, PCSC double knockdown of Oct-4 and Nanog show reduced proliferation, migration, invasion and tumorigenesis[115]. Additionally, Oct-4 contributes to metastasis and cancer multidrug resistance[116]. *De novo* Sox2 expression alone in PC is sufficient to promote self-renewal, differentiation and stemness. Although ESC and PCSC share the property of self-renewal, ESC favors differentiation, while PCSC act more toward proliferation and inhibition of apoptosis. Targeting PCSC may be a viable therapeutic strategy against PC. A better understanding of Oct-4, Sox-2 and Nanog regulation could facilitate the design of individualized therapies for PC patients[117].

Current studies demonstrate that PCSC determine tumor relapse and metastasis following chemotherapy[118]. From a clinical perspective, targeting PCSC populations would ensure tumor eradication. However, PCSC possess escape mechanisms shared with normal stem cells, such as over-expression of multi-drug transporters. These transporters increase the efflux of anticancer drugs, thereby reducing their accumulation inside the cancer cells[118]. ABCB1 protein was significantly augmented in $CD44^+$ cells during acquisition of PC cells resistance to Gemcitabine. CD44 expression in PC was correlated with histologic grade and poor prognosis. These data indicate that cancer stem cells were expanded during the acquisition of Gemcitabine chemoresistance[119]. In line with these findings, the administration of anti-CD44 monoclonal antibody to a human PC xenograft mouse model increased Gemcitabine sensitivity[120]. Additionally, Metformin enhanced the capacity of Gemcitabine to inhibit the proliferation of PC cells by inhibiting the proliferation of $CD133^+$ cells[121]. Side population PCSC identified by Van der Broeck in 2012[111] are resistant to Gemcitabine. Side population PC cells isolated from Panc-1 cell line have been found to express both ABCB1 and ABCG2, which contribute to chemoresistance[122]. Identification of enhanced stem cell populations within PC tumors might be used as biomarkers for personalized therapy.

Pancreatic cancer stem cell regulators

Several factors could affect PCSC. Accumulated evidence suggested that microRNAs are involved in the regulation of PCSC. Specifically, miRNA34 affects the maintenance and survival of PCSC[123]. Obesity is associated with increased severity of acute pancreatitis[124] and decreased survival of PC patients. In obese mice, IL-6 contributes to prolonging inflammation and altering resolution from pancreatic damage, possibly contributing to a microenvironment favorable to tumorigenesis[125]. Cigarette smoking and nicotine, a major risk factor in PC, increase monocyte chemoattractant protein 1 (MCP-1)

Table 2 Pancreatic cancer stem cells markers

Stem cell population	Localization	Ref.
$CD24^+CD44^+$	Extracellular	Li *et al*[106], 2007
$CD24^+CD44^+ESA^+$	Extracellular	Li *et al*[106], 2007
$CD133^+CXCR4^+$	Extracellular	Hermann *et al*[107], 2007
$CD133^+CD44^+$	Extracellular	Ji *et al*[123], 2011
C-Met	Extracellular	Li *et al*[106], 2007
DCLK1	Intracellular	Bailey *et al*[113], 2014
ABCB1	Extracellular	Van den broeck *et al*[111], 2013
Sox2	Intracellular	Herreros-Villanueva *et al*[117], 2014

expression in PC cells. MCP-1 was found in 60% of invasive PC lesions, of whom 66% were smokers[126]. The concentration of six cytokines (IL-1β; IL-6, IL-8, VEGF, TGF, IL-10) were consistently reported to be increased in pancreatic ductal adenocarcinoma (PDAC) patients. These molecules were associated with the severity of PDAC (*i.e*., metastasis, tumor size, and advanced stage) that suggest these cytokines have prognostic biomarker for PC[127]. Additionally, IL-8/CXCR1 axis was associated with cancer stem cell properties in PC[128]. CXCR1 expression in PC patients correlates with lymph node metastasis and poor survival. MMP-13 has been shown to help mediate the effect of leptin on invasiveness and metastasis of PC. In addition, there was a positive correlation between the expression of PCSC markers CD133 and CD44, and CXCR1[129].

P300 is a tumor suppressor gene. However, this factor is also upregulated in various cancer types and associated with worse prognosis. In PC, P300 is associated with chemoresistance from apoptosis upon Gemcitabine-induced DNA damage[130]. TGF-β negatively regulates ALDH1 expression (a PCSC marker) in a SMAD dependent manner in PC cells. This regulatory mechanism might be disrupted by SMAD4 mutations and deletions in PC cells[131]. The binding of stem cell factor (SCF, a protein involved in PC progression) to its receptor, c-kit, determines an increase in HIF-1α synthesis that affects cancerous transformation, chemoradiotherapy resistance, and tumor progression[132].

Additionally, high levels of leptin receptor, Ob-R, are associated with PC stage and lymph node metastasis and overall shorter survival. Ob-R and HIF-1α expression was highly associated in PC tissues. HIF-1α regulated the expression of Ob-R in PC[133]. Leptin binding to Ob-R was earlier found to induce HIF-1α in breast cancer cells. Leptin-induced HIF-1α was involved in the upregulation of VEGFR2 in these cells[55]. Therefore, it is possible that a leptin-induced HIF-1α feedback regulating Ob-R is present in PC. Moreover, robust expression of Ob-R is a characteristic of tumor initiating stem cells and pluripotent stem cells that was mediated directly by Oct-4 and Sox2[91]. Furthermore, the expression of leptin in gastro-esophageal adenocarcinomas was associated with chemoresistance. The use of leptin receptor antagonist SHLA increased the sensitivity to Cisplatin in the resistant gastric cancer cell line, AGS Cis5, and the oesophageal adenocarcinoma cell line, OE33[134].

Chemoresistance and cancer stem cells

In the absence of targeted therapeutic options, chemotherapy, along with surgery and radiotherapy are usually the last and only options for cancer treatment. Thus, resistance to chemotherapy is a vital area of research. Investigations on the mechanisms involved in chemoresistance are essential to overcome this issue. There are several mechanisms related to chemoresistance that have been identified in cancer cells, which include reduction or inhibition of drug-induced apoptosis, overexpression of detoxification and efflux proteins, increased expression of survival factor and pathways as nuclear factor kappa-light-chain-enhancer of activated B cells (NFκB) and PI-3K/Akt, hypoxia and hypoxia inducible factor HIF, and expansion of chemoresistant CSC among others[135-138].

Inhibition of apoptosis

Numerous chemotherapies target the increased DNA synthesis that cancer cells undergo. Classes of chemotherapeutics such as platinum agents (Cisplatin), alkylating agents (Cytoxane) and anthracyclines (Adriamycin or Doxorubicin) inhibit DNA synthesis. A consequence of the action of these agents is increased apoptosis due to DNA damage. The p53 pathway plays an important role in cancer cell avoidance of apoptosis, with mutations in the p53 gene associated with increased drug resistance in cancer cell lines and poor survival in cancer patients[135,139]. In addition, cancer cells have been known to competitively inhibit Caspase 3, a central molecule in the apoptosis pathway. These cells show increased expression of B cell lymphoma 2 (BCL-2) and B cell lymphoma extra-large (BCL-xL) anti apoptotic proteins[140-143].

Detoxification and efflux proteins

Aldehyde dehydrogenases (ALDH) are a class of enzymes that oxidise aldehydes. ALDH isoforms have been implicated in CSC and resistance to chemotherapeutics. ALDH1 is a marker of CSC and progenitor cells[144], whose expression correlated with poor response to Docetaxel therapy in advanced breast cancer[145]. Increased expression of ALDH1A1 and ALDH3A1 lead to greater inactivation of Cyclophosphamide in breast cancer[136].

ATP binding cassette (ABC) transporters are a family of transmembrane proteins involved in the efflux of drugs from cancer cells. ABC (ABCB1, ABCC1 and ABCG2) family of proteins are mainly found on CSC side-population (SP, Hoechst negative). ABCB1, also known as p-glycoprotein, CD243 or MDR1, is an efflux pump protein with broad substrate specificity. It is known to pump out chemotherapeutics such as Doxorubicin and Paclitaxel. ABCC1 is known to give cancer cell resistance to anthracyclines such as Doxorubicin. ABCG2 also called the breast cancer resistance protein or CDw338, allows cancer cell resistance to Mitoxantrone and Doxorubicin[146].

NFκB pathway

NFκB signaling pathway is a survival mechanism that

controls DNA transcription of several genes. In non-malignant cells NFκB signaling plays a central role in immune response to infection. It is responsible for cellular responses to a wide range of stimuli which include reactive oxygen species, ionising radiation, bacterial lipopolysaccharide, IL-β and TNF-α. To drive oncogenesis, NFκB signaling cooperates or crosstalks with signaling pathways, oncogenic or cancer-related proteins such as STAT3, p53, ALDH1, glycogen synthase kinase (GSK-3β), PI-3K, MAPK, PKC, and others[147].

NFκB signaling is a critical mediator of chemoresistance in cancer. Glioblastoma multiforme's resistance to Gemcitabine involves NFκB, ALDH and ROS actions[148]. Anti-ovarian cancer effects of MK5108 compound relied on the inhibition of the Aurora-A kinase and NFkB signaling, which induced polyploidy and cell cycle arrest[149]. In breast cancer, targeting NFκB signaling increased apoptosis and reduced proliferation in drug resistant breast cancer cell lines[150]. In mesothelioma, the STAT3-NFκB signaling crosstalk is essential in ALDH-mediated chemoresistance[151]. Abnormal activation of NFκB signaling is also implicated in cancer resistance to Paclitaxel therapy[152].

HIF and tumor hypoxia

Hypoxia is a term which describes deficient oxygen supply to tissues due to poor vasculature, as it is in the case of obesity and cancer. Proliferation and expansion of adipose tissue induce tissue hypoxia and the expression of HIF. Hypoxia in cancer is associated with poor outcomes and chemoresistance[137,153]. In TNBC, chemotherapeutic treatment with Paclitaxel and Gemcitabine results in expression of HIF, and enrichment of CSC through IL-6 and IL-8 actions. Chemical inhibition of HIF results in the depletion of CSC and tumour abrogation *in vitro* and *in vivo*[154].

In addition, hypoxia promotes survival of TNBC MDA-MB231 from Paclitaxel-induced apoptosis *via* mTOR/JNK pathway[155].

CSC resistance to chemotherapy

The presence of CSC within tumors make them resistant to chemotherapy. CSC are commonly more resistant to chemotherapeutics which target the bulk of the tumour that allow the proliferation of CSC[156]. The CSC stemness phenotype and chemoresistance involve TGF-β signaling, which plays a prominent role in stem biology, facilitating epithelial to mesenchymal transition in mammary cancer cells, which is a property of CSC[138]. TNBC cell lines treated with Paclitaxel showed an enrichment of cancer cells with stem like properties and increased TGF-β signaling both *in vitro* and *in vivo*. Chemical inhibition of TGF-β signaling abrogates tumor formation[157]. CSC show higher expression of ABC family of proteins that increase their capability to efflux chemotherapeutics from cells. CSC also show diminished apoptosis rate, and over activation of detoxification proteins and survival pathways as NFκB and PI-3K[158].

OBESITY, LEPTIN AND DRUG RESISTANCE

Obesity and leptin signaling have been implicated in enhance capabilities of cancer cells to avoid apoptosis. Leptin expression was associated with higher expression of BCL-2 and BCL-xL expression in breast cancer cells[159]. Furthermore, leptin signaling has been reported to activate the PI-3K/Akt pathway that antagonizes apoptosis in various cancers such as colon cancer, liver cancers, endometrial cancers and lymphomas[44,160-163]. Additionally, obesity has been shown to influence breast cancer response to Doxorubicin therapy. Indeed, obese mice treated with Doxorubicin showed more proliferative tumors that also had more CSC as compared with non-obese mice[164]. Leptin increases the expression of ABC protein transporters in glioblastoma[165]. Our preliminary data further show that leptin increases the expression of ABCB1 in breast and pancreatic cancer cells.

Another mechanism involved in obesity-induced chemoresistant is NFκB signaling. It is known that NFκB is activated by leptin signaling and that can increase survival of cancer cells under chemotherapeutic treatment[55]. An additional link between obesity (*via* leptin signaling) and cancer chemoresistance is HIF, which correlates with activation of leptin signaling in several cancers including endometrial, pancreatic, breast and colon cancers[133,166-168]. A potential mechanism involved in obesity-mediated drug resistant is TGF-β signaling. Leptin and TGF-β are commonly co-expressed in breast cancer[34]. It is known that TGF-β signaling induces leptin expression. However, the connection between leptin and TGF-β signaling in breast cancer is still unclear[169].

Leptin increased proliferation and survival of breast cancer estrogen receptor positive cells, MCF-7 cells treated with Cisplatin. These data further assessed that leptin is a survival factor that induces drug resistant in breast cancer[170]. Moreover, leptin was found able to induce CSC expansion in breast[60] and pancreatic cancer[16]. Furthermore, our preliminary data suggest that leptin induces the expression of Oct-4 and Nanog in breast cancer cells. These factors are essential for the upregulation of Ob-R in cancer cells[91]. Thus, leptin can induce a feedback mechanism through Oct-4/Nanog to sustain Ob-R expression and its pro-oncogenic signals in breast cancer.

LEPTIN ANTAGONISTS AND CANCER PROGRESSION

Leptin signaling has numerous protumorigenic effects, including the increase chemoresistance found in several tumors. Therefore, leptin antagonism could be a new strategy to overcome drug resistance in cancer. Several molecules have been described as potential new agents to target leptin-induced cancer growth and drug

resistance. Majority of the leptin antagonists reported are mutated or truncated versions of leptin molecule: Leptin muteins, Allo-aca and D-ser, LDFI, and leptin peptide receptor antagonists (LPrA).

SMLA and SHLA

Leptin muteins or mutant proteins, were generated using random mutagenesis of the leptin sequence and screened for high affinity variants using a yeast surface display. This resulted in the creation and identification of high affinity muteins. Two mutein antagonists named superactive mouse leptin antagonist (SMLA) and superactive human leptin antagonist (SHLA) were made by the introduction of an *Asp23* mutation. These antagonists showing 4 aminoacid residue mutations (D23L/L39A/D40A/F41A) were reported to have 60-fold increased affinity for Ob-R and 14 fold greater antagonistic activity as compared with the original leptin antagonist showing 3 mutations (L39A/D40A/F41A)[171]. These muteins were pegylated at the N terminus to increase bioavailability and stability. However, the pegylated muteins increased BW in mice. Pegylated SMLA induced higher BW gain as compared with the pegylated SHLA[171]. No effects of muteins on leptin-induced chemoresistance in cancer have been reported to date.

Allo-aca and D-ser

Allo-aca is a non-toxic, 9-residue peptide leptin antagonist based on the C terminal Ob-R binding leptin site Ⅲ. Allo-aca was reported to increase survival of CD1 nude mouse hosting TNBC. The effective dose of the peptide was found after 9 to 13 d of treatment by injecting intraperiotoneally between 0.1 and 1 mg Allo-Aca/kg body weight (BW)/day. Allo-aca was nontoxic in C57Bl/6 and CD1 nude mice, but showed hepatotoxicity at 0.2 mg/kg BW/day in SCID mice[172]. Additionally, it induced weight gain of 6% to 10% of BW[172]. Treatment of TNBC MDAMB231 cell line with Allo-aca 50 pM inhibited leptin-induced proliferation *in vitro*[172]. D-ser, peptide inhibitor is an analogue of Allo-aca that at 1 nM concentration inhibited leptin-induced proliferation in Ob-R positive breast and colon cancer cells *in vitro* without exhibiting agonist activity[173]. However, no data on the effects of these antagonists on leptin-induced drug resistance and CSC are available.

LDFI

LDFI is a leptin peptide antagonist composed by amino acid 39 to 42 on the leptin binding site I (Leu-Asp-Phe-Ile). LDFI was reported to inhibit leptin-induced growth of breast cancer cells *in vitro* and *in vivo*[174]. This peptide antagonist inhibited proliferation, colony formation on soft agar and Boyden chamber transmigration of estrogen receptor positive as well as estrogen receptor negative breast cancer cells. LDFI effects correlated with reduced expression of key downstream leptin effectors such as JAK2, STAT3, AKT and MAPK. *In vivo*, the pegylated peptide (LDFI-PEG) was shown to inhibit tumour growth in a murine mammary xenograft model. LDFI-PEG showed no toxicity or effects on BW of mice[174]. No reports on potential effects of LDFI on drug resistance in breast or other cancer types are available.

LPrAs

LPrA1 and LPrA2 were earlier designed and tested *in vitro* and *in vivo* in mouse models[52,53,56,72,175,176]. LPrAs are composed by aminoacid sections of the binding site Ⅰ (LPrA1) and Ⅲ (LPrA2) of the leptin molecule[63]. LPrA2 was conjugate to polyethylene glycol 20 kDa (PEG-LPrA2) or to iron-oxide nanoparticles (IONP-LPrA2) to increase its bioavaibility and effectiveness to block leptin signaling in cancer cells. Unconjugated and conjugated LPrA2 effectively inhibited leptin-induced protumorigenic actions in breast and pancreatic cancer cells[52,53,56,72,175,176]. LPrA2 showed potent effects for the reduction of leptin-induced growth of tumors and expression of inflammatory (IL-1/IL-1R tI), proliferation (Ki67, PCNA), angiogenic factors (VEGF/VEGFR2) and Notch in tumors and endothelial cells[53,56,58,72]. The antagonist effects of LPrA2 on tumor growth and angiogenesis were more evident in obese than in lean mice[53,72]. However, unconjugated or conjugated LPrA2 showed no toxicity and did not affect energy balance (BW or food intake) or general health when it was applied (0.1 mM/i.v. per twice weekly) to many lean and obese mice for two months. Remarkably, LPrA2 negatively impact on leptin-induced expansion of CSC and Notch expression in breast and pancreatic cancer cells, derived tumorspheres and xenografts[16,74]. Moreover, LPrA2 significantly reduced the leptin-induced effects on drug resistance (Cisplatin, Sunitinib, Paclitaxel, Doxorubicin) in breast cancer cells[16,176].

CONCLUSION

Combination of poor dietary habits and low physical activity, which are reinforced by accessibility of low-cost high caloric and fat foods have led to the obesity pandemic. Accumulated evidence supports a negative role of obesity on cancer risk, progression and management. Despite many efforts and social programs to tackle obesity, its effects on morbidity and mortality and its influences on cancer incidence and treatment are in crescendo[1-5]. It is known that obesity and leptin signaling not only affect cancer cells, but also tumor stroma. Moreover, leptin and paracrine factors secreted from cancer and stroma cells (adipocytes, fibroblasts, endothelial cells and inflammatory cells) could affect tumor progression, CSC and chemoresistance[16,176]. In this regards, the use of nontoxic leptin antagonists that do not affect energy balance could be a novel adjuvant therapy for cancer drugs. These compounds can increase chemotherapeutic effectiveness and allow reducing their dosage and undesired side effects in cancer patients.

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REVIEW

Targeted therapies in breast cancer: New challenges to fight against resistance

Viviana Masoud, Gilles Pagès

Viviana Masoud, Gilles Pagès, University Nice Sophia Antipolis, Institute for Research on Cancer and Aging of Nice CNRS UMR7284/INSERM U 1081, 06189 Nice, France

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Correspondence to: Gilles Pagès, PhD, Director of Research INSERM, University Nice Sophia Antipolis, Institute for Research on Cancer and Aging of Nice CNRS UMR7284/INSERM U 1081, Centre Antoine Lacassagne 33 Avenue de Valombrose, 06189 Nice, France. gpages@unice.fr
Telephone: +33-4-92031231
Fax: +33-4-92031235

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Abstract

Breast cancer is the most common type of cancer found in women and today represents a significant challenge to public health. With the latest breakthroughs in molecular biology and immunotherapy, very specific targeted therapies have been tailored to the specific pathophysiology of different types of breast cancers. These recent developments have contributed to a more efficient and specific treatment protocol in breast cancer patients. However, the main challenge to be further investigated still remains the emergence of therapeutic resistance mechanisms, which develop soon after the onset of therapy and need urgent attention and further elucidation. What are the recent emerging molecular resistance mechanisms in breast cancer targeted therapy and what are the best strategies to apply in order to circumvent this important obstacle? The main scope of this review is to provide a thorough update of recent developments in the field and discuss future prospects for preventing resistance mechanisms in the quest to increase overall survival of patients suffering from the disease.

Key words: Breast cancers; Resistance; Human epidermal growth factor receptor 2; Angiogenesis; Triple negative; Immune tolerance

Core tip: There are several reviews in the literature dedicated to breasts cancers. However, our manuscript is an updated review on the current knowledge and particularly on the molecular mechanisms involved in the relapse of patients on the current treatments. A summary of ongoing clinical trials gives a perspective for future therapeutic strategies. Our manuscript represents a working document for researchers/oncologists in the field of breast cancers.

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INTRODUCTION

Breast cancer targeted therapies involve substances or drugs which block the growth of cancer by interfering with the function of specific molecules responsible for tumor cell proliferation and survival[1-21]. Breast cancer cells may overexpress specific receptors which, when activated can initiate downstream signaling resulting in the expression of genes for cancer cell proliferation, growth, survival, migration, angiogenesis and other vital cell cycle pathways[22,23].

There are various types of breast cancer, some have hormone receptors like estrogen or progesterone (some have both) and are called ER+ or PR+ breast cancer respectively.

The estrogen receptor ER is a major driver of the majority of breast cancers as it is expressed in 75% of breast cancers overall. It is more frequently related with postmenopausal women and there is a 99% survival rate at ten years. Hormone sensitive breast cancer has a strong correlation with lower tumor grade; lower levels of amplification of the human epidermal growth factor receptor 2 (*HER2*) oncogene and concomitant loss of *p53* tumor suppressor gene; expression of progesterone receptor (PR), soft tissue and bone metastases and slower rates of disease recurrence. In cases of hormone positive breast cancer along with the expression of ER, multigene tests may be carried out to make treatment decisions particularly for adjuvant therapy and screen those patients who would benefit more from combination of endocrine plus chemotherapy[24-26].

The most common receptors that are overexpressed in breast cancer cells are part of the epidermal growth factor receptor (EGFR) family of receptor tyrosine kinases: EGFR and HER2 are overexpressed in approximately 40% and 25% of breast cancers respectively and are believed to be responsible for more aggressive tumor behavior and poor prognosis[27].

Triple negative breast cancer (TNBC) is defined by the lack of expression of both estrogen and progesterone as well as the HER2 protein and is often associated with an unfavorable prognosis as no treatment is yet available for this particular breast cancer subtype[28].

The rapid acquisition of resistance in breast cancer targeted therapies seems to limit the effectiveness of treatment and even though some of the genetic mutations and epigenetic changes in molecular pathways have been understood, it is sometimes necessary to combine several pathway blockades in order to achieve successful treatment results[29-35].

The identification of new target molecules in breast cancer and the use of combination therapies may have improved the understanding of compensatory pathways which lead to the emergence of resistance mechanisms, nevertheless, breast cancer subtypes like TNBCs seem to exploit alternative proliferative pathways which are not yet fully understood and need urgent attention and elucidation[11] (Figure 1).

TARGETED THERAPIES IN BREAST CANCER

Estrogen and estrogen receptors are key drivers in breast cancer progression. This is the reason why targeting estrogen has been used for many years to inhibit the estrogen signaling pathway in women with estrogen positive breast cancer. Selective estrogen receptor modulators or SERM have been used to suppress tumor growth in estrogen dependent breast cancers and tamoxifen was the first drug to be approved for estrogen positive metastatic breast cancer reducing recurrences by approximately 40%-50%[36].

Aromatase inhibitors (anastrozole, letrozole, exemestane) are also used as an alternative therapy to treat estrogen dependent breast cancers as they block the biosynthesis of androgens through inhibition of the aromatase enzyme resulting in reduction of estrogen levels in tumor cells[36].

Other therapies are available for other forms of breast cancer that are not hormone dependent. The HER2 protein represents the most common overexpressed receptor signature in breast cancer and is considered a relevant biomarker for treatment.

The recombinant antibody trastuzumab (Herceptin) targets HER2 and is the first drug that was approved by the FDA in 1998 for the treatment of HER2 positive breast cancers[37,38].

Other agents that followed such as pertuzumab and lapatinib have not shown immunity to the development of resistance mechanisms with significant side effects for the patients[7,39,40].

The conjugated monoclonal antibody TDM1 (trastuzumab emtansine) may be used in HER2 positive breast cancers as trastuzumab efficiently transports the DM1 drug, a microtubule inhibitor, directly into the breast cancer cells to inhibit growth.

Triple negative cancers lacking hormone receptors and HER2 may respond to agents like PARP1 inhibitors and may have HER1 as a potential target. The monoclonal antibody cetuximab combined with cisplatin chemotherapy has shown promising results in a Phase Ⅱ study, suggesting some subtypes of TNBC may be EGFR inhibition sensitive[41].

The conventional route to treat TNBC patients by taxol derivatives and anthracycline chemotherapy is still widely used until more "druggable" targets are identified[41]. Recent studies suggest that the microtubule-stabilizing agent ixabepilone in combination with capecitabine may

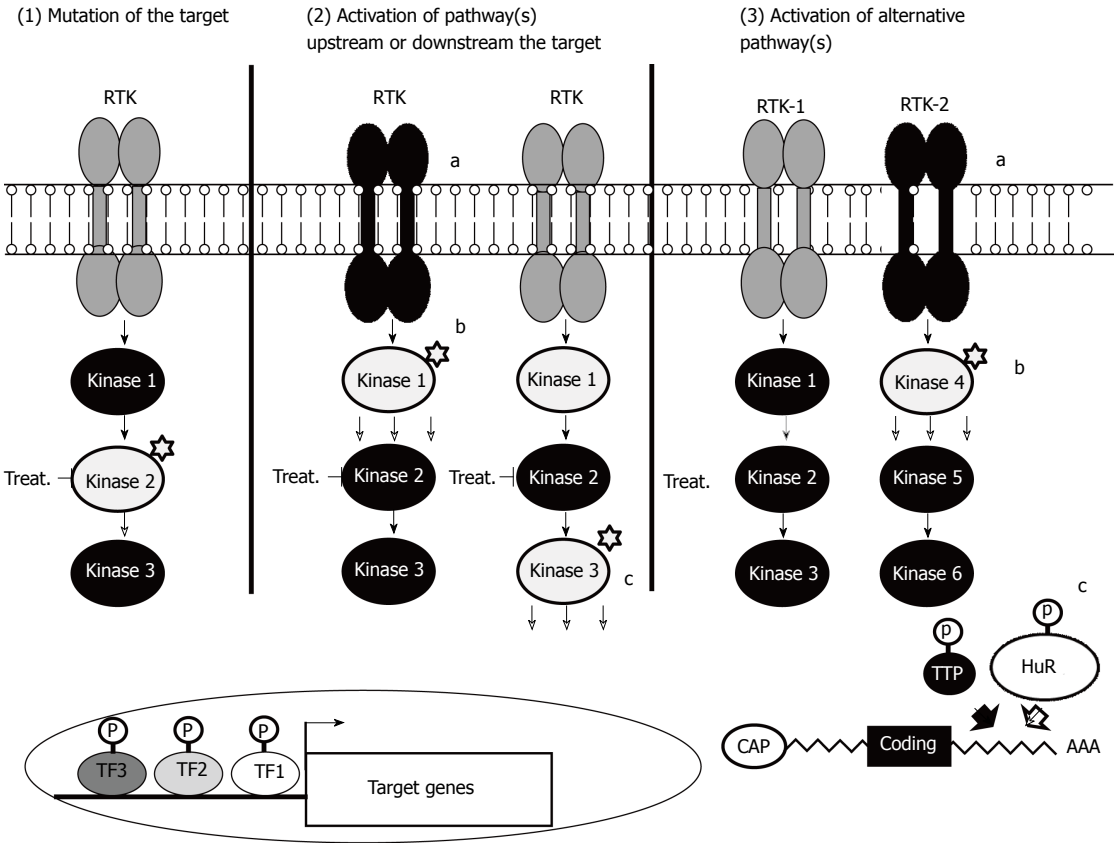

Figure 1 A schematic diagram of the most common resistance mechanisms to targeted therapies. (1) Alteration of the drug target (Treat.): This type of resistance involves mutations as well as amplifications of drug targets such as kinases; (2) Upstream and downstream pathway effect through the activation of receptor tyrosine kinase (RTK) (a) and/or the mutation/amplification of upstream (b) or downstream (c) components; (3) Bypass mechanisms occur as a result of a second receptor tyrosine kinase activation (a), through a mutation of a parallel kinase (b) or modulation of mRNA binding proteins (c). These alternative mechanisms of resistance especially through kinases activation result in the modification of gene expression *via* the phosphorylation or transcription factors (TF).

be effective in TNBC that are resistant to anthracycline and taxane drugs and the PACS08 Phase Ⅲ trial is evaluating this possible treatment strategy[28].

Targeted therapies have also been approved against the vascular endothelial growth factor (VEGF) and the drug bevacizumab has proven effective in the treatment of advanced metastatic breast cancer when used in association with paclitaxel or docetaxel[42,43].

Inhibitors of downstream pathways like PI3K/AKT/mTOR and RAS/MEK/ERK are also available for therapeutic purpose as well as agents directed against other tyrosine kinases like SRC, insulin-like-growth-factor [IGF/IGF-receptor (IGFR)], poly-ADP ribose polymerase (PARP) Inhibitors and also matrix metalloproteases (MMPs) which are involved in cancer cell invasion and metastasis[8,29,31,44-48].

Compensatory survival pathways, increased phosphatydil-inositol-3-kinase (PI3K)[49-52] signaling, receptor tyrosine kinase signaling outside the ErbB/HER family and involvement of other HER receptors[53], may all play a key role in the development of alternative molecular pathways responsible for the development of therapeutic resistance in breast cancer cells.

Indications of breast cancer targeted therapies

Breast cancer targeted therapies are used to treat patients whose breast cancer cells overexpress certain characteristic proteins on their surface allowing an abnormal growth pattern. Antibodies are sometimes used as they work in a similar way as the human immune system.

The most efficient breast cancer targeted therapy today is the one targeting the HER2 protein overexpression on the surface of breast cancer cells. At present, there are seven widely used breast cancer targeted therapies which are effective in blocking several molecular pathways: Afinitor or everolimus, an m-TOR inhibitor, stops cancer cells from getting energy supplies[54-57]; Avastin or bevacizumab inhibits the growth of new blood vessels which supply oxygen and nutrients to cancer cells for growth and function[14,58]; Herceptin or trastuzumab blocks the ability of cancer cells to receive signals which tell them to grow[12,59]; Kadcyla or T-DM1 is a combination of Herceptin and emtansine[7,60]. In this case Herceptin is used as a transport method to deliver the emtansine chemotherapy to cancer cells; Perjita or pertuzumab works by stopping cancer cells from receiving growth signals[12,61]; Tykerb or lapatinib is a HER2 inhibitor that blocks signals of cell growth[4,42].

The HER2 protein

The HER2 proto-oncogene is overexpressed in 10%-12% of over 2500 cases of human breast cancers and this

is associated with malignant transformation and poorer overall survival rates particularly in breast tumors with lymph node metastasis[62].

The HER2 or neu oncogene (erbB2) is part of the EGFR family of tyrosine kinases and is located on chromosome 17 (17q12). It represents the most common overexpressed receptor in breast cancers and is considered a relevant therapeutic target[59,63-69].

The EGFR family is composed of four receptors: EGFR/HER1, ErbB2/HER2, ErbB3/HER3 and ErbB4/HER4. These receptors share common domains: an extracellular region characterized by leucine-rich repeats; cysteine rich repeats in the intracellular domain; a single transmembrane spanning region; a short juxtamembrane region; a kinase region and a cytoplasmic tail with various tyrosine phosphorylation sites[5,10]. Binding of ligands to the extracellular domain of EGFR, HER3 and HER4 allows for the formation of kinase active homo- and heterodimers to which HER2 is recruited as a preferential partner. Heterodimer formation between HER2/HER3 is the most common occurrence in these receptors' preferences. HER3 is often responsible for the activation of the PI3K/AKT signaling pathway *via* six docking sites for the p85 adaptor subunit of PI3K. The HER3/PI3K axis plays a key role in the survival of HER2-dependent cells as the loss of HER3 inhibits the survival of HER2-overexpressing breast cancer cells[70,71].

Trastuzumab resistance mechanisms

The first recombinant antibody approved by the FDA to target HER2-positive breast cancers was trastuzumab or Herceptin followed by other agents like pertuzumab and lapatinib.

Trastuzumab binds to the juxtamembrane region of the HER2 receptor tyrosine kinase resulting in the uncoupling of the HER2/HER3 heterodimers and consequent inhibition of downstream signaling and cytotoxicity.

Resistance mechanisms to trastuzumab develop often as a result of HER2 gene amplification and RNA/protein overexpression. HER2 overexpressing tumor cells continue to depend on the HER2 oncogene even after bypassing trastuzumab action possibly due to signaling from receptor tyrosine kinases (RTK) outside the ErbB family, increased PI3K signaling and the presence of alternative forms of HER2 which are not detected by trastuzumab. Also, the modulation of Cdk inhibitor p27 by IGF-1 may be a key player in resistance to trastuzumab as overexpressed IGF-1 is responsible for the activation of the PI3K downstream signaling pathway and further effects on Akt[72,73]. One of the key players in trastuzumab-resistance in HER2 positive breast cancer was the inhibition of expression of miR-375, a tumor suppressor gene which targets IGF1R[74]. Also, molecular pathway crosstalk may have resulted in increased cell survival and division by interference with HER2 accessibility, independent downstream signaling activation as well as HER2 mutations, particularly the expression of p95HER2, an active truncated form of HER2. Blocking IGFR1 completely resulted in restored sensitivity of HER2 positive cancer cells to trastuzumab *in vitro*. The loss of miR-375 with consequent epigenetic changes such as DNA methylation and histone deacetylation may drive the upregulation of IGFR1 and hence the development of trastuzumab-resistant cancer cells; in this case, targeting miR-375 may prove to be worthy of further investigation as a potential therapeutic target to restore trastuzumab sensitivity in HER2 positive breast cancer cells[74].

The new antibody-drug conjugate trastuzumab-DM1 (TDM1) which has been recently developed for the treatment of HER2 positive cancer has proved to be effective in inhibiting trastuzumab sensitive and resistant HER2 positive breast cancer cell lines *in vitro.* TDM1 drives both apoptosis and mitotic catastrophe in the trastuzumab resistant breast cancer cell line Jimt-1, acting as a potent inhibitor of microtubule assembly. These cells are characterized for having several co-existing trastuzumab resistance mechanisms like a mutation in the PIK3CA gene, low PTEN expression, overexpression of NRG1 and a moderate expression of the HER2 receptor. Interestingly, in the T-DM1 treated Jimt-1 cell line model, an accumulation of HER2 was observed in organelles which resembled enlarged lysosomes, suggesting sequestration of the protein in these intracellular granules[75].

The integrin αvβ6, involved in promoting migration, invasion and cancer cell survival, seems to play a significant role in the development of trastuzumab resistance mechanisms. Targeting αvβ6 with the 264RAD antibody in HER2 positive breast cancer cell lines expressing both HER2 and the integrin seems to slow down the growth of trastuzumab resistant tumors[62].

Resistance of breast cancer cells to trastuzumab mediated cytotoxicity has been implicated in the secretion of soluble factors by adipocytes and preadipocytes in adipose tissue proximal to breast cancer cells. The development of resistance mechanisms in this case occurs by inhibition of trastuzumab-mediated tumor lysis by natural killer cells *in vitro* and by adipose tissue *in vivo*. A reduced antitumor effect was observed in mice which had tumors in close proximity to a lipoma, while in another group of mice which had tumors located distant to the lipoma, the trastuzumab anti-tumor effects were enhanced. The inhibition of antitumor activity was enhanced when the adipocytes were in hypoxic conditions, these factors might suggest a link between patient obesity and development of trastuzumab resistance mechanisms[76].

The dual targeting of HER family receptors with antibody therapy may prove to be a strategy to overcome acquired resistance mechanisms by cancer cells to cetuximab. When both HER3 and EGFR were neutralized by cetuximab and the anti HER3 monoclonal antibody U3-1287, cetuximab sensitive tumor cells showed a significant decrease in proliferation possibly due to inhibition of both MAPK and AKT pathways

and a diminished signaling from all three HER family receptors[77].

The efficacy of trastuzumab in inhibiting proliferation of breast cancer cells might be dependent on the presence of endogenous HER-receptor activating ligands EGF and heregulin-β1; the receptor density of HER-family members and growth ligands are key players in the development of resistance mechanisms to trastuzumab therapy, which interfers with cell cycle kinetics by inducing a G1 accumulation in HER-2 positive breast adenocarcinomas[78].

An unexpected mechanism of resistance is associated with down-stream mutations especially those targeting the mRNA binding protein tristetraprolin (TTP). *ttp* gene germinal mutation generates a form of TTP mRNA which is inefficiently translated in protein. The lack of TTP and the general increase of the TTP competitor the ELAV-like protein 1 (HuR) results in the increase of the half-life of mRNAs encoding oncogenes, inflammatory and angiogenic factors. The mutation of TTP is predictive of trastuzumab resistance[79]. Hence TTP is considered as a tumor suppressor for breast cancers[80-82]. TTP and HuR are phosphorylated by the same kinases and phosphorylation has antagonistic effects on both proteins (inactivation/degradation for TTP and activation/stabilization for HuR). Hence, activation of intracellular signaling pathways results in a general increase of proteins associated with oncogenic properties[83] (Figure 1).

The main drawback in trastuzumab therapy is represented by the emergence of serious cardiac side effects resulting from administration of this monoclonal antibody. Analysis of HER2 specific mutation may predict cardiac toxic effect[84].

HER-2 is expressed in the adult human myocardium and trastuzumab therapy unfortunately carries the risk of inducing cardiac dysfunction and congestive heart failure. When adjacent chemotherapy is applied in addition to trastuzumab, one has to take into consideration anthracyclin-associated cardiotoxicity following the inhibition of the HER-2/erbB2 receptor to ensure safety for patients. Some of the cardiotoxicity side effects of trastuzumab may be reversible over time and in some cases, administering the monoclonal antibody after chemotherapy or radiotherapy may decrease the risk of potential cardiac side effects. Trastuzumab therapy seems to represent clear overall benefits for patients in the long run, therefore, should be still considered as an appropriate standard choice of treatment as a HER-2/erbB2 inhibitor as long as care is taken to minimize its side effects[85].

Endocrine therapy resistance mechanisms

Resistance to hormone therapy is a major challenge within hormone sensitive breast cancers even though ER and PR targeted therapy has proven to be very effective, improving the quality of life of hormone sensitive breast cancer patients. The major pathways responsible for endocrine resistance mechanisms might be several: The HER tyrosine kinase receptor family; receptors for insulin/IGF1, FGF and VEGF, Src, AKT, stress related kinases, might each play a pivotal role in contributing to endocrine therapy resistance when their cognate ligands are amplified or overexpressed.

Cross-talk between the estrogen receptor (ER) and growth factor receptor signaling with hyperactivation of the PI3K pathway have also been associated the development of endocrine resistance[86].

Nuclear receptors and the androgen receptors may also act as alternative growth stimulators by post translational modification, enabling the bypass of ER inhibition. Co-targeting the EGFR and HER2 pathway simultaneously seems to be the most promising way forward in circumventing endocrine resistance as these two seem to be the most important factors responsible in this particular resistance scenario[26].

The mTOR pathway

The mTOR pathway seems to be a master regulator of cell physiology and may be a key player in the targeted therapy of cancer[87]. When the natural product rapamycin was discovered in the early 1970's as an antifungal agent, it emerged in later studies that the molecule could halt growth in many types of eukaryotic cells and have a powerful immunosuppressive function. In 1999 the FDA approved sirolimus as a drug used against rejection of transplanted organs particularly the kidneys. Rapamycin binds to another molecule, FKBP12 and once this complex is formed it associates with a protein called mTOR[88]; a serine/threonine kinase, resembling the kinase domain of PI3 kinase and its related enzymes. The circuitry of the mTOR pathway is of interest as it represents a key element of the mammalian cell cycle integrating incoming signals and vital mechanisms such as glucose import and protein synthesis, as well as phosphorylating two kinases involved in translation: S6 kinase (S6KI) and 4E-BP1[89,90]. The activation of S6KI is followed by the activation of the small 40-S ribosomal subunit which can initiate protein synthesis after associating to the large ribosomal subunit. mTOR is also a key upstream regulator which controls the AKT signaling pathway for the regulation of apoptosis and proliferation; inhibiting the mTOR complex results in a shutdown of the AKT signaling stream resulting in an hyperactivated PI3K/loss of PTEN expression[91,92].

The PI3K/AKT/mTOR pathway is overactivated in 70% of breast cancers and the protein kinases found along these pathways may be potential drug targets for breast cancer therapy. Due to the large scale involvement of this pathway the cell cycle regulation, selectively silencing of the PI3K/AKT/mTOR pathway represents an attractive approach for patients who might have shown resistance mechanisms to previous types of therapy. The combination of mTOR inhibitors with other targeted therapies might be a winning formula to circumvent resistance mechanisms of breast cancer patients.

Inhibition of the mTOR pathway by the drug everolimus in combination with HER-2 or estrogen receptor

inhibitors may be a promising future strategy to apply, in order to reinstate sensitivity of breast cancer cells to traditional therapies and overcome resistance mechanisms which seem to emerge when the mTOR pathway is functioning in hyperactive mode[93]. Molecular alterations like mutations in EGFR, BRAF, AKT, or PI3K are associated with activation of downstream signaling pathways resulting in unrestricted proliferation in cancer cells.

Glaysher *et al*[94] have shown that targeting a breast epithelial cell line after having knocked-in mutations and using EGFR and mTOR inhibitors, there was an increased sensitivity to therapeutic drugs. As development of resistance in breast cancer cells may be a result of the activation of the PI3K/AKT/mTOR pathway, Glaysher *et al*[94] studied the effects of inhibiting both mTOR and EGFR by combined drug action of ZSTK474/sirolimus and erlotinib/gefitinib, observing a more effective signaling blockade, as opposed to use of single agents on the parental cell line and irrespective of the knocked-in mutations in EGFR,KRAS,PI3K,BRAF or AKT[94].

Receptor tyrosine kinase inhibitors resistance mechanisms

Lapatinib is a dual EGFR/HER2 tyrosine kinase inhibitor which acts as an ATP competitor. It is used as a first line monotherapy in patients with HER2 positive metastatic breast cancer in addition to conventional chemotherapy like paclitaxel.

Unfortunately, the activation of compensatory pathways after onset of therapy with lapatinib seems to be responsible for the emergence of resistance mechanisms, particularly when inhibition of AKT phosphorylation leads to increased estrogen receptor-α transcription and estrogen receptor signaling. This mechanism of resistance can be circumvented by administering an ER-down-regulator fulvestrant, which can prevent the proliferation of lapatinib resistant cells. In addition, mutations in the HER2 protein, particularly a YVMA insertion at G776 in exon 20, seems to be responsible for mechanisms of *de novo* resistance to lapatinib as well as trastuzumab[73].

The inhibitory effects of lapatinib may be bypassed as downstream signaling is amplified and upregulation of activated HER3 becomes responsible for compromising the inhibitory effects of tyrosine kinases.

Activation of the PI3K/AKT pathway results from HER3 upregulation with a subsequent nuclear increase in FoxO3A family of transcription factors responsible for control of cell cycle, neoplastic transformation and epithelial-to-mesenchymal transition[30].

Targeting erb-B3 (HER3) with an antibody has proven to be quite effective in both preclinical and clinical studies although tumor cells eventually develop resistance as the antibody is only active in inhibiting signaling without altering the actual expression of the erb-B3 receptors. New strategies which aim at reducing erb-B3 levels are being investigated such as the HDAC inhibitor entinostat and the antisense oligonucleotide EZN-3920[95].

The hepatocyte growth factor receptor HGFR/c-Met tyrosine kinase responsible for cell proliferation, protection from apoptosis and cell invasion, seems to be implicated in the emergence of resistance to targeted therapies particularly lapatinib and trastuzumab and recent preclinical studies suggested that inhibition of c-MET in gastric cancer cell lines circumvented resistance mechanisms as well as restored growth inhibition[96].

The overexpression of the receptor tyrosine kinase AXL is associated with poor prognosis and a more aggressive phenotype in ovarian, breast colon, esophageal, thyroid and lung cancers and may be implicated in the emergence of lapatinib acquired resistance in *in vitro* models of preclinical breast cancer studies.

Lapatinib resistance has been also associated with SRC tyrosine kinase activity; overexpression of SRC in breast cancer cell lines seems to result in an increased interaction with EGFR rather than HER2. According to Formisano *et al*[97], when EGFR was inhibited with the monoclonal antibody cetuximab and SRC was inhibited by the small molecule saracatinib, lapatinib resistant breast cancer cells would not survive and sensitivity was restored. The combined treatment of lapatinib with cetuximab both *in vitro* and *in vivo* resulted in the reduction of EGFR/HER2 signaling and proved to be effective[97].

As observed by Wilson *et al*[98], autocrine tumor cell production might be responsible for increased levels of receptor tyrosine kinase-ligand levels and in breast cancer cell lines the HER3 ligand neuregulin-1 seems to induce complete rescue from lapatinib.

An additional mechanism of resistance to lapatinib may occur as a result of crosstalk between the estrogen receptor and the HER2 pathway. Lapatinib induced upregulation of ER by inhibition of the PI3K/AKT signaling pathway results in overexpression of the anti-apoptotic protein Bcl-2 leading to the emergence of lapatinib resistance and cell death escape[99].

The VEGF

The VEGF and its cell surface receptors represent the main modulators in the emergence of tumor angiogenesis. Avastin or bevacizumab, a humanized anti-VEGF antibody, has played a key role in anti-angiogenic therapy for cancer treatment in concomitance with small molecule VEGF receptor kinase inhibitors[43].

The VEGF ligand presents itself as an antiparallel homodimeric structure in which each monomer is made mostly of β strands stabilized by a disulfide knot and two symmetrically disposed intermolecular disulfide bridges that are responsible for linking the monomers together. On the extracellular domain of each of the three VEGF receptors (VEGF-1, VEGF-2, VEGF-3) there are seven immunoglobulin-like structures (Ig domain)[100,101].

All four members of the VEGF family and the placenta growth factor bind to three endothelial cell tyrosine kinase receptors which have each different functions. VEGFR1 is responsible for promoting differentiation and vascular maintenance, VEGFR2 induction of endothelial

cell proliferation and vascular permeability, VEGFR3 stimulation of lynphangiogenesis. Isoform specific receptors neuropilin-1 and neuropilin-2 may bind to class 3 semaphorins involved in axonal growth and also to some isoforms of VEGF1 as co-receptors which results in additional VEGF binding to VEGFR2[102].

Several other pathways are implicated by the function of VEGF as proteolytic and heparin activation further modulates receptor sites resulting in various cellular effects like the increase of vascular permeability, endothelial cell proliferation, survival and tubular formation. The VEGFR are usually endothelial in origin but in some instances they may be located in the stroma as macrophages and tumor cells themselves. Under abnormally low oxygen conditions (hypoxia), the hypoxia-inducible factor (HIF) plays a central role in transcription of genes like VEGF.

In normoxic conditions, the alpha-subunit of the HIF heterodimer (alpha, beta) is degraded by ubiquitylation as HIF-alpha binds to the von Hippel-Lindau tumor suppressor protein (p-VHL) forming the E3 ubiquitin ligase complex, a recognition component leading to proteasome-dependent degradation. In hypoxic conditions, as the HIF-alpha subunit is stabilized by heterodimerization with HIF-beta and hypoxia response elements (HRE), regulatory elements of HIF target genes are activated including VEGF, genes controlling cell proliferation and cell metabolism[103,104].

VEGF is one of the genes that is upregulated in hypoxia microenvironments eliciting a particular vascular phenotype; the high expression of VEGF is a common prognostic factor in human breast cancer malignancies representing an important therapeutic target. Other family members though play a role in angiogenesis even when VEGF is not expressed, in addition to the function of these homologues, the switching of angiogenic pathways may represent an area for further investigation to be possibly circumvented by multiple pathway inhibition[105].

Emerging patient data suggests that the combination of the anti-angiogenic drug bevacizumab with chemotherapy agents such as paclitaxel has proven to be a very dangerous therapeutic choice in terms of fatal side effects including hemorrhage, neutropenia, perforations of the gastrointestinal tract, blockage of arteries and stroke[106].

VEGF resistance mechanisms

Several mechanisms are implicated in the emergence of resistance mechanisms to anti-angiogenic therapy (Figure 2). The most prominent one relates to the promiscuity of cancer cells to produce many types of alternative angiogenic signals which limit drug efficacy. The rescue of tumor vascularization may occur as escape mechanisms are induced by anti-angiogenic therapy and hypoxia of tumor tissue.

Cancer cells may amplify angiogenic genes which in return do not respond to low doses of anti-angiogenic drugs; they may switch from vessel sprouting to vessel co-option, vasculogenesis or vascular mimicry in order to ensure tumor nutrients. The recruitment of bone-marrow derived cells by cancer cells may result in the secretion of pro-angiogenic factors like angiopoietin, fibroblast growth factor or ephrins. The VEGF receptors may induce the release of a cytokine cascade which results in an inflamed microenvironment allowing for the emergence of tumor extravasation and metastasis.

Some of the alternative targets to overcome drug resistance to anti-angiogenesis therapies might be to target the placental growth factor and Bv8 (Bombina variegata) to reduce tumor inflammation, reduce leakiness of vessels, moderate hypoxia and reduce angiogenesis; the Notch pathway by anti-delta like ligands 4 (DII4) and secretase inhibitors to reduce excessive sprouting and reduce leaky dysfunctional vessels. Vessel normalization may be achieved by PHD2 inhibition improving vessel function and reducing metastasis and hypoxia. Lymphangiogenesis may be targeted by inhibiting neuropilin-2 (Npn2) and by targeting neuropilin-1, tumor growth and angiogenesis can be significantly reduced[107].

Several alternative pathways may take over as resistance develops to anti-angiogenic therapy through intrinsic tumor resistance or acquired resistance: angiogenic redundancy involves the production of redundant pro-angiogenic factors like the fibroblast growth factors (FGFs), platelet derived growth factors (PDGFs), placenta growth factor (PlGF), tumor necrosis factor-α (TNF-α). As these pro-angiogenic factors allow for the growth of tumor vasculature despite the VEGF pathway being inhibited it would be appropriate to target several of them synergistically.

The increase of tumor hypoxia as a result of anti-angiogenic therapy is often implicated in angiogenic redundancy: The overexpression of the hypoxia-induced factor-1 (HIF-1) is correlated with chemotherapy resistance and selection of aggressive cancer cells as it is directly involved in the induction of transcription of genes involved in angiogenesis. The important role of activating the membrane tyrosine kinase receptor c-MET by the hepatocyte growth factor during angiogenesis, allows for downstream activation of SRC, AKT, MEK, STAT3 with an increased expression of VEGF and its receptor by endothelial cells. The HGF/c-MET collaboration is often associated with invasive cancer phenotypes and increased metastasis. In these cases, the selection of more invasive tumor cells may occur as hypoxic environments pressure cancer cells to move rapidly toward normoxic locations. The recruitment of bone marrow derived pro-angiogenic cells and inflammatory cell invasion may contribute to adaptive mechanisms of resistance as low oxygen concentrations induce these cells to release large amounts of pro-angiogenic factors. As alterations in endothelial cells and pericytes may be responsible for crosstalk between angiogenic pathways resulting in the emergence of anti-angiogenesis therapy resistance, inhibiting the VEGF pathway and the platelet derived growth factor receptor with a tyrosine kinase inhibitor simultaneously might be a promising strategy to enhance treatment efficacy. The process of vessel co-

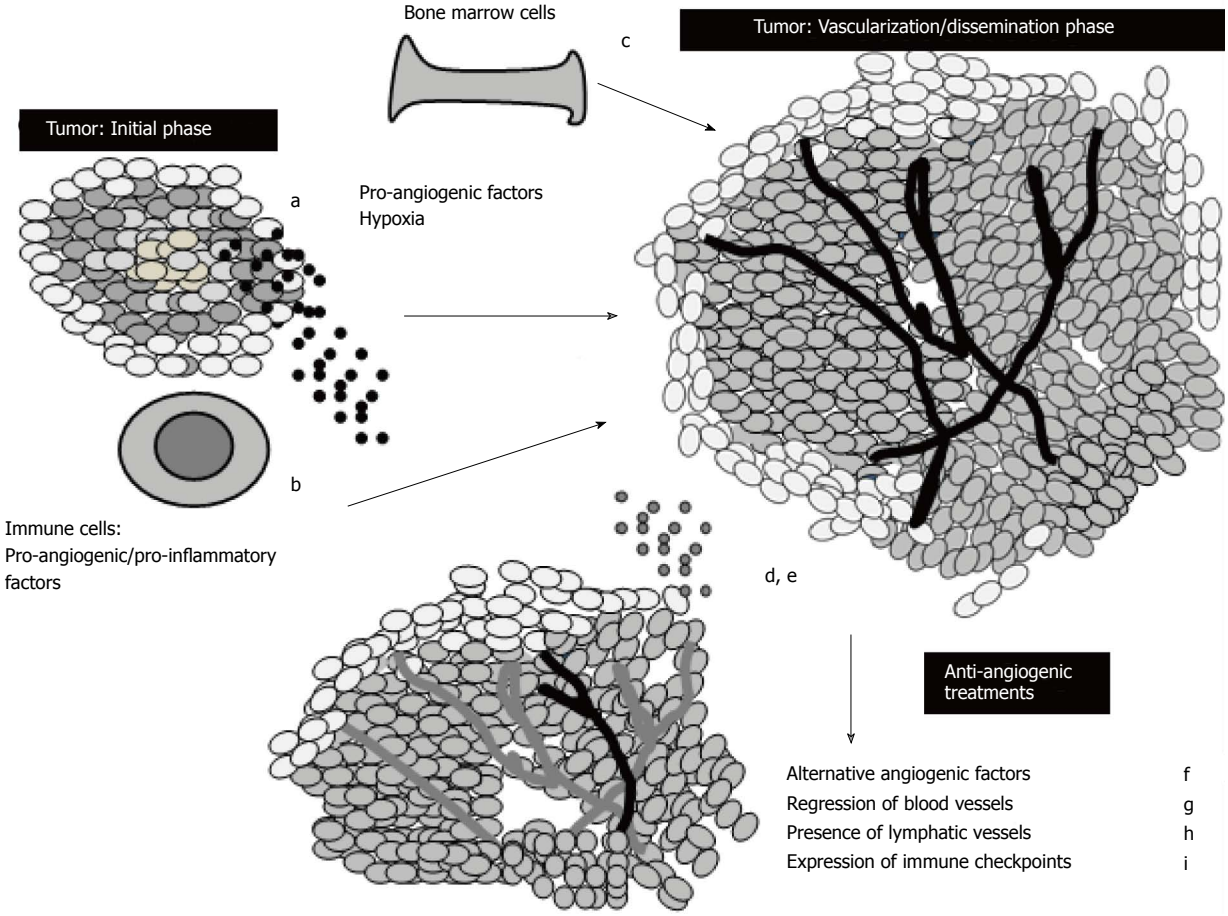

Figure 2 Resistance mechanisms to anti-angiogenic therapy. During the initial development, tumor cells that are in the core of the tumor, become hypoxic and secrete pro-angiogenic factors (a); Proangiogenic factors are also produced by immune cells (b) and bone marrow cell participate in tumor vascularization (c); The amplification of cancer cell genome stimulates high gene expression levels, consequently, requiring an increased anti-angiogenic drug concentration (d); Tumors have evolved to switch from various modes of vascularization, in order to ensure a sufficient supply of nutrients, such as sprouting angiogenesis, vasculogenesis, vessel co-option as well as vascular mimicry (e); Various pro-angiogenic factors that are redundant of VEGF are secreted by tumor and stromal cells in malignant cancers (f); In response to the treatments, blood vessels regress (g) and tumor cell produced alternative proangiogenic prolymphangiogenic factors with the development of a lymphatic network (h); Tumor cells also express immune checkpoints proteins resulting in immune tolerance (i).

option may result in cancer cells displaying a normal looking vasculature which is less sensitive to anti-angiogenic therapy and early stage tumors may escape inhibition as they grow in an angiogenesis independent fashion[108].

The future of anti-angiogenic therapy seems to depend on how different tumors become vascularized and by what alternative pathways these manage to escape therapeutic effects. Elucidation of the complexity of the biology of angiogenesis, coupled with the function of key biomarkers, may prove to be a promising way forward to enhance the function of anti-angiogenic therapy to achieve vascular normalization and increase the effects of complementary chemotherapy.

TNBC and PARP inhibitors

TNBC represent 10%-20% of invasive breast cancers in the general population and have been associated with the African-American ethnic group where a clear prevalence of the disease affects up to 28% of all patients within that group[109].

About 80% of breast tumors which lack the over-expression of the HER-2/erbB2 protein, the estrogen receptor (ER) and the progesterone receptor (PgR) fall under the category of TNBC. They may be characterized by elevated levels of PARP enzymes and often originate from basal-like cell types. TNBC represent the most aggressive phenotype of the disease with no specific targeted therapies available for treatment. Twelve percent of TNBC are characterized by a claudin-low subtype; these can be identified by DNA microarray expression profiling, a method slowly emerging in clinical practice for the detection of this rare form of breast cancer. These tumors seem to respond to molecules which target DNA repair systems to induce synthetic lethality if used in combination with other drugs. PARP inhibitors are an example of therapeutic choice when one of the genes in a synthetic lethal pair, with one gene already defective, is targeted resulting in cell death. PARP iso-enzymes include a group of 18 molecules which are central to base-excision repair pathways of single strand DNA breaks. An example is the BRCA1-2 mutation in breast cancer, this

scenario allows for PARP inhibitors to target and block the only functioning DNA repair system, hence, the selective killing of tumor cells while sparing healthy ones and limiting toxicity for the patient[110]. Nuclear basic fibroblast growth factor (bFGF) is a protein found in a subset of TNBC which contributes to the emergence of resistance following chemotherapy[111]. *In vitro* studies have shown that a residual TNBC subpopulation remains after short-term chemotherapy and this resumes proliferation over time. When bFGF was knocked down in these residual cancer cells using short hairpin RNA, the number of residual TNBC cells decreased. This phenomenon is linked to a down-regulation of DNA-dependent protein kinase (DNA-PK) responsible for accelerated DNA repair. This study might suggest that expression of bFGF in TNBC cells could be a prognostic predictor of incomplete chemotherapy response and future tumor recurrence in TNBC patients[111].

The main challenge of circumventing treatment induced resistance mechanisms and the emergence of alternative escape pathways, significantly lowers the overall survival rate of breast cancer patients belonging to this particular subtype as they often exhibit an incomplete pathological response[93].

Sunitinib seems to suppress angiogenesis, tumor proliferation, migration and growth of basal like breast cancer cells; xenograft models indicate that tumor volumes decrease under sunitinib action but due to its effects on the Notch-1 protein expression and hypoxia through HIF-1, there was an increase in proliferation of breast cancer stem cells. The use of a γ-secretase inhibitor in addition to sunitinib may represent a promising treatment option for TNBC while simultaneously targeting cancer stem cells and angiogenesis[112].

Sunitinib may prove to be an effective treatment choice for patients with TNBC as this breast cancer subtype may express increased levels of VEGF. High levels of VEGF may act as a potential prognostic factor in TNBC as the vascular pathway is a key component when targeting this particularly rare subtype of breast cancer[113].

As targeted therapies have not yet been discovered for TNBC, the conventional route is to treat patients with chemotherapy particularly anthracycline and taxane. The multitude of pathways which drive proliferation of this particular breast cancer subtype need to be further investigated in order to isolate potential therapeutic targets. Patients with BRCA1 and BRCA2 gene mutations which are present in 20% of TNBC, may be sensitive to the function of PARP inhibitors in addition to chemotherapy[6].

In a Phase II clinical trial carried out to evaluate the combined administration of the PARP1 inhibitor iniparib with cisplatin and gemcitabine on patients with TNBC, iniparib seemed to show significant anticancer activity enhancing the antiproliferative and cytotoxic effects of cisplatin and gemcitabine[114]. Combination therapy of cisplatin, gemcitabine and iniparib is currently under Phase Ⅲ clinical trial to see if this association could represent the new standard of care for the treatment of TNBC (ClinicalTrials.gov No.NCT00938652).

Immunotherapy for breast cancer

Breast cancer has been considered non-immunogenic for quite a long time and only recently, data has suggested that TNBC and HER2 positive types are characterized by an immune infiltrate, which might prove to be a promising target to complement the function of other synergistic drugs. Solid tumors like melanoma and lung cancer have already responded to immunotherapeutic agents like ipilimumab and sipuleucel-T has proven a successful vaccine against castration-resistant prostate cancers. Ongoing studies are also evaluating to what extent immune response is correlated to prognosis in breast cancer (Table 1).

The aim of immunotherapy is that of activating the human immune response to recognize tumors as a foreign entity and eventually kill the tumor cells. The tumor microenvironment (TME) including T-regulatory cells (T-Reg) involves a complex structure of intercellular communication which represent a very promising area of research aiming at the isolation of key immunogenic targets which may enhance the function of existing therapies[115].

The immune-checkpoint receptor PD-1 is expressed on tumor-infiltrating lymphocytes (TILs) with the role of inhibiting the activity of effector T-cells, preventing autoimmunity and inflammatory response; it is often upregulated on tumor cell surface in many types of solid tumors. The PD-1 ligand PDL1 engages with T-cells resulting in upregulation of the receptor followed by an immunosuppressive signal, which inhibits kinases involved in the activation of the immune response[116]. Clinical blockade of the PD-1/PDL1 axis should enhance antibody function in cancer patients underlining the importance of further investigation in this particular area of breast cancer research (Table 2). Pro-inflammatory cytokines and the overexpression of PDL1 inhibitory ligand may play a key role in the development of cancer immune resistance mechanisms, resulting in a state of exhausted or tolerant immune T-cell response hence the importance of studying the possible role of PDL1 expression as a resistance biomarker. Overall, main role of PD-1 blockade results in the reversal of chronic antigen response which is often found in cancer and viral infection scenarios[117]. The anti PD-1 antibody nivolumab has shown successful activity in melanoma and lung cancer patients targeting these immunoregulatory proteins and enhancing tumor response. There are several other ligands being investigated at present which might be potential targets like: CD80, CD86, PDL2, ICOS-L, B7-H3, B7-H4 and B7-H6 and future directions in cancer immunotherapy research point towards the effects of combined checkpoint blockade to maximize clinical response[118].

Future direction: Breast cancer combination therapy

Over the last few years, new agents have been intro-

Table 1 Recapitulative breast cancer targeted therapy scheme cited in this article

Target pathway	Current therapy	Combination therapy
HER2 (HER2-positive breast cancer)	Trastuzumab/herceptin Pertuzumab lapatinib	Combination trastuzumab/lapatinib (EPHOS-B trial) trastuzumab/264RAD
m-TOR pathway	Everolimus	Possible combination everolimus/HER2 inhibitor
Angiogenesis (VEGF)	Bevacizumab paclitaxel Docetaxel	Targeting the placental growth factor and Bv8/Targeting the Notch pathway by anti-delta like ligands 4 and secretase inhibitors inhibiting simultaneously the VEGF pathway and the platelet derived growth factor receptor with a TK inhibitor
DNA repair mechanisms (TNBC)	Parp inhibitors/anthracyclins and taxanes	Possible combination cisplatin/gemcitabine/iniparib
Notch-1 protein over-expression/ breast cancer stem cells proliferation (TNBC)		Possible combination of g-secretase inhibitor in addition to sunitinib
Immune system response Cell cycle checkpoints	Immunotherapeutic agents Antibodies against PD-1 T-cell inhibitory molecule or its ligand PD-L1	Nelipepimut-S(human leukocyte antigen)/GM-CSF Pembrolizumab in TNBC/PD-L1 positive (KEYNOTE-086 trial)

HER2: Human epidermal growth factor receptor 2; DII4: Delta like ligands 4; TNBC: Triple negative breast cancer; GM-CSF: Granulocyte-macrophage colony stimulating factor; VEGF: Vascular endothelial growth factor.

Table 2 Some of the current clinical trials in breast cancer targeted immunotherapy (http://www.cancerresearch.org./cancer-immunotherapy/impacting-all-cancers/breast-cancer)

Title of clinical trial	Type of breast cancer
Phase Ⅲ clinical trial: NEUVAX: nelipepimut-S or E75NCT01479244	HER1+ HER2+
Phase Ⅱ clinical trial: NEUVAX NCTO1570036	Node positive or TNBC
Phase Ⅰ clinical trial: Pembrolizumab PD1 antibody + dendritic cell vaccine NCTO2479230	Metastatic breast cancer
Phase Ⅱ trial: Pembrolizumab PD1 antibody + HDAC inhibitor and anti-estrogen therapy NCT02395627	Breast cancer
Phase Ⅱ first line neo adjuvant trial: Atezolizumab + chemotherapy NCTO2530489	TNBC
Phase Ⅰ clinical trial: Atezolizumab and HER2 inhibitors NCTO2605915	HER2+
Phase Ⅰ / Ⅱ clinical trial: PDR001(PD1 antibody)	Advanced breast cancer, TNBC
Phase Ⅰ / Ⅱ clinical trial: MEDI6469 anti OX40 antibody NCTO1642290	Stage 4 breast cancer (patients with prior failure of hormone or chemotherapy)
Pilot study of QBX258 targeting IL-4 and IL-13 NTCO2494206	Advanced TNBC whose cancer cells make a protein called glycoprotein NMB to which CDX-011 binds

IL: Interleukin; HER2: Human epidermal growth factor receptor 2; TNBC: Triple negative breast cancer.

duced in breast cancer targeted therapy resulting in overall improved treatments and greater patient overall survival rates. Some of the most widely used combination therapies involve the use of agents which target the PI3K/AKT/mTOR pathways such as everolimus combined to exemestane. The everolimus-FKBP12 complex that forms when the m-TOR inhibitor binds with high affinity to the intracellular receptor FKBP12, is very effective in inhibiting down strem signaling in cancer cells. The BOLERO study has demonstrated the efficacy of the m-TOR inhibitor everolimus used in combination with exemestane (endocrine therapy) to restore hormonal sensitivity in breast cancer patients[6]. Palbocib has been combined with letrozole in treating women with ER positive (estrogen positive), HER2 negative, advanced breast cancers as a first line endocrine therapy in metastatic cases. Trastuzumab and lapatinib have been used successfully in combination to treat metastatic breast cancers that overexpress HER2[6]. Trastuzumab and pertuzumab have been used in combination for the treatment of HER2 positive metastatic breast cancers and have shown a statistically significant increase in overall survival of patients[6]. The trastuzumab/lapatinib/hormonal therapy combination has proven to be effective in cases of hormonal receptor positive and overexpressed HER2 protein breast cancers like the luminal B/HER2 enriched type. Iniparib, a PARP1 inhibitor, in combination with gemcitabine and carboplatin chemotherapy have been evaluated in a Phase Ⅰ clinical trial for the treatment of metastatic TNBCs and a clinical benefit of 56% was observed in the combined therapy arm, compared to the gemcitabine/carboplatin arm which had a 34% clinical

Table 3 Some of the current clinical trials in breast cancer targeted therapy (http://www.breastcancertrials.org)

Title of clinical trial	Type of cancer
Randomized open label Phase Ⅱ trial: Kadcyla, tykerb and abraxane *vs* herceptin, tykerb and taxol before Surgery for HER2+ tumors NCT02073487	HER2+
Phase Ⅲ randomised, placebo controlled clinical trial: Chemotherapy and a PARP-inhibitor for BRCA1/2+, HER2- advanced breast cancer NCTO2163694	HER2-, BRCA1/2+ metastatic or locally advanced unresectable breast cancer
Phase Ⅱ, multicenter, randomized clinical trial: Alisertib with taxol for advanced ER+/HER2- or TNBC NCTO2187991	ER+/HER2- TNBC
Phase Ⅱ Clinical trial: Gemzar, herceptin and perjeta for HER2+ metastatic breast cancer NCTO2252887	HER2+ metastatic breast cancer
Phase Ⅰ clinical trial: CD-839 for advanced breast tumors NCTO2071862	Advanced breast cancer and solid tumors
Phase Ⅰ clinical trial: Saracatinib and anastrozole for ER-positive disease NCTO1216176	ER+
Randomised Phase Ⅲ clinical trial: Hormone therapy with or without ibrance for HR+, HER2- stage Ⅱ-Ⅲ breast cancer NCTO2513394	HR+, HER2-
Phase Ⅱ clinical trial: CDK-inhibitor for previously treated metastatic disease NCTO1037790	Previously treated metastatic breast cancer
Phase Ⅰ clinical trial: GS-5745 in metastatic HER2- breast cancer and other solid tumors NCTO1813282	Metastatic HER2- breast cancer not responding to other treatments

benefit[114].

A promising area of clinical research for breast cancer targeted therapy involves the use of immune checkpoints inhibitors or immune checkpoint stimulatory molecules. In order to unleash anti-cancer immune responses, inhibitory molecules are blocked or stimulatory molecules are activated to allow the immune system to attack directly cancer cells as foreign invaders. An example would be the anti PD1 antibody pembrolizumab (Keytruda), anti CTLA antibodies, the anti PD-L1 antibody atezolizumab, anti CD73 antibodies or anti OX40 antibodies being tested currently in Phase I/II clinical trials (Table 2).

As the importance of the TME is being discovered with its potential contribution to cancer therapy, novel agents are being developed to target the non-malignant tumor stroma like trabectedin which inhibits macrophage differentiation; other drugs target the tumor necrosis factor-related apoptosis inducing ligand (TRAIL) pathway such as mapatumumab and dulanermin; immunomodulators used alone or in combination to cytotoxic agents should be also investigated as a strategy to decrease the immunosuppression caused by T-effector cell upregulation in the quest to increase the innate immune response against cancer cells, keeping the right balance as immune over-stimulation could be potentially harmful to patients[86].

The main future challenge for breast cancer combination therapy is to design a winning formula that is simultaneously effective against the many subtypes of breast cancers like luminal A, luminal B, basal-like and overexpressing HER2. This approach would represent a hopeful avenue to explore in the quest to inhibit the multitude of pathways being exploited by the various breast cancer subtypes. The phenotype of each breast cancer subtype should be thoroughly investigated as well, to allow researchers to gather a general picture describing in detail the different mechanisms of action for cell survival. Only then, more precise targets can be identified allowing for the discovery of more inclusive breast cancer combination therapies. A more precise and personalized characterization of each cancer as well as the identification of factors involved in resistance for each patient may provide useful improvements in current therapeutic approaches.

CONCLUSION

Decoding of the human genome has allowed for the isolation of key gene signatures for many types of known cancers; unfortunately, targeted therapies to inhibit the function of these genes have proven quite elusive as the quest to circumvent the emergence of resistance mechanisms continues. Breast cancer subtypes, particularly TNBCs, still represent a major challenge; future studies should revolve around the discovery of new prognostic biomarkers as no targets for these rare types of breast cancer have yet been identified.

The EPHOS-B trial carried out by researchers in The Institute of Cancer Research, London, the University of Manchester and University Hospital of South Manchester NHS Foundation Trust investigating the response of HER2 positive breast cancer to dual lapatinib and trastuzumab therapy shortly after diagnosis and surgery to remove the tumors, has released very promising data in which of 257 women who were administered the two drugs synergistically 11 d before surgery, 17% had only minimal residual disease with invasive tumor smaller than 5 mm in size, 11% had a pathological complete response with no biological invasive tumor present in the breast and 3% had a complete response. This dramatic response after only 11 d suggests that combination anti-HER2 targeted therapy prior surgery may reduce the number of breast cancer patients requiring chemotherapy in the future and significantly eliminate long term chemotherapy associated side effects[4,119].

Resistance mechanisms in breast cancer targeted therapies represent the main challenge to current research; the combination of different molecules used to target

different levels of signaling pathways by synergistically blocking cancer cell escape routes and minimizing the emergence of survival mechanisms, could prove to be a promising way forward, keeping in mind that specific molecular profiling particularly for metastatic relapses should be carried out to elucidate further resistance phenotypes and allow for the design of specific new targets. Several clinical trials are underway to try to improve survival of the worse cases (Table 3).

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MINIREVIEWS

How best to manage gastrointestinal stromal tumor

Gandhi Lanke, Jeffrey H Lee

Gandhi Lanke, Jeffrey H Lee, Department of Gastroenterology, Hepatology and Nutrition, University of Texas MD Anderson Cancer Center, Houston, TX 77030, United States

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Correspondence to: Jeffrey H Lee, MD, MPH, FASGE, FACG, AGAF, President-Elect (Texas Society for Gastroenterology and Endoscopy), Professor (Department of Gastroenterology, Hepatology, and Nutrition), Director (Advanced Therapeutic Endoscopy Fellowship), Department of Gastroenterology, Hepatology and Nutrition, University of Texas MD Anderson Cancer Center, 1515 Holcombe Blvd, Houston, TX 77030, United States. jefflee@mdanderson.org
Telephone: +1-713-7945073
Fax: +1-713-5634408

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Abstract

Gastrointestinal stromal tumors (GISTs) are rare but most common nonepithelial tumor of gastrointestinal tract. They are often found incidentally on computed tomography and endoscopic investigations. Increasing knowledge of the pathogenesis of GISTs and the advent of tyrosine kinase inhibitors revolutionized the management of GISTs. The newer advanced endoscopic techniques have challenged the conventional surgery although the true efficacy and safety of endoscopic approach is not clear at this time. This review article focuses on pathogenesis, diagnosis and management of GISTs.

Key words: Gastrointestinal stromal tumor; Endoscopy; Endoscopic ultrasound-fine-needle aspiration; Tyrosine kinase inhibitor; Imatinib

Core tip: Gastrointestinal stromal tumors (GISTs) are most common mesenchymal tumors in the gastrointestinal tract. The management of GISTs is revolutionized with the advent of tyrosine kinase inhibitors (TKIs) and newer advanced endoscopic techniques. Accurate identification and differentiation of GISTs from other submucosal tumors is achieved with the help of endoscopic ultrasound. The management of small to medium GISTs are feasible by newer advanced endoscopic and/or laparoscopic techniques. Team approach involving endoscopist, pathologist, radiologist, medical oncologist and surgeon is key in optimal management of GISTs. This article focuses on role of TKIs and endoscopist perspective in the management of GISTs.

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INTRODUCTION

Gastrointestinal stromal tumors (GISTs) are the most common mesenchymal (sub epithelial) tumor, and are

frequently found in stomach and small intestine[1]. GISTS are hypothesized to originate from interstitial cells of cajal (ICC) which coordinate gut motility[2]. GISTs are rarely found in the peritoneum, mesentery and omentum[3]. GISTs have varied malignant potential, with about 40% of GISTs that are localized at initial diagnosis give rise to metastasis[4], and about 10%-20% of GISTs present with distant metastasis[5,6]. In Europe, the annual incidence of GISTs is about 10 cases per million[7]. In the United States, the annual incidence of GIST ranges from 4000 to 6000 new cases per year (7-20 cases per million population per year)[8]. The mean age at diagnosis is 63 years[9]; men and women are equally affected. The majority of GISTs are sporadic and may be associated with mutations like NF1, C-kit, platelet derived growth factor receptor-alpha (PDGFRA), succinate dehydrogenase (SDH) and deletions in chromosome 1 involving SDH c[10].

PATHOGENESIS OF GIST

Overall, GISTs are defined by the presence of *KIT* gene or PDGFRA mutation. Majority (80%) of GISTs have *KIT* gene mutations and biologic response of KIT receptor is produced without a bound ligand[11]. KIT receptor tyrosine kinase activity in normal cells is regulated by binding of endogenous KIT ligand or stem cell factor (SCF)[12]. In the majority of cases, spontaneous receptor dimerization and activation occurs when exon 11 is affected by *KIT* gene mutation. However, in few cases, a different mechanism results in uncontrolled KIT signaling if mutation occurs in Exon 9, 13 or 17. In cases with NF1, uncontrolled KIT activation may be present even in the absence of *KIT* gene mutation (wild type)[13]. A subset of GISTs which are negative for *KIT* gene mutations are positive for receptor tyrosine kinase PDGFRA mutations. GISTs expressing PDGFRA or *KIT* gene mutations have similar biologic consequences[14]. About 10% of adult GISTs have neither KIT gene nor PDGFRA mutation[15]. SDH-ubiquinone complex 2 is composed of subunits A, B, C and D which is part of Krebs cycle and respiratory chain[16]. In mutant SDH, dysfunction of electron transport chain in mitochondria leads to defective oxidative phosphorylation, which ultimately leads to abnormal stabilization of hypoxia inducible factors (HIF)[17]. Carney-Stratakis syndrome is caused by germline mutation in SDH subunits B, C or D which leads to GIST and paraganglioma[18].

Histologically GISTs are subdivided in to spindle cell (60%-70%), epithelioid (30%-40%) or both (10%). GISTs with spindle cells are compact, highly cellular, arranged in fascicular or whorled pattern with minimal amount of stroma and contain eosinophilic, basophilic or amphophilic cytoplasm. Epithelioid tumors have abundant cytoplasm which is amphophilic to clear and cellular borders are clearly defined[19]. Antibodies to CD34 and CD117 appear in most GISTs[20]. CD34 is a transmembrane glycoprotein present on vascular endothelium and human hematopoietic progenitor cells[21]. CD34 is expressed in a wide variety of tumors and it is detected in about 50%-80% of GISTs[2,11,20]. CD 117 is expressed in 80%-100% of GISTs and it is not expressed in smooth muscle or neural tumors which helps in distinguishing GISTs from other gastrointestinal mesenchymal tumors[20] (Figure 1).

CLINICAL PRESENTATION AND DIAGNOSTIC TOOLS

Clinical manifestations of GISTs are highly variable and it depends on tumor size and location. GISTs are usually asymptomatic and found incidentally by imaging or endoscopy[22]. Symptoms include melena, hematemesis, abdominal pain, discomfort, fullness, early satiety and palpable mass. GISTs in proximal stomach can cause dysphagia and tumors in pylorus can present as gastric outlet obstruction[23]. Rectal GISTs can present with hematochezia[24]. Rarely, they can present as intraperitoneal rupture of large tumor causing hemoperitoneum[25]. GISTs can occur as part of a syndrome; Carneys triad (gastric GIST, pulmonary chondroma, paraganglioma)[26], or neurofibromatosis type1 (mostly spindle cell GIST)[27]. Overall, about 50% of GISTs have local or distant metastasis at the time of presentation[28], with the liver being the most frequent site of metastasis. Other common sites of metastasis include the bone, peritoneum, retroperitoneum, lung, pleura, and subcutaneous (scar) tissue[29].

Computed tomography (CT) is the primary modality of choice for diagnosing GISTs[30,31]. CT tumor characteristics such as size greater than 10 cm, calcifications, irregular margins, heterogeneous, lobulated, regional lymphadenopathy, ulceration, extraluminal and mesenteric fat infiltration are more likely to be associated with metastasis[29]. CT enterography uses large volumes of oral contrast and it is superior to conventional CT. It has advantage of displaying the entire thickness of the small bowel, better visualization of deep ileal loops without superimposition and evaluation of surrounding mesentery[32]. MRI is more accurate than CT for delineating rectal GISTs and in detecting liver metastasis, hemorrhage and necrosis[33].

Esophagogastroduodenoscopy (EGD) shows most sub epithelial lesions as a bulge with a smooth, intact, normal appearing mucosa in the gastrointestinal tract. Hwang *et al*[34] did a prospective study and patients were referred for endoscopic ultrasound (EUS) to evaluate sub epithelial masses diagnosed previously by EGD, sigmoidoscopy or colonoscopy. The size of the mass during endoscopic exam was measured by open biopsy forceps for size reference. Results showed endoscopy was 98% sensitive and 64% specific in identifying intramural lesions. Intramural size measurement of endoscopy correlated with EUS ($r = 0.88$, $P < 0.001$) but, for extramural lesions, it was suboptimal ($r = 0.56$)[34]. Overall, the study concluded endoscopy had a high sensitivity but low specificity in identifying the location of sub epithelial lesions and histologic confirmation by EUS-fine-needle aspiration (FNA) should be obtained for masses originating from 3rd (submucosa) and 4th layer

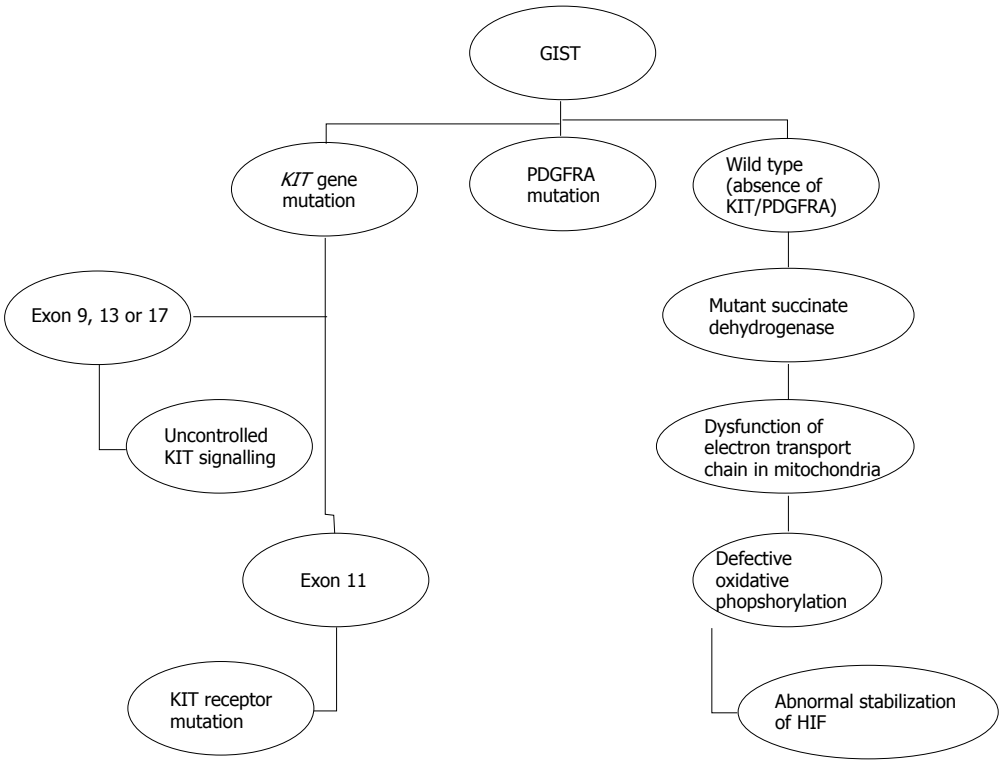

Figure 1 Pathogenesis. GIST: Gastrointestinal stromal tumor; PDGFRA: Platelet derived growth factor receptor-alpha; HIF: Hypoxia inducible factors.

(muscularis propria)[34].

Endosonographically GISTs appear as oval or hypoechoic mass arising from the muscularis propria. EUS features suggestive of malignancy include enlarged lymph nodes, size greater than 4 cm, irregular borders and cystic spaces with in the mass[35]. EUS has 92% sensitivity and 100% specificity in differentiating submucosal tumor from extrinsic compression[36]. Chen *et al*[37], retrospectively evaluated EUS characteristics to predict the malignant potential of GISTs. EUS features of GISTs were compared to National Institutes of Health (NIH) criteria for classification of malignant potential and were divided in to very low/low risk, intermediate/high risk. Results showed that GISTs at high risk for malignancy were associated with EUS characteristics like lesion size ($P < 0.0001$), cystic change ($P = 0.015$) and surface ulceration ($P = 0.036$)[37]. EUS-FNA cannot accurately differentiate benign from malignant GIST due to lack of mitotic activity on smears. The definitive method for assessment of GIST malignant potential requires surgical resection.

Dewitt *et al*[38] evaluated the diagnostic yield and complications of EUS-Trucut biopsy (EUS-TCB) for gastrointestinal mesenchymal tumor (GIMT). EUS-FNA was performed in 33/38 (87%), and was diagnostic on final cytology in 25/33 (76%) and by FNA-immunochemistry (FNA-IC) in 12/24 (50%). EUS-TCB obtained visible tissue specimen in 37/38 (97%), and diagnostic in the final TCB histology in 30/38 (79%) and TCB-IC in 30/31 (97%)[38]. Overall, the authors concluded that EUS-TCB should be considered as an alternative to EUS-FNA when technically feasible[38].

Na *et al*[39] evaluated the yield and utility of 19-gauge (G) TCB *vs* 22-G FNA for diagnosing gastric sub epithelial tumors (SETs). The diagnostic yield of TCB *vs* FNA were 77.8% *vs* 38.7% ($P < 0.0001$). The Accuracy of TCB *vs* FNA for diagnosing GISTs was 90.9% *vs* 68.8%; and for non-GIST SETs was 81.1% *vs* 14.3% respectively. There were 9 technical failures with TCB likely due to stiffness, poor maneuverability of the needle and location of the tumor[39]. The most common procedure associated adverse events were pain, hemorrhage (requiring endoscopic hemostasis) and fever[39]. Procedure related events in TCB *vs* FNA were [3/90 (3.3%) *vs* 5/62 (8.1%); $P = 0.27$] respectively[39].

Positron emission tomography (PET)-CT using ^{18}F-fluorodeoxy glucose (FDG) detects cancer based on changes in tissue metabolism[40,41]. PET-CT is used for initial staging and to monitor disease progression. A baseline ^{18} FDG-PET should be obtained before treatment so that the results can be used to compare with future studies[42]. Liver metastasis from GIST often appear as isodense lesions on CT, but may be detected by PET. Hence PET compliments CT in resolving ambiguity of liver lesions in patients with GISTs[42].

Gayed *et al*[43] showed that the sensitivity and positive predictive value of ^{18}F-FDG PET were 86% and 98% respectively and it is superior to CT in predicting early response to therapy in recurrent or metastatic GISTs[43]. Yoshikawa *et al*[40] evaluated the efficacy of PET-CT to predict the malignant potential of GIST. Standardized uptake value maximum (SUVmax) and GIST parameters

(Ki-67 labeling index and mitotic index) were compared. SUV max and Ki67 labeling index were significantly elevated in high risk group when compared to low/intermediate risk group[40]. Tumor response to treatment with imatinib mesylate may be detected by a decrease in CT attenuation units (Hounsfield units, HU)[44]. However, there may be delay in measurement of cellular and macroscopic changes after treatment with imatinib by CT. In contrast, PET using ^{18}F-FDG can detect early effects induced by imatinib and decrease in FDG uptake after the initiation of imatinib treatment indicates good prognosis[45].

The "Response Evaluation Criteria in Solid Tumors" (RECIST) classification was previously used, however, due to limitations in assessing malignant response to immunotherapy such as imatinib, RECIST has been replaced by the Choi criteria[46]. Limitations of RECIST were primarily because the response to therapy can occur not only in tumor size but also in structure like decreased tumor density and enhancement of intratumoral nodules[31,47]. The Choi criteria of contrast-enhanced CT is based on decrease in tumor size by 10% in any dimension or decrease in structure by 15%, and was found to be more predictive of time to tumor progression (TTP) than RECIST[48].

PROGNOSIS AND RISK STRATIFICATION

Mitotic index, tumor size, location (gastric *vs* non-gastric) and tumor rupture are independent risk factors for GIST metastases[4]. Joensuu *et al*[49] analyzed the association between KIT and PDGFRA mutation and RFS in GIST patients treated with surgery alone. The authors concluded that tumor mutation status should not be interpreted in isolation from other risk factors[49]. The American College of Surgeons Oncology trial (ACOSOG) Z90001 study found that tumor size, location and mitotic rate were important in RFS but not tumor mutation status[50]. Gold *et al*[51] developed a nomogram by calculating concordance probabilities and by comparing three commonly employed staging systems NIH-Miettinen[52], NIH-Fletcher[53] and Armed Forces Institute of Pathology (AFIP)-Miettinen[54]. The investigators concluded that the nomogram can accurately predict RFS after the resection of localized, primary GIST[51].

MANAGEMENT OF GIST

Surgery is the treatment of choice for primary and localized GISTs[55]. The goal of surgery is complete tumor resection (negative microscopic and macroscopic margins) with functional preservation (often accomplished by wedge resection), while avoiding tumor rupture and injury to the pseudo capsule[55]. McCarter *et al*[56] analyzed factors associated with R_0 (grossly and histologically negative margin), R_1 (grossly negative but histologically positive margins), R_2 resection (grossly positive margins) and assessed the risk of recurrence with and without imatinib[56]. Factors associated with R_1 resection included tumor size (> or = 10 cm), tumor rupture and location[56]. The authors concluded there was no significant difference in recurrence free survival (RFS) in patients who underwent R_1 *vs* R_0 resection of GIST with or without adjuvant imatinib[56]. Although the management of R1 resection after complete resection is not clear, options include careful observation (watchful waiting), re-excision and adjuvant imatinib treatment.

Laparoscopic wedge resection (LWR) is recommended for gastric GIST smaller than 5 cm. To prevent tumor seeding in laparoscopy, plastic bag is recommended to collect the tumor sample and direct handling of tumor with forceps is contraindicated. Wedge resection of gastric GIST is considered standard treatment[57] and lymphadenectomy is not indicated as nodal metastasis is rare[28]. LWR has the advantage of early resumption of diet, early return of bowel function, shorter hospital stay and decreased duration of parenteral or epidural analgesia[58]. Lee *et al*[59] study concluded that LWR can be safely performed and have better outcome in terms of recovery after surgery regardless of tumor size and location. Kim *et al*[60] study concluded that LWR is safe and feasible for small to medium sized gastroduodenal tumors irrespective of location in cardia or pylorus. However, they recommended careful consideration of direction of stapling for exogastric resection of submucosal tumors located in antrum, lesser curvature and pylorus to prevent gastric outlet obstruction.

Endoscopic enucleation and other related procedures are more feasible for GISTs less than 5 cm[61]. Complete resection of GIST is indicated with endoscopic enucleation in the presence of a pseudo capsule. According to location in the gastric wall, GISTs are classified in to several types such as type 1 [very narrow connection with muscularis propria (MP) layer which protrudes in to the lumen], type 2 (wide based connection with MP layer and protrudes in the luminal side at obtuse angle), type 3 (located in the middle of gastric wall) and type 4 (protrudes into the serosal surface of gastric wall)[61]. This classification is very important when considering endoscopic enucleation. Endoscopic enucleation is best suitable for type 1 because of narrow connection to the MP layer and can be attempted for type 2. Type 3 and type 4 cannot be completely resected by endoscopic enucleation and hence endoscopic full-thickness resection (EFTR), laparoscopic and endoscopic cooperative surgery (LECS), laparoscopic-assisted endoscopic full-thickness resection (LAEFR) and non-exposed wall-inversion surgery (NEWS) should be considered[61]. Endoscopic enucleation includes various techniques like endoscopic submucosal dissection (ESD)[62], endoscopic muscularis dissection (EMD)[63] and endoscopic submucosal tunnel dissection (ESTD)[64]. Bialek *et al*[62] evaluated the efficacy, safety and outcomes of ESD for gastric sub epithelial tumors. Results showed 47% (17/37) sub epithelial tumors were GISTs, overall rate of R_0 resection was 81.1% (30/37), and perforation rate was 5.4%[62]. Liu *et al*[63] evaluated the feasibility and safety of EMD. Results showed that 51.6% (16/31) were GISTs, 96.8% (30/31) were completely resected, perforation occurred in 12.9% (4/31, all of which were managed by

endoscopic methods)[63]. ESTD procedure involves creation of the submucosal tunnel, dissection of the submucosal tumor (SMT) and closure of mucosal entry with hemostatic clips[64]. Gong *et al*[64] evaluated the feasibility and safety of ESTD in upper gastrointestinal SMTs. Results showed that 58.3% (7/12) were GISTs, complete tumor resection was achieved in all patients, *en bloc* resection in 83.3% (10/12, other 2 lesions were resected in 2 pieces) and 2 patients had both pneumothorax and subcutaneous emphysema which were managed conservatively[64]. Disadvantages of endoscopic techniques include tumor recurrence and peritoneal seeding secondary to perforation. It is unclear whether there is remnant GIST tissue after dissection causing tumor recurrence, although the dissection site is usually ablated with electrical knife or snare. Perforation occurs due to pseudo capsule injury during difficult MP layer dissection which increases the chance of peritoneal seeding. Peritoneal seeding is associated with poor prognosis because of increased tumor recurrence.

EFTR without laparoscopic assistance procedure involves introducing a single-chamber gastroscope into the stomach with a transparent cap attached to its tip. Dots are marked around the lesion and submucosal injection is done using normal saline with 1% indigo carmine and epinephrine (1:100000). Hook knife and IT knife are used to incise superficial layers overlying the SMT and snare is used to remove the mucosal and submucosal layers of gastric wall. Hook knife and IT knife are used to make circumferential dissection around the border of SMT. To visualize the SMT clearly, submucosal injection can be done again in the lower border of the tumor as needed. After the MP layer is reached and root of the tumor is exposed, gastric fluid is extracted as much as possible. Active perforation is made with the help of hook knife. After the tumor is completely exposed, SMT is removed *en bloc* with the snare. Dual channel gastroscope can be used for tumors with a broad basement which has the advantage of passing two snares through the accessory channels in to the gastric cavity. Tumor body is grasped with one snare and the other snare is used to *en bloc* enucleate the tumor along with the attached serosal layer. Titanium clips are used to close the defect in gastric wall. Paracentesis can be performed if there are signs of pneumoperitoneum during the procedure. Feng *et al*[65] evaluated the efficacy and safety of EFTR in 48 patients with gastric SMTs. Results showed that 43/48 had GIST, no post-EFTR complication such as bleeding or peritonitis, 5 had moderate postoperative abdominal distension because of air filtration (3 had abdominal paracentesis and the other 2 were managed conservatively)[65]. Zhou *et al*[66] evaluated the efficacy, feasibility and safety of EFTR for gastric SMTs originating from MP layer. Results showed that 16/26 were GISTs, *en bloc* resection rate was 100% and no major complications[66]. In general, there is a risk of peritoneal seeding with EFTR because it involves creating an active large perforation and hence gentle handling of GIST is necessary to maintain an intact pseudo capsule to prevent peritoneal seeding.

LECS has advantage over LWR especially for gastric SMTs located near esophagogastric junction or pyloric region because SMTs can be located accurately using endoscope and the resection of healthy stomach can be minimized[67]. The best indication for LECS is for gastric GISTs originating from MP layer which are intraluminal[61]. First, Argon plasma coagulation (APC) can be used to mark the periphery of the tumor[67]. A small incision is made on the marked area using standard needle knife after injecting 10% glycerin into submucosal layer. Using the IT knife, three-fourth of the marked area is cut circumferentially. Next, laparoscopic dissection of seromuscular layer is performed by making an artificial perforation and seromuscular dissection is carried out with ultrasonically activated device[67]. The incision is closed with the help of laparoscopic stapling device[67]. Hiki *et al*[67] analyzed seven patients who underwent LECS for gastric GISTs. Results showed that 6/7 were GISTs, no postoperative complications like bleeding, stenosis or anastomotic leakage, and successful tumor resection was done irrespective of tumor location (esophagogastric junction or pyloric ring). Tsujimoto *et al*[68] evaluated the feasibility and surgical outcomes of LECS for gastric SMTs. The authors found 16/20 were GISTs, no postoperative complications like bleeding, stenosis or anastomotic leakage, and there was no recurrence of tumor[68].

NEWS is a new technique developed to prevent peritoneal seeding from large active perforation and minimize resected tissue volume of stomach[69]. Mitsui *et al*[69] evaluated the efficacy and safety of NEWS in 6 patients with suspected gastric GIST. Results showed that 5/6 were GIST, *en bloc* resection was achieved in all GISTs, perforation occurred in 2/6 cases (1 case had muscle injury leading to perforation during mucosal cutting by endoscopic knife and the other case had laparoscopic mucosal injury leading to perforation during seromuscular cutting), and no postoperative complications[69]. Future studies with large cohort are needed to validate the safety of NEWS before it is standardized for GISTs treatment.

IMATINIB AS ADJUVANT THERAPY

Tumor size, location, mitotic index and tumor rupture are the most important independent prognostic indicators to determine RFS[4]. Multiple stratification schema like National Institutes of Health (NIH) consensus criteria, Armed Forces Institute of Pathology (AFIP) criteria and the modified NIH consensus criteria were developed to predict risk of recurrence[4,70-72]. The most commonly used stratification method is AFIP criteria[73]. AFIP groups 3a and above are considered high risk for recurrence. This corresponds to 5-year recurrence rate of 30% based on nomogram evaluation[73]. DeMatteo *et al*[74] evaluated the overall survival (OS) in 106 patients who had undergone complete gross tumor removal but were considered high risk for recurrence. It was a phase II Z9000 trial lead by ACOSOG and all patients were treated with imatinib 400 mg per day for 1 year[74]. Results showed that OS for

1, 3 and 5-year was 99%, 97% and 83% respectively after a mean follow up of 7.7 years[74]. RFS rate for 1, 3 and 5-year was 96%, 60% and 40% respectively[74]. In the subsequent trial, patients were randomly assigned to receive imatinib 400 mg per day or placebo for one year[75]. RFS at the end of 1 year for imatinib *vs* placebo was 98% *vs* 83% respectively and OS for imatinib *vs* placebo was 99.2% *vs* 99.7% respectively[75]. Li *et al*[76] evaluated RFS in Chinese patients after complete tumor resection of GISTs. All patients in treatment group (56/105) were treated with imatinib 400 mg once a day for 3 years and 49/105 were not treated (control group)[76]. RFS for imatinib *vs* control group at the end of 1year, 2 year and 3 years were 100% *vs* 90%, 96% *vs* 57% and 89% *vs* 48% respectively[76]. All GISTs with size ⩾ 3 cm, small bowel site and high mitotic index were shown to benefit from adjuvant imatinib treatment[50,75]. Joensuu *et al*[77] evaluated the RFS and OS in KIT-positive GISTs treated with imatinib for 3 year *vs* 1 year who had undergone complete tumor resection but considered high risk for recurrence. Results showed that RFS for patients treated with imatinib for 3 year *vs* 1 year were 65.6% *vs* 47.9% respectively and OS for 3 year *vs* 1 year were 92% *vs* 81.7% respectively[77]. Kang *et al*[78] evaluated the efficacy of adjuvant imatinib for 2 years in high risk GISTs with KIT exon 11 mutation after complete resection at four South Korean centers. The results showed median RFS was 58.9 mo compared to 22.7 mo in pre-imatinib era[78]. They also concluded that imatinib is effective in GIST recurrence even after completion of adjuvant imatinib therapy[78].

NEOADJUVANT OR PREOPERATIVE IMATINIB THERAPY

National comprehensive cancer network (NCCN) guidelines recommend neoadjuvant imatinib therapy to reduce tumor size before surgery and minimize morbidity in patients with primary GISTs considered unresectable or resectable with high risk morbidity[73]. Eisenberg *et al*[79] evaluated the safety and efficacy of neoadjuvant imatinib (600 mg/d) in patients with KIT positive primary GIST (⩾ 5 cm, 32 patients) or with operable metastatic/recurrent GIST (⩾ 2 cm, 20 patients). It was a prospective nonrandomized trial and imatinib was continued postoperatively for 2 years[79]. In primary GIST group, preoperative response was partial in 2 patients (7%), stable in 25 (83%) and unknown in 3 (10%); in metastatic or recurrent group, partial in 1 (4.5%), stable in 20 (91%), and progression in 1 (4.5%)[79]. Only 7 (13%) patients did not have any surgery (5 inoperable or unresectable, 1 patient refusal and 1 physician refusal)[79]. The estimated 2-year rate of TTP, PFS, OS in primary *vs* metastatic/recurrent GIST was 13.9% *vs* 13.6%, 82.7% *vs* 77.3% and 93.3% *vs* 90.9% respectively[79].

Fiore *et al*[80] prospectively evaluated the PFS in locally advanced or unresectable primary GISTs treated with preoperative imatinib. All patients who were considered high risk or needed extensive surgery (3 considered unresectable underwent complete resection, 7 who were initially considered to undergo extensive surgery were conservatively operated, 4 who were considered high perioperative risk underwent safe surgery) improved after preoperative imatinib therapy. PFS after 3 years was 77% from the time of initial imatinib treatment[80].

IMATINIB IN METASTATIC GIST

The outcome of advanced GISTs treated with imatinib is not clear. Demetri *et al*[81] evaluated the efficacy of imatinib on antitumor response, safety and tolerability in advanced GISTs. Results showed that 79 patients (53.7%) had partial response, 41 patients (27.9%) had stable disease and in 7 patients (4.8%) response could not be evaluated[81]. Adverse effects related to imatinib therapy were diarrhea, edema (periorbital and leg), fatigue and gastrointestinal bleeding[81]. Overall, the therapy was well tolerated. Blanke *et al*[82] conducted a multicenter randomized phase II trial and they evaluated the efficacy and long-term safety of imatinib (group A 400 *vs* group B 600 mg) in advanced GISTs positive for CD117 antigen. In group A (400 mg, 73 patients), the authors observed GISTs with complete response 0 (0%), partial response 50 (68.5%), stable 10 (13.7%), progressive 11 (15.1%) and unknown 2 (2.7%)[82]. In group B (600 mg, 74 patients), the authors reported GISTS with complete response 2 (2.7%), partial 48 (64.9%), stable 13 (17.6%), progressive 6 (8.1%) and unknown 5 (6.8%)[82]. Overall, imatinib was well tolerated[82]. In the subsequent phase Ⅲ trial, Blanke *et al*[83] evaluated PFS or OS with standard imatinib dose (400 mg) *vs* higher dose (400 mg twice daily) in patients with incurable GISTs. After a median follow up of 4.5 years, median PFS for standard *vs* high dose imatinib was 18 mo *vs* 20 mo, median OS for standard *vs* high dose imatinib was 55 mo *vs* 51 mo respectively[83]. Treatment response in standard *vs* high dose imatinib were divided in to complete response (5% *vs* 3%), partial (40% *vs* 42%), stable (25% *vs* 22%), progressive disease (12% *vs* 10%) and inadequate assessment (10% *vs* 15%) respectively[83]. This study concluded that 400 mg twice daily imatinib was more toxic than 400 mg dose in treatment of incurable GISTs[83]. Debiec-Rychter *et al*[84] evaluated the efficacy of standard dose imatinib (400 mg) *vs* higher dose (400 mg two times daily) in advanced GIST based on mutational status (KIT or PDGFRA). There was a 61% relative risk reduction of PFS in GISTs expressing exon 9 mutation treated with high dose imatinib[84]. Overall, this study concluded that tumor genotype determines PFS and OS in advanced GISTs and also GISTs with KIT exon 9 benefited from 400 mg two times daily imatinib[84].

Heinrich *et al*[85] showed that presence of KIT exon-11 mutation (71.7%) had better treatment outcome with imatinib when compared to KIT exon-9 (44.4%) and wild-type mutation (44.6%) in advanced GISTs.

The authors also showed that there was an improved response rate (complete/partial response) in patients with KIT exon-9 mutation treated with imatinib 800 mg *vs* 400 mg (67% *vs* 17%, $P = 0.02$)[85]. GIST meta-analysis group (MetaGIST) evaluated PFS and OS with imatinib (400 mg *vs* 800 mg) in advanced GISTs[86]. The results showed that there was a small but significant PFS ($P = 0.04$) advantage in high dose (400 mg twice daily) group and no difference in OS between both (400 and 800 mg) groups[86].

SUNITINIB AFTER TREATMENT FAILURE WITH IMATINIB IN ADVANCED GIST

Demetri *et al*[87] evaluated patients treated with sunitinib in advanced GISTS who were intolerant or resistant to previous imatinib treatment. They concluded that median TTP with sunitinib *vs* placebo was 27.3 wk *vs* 6.4 wk respectively[87]. Overall, sunitinib was well tolerated and side effects like nausea, fatigue, skin discoloration and diarrhea were common[87].

REGORAFENIB AFTER TREATMENT FAILURE WITH IMATINIB AND SUNITINIB IN ADVANCED GIST

Demetri *et al*[88] evaluated the efficacy and safety of regorafenib after failure of treatment with imatinib and sunitinib. Results showed that the median PFS in regorafenib *vs* placebo group were 4.8 mo *vs* 0.9 mo respectively[88]. There was no statistical significance in terms of OS between regorafenib and placebo group[88]. Drug related adverse events occurred in 130/132 (98.5%) in regorafenib group and 45/66 (68.2%) in placebo group[88]. The most common adverse effects of regorafenib include hypertension (31/132, 23.5%), hand foot skin reaction (26/132, 19.7%) and diarrhea (7/132, 5.3%)[88]. Overall, this study concluded that regorafenib significantly improved PFS in patients with advanced GISTs who failed treatment with imatinib and sunitinib[88].

FOLLOW-UP AFTER TREATMENT

The goal of follow-up after surgery is early detection and treatment of relapse. CT abdomen and pelvis is used for follow-up. Metastasis of GISTs outside the abdomen is infrequent. MRI or PET-CT can be used as an alternative for follow-up. Annual CT abdomen and pelvis for 5 years is recommended for low risk GISTs after surgery[89]. During adjuvant treatment with imatinib for high risk GISTs, CT abdomen and pelvis is recommended every 6 mo[89]. After adjuvant therapy is stopped, CT is repeated every 3-4 mo for first 2 years and there after every 6-12 mo for 10 years[89].

CONCLUSION

With increasing availability of EUS and improved knowledge of the pathogenesis of GISTs, accurate identification and differentiation of GISTs from other submucosal tumors are achieved. Although surgery is preferred, newer endoscopic techniques can be attempted by experienced endoscopists with the assistance of surgeons in suitable candidates. Neoadjuvant imatinib therapy is recommended for primary GISTs considered unresectable or resectable with high morbidity to reduce the tumor size before surgery and minimize morbidity. Adjuvant therapy with imatinib in intermediate and high risk GISTs improves OS and RFS. Sunitinib and regorafenib can be used in advanced GISTs after treatment failure with imatinib. Multidisciplinary approach involving endoscopist, pathologist, radiologist, medical oncologist and surgeon is required for optimal management of GIST.

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MINIREVIEWS

Immunotherapies in sarcoma: Updates and future perspectives

Marwan Ghosn, Elie El Rassy, Hampig Raphael Kourie

Marwan Ghosn, Elie El Rassy, Hampig Raphael Kourie, Department of Oncology, Hotel Dieu de France University Hospital, Faculty of Medicine, Saint Joseph University, Beirut 2038 3054, Lebanon

Author contributions: Ghosn M initiated the review; Ghosn M, El Rassy E and Kourie HR performed the review, analyzed the data and wrote first draft; Ghosn M, El Rassy E and Kourie HR reviewed and commented on the paper and provided final approval.

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Correspondence to: Elie El Rassy, MD, Department of Oncology, Hotel Dieu de France University Hospital, Faculty of Medicine, Saint Joseph University, Monot St, Beirut, PO Box 166830, Beirut 2038 3054, Lebanon. elie.rassy@hotmail.com
Telephone: +961-1-615300
Fax: +961-1-615300

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Abstract

Sarcomas are malignant tumors that are characterized by a wide diversity of subtypes with various cytogenetic profiles. Despite major treatment breakthroughs, standard treatment modalities combining chemotherapy, radiotherapy, and surgery failed to improve overall survival. Therefore, high expectations are foreseen with immunotherapy upon its maturation and better understanding of its mechanism of action. This paper presents a targeted review of the published data and ongoing clinical trials in immunotherapies of sarcomas, mainly adoptive cell therapies, cancer vaccines and immune checkpoint inhibitors.

Key words: Adoptive cell therapy; Cancer vaccines; Immunotherapy; Immune checkpoint inhibitors; Sarcoma

Core tip: This paper is a review that outlines the most recent updates on the immunotherapy treatment of sarcomas. After a brief review of the concept of immunotherapies and the different treatment modalities, we discuss the available data, the limitations and future perspectives of each treatment option.

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INTRODUCTION

Sarcomas are malignant tumors that derive from embryonic mesodermic tissues including fat, muscles, bones, nerves and blood vessels[1]. Epidemiologic studies report its predominance in the pediatric populations and its rare occurrence in adults[2]. Sarcomas are

characterized by a wide diversity of subtypes with various cytogenetic profiles conferring treatment resistances. These findings combined with an advanced stage at diagnosis substantially increase the years of life lost[3]. The standard treatment modalities combining chemotherapy, radiotherapy, and surgery have failed to improve overall survival (OS)[4]. Despite the major breakthroughs in the treatment armamentarium, the recent data reports a relative 5-year survival rate limited to 66% for bone and soft tissue sarcomas, 53.9% for osteosarcomas, 75.2% for chondrosarcomas, and 50.6% for Ewing's sarcomas[5].

Interestingly, Coley described in 1891 a complete regression of sarcomas secondary to severe episodes of erysipelas but failed to regenerate these results in other patients[6]. The Food and Drug Administration thereafter banned the use of toxin therapy without a new drug-approval process. Fortunately, Coley's paper has encouraged scientists to analyze the role of the immune system in carcinogenesis[7].

After more than a century since Coley's research efforts that marked the history of immunotherapy, we present a review on this elegant treatment modality in the management of sarcomas including adoptive cell therapies (ACT), monoclonal antibodies, vaccines, and immune checkpoint inhibitors (ICI).

APPROVED THERAPIES IN SARCOMAS FROM CHEMOTHERAPY TO TARGETED THERAPIES

Specialized centers in the management of sarcomas have demonstrated a better OS and low recurrence rate[8]. Yet, all patients are managed uniformly according to their prognosis dictated by the stage of the disease, which is determined by the grade, depth and size of the tumor[9]. For patients with localized disease, a complete resection with wide 2-3 cm margins followed by adjuvant radiation therapy is the mainstay treatment for a curative approach. However, survival is not only determined by local control since most patients die from systemic disease. The choice of the chemotherapy regimen depends on the tumor chemosensitivity which varies with the tumor subtype and grade, the patient's performance status, and the timing of metastatic disease[10]. Unfortunately, the benefits of adjuvant chemotherapy are limited to rhabdomyosarcomas, osteosacromas and Ewing's sarcomas. Moreover, Trabectidine is showing promising results encountered in the adjuvant and neoadjuvant settings of patients with myxoid liposarcomas[11]. The role of adjuvant and neoadjuvant chemotherapy in the management of soft tissue sarcomas is yet to be clearly established. The actual recommendations by NCCN and ESMO are to address this issue on a case by case basis according to the patient's performance status, comorbid factors, disease location, tumor size, and histologic subtype. In case of advanced and recurrent sarcomas, induction regimens include Cyclophosphamide and Ifosphamide, Vincristine, Doxorubicin, Dactinomycin, and Etoposide[12]. For patients with unresectable or metastatic disease, the management plan is limited to a palliative approach with Trabectedin or Ifosfamide and Doxorubicin based chemotherapy[13,14].

The rationale of using targeted therapies in sarcomas goes back to 1984 when sarcomagenesis was correlated to recurrent translocations[15]. Genetic profiling thus defined two groups of sarcomas. The first group is characterized by a simple karyotype associated with specific tumor genetic alterations that include chromosomal translocations, oncogenetic mutations, and recurrent gene amplifications. The second group is characterized by a complex karyotype associated with nonspecific and nonrecurring genetic alterations[16]. Subsequent to these advances, Pazopanib, a multitargeted tyrosine kinase inhibitor against VEGFR1-3, PDFGRA-B, and KIT was approved for pretreated metastatic nonlipomatous sarcomas based on the phase Ⅲ PALETTE study[17]. Clinical and preclinical mechanistic studies are being conducted to validate a possible therapeutic role of the various targeted therapies available. Among these novel targeted therapies, we report the trials of Cediranib and Sunitinib in alveolar soft part sarcoma, Tivantinib and Cabozantinib in clear cell sarcoma, Imatinib in dermatofibrosarcoma protuberans, Cabozantinib in endometrial stromal tumors, and Everolimus in perivascular epitheloid cell tumor[18].

ADVANCES IN IMMUNO-ONCOLOGY

In fact, the previous cancer treatment approaches addressed distinctive and complementary hallmarks of carcinogenesis that included sustained proliferative signaling, evasion of growth suppressors, resistance of cell death, enabling of replicative immortality, induction of angiogenesis and activation of invasions and metastasis[19]. The well-known conventional cytotoxic drugs and targeted therapies have reached a plateau in effect that required a re-assessment of the six hallmarks of carcinogenesis. Recent conceptual progress has added two new hallmarks, namely reprogramming of energy metabolism and signaling interactions of the tumor microenvironment[20].

The later resides in the concept of the cancer-immunity cycle and is actually a turning point in the history of cancer therapy[21]. This cycle is the result of a counterbalance between immune-stimulatory and inhibitory factors. It occurs physiologically and starts with the release of cancer cell antigens and ends with the apoptosis of cancer cells *via* the activated effectors of the immune system[22]. Subsequently, cancer immunoediting may proceed with any of the three following phases[23]. The elimination phase describes an activation of the innate and adaptive immune effectors in response to cytokine secretion. The equilibrium phase occurs in the setting of a balance between tumor immune destruction and proliferation. The immunologic phase takes place when the tumor cells are capable of evading the immune system[23].

Recent advances recommend addressing only one step of the immune cycle to avoid potential unwanted

activation of autoimmunity mechanism and normal cells damage. Therefore, immunotherapy aims at initiating or maintaining the cancer-immunity cycle by acting on its rate limiting step. Consequently, ICI often address the immunostar function of the tumor microenvironment[24]. The PD-1/PD-L1 axis is a potential therapeutic target in view of the confirmed expression of PD-L1 in various sarcomas[25]. Inhibition of this axis enables the immune system to quickly adapt to cancer resistances thus allowing durable responses with ICI[26].

IMMUNOTHERAPEUTIC MODALITIES EVALUATED IN SARCOMAS

Sarcomas mainly occur either secondary to the activation of oncogenes *via* translocations and inversions, or secondary to the natural expression of germ cell peptides[27,28]. The issuing peptides generate an immune cascade directed against the aberrant cells[29]. Consequently, multiple rationales to immunotherapy including ACT, therapeutic vaccines, and ICI have been assessed in the treatment of sarcomas (Table 1).

Adoptive cell therapy in sarcomas

Adoptive cell therapy is a new therapeutic strategy based on the modulation, manipulation and selection of autologous T-cells *in vitro* to overcome the tolerance of the immune system to the tumor cells. Those T-cells may be harvested from tumor infiltrating lymphocytes (TIL) and re-transfused into the same patient after ensuring their expansion. Lymphocyte T-cells may also be harvested from peripheral blood, and those that recognize tumor antigens are selectively expanded. Alternatively, lymphocyte T-cells may be genetically engineered either by modifying a T-cell receptor for cancer antigen (transgenic TCR) or by adding a chimeric antigen receptor (CAR) that recognizes a specific cancer antigen[30,31]. Apart from T-cells, NK ACT has also been proven efficacious with several advantages over the classical T-cell ACT in the absence of MHC/HLA restriction, namely their NKG2D-dependent cytotoxicity against autologous tumor cells[32,33].

To our knowledge, the use of TIL has never been reported in the treatment of sarcomas whilst the use of NK ACT has been limited to case reports[33]. On the other hand, tumor antigens such as GD2 (93% of sarcomas) and NY-ESO-1 (80% to 100% of different subtype of sarcomas) were found to represent interesting targets for adoptive cells therapies. Moreover, other cancer testis antigens such as LAGE, MAGE-A3 and PRAME were frequently expressed in sarcomas and would be potential immunotherapeutic targets. In this setting, a phase I study evaluated the ability of adoptively transferred autologous T-cells transduced with a T-cell receptor (TCR) directed against NY-ESO-1 to mediate tumor regression in patients with metastatic synovial cell sarcoma expressing NY-ESO-1. The results showed an objective clinical response in 4 out of 6 patients[31].

Two ongoing trials are evaluating genetically engineered NY-ESO-1 T-cells for children and adults in metastatic synovial sarcoma (NCT01343043). Another phase I trial is testing the role of CAR T-cell therapy targeting the GD2 protein in children and young adults with sarcomas and rhabdomyosarcomas (NCT00743496).

Therapeutic vaccines in sarcomas

The therapeutic effects of cancer vaccines rely on the activation of dendritic cells upon the presence of an immunogenic predetermined antigen. However, most of the initial studies of vaccines in sarcomas did not determine specific antigens and used inefficaciously the entirety of the tumor cells[34,35]. Later studies used SYT-SSX, a fusion derived peptide present in 90% of synovial sarcoma, and also failed to demonstrate an objective response[36-38]. Takahashi *et al*[39] personalized the peptide vaccination patients with refractory sarcoma and administered multiple tumor antigens chosen according to preexisting peptide-specific IgG titers. The median OS was 9.6 mo with disease stabilization occurring in 30% of patients but no objective responses were seen. Another vaccination modality used *in situ* vaccination through combining preoperative gamma radiation (50 Gy) with intratumoral dendritic cells injection. The studied population was limited to high risk, localized, and resected extremity soft tissue sarcoma and resulted in 71% progression free survival at one year[40].

Major efforts in this field are being conducted namely in children with Ewing sarcomas. Recent data demonstrated a 75% OS at one year with FANG immunotherapy in adolescent patients with Ewing's sarcoma. The treatment was well tolerated with a favorable OS[41]. A seemingly interesting phase Ⅰ trial designed for the treatment of pediatric patients with relapsed high-risk Ewing sarcoma, osteogenic sarcoma, rhabdomyosarcoma, synovial sarcoma, and neuroblastoma is using a combination of Decitabine demethylating agent and a cancer vaccine composed of dendritic cells pulsed with overlapping peptides of NY-ESO-1, MAGE-A1, and MAGE-A3 (NCT01241162). Another dendritic cell vaccine is also being assessed in combination with Gemcitabine in a phase Ⅰ trial for adults and children with soft tissue and bone sarcomas (NCT01803152).

Immune checkpoint inhibitors in sarcomas

The concept of ICI relies on deactivating the suppressed activity of the immune system. ICI remove the brakes (PD-1 and CTLA4) thus enhancing the immune function of already sensitized T-cells. Effectively, PD-1 and CTLA4 inhibitors are showing interesting results with acceptable response rates in different cancers, including those considered for a long time as non-immunogenic[42]. Unlike CTLA4 inhibitors, the response to PD1 and PDL-1 inhibitors has been correlated with the expression of PD-1 and PDL-1 on tumor cells and to the mutational load of the tumors[42]. Moreover, PD-1 and PDL-1 expression seems to vary between sarcoma subtypes, a finding that may direct immunotherapy management in patients with sarcomas[43].

Table 1 Summary of the phase Ⅰ/Ⅱ trials of immunotherapies in sarcoma

Treatment modality	Ref.	Agent	Phase/Patients	Indication	RR	Survival
Adoptive cell therapy	Robbins *et al*[31], 2011	Adoptively transferred autologous T cells transduced with a T-cell receptor directed against NY-ESO-1	Ⅰ/6	Metastatic synovial cell sarcoma expressing NY-ESO-1	RR: 4/6	N/A
Vaccines	Mahvi *et al*[34], 2002	GM-CSF treated tumor cells	Ⅰ/16	Melanoma and sarcomas	RR: 1/16	N/A
	Dillman *et al*[35], 2004	Autologous tumor cell line-derived vaccines	Ⅰ, Ⅱ/23	Recurrent or metastatic sarcoma	No objective response assessed	10 patients lived more than 1 year
	Kawaguchi *et al*[36], 2005	Vaccination By SYT-SSX junction peptide	Ⅰ/6	Disseminated synovial sarcoma	RR: 0/6	N/A
	Kawaguchi *et al*[38], 2012	SYT-SSX breakpoint peptide vaccines	Ⅰ, Ⅱ/21	Metastatic synovial sarcoma	RR: 1/21 SD: 6/21	N/A
	Takahashi *et al*[39], 2013	Personalized peptide vaccination	Ⅱ/20	Refractory bone and soft tissue sarcoma	SD in all patients	Median OS: 9.6 mo
	Finkelstein *et al*[40], 2012	Combination of external beam radiotherapy with intratumoral injection of dendritic cells	Ⅰ, Ⅱ/17	Neoadjuvant treatment in high-risk soft tissue sarcoma	RR: 9/17	One-year PFS: 70.6%
	Ghisoli *et al*[41], 2015	FANG autologous immunotherapy	Ⅰ/12	Advanced and metastatic Ewing's sarcoma	RR: 1/12	One-year OS: 75%
Checkpoint inhibitors	Makki *et al*[44], 2013	Ipilimumab	Ⅱ/6	Advanced synovial sarcoma	RR: 0/6 (closed prematurely)	Median OS: 8.75 mo

GM-CSF: Granulocyte-Macrophage Colony-Stimulating Factor; N/A: Not available; OS: Overall survival; PFS: Progression free survival; RR: Response rate.

Unfortunately, the efficacy of ICI in sarcomas has been evaluated in only one study so far. It is a phase Ⅱ study that administered Ipilimumab (3 mg/kg intravenously every 3 wk for 3 cycles), a CTLA-4 inhibitor, to six patients with synovial sarcoma. The median OS was 8.75 mo ranging between 0.8 and 19.7 mo. The study was closed prematurely when none of the patients had an objective tumor response. All patients expressed NY-ESO-1 but its titers did not change after treatment administration[44]. PD-1 and PDL-1 inhibitors present a different mechanism of action compared to anti-CTLA4 agents and consequently may present better response rates[43]. Many ongoing phase Ⅰ trials are assessing the role of anti-PD1 agents in sarcomas as single agent or in combination with Ipilimumab and Dasatinib (NCT0 1643278).

PERSPECTIVE

The proof of the immunotherapy concept in sarcomas has been undoubtedly validated with the benefits encountered upon the use of liposomal muramyl-tripeptide-phosphatidyl-ethanolamine, an immunoactivator agent derived from BCG. However, its role remains controversial in view of the discordant results between the preliminary data and final results in both the adjuvant and metastatic setting. Even though the actual trend is moving towards immunotherapy as an essential tool in the treatment of cancer, the recent ASCO 2016 meeting was unfortunately disappointing in this regard. Five studies have been presented, of which one trial of chemotherapy (Busulphan and Melphalan), three trials of tyrosine kinase inhibitors, monotherapy (Anlotinib and Regorafenib) or in combination with chemotherapy (Gemcitabine plus Pazopanib), and one study reporting the evident detrimental impact of disease progression and altered quality of life on the long-term care and survival of patients with sarcomas. The ongoing trials including the promising results of immunotherapies are awaited. The available results reported a failure of Pembrolizumab in multiple soft tissue sarcomas (NCT02301039) and Nivolumab in metastatic uterine leiomyosarcoma (NCT0 2428192) despite the promising findings encountered with Nivolumab in retrospective experiences[45]. In fact, the biological preclinical rationale is not fully elucidated in view of the absence of any correlation between PD-L1 expression and OS[46]. Thus, the actual state of knowledge does not predict the patient profile that might benefit from immunotherapy.

CONCLUSION

The cornerstone treatment for sarcomas consists of complete surgical resection, chemotherapy, and radiotherapy. Unfortunately, these treatment options fall short from achieving an optimal clinical outcome. Immunotherapy is therefore expected to further improve the survival of patients with sarcomas. Until recently, the field of immunotherapy has not yet matured enough to present robust effects. The better understanding of onco-immunotherapy principles is essential to adjust the design of clinical trials and the selection of inclusion criteria. The published data shows that ACT is yet to be more elucidated and evaluated, vaccine therapy requires tailoring and personalization, and ICI, preferably PD-1 and PDL-1 inhibitors, necessitate better patient selection. Such results

would allow more understanding of the antitumor immunity mechanisms and improvement of the treatment arsenal against sarcomas.

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ORIGINAL ARTICLE

Retrospective Study

Bethesda System for Reporting Thyroid Cytopathology: A three-year study at a tertiary care referral center in Saudi Arabia

Mohamed Abdulaziz Al Dawish, Asirvatham Alwin Robert, Aljuboury Muna, Alkharashi Eyad, Abdullah Al Ghamdi, Khalid Al Hajeri, Mohammed A Thabet, Rim Braham

Mohamed Abdulaziz Al Dawish, Asirvatham Alwin Robert, Department of Endocrinology, Prince Sultan Military Medical City, Riyadh 11342, Saudi Arabia

Aljuboury Muna, Department of Pathology, Prince Sultan Military Medical City, Riyadh 11342, Saudi Arabia

Alkharashi Eyad, Abdullah Al Ghamdi, Khalid Al Hajeri, Department of General Surgery, Prince Sultan Military Medical City, Riyadh 11342, Saudi Arabia

Mohammed A Thabet, Department of Radiology, Prince Sultan Military Medical City, Riyadh 11342, Saudi Arabia

Rim Braham, Department of Endocrinology, Henri Mondor Hospital, F-94010 Paris, France

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Correspondence to: Dr. Mohamed Abdulaziz Al Dawish, MD, Department of Endocrinology, Prince Sultan Military Medical City, Alrabwa, P.O. Box 261370, Riyadh 11342, Saudi Arabia. maldawish@psmmc.med.sa
Telephone: +966-50-2048321

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Abstract

AIM

To stratify the malignancy risks in thyroid nodules in a tertiary care referral center using the Bethesda system.

METHODS

From January, 2012 to December, 2014, a retrospective analysis was performed among 1188 patients (15-90 years) who had 1433 thyroid nodules and fine-needle aspiration at Prince Sultan Military Medical City, Saudi Arabia. All thyroid cyto-pathological slides and ultra sound reports were reviewed and classified according to the Bethesda System for Reporting Thyroid Cytopathology. Age, gender, cytological features and histological types of the thyroid cancer were collected from patients' medical chart and cytopathology reports.

RESULTS

There were 124 total cases of malignancy on resection, giving an overall surgical yield malignancy of 33.6%.

Majority of the thyroid cancer nodules (*n* = 57, 46%) in Bethesda VI category followed by Bethesda IV (*n* = 25, 20.2%). Almost 40% of the cancer nodules in 31-45 age group in both sex. Papillary thyroid carcinoma (PTC) was the most common form of thyroid cancer among the study population (111, 89.6%) followed by 8.9% of follicular thyroid carcinoma (FTC), 0.8% of medullary carcinoma and 0.8% of anaplastic carcinoma. Among the Bethesda IV category 68% thyroid nodules were PTC and 32% FTC.

CONCLUSION

The malignancy values reported in our research were constant and comparable with the results of other published data with respect to the risk of malignancy. Patients with follicular neoplasm/suspicious for follicular neoplasm and suspicious of malignancy categories, total thyroidectomy is indicted because of the substantial risk of malignancy.

Key words: Bethesda; Total thyroidectomy; Thyroid nodules; Risk of malignancy; Fine needle aspiration

Core tip: The purpose of this study was to stratify the malignancy risks in thyroid nodules in a tertiary care referral center using the Bethesda system. The study found that there were 124 total cases of malignancy on resection, giving an overall surgical yield malignancy of 33.6%. Majority of the thyroid cancer nodules in Bethesda VI category followed by Bethesda IV. Almost 40% of the cancer nodules in 31-45 age group in both sex. Papillary Thyroid Carcinoma was the most common form of thyroid cancer among the study population followed by follicular thyroid carcinoma, medullary carcinoma and anaplastic carcinoma.

Al Dawish MA, Robert AA, Muna A, Eyad A, Al Ghamdi A, Al Hajeri K, Thabet MA, Braham R. Bethesda System for Reporting Thyroid Cytopathology: A three-year study at a tertiary care referral center in Saudi Arabia. *World J Clin Oncol* 2017; 8(2): 151-157 Available from: URL: http://www.wjgnet.com/2218-4333/full/v8/i2/151.htm DOI: http://dx.doi.org/10.5306/wjco.v8.i2.151

INTRODUCTION

According to epidemiological and clinical studies thyroid nodules are commonly encountered in clinical exams, palpable in 5% of the population on thyroid examination and detectable in nearly 60% of those subjected to thyroid ultrasound. While the majority of the nodules are benign (non-cancerous), they are normally the first indicators of thyroid cancer; therefore, further investigations are required to identify the cancerous nodule[1,2].

The last decades have revealed a constant and remarkable rise in the occurrence of thyroid cancer across the world, including Saudi Arabia[3-5]. The Saudi Cancer Registry (SCR) report has registered 890 thyroid cancer cases, in nearly 8.1% of all the newly diagnosed cases in 2012. However, studies revealed variations in the incidence of thyroid cancer globally. Thyroid cancer is the 5th most common cancer among females in the United States, whereas in Saudi Arabia it is the 2nd commonest identified cancer in females, and 8th among males[6]. However, compared with the developed countries, research regarding the malignancy risks in thyroid nodules is still insufficient due to lack of appropriate studies being conducted in these specified areas.

One of the most widely used diagnostic tools is fine-needle aspiration (FNA) cytology with ultrasound imaging to determine the necessity for the surgical excision of a thyroid nodule. Today, molecular genetic biomarker analyses are employed to increase the diagnostic accuracy of the FNA biopsies, and can at times drastically change clinical decision procedures as they become more commonly available and better assessed. FNA cytology (FNAC) continues to remain the initial investigation mode for malignancy in patients with thyroid nodules and the selection of patients for thyroid surgery[7]. This minimally invasive and useful method is highly effective in identifying a large percentage of thyroid nodules as benign and eliminating unnecessary surgery for patients with benign disease[8]. However, because a standardized reporting system is still unavailable, pathologists have been employing varying terminologies and diagnostic criteria, thus causing misunderstanding among the referring clinicians while interpreting cytopathology reports, resulting in non-definitive clinical management[9-11]. In 2007, the National Cancer Institute (NCI) established guidelines employing a standardized nomenclature to interpret thyroid FNAs called the Bethesda System for Reporting Thyroid Cytopathology (BSRTC) which is now accepted as the proposed diagnostic categories for thyroid cancer[12]. This study attempts to stratify the malignancy risks in thyroid nodules in a tertiary care referral center in Saudi Arabia utilizing the Bethesda system.

MATERIALS AND METHODS

Study design and setting

From January, 2012 to December, 2014 (36 mo), a retrospective analysis was performed among 1188 patients (15-90 years old) who had 1433 thyroid nodules and FNA at Prince Sultan Military Medical City (PSMMC), a 1200 bedded tertiary care center, Riyadh, Saudi Arabia. The PSMMC caters to the patients referred from different regions of Saudi Arabia and considered a worthy representative of Saudi Arabia in general. The study protocol was approved by the Research and Ethics Committee of PSMMC, Riyadh, Saudi Arabia.

Data collection

All thyroid cytopathological slides and ultra sound

Table 1 The Bethesda system

Diagnostic category	Cytological diagnosis	Risk of malignancy, %	Usual management
Ⅰ	Nondiagnostic or unsatisfactory	1-4	Repeat FNA with ultrasound guidance
Ⅱ	Benign	0-3	Clinical follow-up
Ⅲ	AUS/FLUS	5-15	Repeat FNA
Ⅳ	FNS/SFN	15-30	Surgical lobectomy
Ⅴ	Suspicious for malignancy	60-75	Near-total thyroidectomy or surgical
Ⅵ	Malignant	97-99	Near-total thyroidectomy

FNA: Fine-needle aspiration; AUS/FLUS: Atypia of undetermined significance or follicular lesion of undetermined significance; FNS/SFN: Follicular neoplasm or suspicious for follicular neoplasm.

reports were reviewed and classified according to the BSRTC system. Age, gender, cytological features and histological types of the study population were collected from patients' medical chart and cyto-pathology reports.

Bethesda system

Currently, the Bethesda system of reporting thyroid cytology (TBSRTC) is used for reporting FNAC specimens of thyroid. According to Cibas[13], this system was innovated in 2007 and consists of six categories: (1) Unsatisfactory (UNS) or nondiagnostic (ND); (2) Benign and nonneoplastic; (3) Atypia of undetermined significance or follicular lesion of undetermined significance (AUS/FLUS); (4) Follicular neoplasm or suspicious for follicular neoplasm (FNS/SFN); (5) Suspicious for, but not diagnostic of, malignancy; and (6) Malignant (Table 1).

All FNAs were performed by one of five interventional radiologists under ultrasound (US) guidance, performing 3-5 passes by using 25 gauge needles. On-site FNAs stained with the Diff-Quik stain and adequacy assessment was performed for all samples. All slides interpreted by among of five accredited cyto-pathologists.

Histological diagnoses

The histological diagnoses of thyroid nodules were classified into two types: Benign and nonneoplastic and malignant. For papillary thyroid carcinoma (PTC), subtype variants were documented such as the follicular variant, classical variant, conventional variant and tall cell variant. Also were follicular thyroid carcinoma (FTC) subdivided to minimally invasive follicular thyroid carcinoma (MIFTC) and Widely Invasive follicular thyroid carcinoma (WIFTC).

Statistical analysis

All statistical calculations were performed using IBM SPSS Statistics (IBM SPSS Statistics for Windows, Version 22, SPSS Inc. an IBM Company) program and Microsoft Excel 2010 (Microsoft Corporation, Seattle, WA, United States). The descriptive analysis of the epidemiological data presented as frequencies, percentages and mean ± standard deviation (SD). χ^2 test was performed to find out the variables associated with cancer among the surgical patients.

RESULTS

A total of 1188 patients (range 15-90 years) included in this study. The mean age of the study population was 46.3 ± 15.1 (SD), median 45 years, and mode 49 years. Of the 1188 (212 male; 976 female) patients, 245 patients had two thyroid nodules, which resulted in a total of 1433 FNA cases (nodules). Among the study population, a total of 311 patients underwent surgery and 877 patients did not undergo surgery. Of the 311 patients who underwent surgery, 58 patients had two thyroid nodules, which resulted in a total of 369 cases (245 benign and 124 malignant) (Figure 1). Among patients who underwent surgery, no statistically significant differences were observed on the presence of cancer among both gender (P = 0.463), and different age groups (P = 0.928).

As shown in Table 2, the distribution of all cases in the six Bethesda diagnostic categories were as follows: 46 cases (3.2%) of category Ⅰ, 1080 cases (75.3%) of category Ⅱ, 131 cases (9.1%) of category Ⅲ, 71 cases (5%) of category Ⅳ, 32 cases (2.2%) of category Ⅴ and 73 cases (5.1%) of category Ⅵ.

The distributions of follow-up diagnoses for each initial Bethesda diagnostic classification are shown in Table 3. There were 124 total cases of malignancy on resection, giving an overall surgical yield of malignancy of 33.6%. Eight of (2.2%) 369 thyroid nodules were diagnosed as ND, 181 (49.1%) diagnosed as benign, 42 (11.4%) diagnosed as AUS/FLUS, 53 (14.4%) as FNS/SFN. Category Ⅴ (SM) diagnoses (26 cases) reminded benign in 8 cases, but histologically confirmed as carcinoma in 18 case (69.2%). Finally, category Ⅵ diagnoses (59 cases) reminded benign in 2 cases, but histologically confirmed as carcinoma in 57 cases (96.7%).

Table 4 shows the comparison rates of malignancy on surgical resection for FNA diagnostic categories and malignancy risk of the present findings and previously published data. Table 5 shows the age and sex distribution of thyroid cancer. Majority of the thyroid cancer nodules (n = 57, 46%) in Bethesda Ⅵ category followed by Bethesda Ⅳ (n = 25, 20.2%) and Bethesda Ⅴ (n = 18, 14.5%). Among the Bethesda Ⅳ category 17 (68%) were PTC and 8 (32%) were follicular carcinoma. Almost 40% of the cancer nodules in 31-45 age groups in both sex.

Type and variants of thyroid cancer among histopathological diagnosis are shown in Table 6. Papillary carcinoma was the most common form of thyroid cancer among the study population (111, 89.6%). Among PTC (n = 111), four histologic variants exist, with classic variant PTC accounting for 51.4% of PTC followed by follicular-

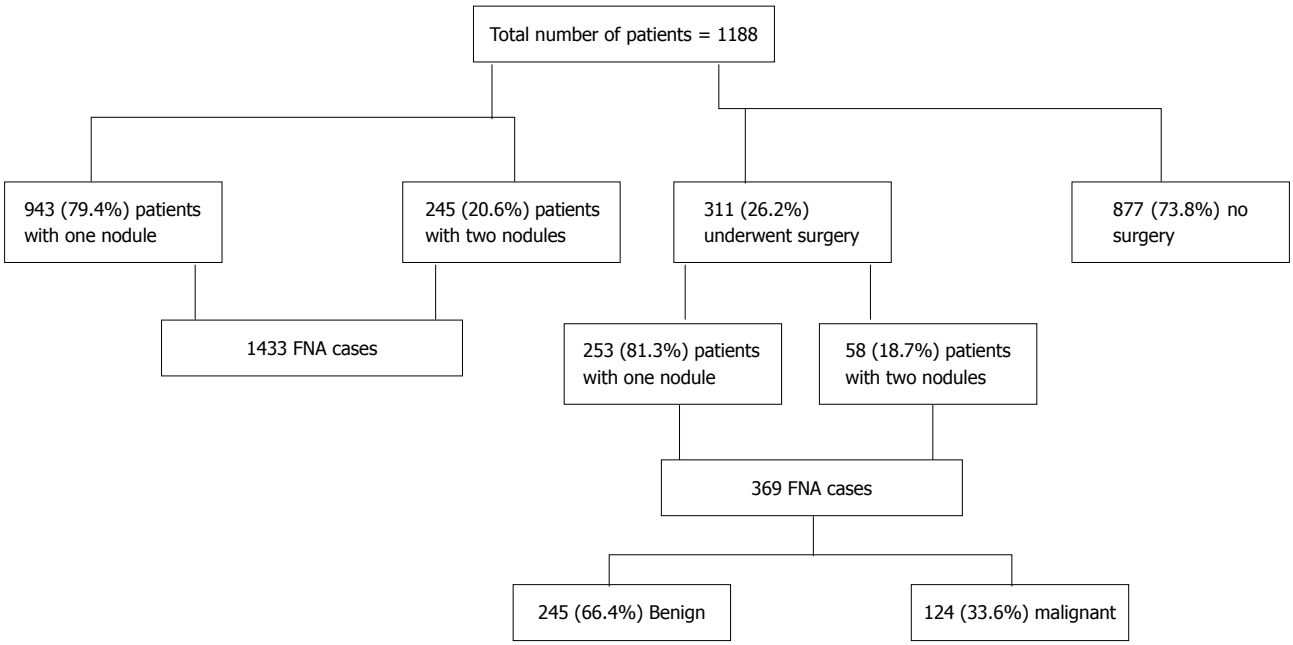

Figure 1 Flowchart of thyroid nodules description among 1188 patients and the risk of malignancy among 311 surgically excised nodules during January, 2012 to December, 2014. FNA: Fine needle aspiration.

Table 2 Age and sex distribution of thyroid lesion (based on fine-needle aspiration cytology according to Bethesda system)

Age (yr)	Total number of patients	Gender	All FNAs (n = 1433) n, %						
		F/M	Bethesda Ⅰ	Bethesda Ⅱ	Bethesda Ⅲ	Bethesda Ⅳ	Bethesda Ⅴ	Bethesda Ⅵ	Total
15-30	176 (14.8)	159/17	9 (4.5)	149 (74.9)	17 (8.5)	12 (6)	4 (2)	8 (4)	199
31-45	420 (35.4)	362/58	12 (2.4)	375 (74.7)	41 (8.2)	28 (5.6)	14 (2.8)	32 (6.4)	502
46-60	374 (31.5)	301/73	15 (3.3)	347 (75.1)	40 (8.8)	22 (4.8)	9 (2)	23 (5)	456
61-75	175 (14.7)	126/49	10 (4.5)	162 (72.3)	33 (14.7)	7 (3.1)	4 (1.8)	8 (3.6)	224
> 75	43 (3.6)	28/15	0	47 (90.4)	0	2 (3.8)	1 (1.9)	2 (3.8)	52
Total	1188	976/212	46 (3.2)	1080 (75.3)	131 (9.1)	71 (5)	32 (2.2)	73 (5.1)	1433

FNA: Fine-needle aspiration; F: Female; M: Male.

Table 3 Cyto-Histopathological correlation of thyroid lesion

Cytopathology	Histopathological diagnosis		Total
	Benign	Malignant, n (%)	
Bethesda Ⅰ	6	2 (25)	8
Bethesda Ⅱ	165	16 (8.9)	181
Bethesda Ⅲ	36	6 (14.3)	42
Bethesda Ⅳ	28	25 (47.2)	53
Bethesda Ⅴ	8	18 (69.3)	26
Bethesda Ⅵ	2	57 (96.7)	59
Total	245	124 (33.6)	369

variant PTC (30.6%). Furthermore, 8.9% of malignancies were FTC (including 0.8% of the highest risk widely invasive phenotype), 0.8% of medullary thyroid carcinoma (MTC) and 0.8% of anaplastic thyroid carcinoma (ATC). Among the Bethesda Ⅳ category 17 (68%) thyroid nodules were PTC and 8 (32%) were FTC.

DISCUSSION

Over the last few decades thyroid cancer has been on the rise considerably, globally, while mortality has steadily dropped, including in Saudi Arabia[14]. This reduction in the mortality resulting from thyroid cancer reflects the variations in the exposure to risk factors and alters the diagnosis and treatment of the disease, while the rise in the incidence is probably due to the improvement in the identification of this neoplasm[14]. However, in comparison with the developed countries, research on the incidence, prevalence and type of thyroid cancer in Saudi Arabia is still inadequate due to the lack of suitable studies being done on this specific aspect. Therefore, the objective of the current study is to stratify the risk of malignancy in the thyroid nodules based on the Bethesda system, which enhances the interpretation of the FNAC reports and enables a more accurate study and diagnosis of such thyroid nodules[13,15]. In this study, the distribution of age and gender among the patients is almost similar to those recorded in identical studies[1,2,16]. Besides, the female/male ratio reported in this study for thyroid cancer (4.7:1) concurs with the concept that thyroid cancer occurs more commonly among women. In the present study we found that the overall

Table 4 Comparison rates of malignancy (%) on surgical resection for fine-needle aspiration diagnostic categories and malignancy risk of recent studies

	Published year	Comparison of diagnostic categories					
		Ⅰ (ND)	Ⅱ (Benign)	Ⅲ (AUS/FLUS)	Ⅳ (FN/SFN)	Ⅴ (SM)	Ⅵ (malignant)
Recent studies							
Park *et al*[22]	2014	13.3	40.6	9.1	0.4	19.3	17.6
Mondal *et al*[10]	2013	1.2	87.5	1	4.2	1.4	4.7
Mufti *et al*[29]	2012	11.6	77.6	0.8	4	2.4	3.6
Wu *et al*[30]	2012	20.1	39	27.2	8.4	2.6	2.7
Bongiovanni *et al*[31]	2012	2	54.7	6.3	25.3	6.3	5.4
Present study		3.2	75.3	9.1	5	2.2	5.1
Comparison of malignancy risk							
Haugen *et al*[32] (meta-analysis)	2016	9-32	1-10	6-48	14-34	53-97	94-100
Pantola *et al*[33] 2016	2016	0	0	8.3	10	100	100
Park *et al*[22]	2014	35.3	5.6	69	50	38.7	98.9
Mondal *et al*[10]	2013	0	4.5	20	30.6	75	97.8
Mufti *et al*[29]	2012	20	3.1	50	20	80	100
Wu *et al*[30]	2012	12	8	27	33	68	100
Present study		25	8.9	14.3	47.2	69.3	96.7

ND: Nondiagnostic; AUS/FLUS: Atypia of undetermined significance/follicular lesion of undetermined significance; FN/SFN: Follicular neoplasm/suspicious for follicular neoplasm; SM: Suspicious for malignancy.

Table 5 Age and sex distribution of thyroid cancer

Age (yr)	Total number of nodules	Gender	All FNAs (n = 124) n, %					
		F/M	Bethesda Ⅰ	Bethesda Ⅱ	Bethesda Ⅲ	Bethesda Ⅳ	Bethesda Ⅴ	Bethesda Ⅵ
15-30	18 (14.5)	3/15	0	3	1	3	4	7
31-45	49 (39.5)	39/10	1	5	2	9	7	25
46-60	43 (34.7)	35/8	1	7	3	9	5	18
61-75	12 (9.7)	8/4	0	1	0	3	2	6
> 75	2 (1.6)	2/0	0	0	0	1	0	1
Total	124	87/37	2 (1.6)	16 (12.9)	6 (4.8)	25 (20.2)	18 (14.5)	57 (46)

FNA: Fine-needle aspiration; F: Female; M: Male.

Table 6 Type and variants of thyroid cancer among histopathological diagnosis

Type of cancer	Total = 124 (n, %)	BETHESDA (n, %)					
		Ⅰ	Ⅱ	Ⅲ	Ⅳ (n = 25)	Ⅴ	Ⅵ
PTC							
Classic variant	57	1	5	1	3	8	39
Follicular variant	34	1	8	2	11	6	6
Conventional	19	0	2	2	3	3	9
Tall-cell variant	1	0	0	0	0	0	1
Total PTC	111 (89.6)	2	15	5	17 (68)	17	55
FTC							
MIFTC	10	0	1	1	7	1	0
WIFTC	1	0	0	0	1	0	0
Total FTC	11 (8.9)	0	1	1	8 (32)	1	0
MTC	1 (0.8)	0	0	0	0	0	1
ATC	1 (0.8)	0	0	0	0	0	1

PTC: Papillary thyroid carcinoma; MTC: Medullary thyroid carcinoma; ATC: Anaplastic thyroid carcinoma; FTC: Follicular thyroid carcinoma; MIFTC: Minimally invasive follicular thyroid carcinoma; WIFTC: Widely invasive follicular thyroid carcinoma.

malignant rate was 33.6% which exactly matches the percentage (33.8%) of 25445 thyroid FNAs used in the meta-analysis done by Bongiovanni *et al*[17], as well as Jo *et al*[18] who reported 30.9%. However, this high malignancy rate is not unusual if it is understood that the FNAC is consistently being performed today for most patients with thyroid nodules. This has resulted in a drop in the number of unwarranted surgeries and thereby to an increase in the percentage for reported malignancies[1]. It is noteworthy that the number of FNA

cases in this study steadily rose from 2012 (n = 357) to 2014 (n = 449). From various studies it was evident that the percentage of cases that were subjected to surgery differed widely among different institutions, reporting a range from 11.8%[19] to 45.1%[20] with an average rate of 25%[17]; the current study identified 26.2% of the study population who had surgical outcome.

Each Bethesda category showed a malignancy rate ranging from 1%-10% ("benign category) to 94%-100% ("malignant" category). This comprehensive range highlights the ability of the Bethesda system to differentiate and determine the likelihood of malignancy. The results recorded in our research concurred closely with the results reported in the American Thyroid Association Management Guidelines and other studies: 25% *vs* 9%-32% ("non-diagnostic or unsatisfactory" category), 9.3% *vs* 1%-10% ("benign and non-neoplastic" category), 14.3% *vs* 6%-48% (AUS/FLUS), 69.2% *vs* 53%-97% ("suspicious for malignancy" category), and 96.7% *vs* 94%-100% ("malignant" category)[13,17]. Among Bethesda, category Ⅳ found 47.2% malignancy risk, a value higher than the meta-analysis results of 14%-34% (FNS/SFN), published recently by Bongiovanni *et al*[17]. However, many studies revealed the greatest variation in the risk of malignancy class Ⅳ, some of which are higher (malignancy rate 50%-67%) than the present values[21-23].

The current study reported PTC (89.6%) as the commonest type of thyroid cancer in the population under study. Studies also reported that overall PTC as the commonest kind of thyroid cancer represents 80% of all the thyroid malignancies and more than 90% of the differentiated thyroid cancers[13,24,25]. A spurt in the occurrence of PTC over the past decades has triggered greater interest in this disease. This is one of the fastest growing kinds of cancer recording over 20000 new cases annually. Although individuals are susceptible to papillary carcinoma irrespective of age, most patients will show the disease prior to 45 years of age[26], a fact corroborated by the current findings (42% PTC between 31-45 years of age). Unfortunately, FTC is not being diagnosed as often, although there is an increasing incidence of well-differentiated thyroid carcinomas everywhere else[27,28], concurring with the results of the current study.

There are a two limitations to this study, mainly the retrospective design and performance in a single center. As the PSMMC is a tertiary center for thyroid lesions, the data of this study may not precisely reflect the general population. More research is warranted to overcome the limitations of the study.

In conclusion, 33.6% of the cases overall among the surgically excised nodules, showed malignancy. The malignancy values reported in our research were constant and comparable with the results of other data with respect to the risk of malignancy. For the FN/SF patients and those with suspicions of malignancy, total thyroidectomy is indicated because of the substantial risk of malignancy. It is clear, that reviewing the thyroid FNAs with the Bethesda system allowed a more precise cytological diagnosis. However, the impact of Bethesda application may vary among different institutions. Clinicians are advised to be aware of the malignancy rate in the Bethesda categories in their respective institutions to improve the investigation and decision regarding patients with thyroid nodules.

COMMENTS

Background

The National Cancer Institute, United States, established guidelines employing a standardized nomenclature to interpret thyroid fine-needle aspirations (FNAs) called the Bethesda System for Reporting Thyroid Cytopathology (BSRTC) which is now accepted as the proposed diagnostic categories for thyroid cancer.

Research frontiers

Compared with the developed countries, research regarding the malignancy risks in thyroid nodules is still inadequate due to lack of appropriate studies being conducted in these specified areas in Saudi Arabia. Hence, this present study attempts to stratify the malignancy risks in thyroid nodules in a tertiary care referral center in Saudi Arabia utilizing the Bethesda system.

Innovations and breakthroughs

The study found that there were 124 total cases of malignancy on resection, giving an overall surgical yield malignancy of 33.6%. Majority of the thyroid cancer nodules in Bethesda Ⅵ category followed by Bethesda Ⅳ. Almost 40% of the cancer nodules in 31-45 age group in both sex. Papillary thyroid carcinoma was the most common form of thyroid cancer among the study population followed by follicular thyroid carcinoma, medullary carcinoma and anaplastic carcinoma.

Applications

Reviewing the thyroid FNAs with the Bethesda system allowed a more precise cytological diagnosis. However, the impact of Bethesda application may vary among different institutions. Clinicians are advised to be aware of the malignancy rate in the Bethesda categories in their respective institutions to improve the investigation and decision regarding patients with thyroid nodules.

Terminology

PTC: Papillary thyroid carcinoma; FTC: Follicular thyroid carcinoma; SCR: Saudi Cancer Registry; FNA: Fine-needle aspiration; FNAC: Fine-needle aspiration cytology; NCI: National Cancer Institute, United States; BSRTC: Bethesda System for Reporting Thyroid Cytopathology; PSMMC: Prince Sultan Military Medical City; TBSRTC: The Bethesda system of reporting thyroid cytology; UNS: Unsatisfactory; ND: Nondiagnostic; AUS/FLUS: Atypia of undetermined significance or follicular lesion of undetermined significance; US: Ultrasound; MIFTC: Minimally invasive follicular thyroid carcinoma; WIFTC: Widely Invasive follicular thyroid carcinoma; ATC: Anaplastic thyroid carcinoma.

Peer-review

The study shows a very exhaustive analysis of the throughput of thyroid cytopathology over a three-year period. The manuscript contains a detailed exposition of the results, including comprehensive tables and a comparison to other recent studies. In my opinion, this manuscript fulfills all the requirements to be published.

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ORIGINAL ARTICLE

Clinical Trials Study

Study of recombinant human interleukin-12 for treatment of complications after radiotherapy for tumor patients

Na Guo, Wen-Qin Wang, Xiao-Jing Gong, Lei Gao, Li-Rong Yang, Wei-Na Yu, Hong-Yu Shen, Ling-Qin Wan, Xi-Feng Jia, Yi-Shan Wang, Yi Zhao

Na Guo, Xiao-Jing Gong, Lei Gao, Li-Rong Yang, Wei-Na Yu, Hong-Yu Shen, Ling-Qin Wan, Xi-Feng Jia, Yi-Shan Wang, Center for Tumor Treatment, People's Liberation Army 107th Hospital, Yantai 264002, Shandong Province, China

Wen-Qin Wang, Academy of Military Medical Sciences, Sanatorium for Retired Cadres, Beijing 100039, China

Yi Zhao, Kang Litai Pharmaceutical Co. Ltd., Qingdao 266001, Shandong Province, China

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Institutional review board statement: This study was reviewed and approved by the scientific ethical committee of the Hospital. All operations were performed according to international guidelines concerning the care and treatment of cancer patients.

Informed consent statement: Patients were informed of the purpose of the experiment and agreed to treatment with rhuIL-12. Informed consent was obtained in all cases, and protocols were approved by the scientific ethical committee of the Hospital.

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Correspondence to: Dr. Yi-Shan Wang, Center for Tumor Treatment, People's Liberation Army 107th Hospital, 7 Zhichu South Road, Yantai 264002, Shandong Province, China. wangyishan288@163.com
Telephone: +86-535-2933528
Fax: +86-535-2933500

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Abstract

AIM

To evaluate the treatment effects of recombinant human interleukin-12 (rhIL-12) on radiotherapy complications, such as severe myelosuppression or pancytopenia, the decline or imbalance of immune function, *etc*.

METHODS

The patients received high-dose and short-course precise radiotherapy, such as Cyber knife and image-guided radiotherapy (IGRT), which can cause myelosuppression or pancytopenia and immune function decline within a short time. One-hundred subjects were enrolled in the study, and 50 were randomized to a treatment group which used rhIL-12 and 50 were randomized to a control group which used symptomatic and supportive therapy after radiotherapy. The 50 subjects in the treatment group were further divided into five subgroups and intervened

with rhIL-12 at a dose of 50, 100, 150, 200 or 250 ng/kg respectively. The dose-effect relationship was observed.

RESULTS

RhIL-12 significantly attenuated the decrease of peripheral blood cells in the treatment group, and immune function was improved after treatment. Due to the different radiation doses, there was a fluctuation within 12 h after treatment but mostly showing an increasing trend. As to the clinical manifestations, 2 patients in the 250 ng/kg subgroup showed low fever after administration, 1 patient in the 200 ng/kg subgroup and 2 patients in the 250 ng/kg subgroup showed mild impairment of liver function during the observation period.

CONCLUSION

RhIL-12 has effective therapeutic and protective effects on complications following radiotherapy, such as the decline of blood cells, myelosuppression and the decline or imbalance of immune function, which indicated good prospects for development and application.

Key words: Recombinant human interleukin-12; Cancer prevention; Radiotherapy complications; Clinical research

Core tip: Recombinant human interleukin-12 (rhIL-12) is a new kind of biological agent secreted by Chinese hamster ovary cells. Study has shown that it has the advantage of promoting recovery of hematopoietic function, regulating the body's immunity and inhibiting angiogenesis growth. At present, the research of rhIL-12 stays in the foundational realm and in animal experimentation. In our study, however, there were 100 patients with large or numerous tumors (more than two) and who received precision radiotherapy (Cyber knife or image-guided radiotherapy). The results showed that rhIL-12 can prevent radiation damage, improve hematopoietic function, regulate immunity, reduce the side effect of radiotherapy and improve the quality of life of patients.

Guo N, Wang WQ, Gong XJ, Gao L, Yang LR, Yu WN, Shen HY, Wan LQ, Jia XF, Wang YS, Zhao Y. Study of recombinant human interleukin-12 for treatment of complications after radiotherapy for tumor patients. *World J Clin Oncol* 2017; 8(2): 158-167 Available from: URL: http://www.wjgnet.com/2218-4333/full/v8/i2/158.htm DOI: http://dx.doi.org/10.5306/wjco.v8.i2.158

INTRODUCTION

Interleukin-12 (IL-12) is an immunoregulatory protein produced by macrophages, B cells, mononuclear cells, keratinocytes and dendritic cells, and its target point lies in early undifferentiated pluripotent hematopoietic stem cells. The studies of IL-2 trace back to early in 1986. Subsequently, many studies have confirmed that IL-12 can contribute to enhancing immunity. For example, Zhang *et al*[1] found a cytokine which can promote secretion of cytotoxic T cells (CTLs) and lymphatic factor-activated killer cells (LAK) in synergy with IL-2. In 1989, Bellone and Trinchieri[2] found a cytokine called natural killer cell-stimulating factor (NKSF), which can stimulate the production of IFN-γ. Eventually, it became known that the two cytokines were the same substance, now known as IL-12.

Based on subsequent research studies, IL-12 seems to serve as an immunoregulatory anti-cancer agent for oncology patients. However, the adverse events related to IL-12, including fever, chills, decreased peripheral blood cells and organ dysfunction, have limited the clinical application of IL-12[3]. Recombinant human interleukin-12 (rhIL-12) is an immunoregulatory protein produced by gene engineering technology. RhIL-12 has similar biological activity to IL-12. With the advantage of high purity (> 98%), high activity and low therapeutic dose, rhIL-12 became the only agent which could not only restore hematopoietic function but also improve immune function[4].

The basic experimental studies have found that the recovery and reconstitution of hematopoiesis system after radiotherapy is helpful to avoid the rapid increase of single blood cells, which lead to high fever, conjunctival hemorrhage, abnormal immune response, embolism and other detrimental side effects[5]. But a large number of studies are only based on animal experiments. The aim of our study, then, was to explore the interventional effects of rhIL-12 in tumor patients receiving radiotherapy, including the complications after radiotherapy, the curative effects on hematopoietic function and immune function as well as dose-effect relationship, and to provide scientific basis for clinical application.

MATERIALS AND METHODS

Objectives

To observe 100 patients with mid-advanced tumors who were treated with Cyber knife or image-guided radiotherapy (IGRT) in the People's Liberation Army 107th Hospital, from October 2014 to June 2016. Inclusion criteria were: (1) Tumor confirmed by pathology, CT or MRI diagnosis, for which the clinical staging criteria were Ⅲ-Ⅳ according to the World Health Organization (WHO); (2) ECOG score of 1 to 4 points; (3) Postoperative recurrence or lymphocytes invasion and metastasis, and need for radiosurgery; and (4) Provision of written informed consent for research and therapy. Exclusion criteria were: (1) Illness combined with tuberculosis or serious failure of important organs, such as heart, liver, kidney, lung, *etc*.; (2) Presence of benign tumors; (3) History of organ transplantation or allergies; and (4) Pregnant or lactating women or women of childbearing age. The experimental study was approved by the hospital's ethics committee.

In the treatment group, 34 of the subjects (68%) were

males and 16 (32%) were females; the mean age was 58.5-year-old (range: 27-83 years). Fifty subjects (30%) had lung cancers, 12 (24%) had liver cancers, 8 (16%) had head tumors, 1 (2%) had pancreatic cancer and 14 (28%) had other tumors. Solid relapse and metastasis tumor, for which size of the nidus could be assessed, accounted for 98% (n = 49) of the cases. Diffuse invasive metastatic tumor accounted for 2% (n = 1) of the cases, and the tumor diameter ranged from 3 cm to 16 cm. The patients who were classified as recurrent after the surgery or with more than 2 lesions accounted for 96% (n = 48) of the cases. In the control group, 28 of the subjects (56%) were males and 22 (44%) were females; the mean age was 57.4-year-old (range: 25-79 years). Twenty subjects (40%) had lung cancers, 15 (30%) had liver cancers and 15 (30%) had pancreatic cancers.

Main equipment, drugs and reagents

Equipment: Cyber knife, third-generation model produced by a United States' accuracy company; IGRT, Eiekta Synergy model produced by a Swedish medical company; Flow cytometer produced by the United States' BD Biosciences; Enzyme-mark instrument produced by a United States' automation company; Chemiluminescence apparatus and automatic biochemistry analyzer produced by Roche Company; Hematology analyzer produced in Japan.

Drugs and reagents: RhIL-12 (for injection) produced by Qingdao Litai Kang Pharmaceutical Co. Ltd. Antibodies used in the study were purchased from BD Biosciences, including anti-human-IgG-FITC, anti-human-CD45-FITC, anti-human-CD56-PE and anti-human-CD3-PerCP-CD4-FITC-CD8-PE. Hemolysin was produced by an American research and development company.

Methods: After being admitted to hospital, all patients' data were recorded for the three routine(liver and kidney function, heart function, bleeding and clotting time) and the imaging examination (such as electrolytes, color Doppler, CT and IMT), as well as adverse reactions, *etc*. All patients signed "consent form of precise radiotherapy", "consent form of experimental study" and "agreement about the clinical application of rhIL-12 for prevention and treatment of malignant tumor radiotherapy complications".

According to the research program, the patients were divided into 50, 100, 150, 200 and 250 ng/kg different-dose subgroups, and injected with rhIL-12 subcutaneously. Peripheral blood samples (for red blood cell (RBC), white blood cell (WBC) and platelet (PLT) assessment) and the immunophenotypes (CD4/8, CD45 and CD56) were collected before dosing (0 d) and at 12 h, 3 d, 7 d, 10 d, 14 d, 21 d, 28 d after dosing respectively. The effects on hematopoietic function and immune function were observed, as well as the dose-effect relationship. The control group used symptomatic supportive treatment.

Efficacy evaluation criteria

Test evaluation: The patients who accepted high-dose and short-course of accurate radiotherapy, such as cyber knife and IGRT, could experience induction of decrease of peripheral blood cells and decline or imbalance of immune function. In this study, rhIL-12 was given to explore the interventional effects on radiation oncology surgery patients, including effects on hematopoietic function and immune function as well as dose-effect relationship and to provide scientific basis for drug development and clinical application.

WHO objective evaluation criteria: Complete remission (CR) indicated all symptoms and signs disappearing for 4 wk; partial remission (PR) indicated the tumor size was estimated to have reduced by more than 50% for at least 4 wk. No change (NC) indicated the patient's condition had no obvious change for at least 4 wk, the tumor size has increased less than 25% and decreased less than 50%. PD (worsen) indicated new lesions having appeared or lesions had increased by more than 25%.

Zubrod-ECOG-WHO score: 0 score stood for normal activities; 1 score stood for mild symptoms and almost normal activities; 2 score stood for the time staying in bed as less than 50% of the daytime; 3 score stood for the time staying in bed as more than 50% of the daytime; 4 score stood for completely bedridden; 5 score stood for death. Total efficiency = (CR + PR)/total number of cases × 100%.

Statistical analysis

SPSS16.0 software was used for statistical analysis. Continuous variables are expressed as a mean and standard deviation; the mean differences between the groups were compared by independent t-test and ANOVA, and χ^2 test was used to compare classified variables. Two values of data used two distribution tests. $P < 0.05$ indicated that the difference was statistically significant. Charts and tables were made by Prism GraphPad 4 software.

RESULTS

Analysis of research subjects' number

All of the 100 patients completed the study.

Results of whole blood test

Treatment group: There was a transient decline of WBC and PLT within 12 h after treatment and by 3 d the lowest level was reached; the recovery rate decreased after 7 d, and the trend became stable after 21 d until the end of observation. This trend was relatively significant for WBC.

Control group: The whole blood cells declined on 3 d after radiotherapy, decreased significantly after 7 d, and reached the lowest point on 14 d. The degree of decrease was related to the radiation dose and tumor size. The difference between the treatment group and the control

Table 1 Results of whole blood test for the treatment group and the control group (mean ± SD, *n* = 10)

Group	Indicator	0 d	12 h	3 d	7 d	14 d	21 d	28 d
Control	WBC (× 10^9/L)	7.66 ± 0.82	5.5 ± 0.67	4.2 ± 0.39	4.3 ± 0.48	4.21 ± 0.62	4.69 ± 0.38	4.89 ± 0.63
	RBC (× 10^{12}/L)	3.92 ± 0.31	4.26 ± 0.43	3.9 ± 0.41	3.93 ± 0.22	4.38 ± 0.36	3.86 ± 0.34	3.89 ± 0.28
	PLT (× 10^9/L)	358 ± 0.43	339 ± 31.45	232 ± 20.43	258 ± 19.2	275 ± 0.31	296 ± 0.29	321 ± 0.26
50 ng/kg	WBC (× 10^9/L)	6.31 ± 0.59	3.6 ± 0.35	4.27 ± 0.46	4.85 ± 0.35	4.52 ± 0.42	4.72 ± 0.39	5.18 ± 0.52
	RBC (× 10^{12}/L)	5.43 ± 0.54	4.92 ± 0.53	4.16 ± 0.38	4.59 ± 0.82	4.73 ± 0.32	5.26 ± 0.37	5.63 ± 0.41
	PLT (× 10^9/L)	231 ± 20.81	185 ± 18.24	195 ± 18.97	205 ± 18	226 ± 18.2	246 ± 17.5	229 ± 19.4
100 ng/kg	WBC	8.4 ± 0.45	7.7 ± 7.89	5.3 ± 10.64	6.6 ± 0.98	6.1 ± 0.64	5.73 ± 0.47	5.9 ± 0.21
	RBC (× 10^{12}/L)	6.3 ± 0.72	3.37 ± 0.43	3.2 ± 0.34	4.2 ± 0.37	4.78 ± 0.46	4.5 ± 0.42	4.6 ± 0.34
	PLT (× 10^9/L)	231 ± 27.2	185 ± 10.7	178 ± 12.6	195 ± 12.8	166 ± 10.9	182 ± 12.5	205 ± 15.3
150 ng/kg	WBC (× 10^9/L)	6.6 ± 0.73	5.4 ± 0.76	3.8 ± 0.35	5.2 ± 0.37	5.7 ± 0.42	6.2 ± 0.54	6.3 ± 0.54
	RBC (× 10^{12}/L)	3.4 ± 0.36	3.5 ± 0.37	3.2 ± 0.29	3.3 ± 0.28	3.6 ± 0.24	3.6 ± 0.26	3.44 ± 0.21
	PLT (× 10^9/L)	367 ± 35.75	352 ± 32.45	306 ± 30.12	316 ± 16	357 ± 17	348 ± 26	317 ± 16
200 ng/kg	WBC (× 10^9/L)	5.3 ± 0.8	4.2 ± 0.3	3.2 ± 0.1	3.5 ± 0.32	3.6 ± 0.27	4.3 ± 0.31	5.4 ± 0.43
	RBC (× 10^{12}/L)	4.6 ± 0.51	4.2 ± 0.41	4.1 ± 0.45	4.2 ± 0.3	4 ± 0.2	4.12 ± 0.34	4.5 ± 0.42
	PLT (× 10^9/L)	278 ± 36	183 ± 19	149 ± 14	208 ± 22	259 ± 24	267 ± 25	271 ± 21
250 ng/kg	WBC (× 10^9/L)	3.6 ±	3 ± 0.37	2.7 ± 0.24	3.4 ± 0.4	4.2 ± 0.3	4.5 ± 0.4	4.2 ± 0.4
	RBC (× 10^{12}/L)	3.6 ± 0.3	3.3 ± 0.2	3.1 ± 0.2	3.2 ± 0.3	3.4 ± 0.3	3.7 ± 0.2	4.1 ± 0.3
	PLT (× 10^9/L)	364 ± 35	235 ± 21	240 ± 20	276 ± 22	314 ± 19	342 ± 21	312 ± 20

WBC: White blood cell; RBC: Red blood cell; PLT: Platelet.

Table 2 Results of immunologic detection for the treatment group and the control group (mean ± SD, *n* = 10)

Group	Indicator	0 d	12 h	3 d	7 d	14 d	21 d	28 d
Control	CD4/8	20.4 ± 2.6	18 ± 1.2	17.5 ± 1.2	17.8 ± 1.3	18.4 ± 1.2	17.3 ± 1.4	16.5 ± 0.4
	CD45	75.2 ± 7.5	68.1 ± 5.2	65.4 ± 4.2	83.3 ± 5.2	79.3 ± 3.7	62.4 ± 5.1	60.3 ± 3.6
	CD56	12.3 ± 1.2	8.7 ± 0.6	7.6 ± 0.5	11.4 ± 1.3	10.4 ± 0.9	8.2 ± 0.6	6.7 ± 0.5
50 ng/kg	CD4/8	28.2 ± 1.8	22.4 ± 1.6	19.8 ± 1.6	19.6 ± 1.6	21.4 ± 1.8	18.8 ± 1.2	16.9 ± 1.4
	CD45	81.9 ± 2.4	78.8 ± 5.1	75.2 ± 3.6	82.9 ± 3.8	79.8 ± 5.6	62.4 ± 4.6	51.8 ± 4.3
	CD56	27.9 ± 2.2	21.9 ± 1.8	19.2 ± 1.2	22.1 ± 1.8	19.7 ± 2.1	21.6 ± 1.3	22.4 ± 0.9
100 ng/kg	CD4/8	15.4 ± 1.5	12.7 ± 1.2	11.5 ± 1.1	10.6 ± 1.2	10.1 ± 0.3	9.2 ± 0.4	8.7 ± 0.2
	CD45	84.3 ± 2.6	66.1 ± 3.8	68.7 ± 3.5	67.9 ± 4.4	57.6 ± 4.6	63.1 ± 3.6	70.2 ± 3.1
	CD56	14.8 ± 1.8	8.9 ± 0.6	10.8 ± 0.9	12.3 ± 1.5	10.4 ± 4.6	12.9 ± 0.3	11.6 ± 0.4
150 ng/kg	CD4/8	16.5 ± 1.2	12.8 ± 1.2	11.6 ± 0.9	13.1 ± 4.6	12.6 ± 1.5	10.8 ± 0.9	7.6 ± 0.5
	CD45	74.2 ± 3.6	63.2 ± 3.2	66.1 ± 4.8	82.7 ± 5.2	68.1 ± 4.6	75.2 ± 4.2	65.1 ± 4.1
	CD56	6.2 ± 0.3	6.5 ± 0.3	5.9 ± 0.3	7.9 ± 0.6	8.2 ± 7.4	8.8 ± 0.9	6.5 ± 0.1
200 ng/kg	CD4/8	9.6 ± 0.4	8.3 ± 0.61	7.6 ± 0.6	7.2 ± 0.9	8.6 ± 4.6	8.2 ± 1.2	6.1 ± 0.2
	CD45	64.5 ± 4.2	60.7 ± 4.2	65.8 ± 4.4	76.6 ± 5.9	74.3 ± 4.6	73.2 ± 5.2	55.9 ± 5.0
	CD56	3.8 ± 0.2	2.8 ± 0.2	4.4 ± 0.4	4.9 ± 0.9	6.2 ± 0.6	5.9 ± 0.3	4.5 ± 0.1
250 ng/kg	CD4/8	6.4 ± 0.2	5.7 ± 0.2	4.2 ± 0.3	4.7 ± 0.3	3.5 ± 0.3	3.4 ± 0.9	3.1 ± 0.9
	CD45	46.5 ± 3.9	40.2 ± 3.2	57.9 ± 4.1	66.5 ± 3.8	54.9 ± 4.2	50.4 ± 4.9	35.9 ± 3.2
	CD56	4.3 ± 0.2	3.9 ± 0.2	5.4 ± 0.4	6.3 ± 0.5	6.6 ± 0.3	7.2 ± 0.6	6.4 ± 0.8

group was statistically significant (Table 1 and Figure 1).

Results of immunologic detection

The aim was to observe the immune indexes, including CD4/8, CD45 and CD56.

Treatment group: There was a transient decline of CD4/CD8 within 12 h in the 150, 200 and 250 ng/kg subgroups. There was volatility rise between 3-14 d but the level remained below the pre-medication level, and went down after 21 d. There was a transient decline of CD45 and CD56 within 12 h, which rose after 3 d and went down after 21 d. The overall recovery improvement trend was obvious.

Control group: The trend of the immune indexes showed rebound on 3 d and a continuous downward trend after 7 d. There was no significant difference in these immune indexes between the other two groups (50 and 100 ng/kg subgroups) and the control group (Table 2 and Figure 2).

Objective evaluation results

The remission rate in the treatment group (84%) was obviously higher than that in the control group (60%), and the difference was statistically significant ($P < 0.05$). During the observation period, there were no recurrence, metastasis or death, and the survival time of patients was significantly prolonged. There were 2 patients in the 250 ng/kg subgroup that had low fever after administration, 1 in the 200 ng/kg subgroup and 2 in the 250 ng/kg subgroup that had mild impairment of liver function during the observation period. There was no other adverse event

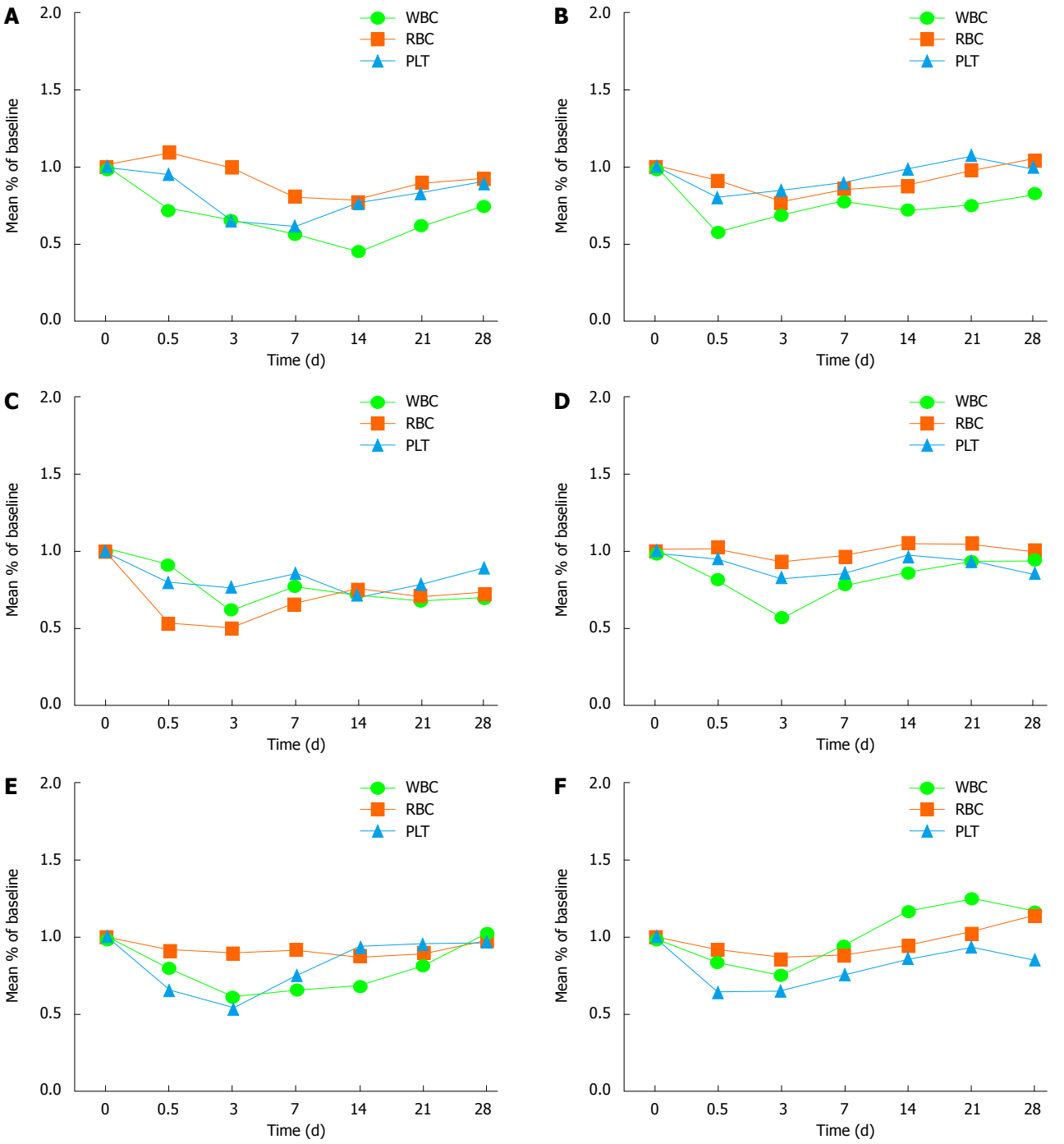

Figure 1 Count changes of the whole blood test. A: Control group; B: 50 ng/kg subgroup; C: 100 ng/kg subgroup; D: 150 ng/kg subgroup; E: 200 ng/kg subgroup; F: 250 ng/kg subgroup. WBC: White blood cell; RBC: Red blood cell; PLT: Platelet.

found (Table 3).

ECOG score results

The aim was to observe the ECOG score for a period of a month after rhIL-12 intervention.

Treatment group: There were 26 patients (52%) with normal activities after treatment, and the difference was statistically significant as compared with the 10 cases (20%) before treatment ($P < 0.05$). The life quality of patients was significantly improved.

Control group: There were 20 patients (40%) with normal activities after treatment, and the difference was not statistically significant as compared with the 12 cases (24%) before treatment ($P > 0.05$) (Table 4).

Imaging evaluation results

There were two CT pictures, including 1 case of pancreatic cancer and 1 case of lung cancer in the treatment group, before and after treatment. The results showed that the original lesion was significantly reduced after treatment and no new lesions appeared (Figures 3 and 4).

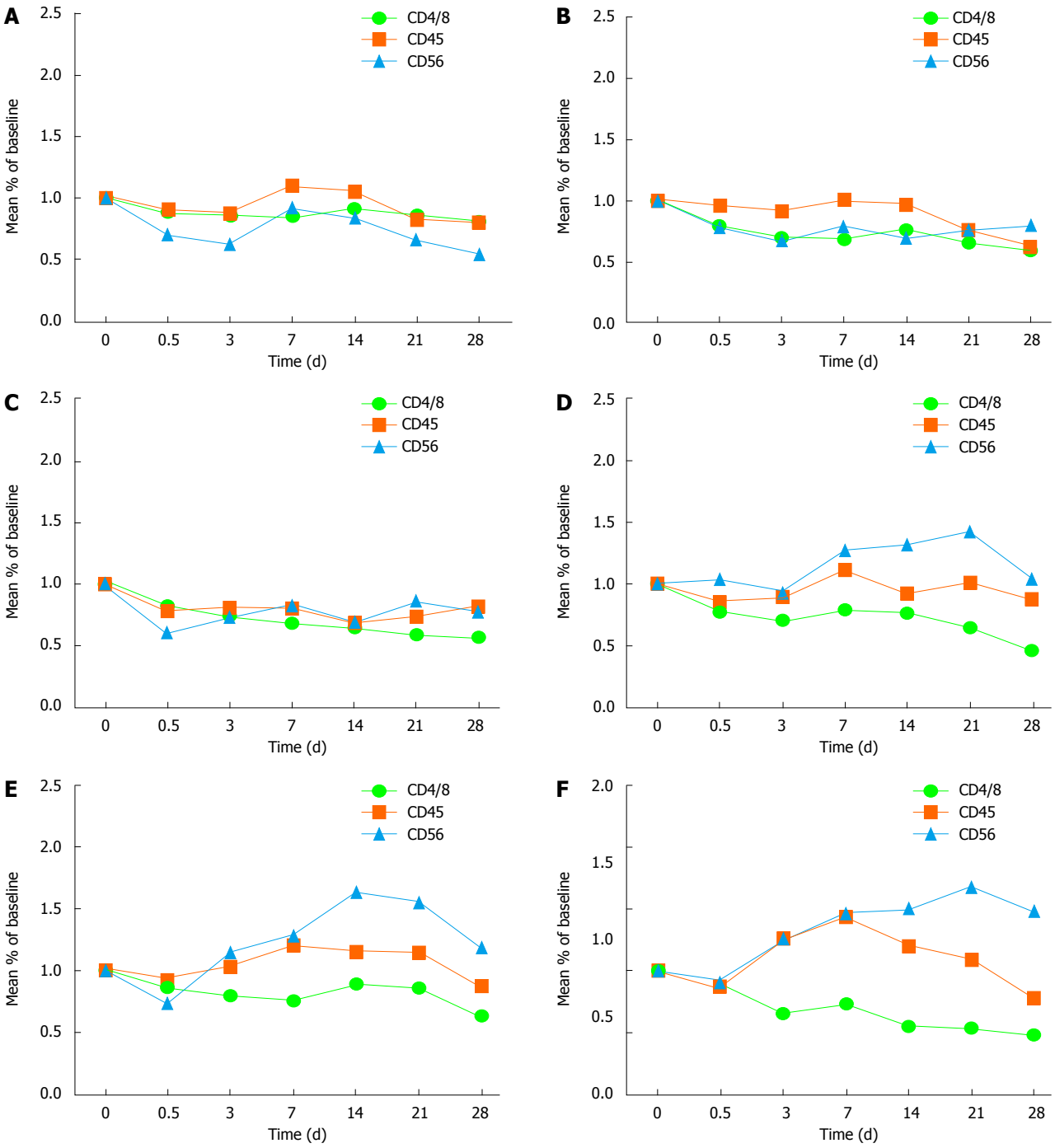

Figure 2 Changes of immune indexes. A: Control group; B: 50 ng/kg subgroup; C: 100 ng/kg subgroup; D: 150 ng/kg subgroup; E: 200 ng/kg subgroup; F: 250 ng/kg subgroup.

DISCUSSION

The incidence of cancer is rising, and cancer has become one of the main causes of death[6]. In recent years, radiotherapy of malignant tumors has developed rapidly, especially for accurate radiotherapy. However, the clinical curative effect for patients who have larger or more numerous tumor lesions are often reduced due to adverse reactions after radiotherapy, such as immune injury and myelosuppression. Under normal circumstances, the immune systems maintain the physiological balance and stabilization of the body.

Immune cells are the first line of the anti-tumor system. Immune regulatory factors or cytokines participate in immune regulation by means of signal transduction[7]. Studies have shown that tumor cells can escape immunosurveillance through a number of special mechanisms. The immune system is critical to the body's surveillance against cancer.

The immune function of about 86% of patients has been shown to be on the decline in the early stage of cancer, and to further decline after treatment, which is

Table 3 Results of objective evaluation for the treatment group and the control group

Group	*n*	CR	%	PR	%	MR	%	PD	%
Treatment	50	12	24[a]	30	60	5[a]	10[a]	3[a]	6[a]
Control	10	5	10	25	50	10	20	10	20

Compared with the control group, [a]$P < 0.05$. CR: Complete remission; PR: Partial remission.

Table 4 Comparison of ECOG scores before and 1 mo after intervention for the treatment and control groups

ECOG scores before treatment	after treatment	
Treatment group (n = 50)		
0	0	11[a]
1	10	15[a]
2	15	19[a]
3	20	5[a]
4	5	0[a]
Control group (n = 50)		
0	1	4
1	11	16
2	17	20
3	15	10
4	6	0

There was significant difference between the two groups, [a]$P < 0.05$.

the main cause of tumor metastasis and recurrence[8]. What's more, the complications including infection and bleeding that are caused by the decrease of peripheral blood cells counts are also common causes of death in patients with cancer.

Therefore, improving immune function and reducing myelosuppression are indispensable auxiliary treatments in the process of tumor radiotherapy. At present, the treatment of cancer has entered into the era of personalized multidisciplinary treatment. The research shows that the combination of radiotherapy and immunotherapy has a unique advantage[9]. In this respect, IL-12 has received more and more attention.

IL-12 is an immunoregulatory protein produced by macrophages, B cells, mononuclear cells, keratinocytes and dendritic cells. IL-12 mainly functions to mediate cellular immunity, and it can induce the differentiation of T helper cell 1 (Th1), as well as promote the proliferation of NK cells and T cells, further stimulate IFN-γ secretion, and enhance the ability to kill target cells. IL-12 also can promote the formation of interferon-inducible protein-10 (IP-10). IP-10 can prevent the formation of tumor blood vessels, thereby reducing and blocking the nutrition source of tumor cells and inhibiting their growth[10,11].

It has been nearly 30 years since IL-12 was discovered, and a large amount of the related research is still at the stage of basic study and animal study. Many studies have found its abilities to improve immunity capability, adjust the immune function and inhibit the production of tumor blood vessels, but the adverse reactions such as chills and fever, nausea and vomiting, pulmonary edema and allergic reactions limit its clinical application and temper its promotion.

RhIL-12 is a new kind of biological agent secreted by Chinese hamster ovary cells through gene engineering. Its biological activity is similar to that of IL-12. The advantage of rhIL-12, however, is its high purity (> 98%), high activity (≥ 10000 IU/μg), low therapeutic dose and dosage that can be tolerated. Preliminary experimental study[12-14] found that rhIL-12 is currently the only biological agent with the advantage of comprehensive recovery of hematopoietic function, regulation of the body's immunity, inhibition of tumor angiogenesis and inhibition of tumor growth, thereby improving the life quality of patients with cancer[15]. As an immune regulatory factor, it plays an important role in both primary and secondary immunity[16]. Especially for radiotherapy patients who present with larger or more numerous tumor targets (more than two), it has important research value.

In our study, there were 100 patients who had larger or more numerous tumor s (more than two) and who received precision radiotherapy (Cyber knife or IGRT). The following conclusions are drawn. In the treatment group, the whole blood cells showed a transient decline within 12 h after treatment. The reason for this may be that the cell changes into the microcirculation or the bone marrow microenvironment, which may affect the proliferation of hematopoietic stem cells. The whole blood cells reached the lowest level at 3 d. People have always stopped treatment at this time, which represents a misunderstanding of the early research. The recovery rate decreased after 7 d, and the trend became stable after 21 d until the end of observation. This trend is relatively significant for WBC. Compared with the control group, the difference was significant, which indicated that the rhIL-12 was effective.

Observation of the immune indexes, including CD4/8, CD45 and CD56, showed a transient decline of CD4/CD8 within 12 h in the 150, 200 and 250 ng/kg subgroups in the treatment group, with volatile rises between 3-14 d, but remaining below the pre-medication level, and then decreasing after 21 d. There was a transient decline of CD45 and CD56 within 12 h, which rose up after 3 d and went down after 21 d. Compared with the control group, the difference was significant. The improvement tendency was obvious, which suggested that rhIL-12 could promote the immune function of the patients after radiotherapy.

From our observations of the clinical manifestations, 2 patients in the 250 ng/kg subgroup showed low fever after administration, which could be returned to normal after physical cooling. One patient in the 200 ng/kg subgroup and 2 in the 250 ng/kg subgroup showed mild injury of

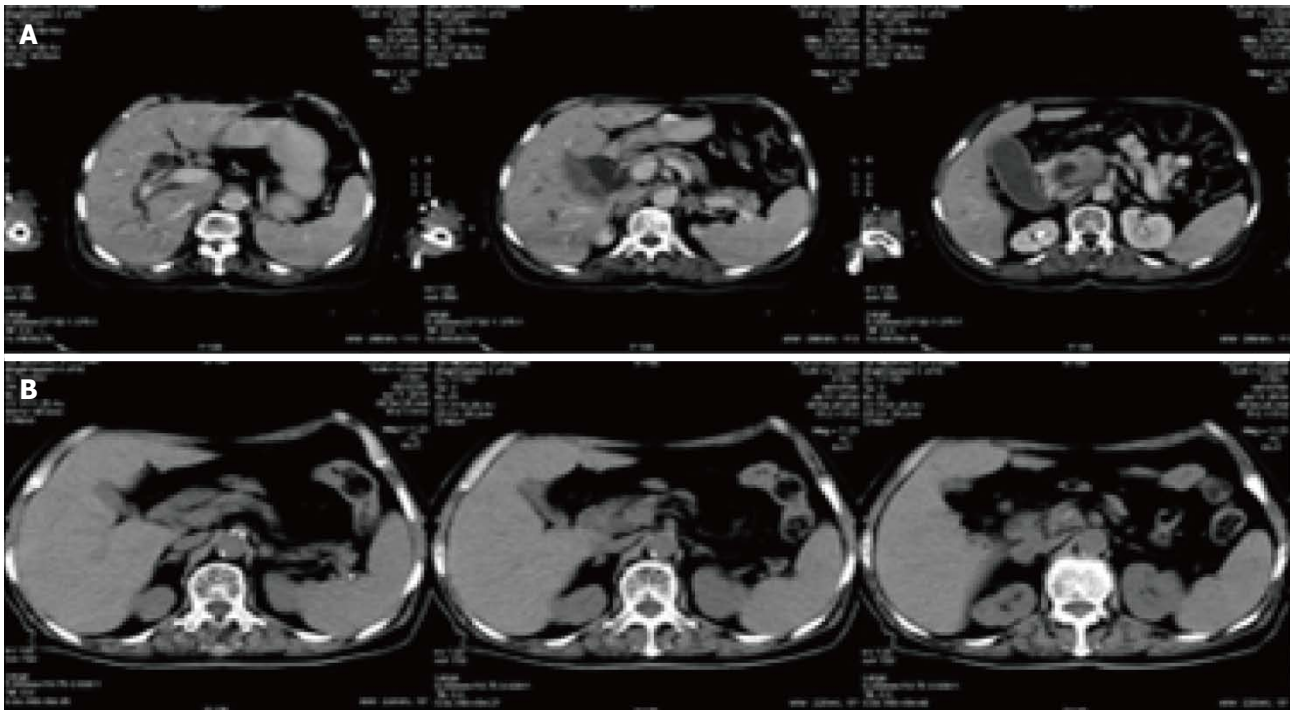

Figure 3 Changes of computed tomography slice before and after treatment in pancreatic cancer patients. A: Pancreatic head tumor mass (4.4 cm × 3.6 cm × 5.3 cm) accompanied by dilation of intrahepatic and extrahepatic bile duct, pancreatic duct and gallbladder before treatment; B: Most of the pancreatic head mass disappeared in 2 mo after B treatment. Low obstruction disappeared.

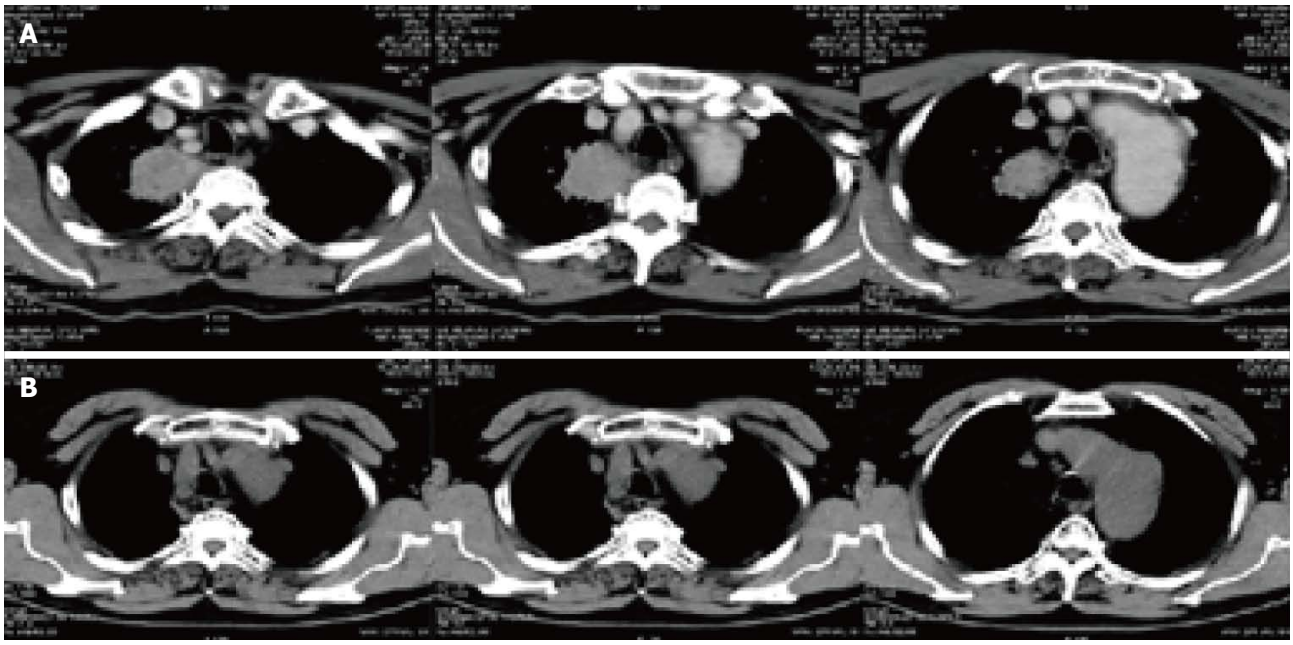

Figure 4 Changes of computed tomography slice before and after treatment in lung cancer patients. A: Right peripheral lung tumor mass (4.5 cm × 4.8 cm × 4.0 cm) located in the right upper lobe of the right lung before treatment; B: The mass disappeared 1 year after treatment.

liver function during the observation period, which could be returned to normal after liver-protecting therapy. The injury related to radiation or rhIL-12 needs to be further studied.

In addition, this study explored the relationship between biological activity and concentration. The result showed that there was no statistical difference among the 50 ng/kg subgroup, the 100 ng/kg subgroup and the control group for immune response. What's more, the adverse reactions were mainly concentrated in the 200 and 250 ng/kg subgroups, which suggests that the suitable clinical dosage concentration in our study is 150 ng/kg. The anti-tumor activity of rhIL-12 has been shown in clinical trials, but its toxicity to some extent

limits the application. Therefore, rhIL-12 still needs to be further researched.

In conclusion, rhIL-12 can prevent radiation damage, improve hematopoietic function, regulate immunity, reduce the side effect of radiotherapy and improve the quality of life of patients. It has good clinical application value and good development prospect as tumor auxiliary treatment.

COMMENTS

Background

Interleukin-12 (IL-12) is an immunoregulatory protein produced by macrophages, B cells, mononuclear cells, keratinocytes and dendritic cells, and its target point lies in early undifferentiated pluripotent hematopoietic stem cells. However, the adverse events related to IL-12, including fever, chills, decrease of peripheral blood cells and organ dysfunction, have limited its clinical application. Recombinant human interleukin-12 (rhIL-12) is an immunoregulatory protein produced by gene engineering technology. RhIL-12 has similar biological activity to IL-12, but with the advantage of high purity (> 98%), high activity and low therapeutic dose. RhIL-12 has become the only available agent which can not only restore hematopoietic function but also improve immune function. Basic experimental study has found that the recovery of hematopoietic function after radiotherapy is helpful to avoid the rapid increase of single blood cells, which can lead to high fever, conjunctival hemorrhage, abnormal immune response, embolism and other detrimental side effects. But a large number of studies are basic in nature and based on animal experiments. The aim of the study was to explore the interventional effects of rhIL-12 on tumor patients receiving radiotherapy, including the complications after radiotherapy, the curative effects on hematopoietic function and immune function as well as dose-effect relationship, and to provide scientific basis for drug development and clinical application.

Research frontiers

Some studies have shown that rhIL-12 can stimulate various kinds of cytokines through stimulating the bone marrow microenvironment, either directly or indirectly, further promoting long-term hematopoietic reconstitution progenitor cells' differentiation and maturation, and instigate short-term hematopoietic reconstitution progenitor cells. These help to achieve a comprehensive recovery of hematopoietic function. In addition, in the circumstances of no supportive treatments, primate experiments demonstrated that using a low dose of rhIL-12 within 24 h after lethal dose irradiation could significantly improve (4-times) the animal's survival rate. What's more, rhIL-12 could promote the healing of skin wounds after radiation injury. As a radiation injury prevention drug, rhIL-12 is still effective at 24 h to 48 h after radiation. At the same time, a large number of animal experiments have shown that IL-12 can significantly inhibit the growth and metastasis of malignant tumors, prolonging the survival time of tumor-bearing animals. IL-12 can enhance the natural killer (NK) cell and cytotoxic T lymphocyte (CTL) cell response and the ability to induce production of IFN-C, which indicates that it may have anti-tumor activity. IL-12 enhances the binding ability of NK cells and K562 target cells and tumor cell monolayer, and enhances the cytotoxicity of NK cells to tumor cells. Because rhIL-12 and IL-12 have similar biological activities, some studies have shown that low dose of rhIL-12 can inhibit tumor cell growth, and rhIL-12 has synergistic anti-cancer effect on radiotherapy and chemotherapy, which needs further clinical validation.

Innovations and breakthroughs

The study found that low-dose rhIL-12 has the effect of prevention and treatment for the decrease of blood cells after radiotherapy, and could effectively improve the immune function and reduce the complications of radiotherapy.

Applications

RhIL-12 can prevent radiation damage, improve hematopoietic function, regulate immunity, reduce the detrimental side effects of radiotherapy and improve the quality of life of patients.

Terminology

IL-12: Interleukin-12 is an immunoregulatory protein produced by macrophages, B cells, mononuclear cells, keratinocytes and dendritic cells, and its target point lies in early undifferentiated pluripotent hematopoietic stem cells; RhIL-12: Recombinant human interleukin-12 is an immunoregulatory protein produced by gene engineering technology; its biological activity is similar to IL-12.

Peer-review

The authors conducted an interesting clinical study on rhIL-12 for the prevention and treatment of complications after radiotherapy in patients with malignant tumors. The manuscript was well written. The methodology was clear and accurate. The results section was adequate.

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ORIGINAL ARTICLE

Observational Study

Gastric and duodenal polyps in familial adenomatous polyposis patients: Conventional endoscopy *vs* virtual chromoendoscopy (fujinon intelligent color enhancement) in dysplasia evaluation

Gabriele Lami, Andrea Galli, Giuseppe Macrì, Emanuele Dabizzi, Maria Rosa Biagini, Mirko Tarocchi, Luca Messerini, Rosa Valanzano, Stefano Milani, Simone Polvani

Gabriele Lami, Andrea Galli, Maria Rosa Biagini, Mirko Tarocchi, Stefano Milani, Simone Polvani, Gastroenterology Research Unit, Department of Experimental and Clinical Biomedical Sciences "Mario Serio", University of Florence, 50139 Florence, Italy

Andrea Galli, Giuseppe Macrì, Maria Rosa Biagini, Mirko Tarocchi, Stefano Milani, Clinical Gastroenterology Unit, Careggi University Hospital, 50134 Florence, Italy

Emanuele Dabizzi, Gastroenterology and Gastrointestinal Endoscopy, San Raffaele Hospital, 20132 Milan, Italy

Luca Messerini, Division of Pathological Anatomy, Department of Experimental and Clinical Medicine, University of Florence, 50139 Florence, Italy

Rosa Valanzano, Surgical Unit, Department of Translational Surgery and Medicine, University of Florence, 50139 Florence, Italy

Author contributions: Lami G, Macrì G, Galli A and Dabizzi E contributed to the study conception, design and supervision; Lami G, Galli A, Macrì G, Dabizzi E and Valenzano R performed the patients recruitment and samples collection; Messerini L supervised the histological examination; Lami G and Biagini MR collected the data and performed the statistical analysis; Milani S reviewed the statistical methods; Lami G, Galli A, Macrì G, Milani S, Dabizzi E, Biagini MR, Messerini L and Polvani S contributed to the interpretation of the data and discussion of the results; Tarocchi M critically revised the manuscript; Lami G, Galli A and Polvani S drafted the manuscript.

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Correspondence to: Simone Polvani, PhD, Gastroenterology Research Unit, Department of Experimental and Clinical Biomedical Sciences "Mario Serio", University of Florence, viale Pieraccini 6, 50139 Florence, Italy. simone.polvani@unifi.it
Telephone: +39-055-2758115

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Abstract

AIM

To test the fujinon intelligent color enhancement (FICE) in identifying dysplastic or adenomatous polyps in familial adenomatous polyposis (FAP) patients.

METHODS
Seventy-six consecutive FAP patients, already treated by colectomy and members of sixty-five families, were enrolled. A FICE system for the upper gastro-intestinal tract with an electronic endoscope system and a standard duodenoscope (for side-viewing examination) were used by two expert examiners. Endoscopic resection was performed with diathermic loop for polyps ≥ 6 mm and with forceps for polyps < 6 mm. Formalin-fixed biopsy specimens were analyzed by two expert gastrointestinal pathologists blinded to size, location and number of FAP-associated fundic gland polyps.

RESULTS
Sixty-nine (90.8%) patients had gastric polyps (34 only in the corpus-fundus, 7 only in the antrum and 28 in the whole stomach) and 52 (68.4%) in duodenum (7 in the bulb, 35 in second/third duodenal portion, 10 both in the bulb and the second portion of duodenum). In the stomach fundus after FICE evaluation, 10 more polyps were removed from 10 patients for suspicious features of dysplasia or adenomas, but they were classified as cystic fundic gland after histology. In the antrum FICE identified more polyps than traditional endoscopy, showing a better tendency to identify adenomas and displastic areas. In the duodenum FICE added a significant advantage in identifying adenomas in the bulb and identified more polyps in the Ⅱ/Ⅲ portion.

CONCLUSION
FICE significantly increases adenoma detection rate in FAP patients but does not change any Spigelman stage and thus does not modify patient's prognosis and treatment strategies.

Key words: Fujinon intelligent color enhancement; Familial adenomatous polyposis; Spigelman; Endoscopy; Polyp; Adenoma; Stomach; Duodenum

Core tip: Colon endoscopic surveillance and prophylactic colectomy have strongly reduced mortality due to colorectal carcinoma and have improved survival of familial adenomatous polyposis (FAP) patients, leading to the development of surveillance for extra-colonic cancers. Polyps in the duodenum and stomach are frequent findings in FAP. The timing of endoscopic and histology surveillance is currently a great challenge. Spectral estimation by fujinon intelligent color enhancement (FICE) may identify dysplasia and discriminate between adenomatous and non-adenomatous polyps. Interestingly, application of FICE to FAP patients significantly increases the detection of adenomas but does not yet change the prognosis, surveillance program and treatment strategies.

Lami G, Galli A, Macrì G, Dabizzi E, Biagini MR, Tarocchi M, Messerini L, Valanzano R, Milani S, Polvani S. Gastric and duodenal polyps in familial adenomatous polyposis patients: Conventional endoscopy *vs* virtual chromoendoscopy (fujinon intelligent color enhancement) in dysplasia evaluation. *World J Clin Oncol* 2017; 8(2): 168-177 Available from: URL: http://www.wjgnet.com/2218-4333/full/v8/i2/168.htm DOI: http://dx.doi.org/10.5306/wjco.v8.i2.168

INTRODUCTION

Familial adenomatous polyposis (FAP) is an autosomal dominant inherited syndrome characterized by the development of colorectal cancer by the age of 40 years in nearly 100% of individuals[1]. Colon Endoscopic surveillance and prophylactic colectomy have strongly reduced mortality due to colorectal carcinoma and have improved survival of FAP patients with minimal consequences[2,3], leading to the introduction of surveillance strategies for the prevention of other extra-colonic malignancies[4].

The duodenum is the second most common site of polyps development after colon, with a life-time risk of duodenal adenomas that approaches 100% in FAP affected individuals[5,6]. The cumulative risk of duodenal cancer or high grade of dysplasia by the age of 60 years is 4%-10%[6-8].

Endoscopic surveillance and removal of neoplastic tissue is useful in the prevention of duodenal cancer[8]. However, the choice of treatment and the optimal timing of surveillance based on endoscopic and histopathology examination for each patient is currently a great challenge. Currently the surveillance of duodenum is based on the Spigelman classification of duodenal adenomatosis (Table 1); however, this staging system has low predictive values and has never been validated[6,8].

Gastric polyps are also a common finding in patients with FAP: they mostly consist of FAP-associated fundic gland polyps (FGPs) reported to occur at variable rates, up to 88%[9,10], against a strongly smaller rate (0.8%-5.0%)[11,12] in non-FAP subjects who undergo an esophagogastroduodenoscopy (EGD).

FGPs have historically been considered non-neoplastic lesions without malignant potential[13]; however recent studies have questioned this assumption reporting high rates of low and high grade dysplasia (up to 54%)[9,14,15]. In particular, European and Asian registries of FAP patients proved the presence of gastric carcinoma arising from FGPs in FAP patients and an incidence of gastric adenocarcinoma between 0.6% and 4.0%[16-19].

Other common types of gastric polyps are represented by adenomas (gastric foveolar or intestinal type-gastric adenomas and pyloric gland adenomas) which are reported in approximately 10% of gastric polyps in FAP patients[10,20,21] and which can arise in the gastric antrum, in the gastric body-fundus or in the context of FGP[22]. So, identification of dysplastic lesions or adenomatous tissue in these patients is often made difficult by the great number of polyps (up to hundreds) and by the patchy

Table 1 Demographic features

Features	Patients
Total	76
Age (yr)	Mean 40.3 (24-64)
Gender	
Male	41 (53.9%)
Female	35 (46.1%)
Prior surgery	
IRA	10 (13.2%)
IPAA	66 (86.8%)
Chemoprevention	16 (21.1%)
NSAIDS intake	17 (22.4%)
Tobacco exposure	21 (27.6%)
PPI/anti-H2 intake	14 (18.4%)
Family history of GI malignancies	
None	31 (40.8%)
1 member	32 (42.1%)
2 members	10 (13.2%)
3 members	3 (3.9%)
Spigelman duodenal stage	
0	28 (36.8%)
I	7 (9.2%)
II	34 (44.8%)
III	7 (9.2%)
IV	0 (0.0%)

IRA: Total colectomy and ileo-rectal anastomosis; IPAA: Total proctocolectomy and ileopouch-anal anastomosis; NSAIDS: Non steroidal anti-inflammatory drugs; PPI: Proton pump inhibitors.

distribution of dysplasia.

By now, dysplasia finding in this subgroup of subjects is made on the basis of random biopsies[9] which lead to a time consuming, laborious and poorly performing procedure, that can result in a high rate of missed lesions. According to that, it would be useful identifying FGPs at risk of malignant degeneration.

A better characterization of patients, an optimized program of surveillance and a good survival are possible with a selective and complete asportation and with a careful histological evaluation.

It is well known that is possible to predict the histology of a mucosal lesion by observing the crypt orifices (the so called pit pattern) of mucosal glands[23] and the capillary pattern of the mucosa[24]. Several endoscopic imaging techniques have been proposed to enhance the details of these patterns[25]. Among these, chromoendoscopy is a widely applied staining method that uses biocompatible dye agents which accumulate within crypt orifices during endoscopy[26]. Although chromoendoscopy is effective for many applications, it still has some problems, such as difficulty in achieving complete and even coating of the mucosal surface with the dye, the extra cost of the equipment needed for dye spraying and the extra time required to perform the procedure. Moreover, traditional chromoendoscopy isn't able to enhance the capillary pattern, whose evaluation is essential in early diagnoses of malignant lesions[24]. In attempt to resolve these problems, other endoscopic technologies have been developed. Fujinon intelligent color enhancement (FICE™, Fujinon Corp, Saitama, Japan) represents a spectral estimation technique based on arithmetically processing of a white-light image captured by a video endoscope and sent to the spectral-estimation matrix-processing circuit. The image of the white-light endoscopic observation is resolved in each color image of the red, green and blue signal. Next, each resolved image is converted into various presumed wavelength images by a pixel unit. The images of an arbitrary single wavelength are then extracted and reconstructed. Due to its variable setting functions (up to 10) it is possible to select flexibly the most suitable wavelengths required for examination. Preliminary studies suggested that FICE successfully achieved enhancements of real-time observations of mucosal and microvascular patterns[27,28].

The light penetration into the mucosa varies according to the wavelengths: Those in the 400-500 nm range are ideal for analyzing surface structures whereas, because of the absorption properties of hemoglobin, longer wavelengths of about 550 nm are more effective for the visualization of blood vessels.

FICE seems able to discriminate between adenomatous and non-adenomatous polyps and to identify the presence of dysplasia[29-32]. Few studies have been conducted to determine the efficacy of chromoendoscopy, both traditional and virtual, in the evaluation of duodenal and peri-ampullary adenomatous polyps in FAP patients[33-35]. Interestingly, FICE application in the discrimination between neoplastic and non-neoplastic gastric lesions has not been throughly investigated[36-39], and no data are available about FICE in evaluating FGPs dysplasia or application of FICE for the screening of FAP patients.

In FAP cohort, the specific identification of who is at a greater risk of cancer development could be of paramount importance to assure a personalized program of surveillance or a therapeutic intervention.

The primary aim of this study was to assess the capability of FICE in identifying gastric polyps with dysplastic or adenomatous tissue in comparison to traditional endoscopy and in identifying a greater number of duodenal adenomas with advanced histological features.

Secondary aim was to assess the capability of FICE in identifying adenomas not seen on white light evaluation.

MATERIALS AND METHODS

Patients

Seventy-six consecutive FAP patients, already treated by colectomy and members of sixty-five families, were enrolled. Exclusion criteria were: Uncorrectable coagulopathy, inability to give informed consent, age < 18 years, prior gastro-duodenal surgery or a personal history of gastro-duodenal cancer. All patients underwent a surveillance esophagogastroduodenoscopy (EGD) in deep sedation at the Gastroenterology U.O. of the Azienda Ospedaliero Universitaria di Careggi, Firenze, Italy. Written informed consent were obtained before EGD and sedation.

Endoscopy

A FICE system (EG-590ZW; Fujinon Corp, Saitama,

Japan) for the upper gastro-intestinal tract with an electronic endoscope system (EPX-4400; Fujinon Corp, Saitama, Japan) was used for this study. In this system, ten channels with different predefined combinations of absorption wavelengths are available. We used channel 5, corresponding to R 500 nm, G 480 nm, B 420 nm, on the basis of previous studies.

A standard duodenoscope was used for side-viewing examination. Because this model of duodenoscope does not support FICE system, ampullary and periampullary evaluations were not included in the analysis. All of the endoscopic procedures were performed by two experts examiners.

"A" operator performed the exam on white light, while "B" operator used only FICE system for gastroduodenal visualization. Each EGD was divided into three phases.

During the first phase, "A" operator observed stomach and duodenum by white light recording photographic images of suspected polyps and pointing them. We intended suspected polyps on white light those larger than 1 cm and those with irregular shape or surface features.

During the second phase, "B" operator performed the exam using FICE and, like "A" operator, recorded photographic images of suspected polyps on the basis of Kudo classification[23] and capillary pattern, and pointed them.

Kudo classification classifies mucosal crypt patterns into five types, with type Ⅰ and Ⅱ predicting non-adenomatous lesions and type Ⅲ-Ⅴ predicting adenomatous lesions.

Hyperplastic polyps were suspected when the surface showed pale color with only minute thin superficial (couperose-like) vessels and round or asteroid pattern (type Ⅰ and Ⅱ). Adenomas were suspected in the presence of increased vascular density (darkening of the mucosal pattern or a fine meshwork of brownish/bluish vessels) and a typical tubular or gyrus-like pattern (type Ⅲ-Ⅳ). Finally, type V have a non structural pattern which identifies high dysplastic or yet carcinomatous lesions.

During this phase we intended suspected those lesions with a pit pattern type Ⅲ-Ⅴ and those with an increased capillary density.

During the third phase, after the two endoscopists' cross-evaluation, lesions which seemed suspected only by FICE, only by white light or by both methods were resected or biopsied according to Kudo class.

Endoscopic resection was performed with diatermic loop for polyps ≥ 6 mm and with forceps for polyps < 6 mm. The size was estimated using on open biopsy forceps (8 mm) for comparison and recorded as smaller than 6 mm, 6 to 10 mm, 11 to 20 mm and greater than 20 mm.

The total number of FGPs was documented as below: 0 to 2 polyps, 3 to 20, 21 to 30 and more than 30 polyps. On the basis of location we identified: Fundus-corpus, antrum, duodenal bulb, Ⅱ°/Ⅲ° duodenal portion.

For fundic polyps seen on white light, the number of FGPs from which a biopsy specimen was taken was based on the total number of FGPs present: 3 biopsies if 3-20 polyps, 5 biopsies if 21-30 polyps, 7 biopsies if > 30 polyps[9].

On FICE, only suspected polyps (Kudo Ⅲ-Ⅴ, high capillary density) were removed.

For antral and bulbar polyps, all of them were removed or biopsied both on withe light than on FICE.

In the second and third duodenal portion, on white light only suspected polyps were resected or biopsied, while on FICE were biopsied those with Kudo Ⅴ and those with Kudo Ⅳ and high capillary density.

Macroscopic classification of lesions followed the Paris classification[40] as polyp, superficially flat or depressed lesion, and lateral spreading tumor.

Histology

All biopsy specimens, fixed in 10% neutral buffered formalin, were analyzed by two expert gastrointestinal pathologists blinded to size, location and number of FGPs.

In the case of multiple lesions in the same patient, each lesion was identified and placed in different flasks. Lesions were histological classified in adenomatous, hyperplastic or inflammatory polyps, fundic gland polyps, and metaplastic areas.

Adenomatous polyps were classified according to OMS classification: Tubular if holding more than 75% of tubular glands, villus if holding more than 75% of villus glands, tubulo-villus if not prevailing none of the two patterns.

Dysplasia was classified according to Vienna criteria[41] in low grade if holding nuclear enlargement, stratification and hyperchromasia with overall preservation of nuclear polarity; high grade as above but with nuclear polarity loss and glandular crowding; indefinite for dysplasia if present mild nuclear enlargment and insufficient hypercromasia to be classified as dysplasia or if present a significant obscuring background inflammation.

The stage of duodenal polyposis was graded according to Spigelman classification modified sec. Saurin[42], which take into account duodenal polyp number, size, histological type and grade of dysplasia. It was noted before and after FICE evaluation.

Statistical analysis

The diagnostic performances (sensitivities, specificities, positive and negative predictive values) of FICE and WL were determined by comparing the endoscopic diagnoses with the histo-pathological findings. To identify associations of demographic, clinical and endoscopic features with the presence of FGP dysplasia or with adenomas, the Fisher exact test was used to study univariable associations of categoric demographic and endoscopic factors with the presence of dysplasia or adenomatous tissue. The Student t test was used for continuous factors. A *P* value "two tailed" < 0.05 was considered statistical significant. The strength of association was calculated by odds ratio (OR). The statistical methods of this study were reviewed by S. Milani, University of Florence.

Table 2 Stomach and duodenum polyps

	Patients		Patients
Fundus	34 (49.3%)	Bulb	7 (13.5%)
Antrum	7 (10.1%)	Ii°/iii° portion	35 (67.3%)
Fundus + antrum	28 (40.6%)	Bulb+ii°/iii°	10 (19.2%)
Total stomach	69 (100%)	Total duodenum	52 (100%)

Table 3 Features of fundic polyps identified by white light endoscopy

	P1-P3	P4-P10	P11-P24	P25-P55	P56-P397
Kudo	I	II	II	II	I
Size (mm)	5	5	6-10	5	5
Paris CL	Is	Is	Is	Is	Is
Histology	HYP	IN	FGP	FGP	FGP

IN: Inflammatory; FGP: Fundic gland polyp; IP: Hyperplastic.

RESULTS

Seventy-six consecutive FAP patients (41 male and 35 female; mean age 40.3 years old, range 24-64) underwent EGD. Among all patients, 69 (90.8%) had gastric polyps (34 only in the corpus-fundus, 7 only in the antrum and 28 in the whole stomach) and 52 (68.4%) in duodenum (7 in the bulb, 35 in second/third duodenal portion, 10 both in the bulb and the second portion of duodenum) (Table 2).

Identification of polyps in the stomach

Fundus: 62 patients had a widespread fundic polyposis (81.6%); 52 of them had more than 30 fundic polyps (68.5%), 3 between 21 and 30 (3.9%) and 7 between 5 and 20 (9.2%).

On white light visualization, 397 polyps in 62 patients (6.4 polyps per patient) were removed. Three were hyperplastic polyps, 7 inflammatory while the rest were cystic fundic gland polyps. No polyp harboured dysplasia nor adenomatous foci (specificity 100%, sensitivity NV, positive predictive value NV, negative predictive value 100%, 95%CI) (Table 3).

After FICE evaluation, 10 polyps were removed from 10 patients on the basis of suspicious features of dysplasia or adenomatous tissue. All of them were cystic fundic gland polyps, none of them harboring dysplasia or adenomatous foci (specificity 97%; sensitivity NV; positive predictive value 0%; negative predictive value 100%) (Table 4).

Thirty-eight patients with fundic polyposis had also duodenal polyposis (61.2%), while among the 14 patients without fundic polyps, 10 had polyps in the duodenum (71.4%). Thus the presence of fundic polyps doesn't correlate with a higher risk to develop duodenal polyps (P = 0.55; OR = 0.6).

Antrum: A total of 56 polyps were identified and removed in the antrum of 35 patients (average 1.6 polyps per patient). Twenty-four polyps were identified in 35 patients by white light endoscopy (0.7 polyps per patient); 21 of them were tubular adenomas with low grade dysplasia while 3 were inflammatory polyps (specificity 88.0%; sensitivity 67.7%; positive predictive value 87.5%; negative predictive value 68.7%) (Table 5).

Beside polyps seen with conventional endoscopy, FICE was further able to identify 32 polyps in 28 patients. They were 7 tubular adenomas with low grade dysplasia, 14 inflammatory polyps, 3 areas with low grade dysplasia in the context of flogistic mucosa, 8 metaplasic areas (specificity 12.0%; sensitivity 100%; positive predictive value 58.5%; negative predictive value 100%) (Table 6).

FICE identified a higher number of polyps than traditional endoscopy (56 *vs* 24; $P < 0.0001$), showing a better, but not statistically significant, tendency to identify adenomas and displastic areas (31 *vs* 21; $P = 0.0857$). All but 4 polyps missed out by white light, were flat.

Eighteen of patients with antral lesions (51.4%) had polyps also in the duodenum. There is no relationship between presence of dysplasia in antral stomach and Spigelman advanced stages (P = 1; OR = NV).

Table 4 Features of fundic polyps identified by fujinon intelligent color enhancement

	P1-P6	P7-P10
Kudo	IIIS	IIIL
Size (mm)	5	5
Paris CL	Is	Is
Histology	FGP	FGP

FGP: Fundic gland polyp.

Table 5 Features of antral polyps identified by white light endoscopy

	P1-P3	P4	P5-P10	P11-P17	P18-P24
Kudo	II	IIIS	IIIS	IIIS	IV
Size (mm)	6-10	6-10	5	5	5
Paris CL	IIa	IIa + IIc	Is	Is	Is
Histology	IN	TA, LGD	TA, LGD	TA, LGD	TA, LGD
Spigelman	0	0	I°	0	0

IN: Inflammatory; TA: Tubular adenoma; LGD: Low grade dysplasia.

Identification of duodenal polyps

Bulb: 21 polyps were seen in 17 patients (1.2 polyps per patient). All of them were endoscopically removed. White light endoscopy identified 14 polyps in 12 patients; 8 polyps were inflammatory, while 6 were tubular adenomas with low grade dysplasia (specificity 0%; sensitivity 46.2%; positive predictive value 42.9%; negative predictive value 0%) (Table 7).

During FICE evaluation, beside polyps seen with conventional endoscopy, 7 more polyps in 7 patients, five

Table 6 Features of antral polyps identified by fujinon intelligent color enhancement

	P25	P26	P27	P28-P30	P31	P32-P33	P34-P35	P36-P38	P39-P44	P45-P49	P50-P53	P54-P56
Kudo	V	V	IIIS	IIIL	IIIL	IV	IV	IIIL	IIIL	IIIS	IIIS	V
Size (mm)	5	5	5	6-10	5	5	6-10	5	5	5	6-10	5
Paris CL	IIb	IIa	IIb	IIb	IIa	IIb	IIa	IIa	IIb	IIb	Is	IIb
Histology	IN LGD	IN LGD	IN LGD	MET	MET	MET	MET	IN	IN	IN	TA LGD	TA LGD
Spigelman	III°	II°	I°	II°	II°	0	II°	0	0	0	I°	II°

IN: Inflammatory; TA: Tubular adenoma; LGD: Low grade dysplasia; MET: Metaplasia.

Table 7 Features of bulbal polyps identified by white light endoscopy

	P1-P5	P6-P8	P9	P10-P12	P13	P14
Kudo	II	IIIS	IIIS	IIIL	IV	IV
Size (mm)	5	6-10	6-10	5	6-10	6-10
Paris CL	Is	Is	Is	IIa	Is	Is
Histology	IN	TA LGD	TA LGD	IN	TA LGD	TA LGD
Spigelman	0	II°	I°	0	III°	II°

TA: Tubular adenoma; LGD: Low grade dysplasia.

Table 8 Features of bulbal polyps identified by fujinon intelligent color enhancement

	P15	P16-P17	P18	P19	P20-P21
Kudo	IIIS	IV	IIIS	IIIL	IIIS
Size (mm)	5	6-10	6-10	5	5
Paris CL	IIa	IIb	IIa	IIb	IIb
Histology	TA LGD	TA LGD	TA LGD	TA LGD	TA LGD
Spigelman	I°	II°	II°	I°	I°

TA: Tubular adenoma; LGD: Low grade dysplasia.

of them new, were discovered. All of them were tubular adenomas with low grade dysplasia (specificity 62.5%; sensitivity 100%; positive predictive value 81.3%; negative predictive value 100%) (Table 8).

FICE was able to see further 7 polyps than traditional endoscopy, and it was able to identify a quite significant higher number of polyp in the duodenal bulb (21 *vs* 14; $P = 0.069$). FICE added a statistical significant advantage in identifying adenomas (13 *vs* 6; $P = 0.03$). All FICE identified polyps were flat lesions. All patients with bulbal polyps had also lesions in the gastric fundus and no adenoma in the antrum.

All patients with bulbal adenomas had polyps in the second/third portion of duodenum, while patients with inflammatory polyps had a Spigelman's stage 0.

II°/III° duodenal portion: Totally, 391 polyps in 45 patients (8.7 polyps per patient) were identified. Of them, 105 were removed or biopsied (26.5%). Conventional endoscopy identify 324 polyps in 45 patients (7.2 polyps per patient). Of them, 94 were removed or biopsied (2.1 polyps per patient) and they resulted: 80 tubular adenomas with low grade dysplasia, 10 inflammatory polyps and 4 tubulo-villous adenomas with low grade dysplasia. No case of high grade dysplasia (3 suspected). (Table 9). FICE identified further 67 polyps in 35 patients and 11 were removed or biopsied in 11 subjects. All of them were tubular adenomas with low grade dysplasia. No case of high grade dysplasia (Table 10). FICE was able to identify a higher number of polyps than traditional endoscopy (8.7 *vs* 7.2; $P < 0.001$). All polyps not seen on white light were flat lesions.

Thirty-five of patients with duodenal polyposis had also polyps in the fundus, 4 had adenomas and 2 dysplastic areas in the antrum, thus FICE didn't change any Spigelman stage just defined with conventional endoscopy.

DISCUSSION

Duodenal adenomatous polyps are common manifestations of FAP found in 30% to 90% of patients, with a life time risk approaching 100%[5,6,43]. While rare in the general population (0.01%-0.04% of incidence at an average age of 65 years)[43], the risk of duodenal or periampullary cancer is increased several hundreds fold in FAP patients (estimated cumulative risk of 4.5% by age 57 and a median age at presentation of 52 years)[6,8]. Duodenal cancer is the second most common cause of disease-related mortality in patients with FAP, only the

Table 9 Features of duodenal polyps identified by white light endoscopy

	Kudo	Size (mm)	Paris CL	Histology	Spigelman
P1-P4	Ⅳ	11-20	Ⅱa	TA LGD	Ⅲ°
P5-P7	Ⅴ	6-10	Ⅱa	TA LGD	Ⅱ°
P8-P11	Ⅳ	5	Ⅱb	TA LGD	Ⅱ°
P12-P16	ⅢS	6-10	Ⅱa	TVA LGD	Ⅲ°
P17-P27	ⅢS	5	Ⅱa	TA LGD	Ⅱ°
P28-P34	ⅢS	5	Ⅱa	TA LGD	Ⅰ°
P35-P40	ⅢS	5	Ⅱb	TA LGD	Ⅱ°
P41-P43	ⅢS	5	Ⅰs	TA LGD	Ⅱ°
P44-P47	ⅢS	5	Ⅰs	TA LGD	Ⅰ°
P48-P50	ⅢS	11-20	Ⅱa	TA LGD	Ⅱ°
P51-P58	ⅢL	5	Ⅱb	TA LGD	Ⅱ°
P59-P65	ⅢL	5	Ⅱa	TA LGD	Ⅱ°
P66-P68	ⅢL	6-10	Ⅱa	TA LGD	Ⅱ°
P69-P72	ⅢL	6-10	Ⅱa	TA LGD	Ⅲ°
P73-P79	ⅢL	6-10	Ⅱb	TA LGD	Ⅱ°
P80-P84	Ⅱ	5	Ⅱa	TA LGD	Ⅲ°
P85-P91	ⅢL	5	Ⅱb	IN	Ⅱ°
P92-P94	ⅢS	5	Ⅱa	IN	Ⅰ°

IN: Inflammatory; TA: Tubular adenoma; LGD: Low grade dysplasia.

Table 10 Features of duodenal polyps identified by fujinon intelligent color enhancement

	P95-P97	P98-P100	P101-P102	P103-P105
Kudo	Ⅳ	Ⅳ	Ⅴ	Ⅳ
Size (mm)	5	6-10	6-10	5
Paris CL	Ⅱa	Ⅱb	Ⅱb	Ⅱb
Histology	TA LGD	TA LGD	TA LGD	TA LGD
Spigelman	Ⅰ°	Ⅱ°	Ⅱ°	Ⅱ°

TA: Tubular adenoma; LGD: Low grade dysplasia.

second to advanced and metastatic colorectal cancer. A regular and careful program of endoscopic surveillance is worthwhile in identifying early pre-malignant lesions.

Gastric polyps, particularly fundic polyps, are considered always non-neoplastic lesions, also in FAP and non-FAP patients; nonetheless high rate of their prevalence (20%-88%)[6,9-11] and several cases of dysplasia in FGPs in FAP have been recently reported, with rate of incidence up to 54%[9-11,18].

Chromoendoscopy, both traditional and virtual, has been proven to be a good tool to increase polyps identification rate and to predict their histology[29-32]. Only one study was published on the use of FICE in the evaluation of duodenal lesions[44]. This study was conducted using a double balloon enteroscopy on patients with duodenal lesions. In this study only two FAP patients were included and FICE enhanced mucosal pattern of these polyps, and it correlated with the increase of detection of more lesions.

However, neither previous studies using traditional chromoendoscopy nor FICE, were conducted in evaluation of gastric polyps in FAP patients. To the best of our knowledge, our study is the first that has assessed the role of FICE in FGPs dysplasia identification and in the gastroduodenal polyps characterization in FAP subjects.

In agreement with the literature's data, the prevalence of gastric polyps was relatively elevated (90.8%), while duodenal polyps were diagnosed in 68.4% of patients, slightly lower than the reported literature value.

In the majority of FAP subjects (62/76; 83.3%), gastric polyps were so numerous that they carpeted the fundic mucosa, making difficult identifying dysplasia by random biopsies on the basis of the total number of polyps, as indicated in a recent study conducted by Bianchi *et al*[9]. Consequently, having an endoscopic technique able to target fundic biopsies is important to overcome this issue. Moreover, Bianchi *et al*[9] reported a prevalence of dysplasia in fundic polyps of 42%, while we have found only fundic gland polyps without displastic or adenomatous areas, although we have followed their sampling method. A possible explanation to this marked mismatch, could lie in the size of the polyps removed: we did found only subcentimetric polyps, while Bianchi *et al*[9] have demonstrated that the risk of dysplasia correlated with polyp size. No polyps removed had suspected superficial features according to Kudo classification, while Bianchi *et al*[9] did not adopted any classification to describe mucosal and vascular pattern; consequently we don't know if their removed polyps had or not a suspected pattern.

FICE pointed our attention on 10 fundic polyps, that seemed suspected for harboring adenomatous tissue;

however histological results did not confirmed this suspect and all of polyps resulted fundic gland polyps. In this case, FICE has not increased the identification rate of dysplasia or adenomatous tissue in fundic polyps.

Prevalence of patients with antral adenomas was about 21.1% (16/76), more than reported in the Western World data, but consistent with Japanese findings. The use of FICE could explain our result, since it has increased the identification rate of antral adenomas compared to white light, with a difference near to statistical significance (P = 0.0857).

The very low specificity of the method (12.0%) could be explained by the presence of phlogosis (in fact almost all false positive harbored flogistic areas) able to distort the mucosal and vascular pattern, specifically enhanced by virtual chromoendoscopy.

Therefore, FICE allows to identify a greater number of adenomas to the detriment of a greater number of biopsies. Anyway this approach didn't determine a different timing in the surveillance program, but changed the attention on the antral evaluation during the following endoscopies. In duodenal bulb FICE was able to identify more adenomas than traditional endoscopy (P = 0.03). Furthermore, all patients with FICE-identified adenomas had polyps in the duodenum too, thus the identification of bulbar adenomas didn't modify surveillance timing.

Taking into account also bulbar polyps, duodenal adenomas prevalence in FAP patients was 68.4%, with low Spigelman stages (9.2% stage Ⅲ e 0% stage Ⅳ). In duodenum, FICE has allowed to see a greater number of adenomas than white light (P < 0.001), without no modifications of Spigelman stage neither identification of high grade dysplasia.

Among FICE identified polyps, 4 lesions were suspected for high grade dysplasia, but three were inflammatory polyps at histopathological examination and one was a tubular adenoma with low grade of dysplasia. Other 7 polyps (Kudo IV) had an increased capillary density but they were tubular adenomas with low grade of dysplasia.

Finally, in duodenum, FICE increased the polyps detection rate but didn't change any Spigelman stage determined with conventional endoscopy. These data are in agreement with the little size and the absence of high grade dysplasia. Moreover this method wasn't able to modify FAP patients' prognosis, polyps' surveillance program and their therapeutic management. We did not find any relationship between the presence of gastric polyps, duodenal polyposis and high Spigelman stage (P = 1).

Adenomas were 435 and 81 of them were diagnosed only by FICE that was able to identify a significative higher number of adenomas (P = 0.0062). Overall, FICE has specificity, sensitivity,positive and negative predictive values higher than traditional endoscopy referring to adenomas (96.0% *vs* 7.1%; 98.8% *vs* 80.2%; 90.3% *vs* 44.9%; 98.8% *vs* 27.6%, respectively; P < 0.0001). Conversely, it wasn't possible to correlate for high grade dysplasia due the absence of dysplastic lesions according to the histopathological examination.

The FICE identified lesions (106/468; 22.6%) were mostly flat (67.9%; P = 0.029) and small (all below 1 cm). According to already published data, FICE was particularly able to identify polyps with these features. It isn't clear if this ability might have clinical and procedural consequences.

In summary, in our study, FICE, like traditional endoscopy, could not identify any adenoma at risk of malignant transformation probably as a consequence of patients features (*e.g.,* favorable genotype, recent EGD).

Nonetheless FICE significantly increases adenoma detection rate (P = 0.0062) but does not change any Spigelman stage and thus does not modify patient' s prognosis, surveillance program and treatment strategies. Probably a careful patient selection, an accurate histological examination, a concomitant use of lateral viewing endoscope, could make FICE gain that role who everybody expects in FAP patient.

COMMENTS

Background

Familial adenomatous polyposis (FAP) is an autosomal dominant inherited syndrome characterized by the development of colorectal cancer by the age of 40 years in nearly 100% of individuals. The use of colon endoscopic surveillance and prophylactic colectomy have strongly reduced mortality in FAP patients leading to the introduction of surveillance strategies for the prevention of other extracolonic malignancies (*e.g.,* in the duodenum and in the stomach). Duodenal adenomatous polyps are common manifestations of FAP found in 30% to 90% of patients. Duodenal cancer is the second most common cause of disease-related mortality in patients with FAP, only the second to advanced and metastatic colorectal cancer. Gastric polyps, particularly fundic polyps, are considered always non-neoplastic lesions, also in FAP and non-FAP patients; nonetheless high rate of their prevalence (20%-88%) and several cases of dysplasia in FGPs in FAP have been recently reported, with rate of incidence up to 54%.

Research frontiers

The observation of the pit and capillary patterns of the mucosal glands and the mucosa, respectively, by chromoendoscopy might predict the histology of mucosal lesions.

Innovations and breakthroughs

Chromoendoscopy is a staining method that uses biocompatible dye agents which accumulate within crypt orifices during endoscopy. Chromoendoscopy has difficulty in achieving complete and even coating of the mucosal surface with the dye, requires the extra cost for the of the dye spraying equipments and extra time to perform the procedure. Fujinon Intelligent Color Enhancement (FICE™, Fujinon Corp, Saitama, Japan) is a spectral estimation technique based on arithmetically processing of a white-light image captured by a video endoscope and sent to the spectral-estimation matrix-processing circuit. Preliminary studies suggested that FICE successfully achieves enhancements of real-time observations of mucosal and microvascular patterns and may discriminate between adenomatous and non-adenomatous polyps and it may identify the presence of dysplasia. In the study, FICE, like traditional endoscopy, could not identify any adenoma at risk of malignant transformation probably as a consequence of patients features. However FICE significantly increases adenoma detection without changing patient's prognosis, surveillance program and treatment strategies. Probably a careful patient selection, an accurate histological examination, a concomitant use of lateral viewing endoscope, could make FICE gain that role who everybody expects in FAP patient.

Applications

The timing of endoscopic and histology surveillance is currently a great challenge. Spectral estimation by fujinon intelligent color enhancement (FICE) may identify dysplasia and discriminate between adenomatous and non-adenomatous polyps.

Terminology

FAP is an autosomal dominant inherited syndrome who invariably develops to colorectal cancer by the age of 40 years in nearly 100% of individuals. Several endoscopic imaging techniques have been proposed to enhance the detail of these patterns. Among these, chromoendoscopy is a staining method applied in endoscopy that uses biocompatible dye agents which accumulate within crypt orifices. FICE is a modern endoscopic spectral estimation technique that successfully enhances the observation of mucosal and micro-vascular patterns.

Peer-review

The presented results, obtained with 76 FAP patients, indicate that FICE assay offers considerable advantage over traditional chromoendoscopy to discriminate between adenomatous and non-adenomatous polyps. The authors, however, caution that the application of FICE to FAP patients while helpful in prediction the histology of the mucosal lesion and significantly increases the detection of adenomas, do not change the prognosis and treatment.

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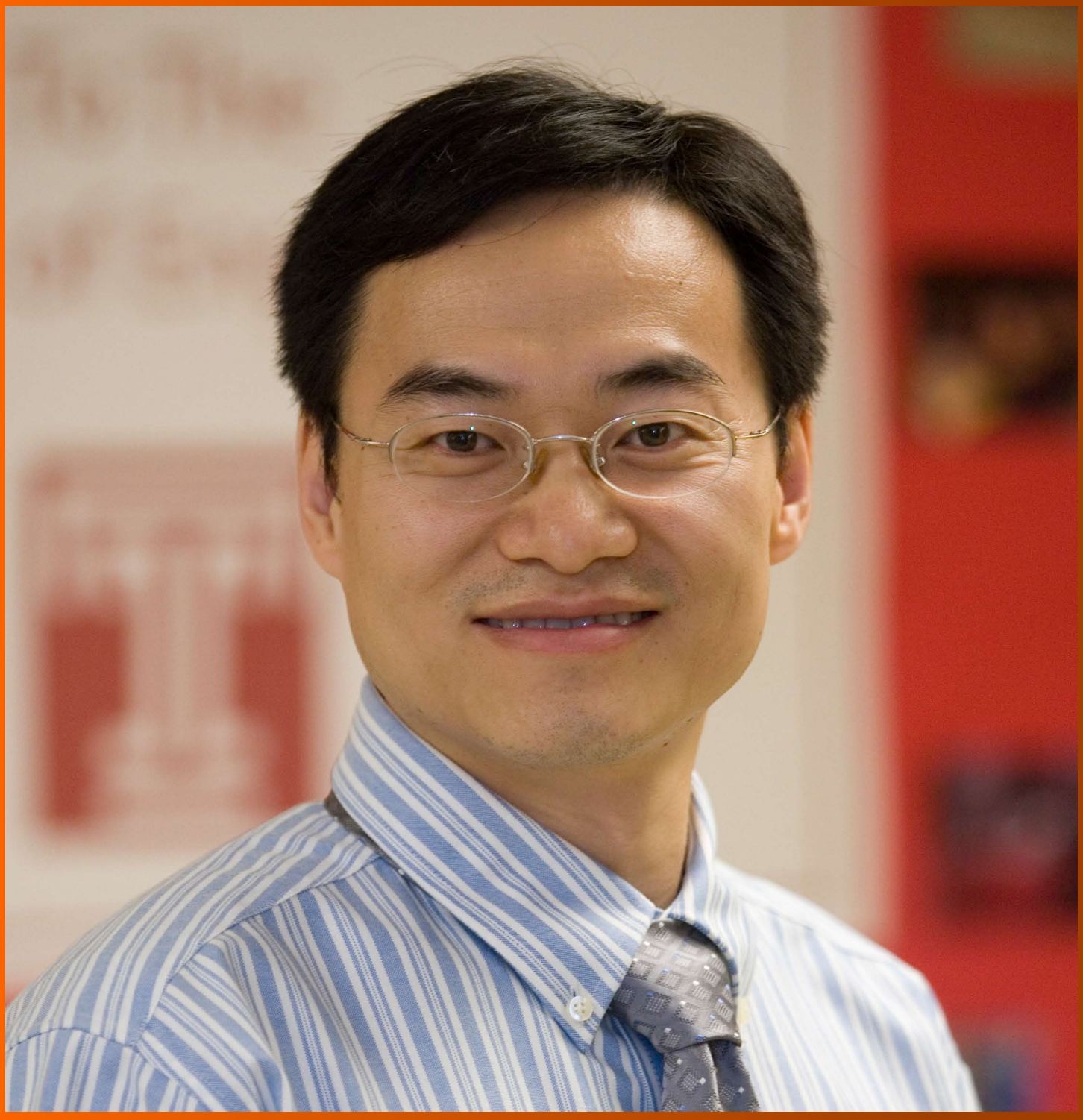

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Contents

Bimonthly Volume 8 Number 3 June 10, 2017

REVIEW

MINIREVIEWS

ORIGINAL ARTICLE

Case Control Study

SYSTEMATIC REVIEWS

META-ANALYSIS

CASE REPORT

Contents

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REVIEW

Histone deacetylases, microRNA and leptin crosstalk in pancreatic cancer

Cynthia I Tchio Mantho, Adriana Harbuzariu, Ruben R Gonzalez-Perez

Cynthia I Tchio Mantho, Adriana Harbuzariu, Ruben R Gonzalez-Perez, Department of Microbiology, Biochemistry and Immunology, Morehouse School of Medicine, Atlanta, GA 30310, United States

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Correspondence to: Dr. Ruben R Gonzalez-Perez, Department of Microbiology, Biochemistry and Immunology, Morehouse School of Medicine, 720 Westview Drive SW, Hugh Gloster Bldg., Room 329, Atlanta, GA 30310,
United States. rgonzalez@msm.edu
Telephone: +1-404-7521581
Fax: +1-404-7521179

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Abstract

Because pancreatic cancer (PC) historically has had poor prognosis and five year survival rates, it has been intensely investigated. Analysis of PC incidence and biology has shown a link between different risk factors such as smoking, alcoholism, and obesity and disease progression. Important factors affecting PC include the epigenomic changes driven by DNA methylation and histone acetylation, and actions of microRNA inducing oncogenic or tumor suppressor effects. Studies have identified markers whose dysregulation seem to play important roles in PC progression. PC markers involve classical histone deacetylases (HDAC), PC stem cell (PCSC), and leptin. In this review, we discuss the role of several PC biomarkers, and the potential crosstalk between HDAC, microRNA, and leptin in PC progression. Dysregulated expression of these molecules can increase proliferation, survival, PCSC, resistance to chemotherapy and tumor angiogenesis. The potential relationships between these molecules are further analyzed using data from The Cancer Genome Atlas and crosstalk pathways generated by the Pathway Studio Platform (Ariadne Genomics, Inc.). Oncogenic miRNA21 and tumor suppressor miRNA200 have been previously linked to leptin signaling. Preliminary analysis of PC biopsies and signaling crosstalk suggests that the main adipokine leptin could affect the expression of microRNA and HDAC in PC. Data analysis suggests that HDAC-microRNA-leptin signaling crosstalk may be a new target for PC therapy.

Key words: Pancreatic Cancer; MicroRNA; Histone

deacetylases; Pancreatic cancer stem cell markers; Leptin; Obesity

Core tip: Pancreatic cancer has no targeted therapy. Obesity is a risk factor for pancreatic cancer, characterized by high levels of leptin. In this review, we discuss the potential crosstalk between histone deacetylases, microRNA, and leptin in disease progression. Crosstalk among these molecules increases proliferation, survival, cancer stem cells and resistance to chemotherapy. The potential relationships between these molecules are analyzed using data from the Cancer Genome Atlas and the Pathway Studio Platform. The crosstalk among these molecules could be a novel target for pancreatic cancer prevention or treatment, particularly in obese patients that show elevated levels of leptin.

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INTRODUCTION

Pancreatic cancer (PC) is a malignant disease, which is difficult to treat. It is a silent disease that can go undetected for long periods of time; however, when diagnosed, it is often in advanced stages (Ⅲ or Ⅳ)[1]. PC incidence and mortality rates vary across different racial/ethnic groups, with the highest rates found in African Americans, and the lowest in Asian Americans/Pacific Islanders. Moreover, PC incidence rate is higher in African Americans when compared to European Americans at every age[1]. Risk factors for the development of PC include tobacco usage, continuous exposure to such chemicals as dyes and pesticides, family history, age, epigenetic changes, and obesity[1-5]. The best outcomes from PC treatments are obtained after complete surgical resection, with no residual disease; this can improve 5 year survival, but only from 5% to about 20%-25%[6]. Even patients who are eligible for surgical treatment with tumor free margins often experience recurrence and eventually require palliative treatment[7,8]. Surgical resection of PC is performed on patients with locally advanced or borderline resectable tumors. Improved outcomes may be achieved with a multimodal approach, combining neo-adjuvant chemotherapy with radiation therapy and surgery. Adjuvant therapy includes 5-fluoruracil (5-FU) or capecitabine; gemcitabine induction followed by concomitant chemoradiation with either gemcitabine or 5-FU; FOLFIRINOX (an aggressive chemotherapeutic regimen, including several chemotherapeutic agents) or gemcitabine-Nab paclitaxel (albumin bound) with or without subsequent chemoradiation. For patients with metastatic PC, the treatment options are very limited. It mainly consists of palliative care (pain and nutrition management), as well as chemotherapy. The chemotherapeutic regimens for metastatic PC (gemcitabine alone or in combination with other agents for example, FOLFIRINOX, Nab-paclitaxel) have only modest results, improving the survival of these patients by only a few months[9,10].

Important factors affecting PC are the changes in the epigenome driven by DNA methylation and histone acetylation. Epigenetic changes are alterations in gene expression or cellular phenotype that occur without changes in the DNA sequence. Some of the epigenetic changes are DNA methylation and histone acetylation. This last process is characterized by the addition of acetyl groups to the lysine residues of the histones *via* histone acetyltransferases (HAT). Histone acetylation is essential to gene regulation, and is usually associated with the relaxed form of chromatin. Lysine residues can also be deacetylated by histone deacetylases (HDAC). These enzymes are involved in cancer progression by increasing proliferation, survival and resistance to chemotherapy of cancer cells as well as angiogenesis[11-15].

The dysregulation of microRNAs is another factor involved in cancer progression[16-18]. MicroRNAs (miRNA or miR) are noncoding endogenous RNAs that regulate protein expression. Accumulating data show important relationships between dysregulated miRNAs and cancer[16-19]. The effect that miRNAs dysregulation has on the cancer cells determines whether these molecules are considered oncogenics or tumor suppressors. Oncogenic miRNAs promote cancer development through various signaling mechanisms while tumor suppressor miRNAs have contrary effects and their expression is decreased in cancer[19,20]. There are many oncogenic microRNAs (*e.g.,* miR21) that have been reported to play a role in cancer progression[20-23]. Furthermore, the decreased expression of tumor suppressor miR200 family has been associated with PC progression[24,25].

Obesity is one the most observed risk factors for cancer progression. Obesity is a growing pandemic, and is associated with more than 100000 incidences of various cancers in the United States, particularly breast, colon, endometrium and PC[26-28]. Obesity is characterized by the accumulation of excessive body fat, and a body mass index (BMI) value greater than 30. Obesity is also characterized by high levels of leptin, which has been consistently associated with many cancers, including PC[29-33]. Preliminary analyses suggest that leptin could affect the expression of microRNA and HDAC in PC.

Because of the absence of targeted therapies for obese PC patients, there is a need to better understand the mechanisms behind the disease progression in order to develop better treatment strategies. Thus, in this review, we will discuss the potential relationships between HDAC, microRNA, cancer stem cells, and leptin

signaling in PC.

PC TYPES

There are two types of PC - those that comprise tumors arising from the endocrine pancreatic cells and those that arise from the exocrine pancreatic cells. Cancers of the endocrine pancreas are rare and represent less than 4% of all PC cases[1]. Pancreatic Adenocarcinoma (PA) is the most common type of PC and usually begins in the ducts of the pancreatic glands. PC has been recently classified into four main subtypes based on their genomic analysis (*e.g.,* squamous, pancreatic progenitor, immunogenic, and aberrantly differentiated endocrine exocrine or ADEX)[34].

PC squamous subtype is characterized by four core genes programs involved in inflammation, hypoxia response, metabolic reprogramming, transforming growth factor-β (TGF-β) signaling, autophagy, and upregulated expression of TP63ΔN and its target genes[34]. The pancreatic progenitor subtype is characterized by the transcriptional networks of pancreatic and duodenal homeobox-1 (PDX1), motor neuron and pancreas homeobox- 1 (MNX1), hepatocyte nuclear factor-4-α (HNF4A), hepatocyte nuclear factor-1-β (HNF1B), hepatocyte nuclear factor-1-α (HNF1A), forkhead box-A2 (FOXA2), forkhead box-A3 (FOXA3), and hairy and enhancer of split-1 (HES1) transcription factors[34,35]. The immunogenic subtype of PC is characterized by changes in the programs of immune genes that include antigen presentation, $CD4^+$ and $CD8^+$ T cells, and toll-like receptor and B cell signaling pathways[8]. ADEX is characterized by the upregulation of transcriptional networks of both exocrine and endocrines lineages that are important in later stages of pancreatic development and differentiation[34].

In addition, some hereditary factors play roles in the development of PC. Individuals with a high risk of developing PC can be divided into underlying gene defect, like cyclin-dependent kinase Inhibitor 2A (CDKN2A), breast cancer gene 1 and 2 (BRCA1/2), partner and localizer of BRCA2 (PALB2), and serine/threonine kinase 11 (STK11) mutations[35,36]. In a study performed by Vasen *et al*[36], the longterm outcome of prospective surveillance of a large group of CDKN2A/p216-Leiden carriers and, BRCA1/2 and PALB2 mutation carriers, and individuals at risk (IARs) for familial PC (FPC) was evaluated. The main goal of the study was to determine whether or not surveillance will lead to the detection of early stage PC or the detection of relevant precursors lesions (PRLs) as well as to assess if their program leads to improvements in prognosis[36]. Based on the surveillance, it was determined that PRLs were more frequent in patients with FPC than those with CDKN2A/p216-Leiden mutation[36]. The surveillance study also reveals that the resection of screen detected PC with CDKN2A/p216-Leiden mutation carriers was 75%, which is higher than that reported for patients with sporadic PC (15%-20%)[36]. Overall, the study demonstrated that the surveillance of CDNK2A mutation carriers was successful for the detection of PC at the resectable stage[36].

PC BIOMARKERS

PC is generally diagnosed when approximately 30% of patients present a locally advanced disease[1]. Because there is no effective treatment for advanced PC, this disease should be detected in the early stages when treatment could significantly increase the percentage of patients with five years of survival. The best way to PC early diagnosis would be *via* the usage of screening biomarkers with high specificity and sensitivity. Currently, the most established and used biomarker is CA19-9. However, CA19-9 detection is not highly specific for PC, as it can also be detected in colorectal cancer, stomach, and biliary epithelium and chronic pancreatitis[1,37,38].

A vast array of other PC biomarkers has been investigated, but so far none are as yet widely used clinically. It has recently been shown that exosomes could potentially impact on the pathogenesis of PC through the modulation of tumor growth, microenvironment, and immune response. This suggests that exosomes could be used as biomarkers for PC[39]. An additional PC marker could be the leptin receptor, OB-R, which has been detected in PC cell lines[40]. Moreover, OB-R expression was positively correlated with the matrix metalloproteinase-13 (MMP-13) in human PC tissues. The increased expression of either OB-R or MMP-13 was significantly associated with lymph node metastasis; it also tends to be associated with the TNM stage in PC patients[40].

Likewise, it has been proposed that the detection of PC cells in blood could be used as a surrogate for PC detection[41,42]. Circulating tumor cells (CTC) could be related to metastatic and more aggressive PC disease, according to the results from an international multicenter randomized study that included 79 patients. A subgroup of PC patients was screened for CTCs before the start of the chemotherapy, and after two months of treatment. Overall, CTC detection was found in 11% of PC patients and associated with poor tumor differentiation ($P = 0.04$), and with shorter overall survival (RR = 2.5, $P = 0.01$). Therefore, CTC detection might be a new way to detect PC[38].

HISTONE DEACETYLASES IN PC

HDAC play a major role in the regulation of gene expression *via* epigenetics changes. HDAC catalyze the removal of an acetyl group, which stimulates chromatin condensation, thus suppressing transcription. Currently, 18 HDAC family members have been identified in the human genome, which are grouped into four classes (Ⅰ-Ⅳ)[43]. HDAC are also classified into two major types: Sirtuins (SIRT) and classical HDAC. Classical HDAC include Classes Ⅰ, Ⅱ, and Ⅳ, whereas the sirtuins comprise Class Ⅲ[43,44] (Table 1). HDAC classes Ⅰ, Ⅱ, and Ⅳ are zinc dependent metalloproteins, while class Ⅲ are nicotinamide adenine dinucleotide (NAD^+) dependent

Table 1 Classification of classical histone deacetylases

Class	Members	Cellular localization	Function in cancer[13,14,45,46,104]	Substrates[43,44,104]
Ⅰ	HDAC1	Nucleus	Proliferation, survival and resistance to chemoresistance	P53, E2F-1, Stat3, and androgen
	HDAC2	Nucleus	Proliferation and survival	Bcl-6, Stat3. YY-1, and glucocorticoid receptor
	HDAC3	Nucleus	Proliferation and anti-differentiation	GATA-1, RelA, Stat3, MEF2D, YY-1, and SHP
	HDAC8	Nucleus	Proliferation and anti-differentiation	ERRα, Inv (16), and CREB
ⅡA	HDAC4	Nucleus/cytoplasm	Angiogenesis and anti-differentiation	GCMa, GATA-1, and HP-1
	HDAC5	Nucleus/cytoplasm	Anti-differentiation	Smad7, HP-1, and GCMa
	HDAC7	Nucleus/cytoplasm	Angiogenesis and migration	FLAG-1, and FLAG-2
	HDAC9	Nucleus/cytoplasm	Cell survival	ATDC (TRIM29)
ⅡB	HDAC6	Cytoplasm	Angiogenesis and migration	Alpha-Tubulin, HSP-90, SHP, Smad7
	HDAC10	Cytoplasm	Angiogenesis	HSP90
Ⅳ	HDAC11	Nucleus/cytoplasm	Tumor immune response	OX40L

HDAC: Histone deacetylases.

enzymes[43]. Class Ⅰ HDAC family consists of HDAC 1, 2, 3, and 8. These enzymes are mainly located in the cellular nucleus. Class Ⅱ HDAC family is divided into two groups - Classes ⅡA and ⅡB. These HDAC are mainly located in the cytoplasm, but can also be found in the nucleus, which is dependent on their phosphorylation status influencing their shuttle mechanism[43,44]. Subclass ⅡA HDAC family consists of HDAC 4, 5, 7, and 9; while subclass ⅡB consists of HDAC 6 and 10. HDAC Class Ⅳ is only made of HDAC11 that is mainly located in the nucleus. Class Ⅲ is composed of SIRT1, SIRT2, SIRT3, SIRT4, SIRT5, SIRT6, and SIRT7, which are located in the nucleus, cytoplasm, and mitochondria[38]. Due to the role of HDAC in epigenetic regulations and their effect on chromatin structures, many studies have found them linked to cancer progression[13,14,45,46]. The classical HDAC have been associated with cancer progression through the increase of proliferation, survival and resistance to chemotherapy of cancer cells, and angiogenesis. More studies suggest the roles of HDAC in PC progression. The use of HDAC inhibitors is a novel avenue toward targeted therapy for PC. Several HDAC inhibitors are currently under clinical trials for cancer-targeted treatment. However, currently there are only three FDA approved HDAC inhibitor drugs available [Vorinostat or Suberoylanilide Hydroxamic Acid (SAHA), Zolina], Romidepsin (Depsipeptide, ISTODAX), and Belinostat (Beleodaq)[47-50].

Vorinostat was the first FDA approved anti-HDAC drug[47,49,51]. It is a hydroxamic acid based drug that inhibits Class Ⅰ, Ⅱ, Ⅳ HDAC by chelating the zinc cofactor. This drug shows apoptotic and anti-proliferative effects by modifying the expression of specific genes related to insulin-like growth factor-1 receptor signaling receptor (IGF-1R)[47,49,52]. The second FDA approved HDAC inhibitor drug is Romidepsin, which was effective in phase Ⅱ clinical trials when used with gemcitabine for treatment of advanced PC[49,53-55]. The third FDA approved HDAC inhibitor drug is Belinostat. It showed a dose dependent growth inhibitory or pro-apoptotic effects promoting cell cycle arrest at the G0/G1 or S phase transition[56-58]. Additionally, positive results in the treatment of PC have been reported with the use of benzamide derivative HDAC inhibitor (Class Ⅰ HDAC inhibitor MGCD0103) selective for Class Ⅰ and Ⅳ HDAC[59]. PC cell lines treated with MGCD0103 showed dose dependent growth arrest, apoptosis, and induction of p21, which mediated cell cycle arrest in G2/M phase[59].

TUMOR SUPPRESSOR AND ONCOGENIC MIRNAS IN PC

MicroRNAs (miRNA or miR) are noncoding endogenous RNAs of 14-24 nucleotides that have the ability to regulate protein expression at the post-transcriptional level. Many studies have found strong correlations between dysregulated microRNAs and cancer[17,18,60]. According to the effect that miRNAs dysregulation has on the cancer cells, these molecules are considered oncogenic or tumor suppressors. There are many oncogenic microRNAs, such as miR1290, miR24, miR134, miR146a, miR378, miR484, miR628-3p, miR1825[61] and miR21[20-23] that have been reported to play a role in cancer progression. It was reported that serum levels of miR1290 distinguished patients with low-stage PC from controls better than CA19-9 levels[61]. Furthermore, decreased expressions of miRNA34[62] and miR200[20] family have been associated with PC progression.

A study found that oncogenic miR21 was expressed in the early stage of PA[63]. Furthermore, knockdown of miR21 using lentiviral vectors inhibited cell proliferation in PC derived cell lines. In addition, miR21 was found to protect PC cell from apoptosis, and its knockdown resulted in the activation of mitochondrial pathway apoptosis *via* the downregulation of Bcl9 (a protein involved in Wnt Pathway), upregulation of Bax, and induction of Bim[63]. Targeting miR21 *in vivo* strongly inhibited PC growth, which led to the suggestion that simultaneous standard gemcitabine chemotherapy combined with miR21 targeting could improve the prognosis of PC

MiRNA200 family consists of five members (miR200a, b, c and miR429, and miR141)[64]. *In vitro* studies suggested that miR200c expression was related to low cancer invasion[20]. MiRNA200 activities include inhibition of epithelial-mesenchymal-transformation (EMT), repression of cancer stem cell (CSC) self-renewal and differentiation,

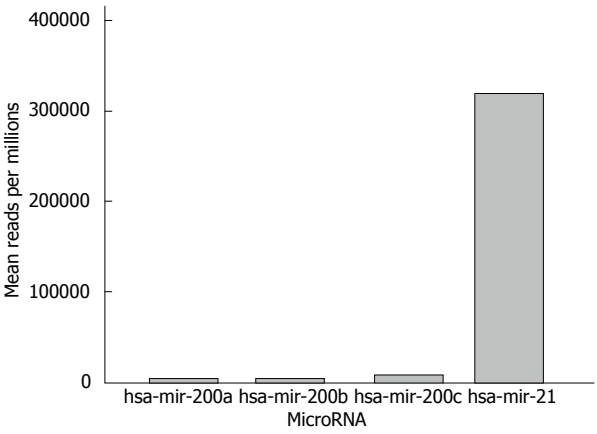

Figure 1 IlluminaHiSeq miRNA expression of tumors tissues biopsies from pancreatic cancer patients. The data sets used were generated from the the Cancer Genome Atlas Database[71]. The oncogenic miR21 is highly expressed in PC while there is a low expression of tumor suppressor miR200a/b/c (pancreatic cancer samples n = 45).

modulation of cell division and apoptosis, and involvement in chemoresistance. High level of miR200c correlated to better survival rates[20]; however, miR200a and miR200b, were hypomethylated and overexpressed in PC[65]. It has been suggested that targeting miRNA200 upregulation could improve PC prognosis if used together with the chemotherapeutic drug gemcitabine[20]. Indeed, treatment of PC cells with a curcumin analoge, CDF, improved gemcitabine effects by upregulating miR200 and downregulating miR-21 expression. These effects were found together with the downregulation of Akt, cyclooxygenase-2, prostaglandin E2, vascular endothelial growth factor, and nuclear factor-κB DNA binding activity, and induction of PTEN[66].

Transcriptor factor ZFH family (ZEB1 and ZEB2)[67] represses the expression of epithelial genes. miR200 members increased Notch activation by ZEB1 that regulates the expression of Jagged1 and the mastermind-like coactivators Maml2 and Maml3. Moreover, in PC and breast cancer cells, decreased miR200 expression was associated with increased Jag1 and ZEB1 levels[68]. Therefore, MiR200 inhibits EMT by interacting with ZEB1/2 and the Notch pathway, represses self-renewal and differentiation in CSCs, and is involved in the regulation of cell division and apoptosis[64]. In turn, ZEB1 suppresses the expression of miR200 family, which inhibits the translation of ZEB1 mRNA, resulting in the double-negative ZEB/miR200 feedback loop[69]. Additionally, in lung cancer Jagged2 inhibits the expression of miR200 family by induction of GATA transcription factors, which promotes tumor metastasis[70].

We preliminarily analyzed PC biopsies using TCGA databank[71]. Data analysis shows higher miR21 expression compared with miR200 in PC (Figure 1). These data suggest that progression of PC could be positively associated with upregulation of miR21 and downregulation of miR200.

PC STEM CELLS

PC is usually diagnosed in the advanced stages after distant metastasis has already occurred in most cases. PC shows high frequency of local relapse, even after surgical resection. Treatment of PC *via* surgery and chemotherapy has historically had little success. Patients still have a poor survival rate with chemoresistance and reoccurrence of the disease as significant factors. These features of PC could be related to the action of PC stem cell (PCSC)[72]. Cancer stem cells are a small population that have the capacity to self-renew, and generate cells with identical tumorigenic potential that could also differentiate to form the bulk of the tumor cells, thereby contributing to the formation of heterogenic cellular composition of cancers[55]. A highly tumorigenic PCSC population ($CD24^+CD44^+ESA^+$) was described in PC for the first time by Li *et al*[73] in a xenograft human model. PCSC are resistant to chemotherapy and contribute to tumor initiation, growth and metastasis[74]. PCSC were also identified as $CD133^+$ population that is highly resistant to standard chemotherapy. A subgroup of these cells, $CD133^+CXCR4^+$ was found to be involved in metastasis[75].

C-Met, also known as hepatocyte growth factor receptor (HGFR), is an oncogene involved in the progression of cancer. C-Met was earlier described as a potential PCSC marker[75-77]. $C\text{-}Met^+$ PCSC showed similar tumorigenic capacity as $CD24^+CD44^+ESA^+$ population[78-80]. C-Met is a heterodimer that consist of an extracellular α-chain bound through a disulphide bridge to a transmembrane β-chain[79]. It is also a tyrosine kinase found in the cell membrane. HGF ligand binding to C-Met immunoglobulin like-domain induces C-Met dimerization, leading to autophosphorylation of the two tyrosine residues within the catalytic loop[79]. Subsequently, further autophosphorylation of two more tyrosine residues occurs in the C-terminal of c-Met receptors, which provides the platform for the recruitment of other molecular factors and signal conveyors like Grb2-associated binding protein 1 (Gab1). This provides a binding site for such SH2-containing effectors as SHP2, PLCγL, STAT3, Ras GTPase, and PI3K9[79]. With the emerging evidence of c-Met as a stem cell marker, some studies were able to identify part of the c-Met cell population that also expresses CD44, CD24, CD133, and ALDH1[78,81]. However, in a study $c\text{-}Met^+$ PCSC produced tumors in 35% of cases when compared to PCSC $CD133^+$ (16%) and $CD44^+$ (25%) of cases[78-80].

Notch signaling pathway is another factor that affects the maintenance of PCSC. Notch signaling pathway is a known regulator of the balance between cell self-renewal and cell differentiation. Abel *et al*[82] found that components of Notch signaling were upregulated in PCSC. Moreover, the inhibition of Notch signaling pathway with gamma secretase inhibitors or Hes1 shRNA in PCSC reduced the percentage of PCSC and their ability to form tumorspheres. Furthermore, these authors found that the activation of Notch signaling pathway

using an exogenous peptide ligand greatly increased the percentage of PCSC and formation of tumorspheres[82].

Due to PCSC role in chemoresistance and disease reoccurrence, c-Met, CD44, CD24, CD133, and ALDH1 could potentially be used as biomarkers to detect PC progression. Moreover, developing therapies that would target PCSC markers could be adjuvant to the standard gemcitabine chemotherapy, which could improve PC survival rate[72,78].

OBESITY AND PC

Obesity is mainly the result of unhealthy diets and lifestyles, and has proven to be a contributing factor to higher risk and poor prognosis of cancer[1,83,84]. Several studies have examined the impact that obesity has on the overall survival rate of PC patients[84,85]. Some studies have determined that obesity in adulthood significantly shortened the overall survival of PC patients, whereas obesity at diagnosis was not associated with increased risk of death[84,85]. In another study, Sandini *et al*[86] assessed whether the evaluation of different body compartments and their relationships were associated with the development of major postoperative complications after pancreatoduodenectomy for cancer. It was found that the prevalence of sarcopenia (loss of muscle tissue related to aging) was 24.2%. Overall, sarcopenic obesity[86] and non-sarcopenic obesity[87] are strong predictors of major complications after pancreatoduodenectomy for cancer.

Obese PC patients have the poorest prognosis, and often develop chemoresistance. Obesity is recognized as a co-morbidity factor to cancer and there is great interest in understanding the mechanism linking this condition and cancer. In this regard, a recent study has found that obesity promoted desmoplasia associated with accelerated PC growth and impaired delivery/efficacy of chemotherapeutics through reduced perfusion *in vivo*[87]. Furthermore, the inhibition of angiotensin-Ⅱ type-1 receptor (AT1) reversed obesity-augmented desmoplasia and PC growth and improved response to 5-FU chemotherapeutic *in vivo*[87]. In addition, clinical studies have shown that excess weight alters PC microenvironment to augment the crosstalk between cancer associated adipocytes, tumor associated neutrophils, and pancreatic stellate cells, which subsequently lead to increased tumor progression and survival[87].

LEPTIN AND PC

A potential link between obesity and PC could be the major adipokine leptin. A crosstalk between leptin and Notch (an embryonic signaling pathway altered in PC) has been reported in PC lines. Moreover, leptin induces PC tumorspheres formation and expansion of PCSC[81,88]. Leptin is a small cytokine secreted by adipose tissue that is coded by the obese (*ob*) gene. Leptin has been the most studied adipokine since it was first cloned in 1994[89]. Leptin is an adipokine that regulates appetite, energy intake and expenditure. Leptin plays many roles, some of which involve regulation of glucose homeostasis, growth response, reproduction and immune response[90]. The level of circulating leptin is proportional to total body fat. Obese patients exhibit high circulating levels of leptin due to leptin resistance[91]. Leptin is a pleiotropic adipokine and pro-inflammatory molecule that belongs to the family of helical cytokines. It is structurally similar to interleukin (IL)-6, IL-12, IL-15, prolactin, GH, oncostatin M, and granulocyte CSF[92].

Leptin receptor, OB-R, is a product of diabetic (*db*) gene that shows six alternatives spliced isoforms, including a long isoform (OB-RL, OB-Rb or LEPR) with full intracellular signaling capabilities, shorter isoforms with less biological activity (OB-Rs or OB-Ra) and a soluble isoform (OB-Re or sOB-R)[93,94]. Both the long and short isoforms of OB-R are expressed in PC cell lines[4]. Moreover, PC cells secreted leptin and expressed OB-R, which indicates a leptin autocrine/paracrine signaling loop could also affect tumor progression[95]. The binding of leptin to OB-R activates a cascade of events that promotes tumor progression and cancer cell survival[3,30,31]. Leptin binding to its receptor triggers an activation cascade of several canonical (JAK2/STAT3, MAPK, PI-3K/AKT1) and non-canonical signaling pathways (p38MAK, JNK and AMPK)[29,88,96].

A nested case control study from three cohort studies of middle-aged adults showed that high pre-diagnostic circulating leptin concentrations were associated with an increased PC risk among those with longer follow-up[97]. In another study, Mendonsa *et al*[4] showed the contribution of obesity and leptin to PC growth by using an *in vivo* orthotopic murine PC model. These studies revealed the increase of tumor growth in diet-induced obese mice when compared to lean mice.

We have recently showed that leptin and Notch crosstalk could influence PC progression. Our data suggest that a functional leptin-Notch axis affects PC progression and expansion of cancer stem cells (PCSC) in PC cell lines (BxPC-3, MiaPaCa-2, Panc-1, AsPC-1) and derived tumorspheres. Leptin treatment increased cell cycle progression and proliferation, and the expression of Notch receptors, ligands and targeted molecules (Notch1-4, DLL4, JAG1, Survivin and Hey2), PCSC markers (CD24/CD44/ESA, ALDH, CD133, Oct-4), ABCB1 protein, as well as tumorsphere formation. PC has no targeted therapy and is mainly treated with chemotherapy, whose efficiency could be decreased by leptin and Notch activities. Thus, the leptin-Notch axis could be a novel therapeutic target, particularly for obese PC patients[95].

LEPTIN-HDAC-MICRORNA-CANCER STEM CELLS CROSSTALK

Resistance to leptin is observed in obese people who show high levels of leptin. Precise reasons explaining why some obese patients are leptin-resistant are not fully known. Some studies have suggested that leptin resistance could be due to abnormalities of the leptin molecule while others believe the resistance might be due to impairment of OB-R

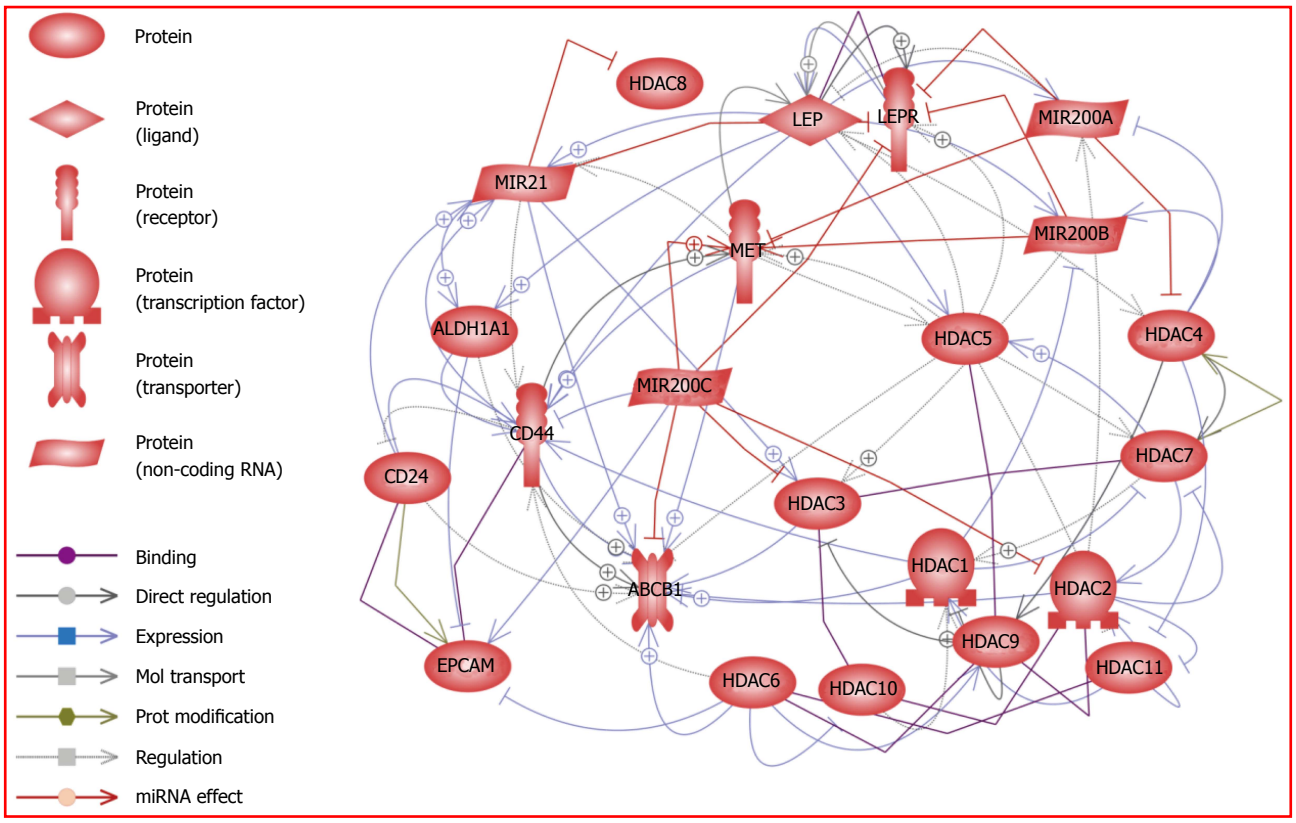

Figure 2 Potential crosstalk between leptin signaling, cancer stem cells, histone deacetylases and microRNA. Leptin and its receptor LEPR (OB-R) are involved in the regulation of PCSC markers, Classical HDAC, miR21, and miR200a/b/c. HDAC 1, 2, 3, 8: Histone Deacetylases Class I; HDAC 4, 5, 7, 9: Histone Deacetylases Class IIA; HDAC 6,10: Histone Deacetylases Class IIB; HDAC11: Histone Deacetylase Class IV. MiR200a, miR200b, and miR200c: Tumor Suppressors MicroRNA; MiR21: Oncogenic MicroRNA. CD24, CD44, ALDH1A1, ABCB1, MET, and EPCAM: Pancreatic Cancer Stem Cell Markers. HGF: Hepatocyte growth factor cytokine; LEP: Leptin adipokine; LEPR: Leptin receptor. Data generated from Pathway Studio (Pathway Studio – web; Ariadne Genomics, Inc.). Genes were analyzed by Pathway Studio 11 software (Elsevier, Inc., Atlanta, GA, United States) for disease, cellular processes and miRNA interactions. Only genes that had a P value of 0.05 were reported in this study. Specific references supporting these relationships are shown in Supplemental Table 1. References found by Pathway Studio were exported into an Excel file, column D, that contains the PMID number for the citations.

function or deficient leptin transport. Leptin is a known proliferation factor for cancer[3,29,31,96]. Analysis of data from Pathway Studio Platform shows that leptin signaling could promote PC through crosstalk mechanisms that involve PCSC, classical HDAC, oncogenic microRNA21, and tumor suppressor microRNA200a/b/c (Figure 2 and Supplemental Table 1).

A relationship between leptin signaling and miR21 in cutaneous wound healing was earlier reported[98]. The expression of miR21 and miR200 was previously linked to leptin hypothalamic signaling[16,99,100]. It was shown that the use of a pegylated leptin antagonist predisposed the rats to obesity and promoted leptin resistance in the both hypothalamus and liver. RT-PCR data from these studies showed that miR200 was upregulated in rats treated with leptin antagonist[16]. Additionally, miR21 (oncogenic) and miR200 (tumor suppressor) have been shown to affect PC progression[20,63]. The potential relationships between leptin signaling and miR21 and miR200a/b/c regulation in PC are shown in Figure 2. Leptin signaling is involved in the crosstalk to many important oncogenic and tumor suppressor molecules. Previous studies have determined that leptin increases the expression of PCSC markers ALDH1 and CD44. Leptin has also been found to increase the expression of miR21 while the tumor suppressors miR200a, miR200b, and miR200c decrease the expression of OB-R. Interestingly, these tumor suppressors could also interact with some of PCSC markers (Met, ABCB1, CD44), which decrease their expression. In contrast, oncogenic miR21 increases the expression of ALDH1, ABCB1 and CD44 markers. With regard to the classical HDAC, only HDAC5 and HDAC4 were reported to be directly regulated by leptin signaling (Figure 2). However, leptin signaling could indirectly affect the expression of some of HDAC *via* microRNA or PCSC markers. Further analysis suggests that leptin increases the expression of miR21, which, in turn, could increase the expression of HDAC3. The combined action of these factors could promote cancer proliferation and the expression of an anti-differentiation phenotype (Supplemental Table 1).

Our published data show that leptin increased PCSC populations that correlated with growth of PC tumorspheres and resistance to gemcitabine[88,95]. Furthermore, leptin induced PCSC populations ($CD24^+CD44^+ESA^+$, $CD133^+$, $ALDH^+$) in MiaPaCa-2 PC cells. Additionally, in Panc-1 cells, leptin increased mostly $CD133^+$ PCSC. Moreover, leptin increased ABCB1 (an ATP Binding Transporter Protein linked

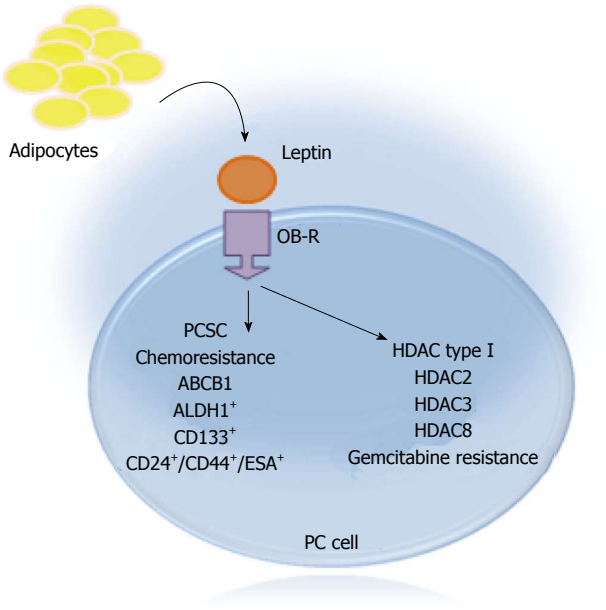

Figure 3 Leptin effects on pancreatic cancer stem cells and histone deacetylases in pancreatic cancer tumorspheres. Representative cartoon of the effects of leptin on PC cells *in vitro*. Leptin induced the expression of PCSC markers ($CD24^+/CD44^+/ESA^+$, $CD133^+$ and $ALDH1^+$). Leptin also increased the levels of ABCB1 [P-glycoprotein 1 or multidrug resistance protein 1 (MDR1) or ATP-binding cassette sub-family B member 1], which is involved in chemoresistance. Additionally, leptin induced the expression of HDAC type I (HDAC 2, 3 and 8). Leptin attenuates the cytotoxic effects of gemcitabine on PC. PC cells were cultured in low attachment plates containing mammocult media (Stem Cell Technol.), which allow the growth of tumorspheres. The tumorspheres were treated for 6 d with leptin (1.2 nmol/L), IONP-LPrA2 (a leptin antagonist bound to iron oxide nanoparticles; 0.0072 pmol/L), and gemcitabine (2 μmol/L). PC viability, PCSC markers and HDAC expression were determined by flow cytometry. Experiments were repeated three times[32,33,81,95].

to chemoresistance) expression in PC tumorspheres[95]. These data suggest that leptin could play a role in the induction of PCSC and PC chemoresistance (Figure 3).

Several studies have found that classical HDAC are overexpressed in PC. Therefore, HDAC inhibition has become a potential target therapy for cancer[101,102]. Intriguingly, high expression of HDAC Class Ⅰ and Ⅱ in PC could be associated with obesity. It was found that the hypothalamic expression of classical HDAC was increased in obese mice fed a high fat diet[100,103]. Thus, it is possible that the increase in classical HDAC in obese mice could be related to leptin signaling. To initially explore the potential relationships between leptin signaling and HDAC, leptin effects on HDAC expression was preliminary determined in PC tumorspheres. Results from these experiments show that leptin increased the expression of HDAC3 and HDAC8 in BxPC-3 tumorspheres (Figure 3). Furthermore, preliminary data suggest that gemcitabine decreased the expression of HDAC2, HDAC3 and HDAC8 that was reverted by leptin. This suggests that leptin could affect the expression of HDAC in PC, which might be associated with chemoresistance.

CONCLUSION

PC is an aggressive disease commonly detected in its late stages, continues to show poor prognosis, and has no targeted treatment. Surgical tumor removal is the best option to eliminate PC, but only in limited number of cases. Therefore, most PC patients are treated with chemotherapeutics, but survival rates have historically been poor. Obesity is a modifiable risk factor of PC that is characterized by inflammation and high levels of the adipokine leptin, which is a cancer proliferation factor that can also contribute to chemoresistance. Studies have identified that the dysregulation of HDAC, miR21, miR200, leptin, and PCSC could play important roles in PC progression. Previous reports showed that leptin signaling can induce PC proliferation, PCSC expand and regulate miR21, miR200, and HDAC levels. Moreover, the analysis of data from PC biopsies (Cancer Genome Atlas)[71] showed inverse expression profiles for miRNA21 and miRNA200 that suggests these molecules could be involved in PC development. Furthermore, HDAC, miRNA21/200, and leptin could have complex signaling crosstalk, according to Pathway Studio analysis. Therefore, leptin, miR21, miR200 and HDAC could be involved in PC progression. Thus, the potential crosstalk among these molecules could be a novel target for PC prevention or treatment, particularly in obese patients who show elevated levels of leptin.

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REVIEW

Multidisciplinary approach of colorectal cancer liver metastases

Giammaria Fiorentini, Donatella Sarti, Camillo Aliberti, Riccardo Carandina, Andrea Mambrini, Stefano Guadagni

Giammaria Fiorentini, Donatella Sarti, Onco-Ematology Department, Azienda Ospedaliera "Ospedali Riuniti Marche Nord", 61122 Pesaro, Italy

Camillo Aliberti, Riccardo Carandina, Oncology Radiodiagnostics Department, Oncology Institute of Veneto, Institute for the Research and Treatment of Cancer, 35128 Padova, Italy

Andrea Mambrini, Oncology Department, Carrara General Hospital, 54033 Carrara, Italy

Stefano Guadagni, Department of Applied Clinical Sciences and Biotechnology, Section of General Surgery, University of L'Aquila, 67100 L'Aquila, Italy

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Correspondence to: Dr. Giammaria Fiorentini, Onco-Ematology Department, Azienda Ospedaliera "Ospedali Riuniti Marche Nord", Via Lombroso 1, 61122 Pesaro, Italy. g.fiorentini@alice.it
Telephone: +39-0721-364005
Fax: +39-0721-364094

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Abstract

Large bowel cancer is a worldwide public health challenge. More than one third of patients present an advanced stage of disease at diagnosis and the liver is the most common site of metastases. Selection criteria for early diagnosis, chemotherapy and surgery have been recently expanded. The definition of resectability remains unclear. The presence of metastases is the most significant prognostic factor. For this reason the surgical resection of hepatic metastases is the leading treatment. The most appropriate resection approach remains to be defined. The two step and simultaneous resection processes of both primary and metastases have comparable survival long-term outcomes. The advent of targeted biological chemotherapeutic agents and the development of loco-regional therapies (chemoembolization, thermal ablation, arterial infusion chemotherapy) contribute to extend favorable results. Standardized evidence-based protocols are missing, hence optimal management of hepatic metastases should be single patient tailored and decided by a multidisciplinary team. This article reviews the outcomes of resection, systemic and loco-regional therapies of liver metastases originating from large bowel cancer.

Key words: Colorectal cancer; Chemoembolization; Liver metastases; Hepatic resection; Colorectal cancer liver metastases; Chemotherapy; Arterial infusion chemotherapy; Radioembolization

Core tip: Improvements of colorectal cancer liver

metastases (CRC-LM) treatment allows the down-staging of several patients. There is currently no agreement in the correct sequence of surgical resection of the primary cancer and metastatic disease. Surgical resection can be performed if the complete removal of cancer is achievable, leaving an adequate normal liver tissue. Neoadjuvant chemotherapy is widely accepted as primary therapy. Chemotherapy may lead to disease regression for unresectable CRC-LM, allowing resection and cure. The application of loco-regional therapies is increasing. They are recommended as third-line treatment for unresectable CRC-LM and have a palliative intent.

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INTRODUCTION

Colorectal cancer (CRC) is an increasing global health issue[1,2] It is the most common gastro-intestinal tumor and the third most frequently diagnosed malignancy worldwide. It has a mortality rate of up to 10%[1,2]. Most recent epidemiological data show more than 1.4 million newly diagnosed CRC each year[1,2].

The liver is the most common site of CRC metastases with an incidence of 15%-20% at diagnosis. CRC patients have a > 50% probability of liver metastases development[3]. The majority of CRC liver metastases (CRC-LM) were defined not resectable in the past century. Surgery methods are considerably improved nowadays, resulting in cure or survival increase. CRC-LM resection rates are also increased[4]. Recent updating of resectability criteria of CRC-LM considerably improves outcomes, resulting in 5 and 10-year survival rates of 40% and 25% respectively[5,6].

Notwithstanding these good outcomes, the recurrence rate one year after metastasis resection is 30% and a recent study on CRC-LM survival after resection shows a 5-year survival of 16%-71%[7].

Neoadjuvant chemotherapy allow initially unresectable CRC-LM patients to have long term survival similar to those of resectable patients[8-12]. Chemotherapy efficacy, in terms of tumor reduction, is strongly correlated to resectability[10-13]. For this reason, chemotherapy associated to biological agents is increasingly used as resectability conversion of CRC-LM from unresectable to resectable. This method can efficiently increase downsizing rates[14,15].

Candidate selection for resection is still difficult and several CRC-LM patients are never referred to hepatobiliary multidisciplinary group[10,13]. For this reason CRC-LM patients need a multidisciplinary team for treatment decision. This team should include specialists from different disciplines: Oncology, surgery, radiology and radiotherapy. The purpose of this review is to examine the current management of CRC-LM, in order to better define potential advantages and limitations of the several available treatments.

PERIOPERATIVE EVALUATION

The perioperative evaluation of a patient's global health and liver function is essential to reduce postoperative complications. A dedicated multidisciplinary team should assess co-morbidities and patient's performance status in order to decide a future treatment plan. Complete blood examination should be performed before surgery, to assess liver function [alanine aminotransferase (ALT), glutamic-oxalacetic transaminase (AST)], coagulation profile, bilirubin, creatinine and tumor markers, such as carcinoembryonic antigen (CEA).

Exclusion criteria for surgery include several factors to guarantee patient safety. They include advanced age, male gender, low serum albumin, presence of liver disease (hepatitis or alcoholic hepatitis), ascites, kidney or cardiologic impairment, bleeding syndromes, and chronic obstructive pulmonary disease[16-18].

Morbidity and mortality after liver resection is often due to inadequate function of remnant liver, leading to liver failure. Morbidity and mortality rates are around 61% and 11%, respectively[16,17].

The remnant liver cannot sustain metabolic, synthetic, and detoxifying functions if reduced below a critical liver volume[18]. Liver volume is not the best index for liver functionality assessment[16-20]. Patients with concomitant liver disease may have impaired liver regeneration capacity due to cirrhosis, steatosis, or jaundice obstruction[20].

Most chemotherapeutic agents (5-fluorouracil, irinotecan, oxaliplatin) can result in hepatic damage and modification of liver regeneration[11,19-22].

Morbidity and mortality after liver resection may be improved by measuring the intake of 99mTc mebrofenin of tumor-free liver in a pre-operative setting, in order to assess the risk of liver failure and liver failure-related mortality after partial liver resection[17].

During liver regeneration induced by partial hepatectomy, normally quiescent hepatocytes start to replicate in order to restore the original liver. Several genes are involved in liver regeneration, including cytokine, growth factor and metabolic genes[21]. Several studies show that recurrence and progression are directly proportional to the amount of liver resected[22,23].

Neoadjuvant chemotherapy may induce hepatic changes, such as steatohepatitis, hepatic sinusoidal obstruction and periportal inflammation, negatively affecting patient outcome[20,21] and increasing the risk of liver failure and death after major liver resection. A normal liver can bear an extensive resection. Severely compromised livers, on the contrary, cannot tolerate even a minor hepatectomy[8,9,19]. For this reason, monitoring the functionality of surrounding tumor-free liver needs to be highly considered for selection of surgical method.

RADIOLOGICAL ASSESSMENT

CRC-LM radiological study is necessary for assessment of surgical resectability. This can be performed using any of these main radiological methods: Magnetic resonance imaging (MRI), computed tomography (CT) and positron emission tomography (PET) scan[24]. Liver metastases can be detected as hypoattenuating lesions, when using contrast-enhanced normal or multidetector CT scans with a sensitivity rate of 85% and 90%, respectively[25]. MRI performed with liver-specific contrast agents has > 90% sensitivity in cases of underlying liver disease (steatosis, cirrhosis) or very small lesions (< 1 cm). For this reason MRI is better than CT for metastasis detection[26].

Specificity of CT, MRI and PET is very high: 95%, 93%, and 97% respectively. PET scan is useful to obtain whole body map, to identify extrahepatic disease (EHD) and to assess resectability[24]. A recent study showed that the FDG PET scan is the best radiological modality for detecting CRC-LM. It can have high false negative rates in patients recently treated with chemotherapy[17,18]. The association of CT to FDG PET scans is highly recommended because it improves the sensitivity up to 97%[24].

Nowadays, also intraoperative ultrasound (IOUS) is a mandatory surgical tool to confirm preoperative investigations by CT or MRI and for detection of missed lesions[27].

CRITERIA FOR RESECTABILITY

The preferred therapy for CRC-LM is surgery, providing up to 50% survival at five-years[28]. Patient selection criteria for resectability are not standardized and still controversial in clinical practice. The American Hepato-Pancreato-Biliary Association (AHPBA) consensus on definition of resectability is currently accepted by most liver surgeons[28,29]. Main CRC-LM resection criteria of AHPBA are: Presence of disease confined to the liver as identified after surgery of primitive cancer; disease in a single hepatic lobe; < 3 nodules; the largest size of nodules < 5 cm in diameter; margin FLR > 1 cm. According to these criteria, however, less than 10% of patients would be indicated for resection.

The classification of resectable disease is broader nowadays, increasing the number of resections[30]. Current guidelines generally agree that resection should be performed for liver metastases only[12,30,31], but hepatectomy and resections of concomitant extrahepatic disease are considered[32]. The remaining liver must be undamaged and at least 20% or 25% of the whole hepatic volume, and have a full functional vascular and biliary in- and out- flow. In this case also multiple resection can be performed[8,14,30]. The survival advantage of repeated resection is close to that after the surgery of primary hepatic disease[33]. Hepatic resection safety depends on: Age of patients, performance score, and concomitant hepatic impairments. Resection is contraindicated when the following are observed: Non resectable extra hepatic tumor; wide involvement of parenchyma; or patient's poor general conditions.

Possible prognostic factors of resection outcome of CRC-LM are: Age, sex, synchronous or metachronous hepatic metastases, tumor size, number and distribution of LM, primary tumor stage, extrahepatic distant metastases, surgical margin, type of primary hepatic tumor surgery and previous tumor pharmacological therapy, levels of tumor markers.

Fong *et al*[34] report data from 1001 CRC-LM patients who were candidates for resection. These data led to the identification of seven criteria for worse prognosis prediction after resection. Five of these criteria are actually used for the Clinical Risk Score (CRS) that is a preoperative scoring system. These criteria are: Disease-free interval from primary to metastases < 12 mo; largest hepatic tumor > 5 cm in diameter; node-positivity; number of lesions > 1; and CA 19-9 > 200 ng/mL. Positive prognosis after surgery corresponds to a score < 2. Scores of 3-4 indicate that patients are candidates for resection followed by adjuvant therapy. Prognosis is poor when the score is five. The appropriateness of CRS is proved. CRS can predict patients' response and OS[35].

A new method has been recently introduced in the CRC-LM resectability criteria assessment[5]. Resection criteria are different. They depend less on the size, number, and location of the metastases. They give more importance to the volume and function of the future liver remnant (FLR), which should be > 25% estimated normal liver parenchyma or 30% in the presence of impaired liver function[36]. Metastases are considered resectable if the excision of all metastatic lesions can be obtained with an adequate FLR[37] and the presence of EHD is currently no longer considered as a contraindication[5]. The new requirements for LM resection are: R0 resection achievement of intrahepatic and extra hepatic disease; adequate FRL; and > 2 adjacent liver segments to be spared with blood and bile inflow and outflow preservation[31,37].

TIMING OF COLON AND LIVER RESECTION

The best sequence and timing of CRC-LM resection is still under debate and many options are available. The use of up front chemotherapy is increasing. Strong evidence is missing and there are currently no randomized controlled trials comparing the different approaches[38].

The classic surgical method is "primary first", whose suggested sequence is to firstly resect the primary CRC, then to administer the chemotherapy and after 3-6 mo to eventually resect the LM. This approach is indicated for patients with advanced or symptomatic CRC, important comorbidities, or inadequate FLR. In cases of advanced CRC, indeed, the chemotherapy may be associated with high complication rates and the insurgence of disease progression may lead to unresectability[39]. Any delay correlated to complications during surgery of CRC may also increase the risk of progression occurrence for some patients[40,41]. A possible benefit of this method can be

Table 1 Recommendations for perioperative and conversion therapy (adapted from ESMO 2016[110])

Perioperative treatment
It is defined by technical criteria for resection and prognostic considerations
It may not be necessary in patients with clearly resectable disease and favourable prognosis, in this case upfront resection is justified
It should administer FOLFOX or CAPOX to patients with resectable disease and unclear (probably unfavourable)
Targeted agents should not be used in resectable patients with prognostic indication for perioperative treatment
It should be considered when prognostic and resectability criteria are unclearly defined, and in patients with synchronous onset of metastases
Adjuvant chemotherapy is not strongly indicated for patients with favourable oncological and surgical criteria, who did not receive any neoadjuvant chemotherapy
Adjuvant chemotherapy is indicated for patients with unfavourable criteria
Adjuvant treatment with FOLFOX or CAPOX is recommended for patients who have not received any previous chemotherapy, unless patients already received oxaliplatin-based adjuvant chemotherapy
The choice of chemotherapy type should consider patients' clinical conditions and therapy preferences
Conversion therapy
A chemotherapy regimen leading to high response rates and/or a large tumour shrinkage is recommended for potentially resectable patients
The best drug combination to use is still not clear because only few trials have addressed this issue:
RAS wild-type patients may benefit from a cytotoxic doublet plus an epidermal growth factor receptors agents antibody (best benefit/risk), and from the combination of FOLFOXIRI plus bevacizumab and, to a lesser extent, from a cytotoxic doublet plus bevacizumab
RAS mutant patients may benefit from a cytotoxic doublet plus bevacizumab or FOLFOXIRI plus bevacizumab
Patients must be re-evaluated regularly (every 2-3 mo) to prevent the overtreatment of resectable patients

the possibility to identify previously occult LM that may become visible during adjuvant chemotherapy. This allows avoidance of the morbidity of a liver resection.

Another surgical method is the "synchronous resection of LM and primary CRC". This approach can avoid delays in chemotherapy treatment that can be started earlier if no complications occur after surgery. The possible disadvantage of this method is the increased postoperative morbidity and mortality because of infection resulting from bacterial contamination of the surgical field[36]. For this reason this approach is indicated for patients who can tolerate long operative times[6].

The third available surgical method is the "alternative staged liver-first" approach that firstly resect the LM, then administer 3-6 cycles of chemotherapy, and at last resect the primary CRC. Adjuvant chemotherapy can be administered in between both procedures. Recent data report that this method is indicated for selected patients with advanced CRC-LM, and when neo-adjuvant and adjuvant chemotherapy may have better results[9,12].

CHEMOTHERAPY FOR RESECTABLE CRC-LM

Neo-adjuvant chemotherapy

The utility of neoadjuvant chemotherapy for CRC-LM is unclear even if there is the tendency to use it frequently[15]. There are many advantages of neo-adjuvant treatment such as increasing tumor sensitivity, downstaging large or multiple liver lesions, increasing resectability, and treating micrometastases[8,9,11]. This therapy also allows better planning for the date of surgical resection.

On the other hand, neo-adjuvant chemotherapy can delay surgical treatment, which may be detrimental for patients, increasing the risk of disease progression[12,15]. This chemotherapy can also induce liver toxicity, such as steatohepatitis, increasing postoperative mortality. It can also mask metastases on preoperative imaging, as is observed in 5%-25% of cases[42].

Perioperative chemotherapy is widely used for patients with unresectable disease (Table 1 and Figure 1) with the purpose of reducing disease progression, which occurs in 50%-70% of patients after surgery[3]. A multicentre randomized trial compared surgery alone with perioperative chemotherapy (6 cycles of preoperative and post operative FOLFOX4) in 364 unresectable CRC-LM patients. The results of this study showed no significant differences in five-year OS for the two groups; nevertheless, progression-free survival (PFS) increased by 7.3% at 3 years in the perioperative chemotherapy group[43]. The rate of post-operative complications is also increased and is directly proportional to the length of therapy. For this reason, it is suggested that only 6 cycles of chemotherapy for no longer than 3 mo should be performed, in order to reduce toxicity[28], especially for patients who need a major hepatectomy[44].

Patients with more than 3 lesions, and tumor diameter greater than 3 cm are clearly indicated for this treatment. The surgery of lesions should be done 4-8 wk after the neo-adjuvant chemotherapy. In summary, the advantages of neo-adjuvant chemotherapy outnumber the disadvantages, and we are in favor of its utilization.

Adjuvant chemotherapy

The ultimate dilemma after complete CRC-LM resection is the rate of recurrence that is reported as high as 60% after complete surgical excision. Several studies show the benefits of adjuvant therapy such as FOLFOX4 (folinic acid, fluorouracil, and oxaliplatin), resulting in longer disease-free-survival (DFS)[45] than liver resection alone.

Adjuvant chemotherapy also increases OS when compared to surgery alone, even if the difference is not statistically significant[46,47].

The classic adjuvant chemotherapeutic drugs are: 5-fluorouracil/leucovorin (5-FU/LV), capecitabine, oxaliplatin and irinotecan[47]. New molecular-targeted agents are now available. They include anti-angiogenic drugs (bevacizumab, regorafenib and aflibercept) and anti-

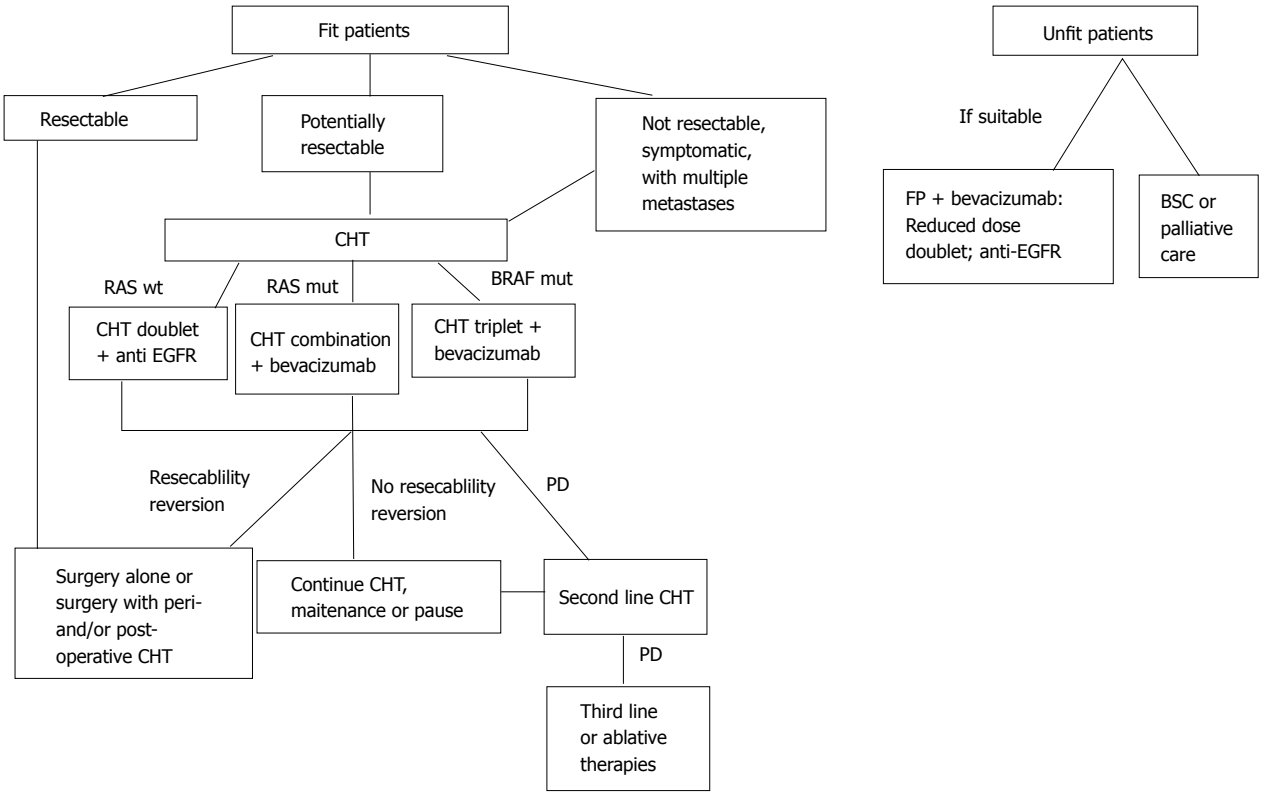

Figure 1 Treatment indications for fit and unfit colorectal cancer liver metastases patients. BSC: Best supportive care; CHT: Chemotherapy; EGFR: Epidermal growth factor receptors agents; mut: Mutated; FP: Fluoro pyrimidine. Adapted from ESMO 2016[110].

epidermal growth factor receptors agents (anti-EGFR), such as cetuximab and panitumumab. These agents are widely used as adjuvant treatment without any evidence of clinical benefit[48].

Adjuvant chemotherapy after metastasectomy is generally recommended by clinicians, even if the best regimen protocol is still unclear, and should be considered in a patient dependent manner[24]. There, efficacy of adjuvant chemotherapy on OS for resectable CRC-LM is still under discussion[45]. The National Comprehensive Cancer Network (NCCN) guidelines suggests the use of more than one chemotherapy line[48]. Most study agree that 5-FU/LV with or without oxaliplatin should always be used as first-line[47]. More recently, however, the use of combination therapy is increasing, and several combinations have emerged.

A recent study on FOLFIRI (5-FU/LV and irinotecan) *vs* 5FU/LV after R0 (complete resection) of CRC-LM does not report any difference in OS and median DFS. FOLFIRI improves DFS, but causes more frequent grade 3/4 toxic adverse events (47% *vs* 30%)[49].

We suggest the use of adjuvant chemotherapy in patients with multiple lesions that are found in more than 3 liver segments, where the surgery, even if radical, may not be able to remove undetected tumor deposits.

CHEMOTHERAPY FOR UNRESECTABLE CRC-LM

Patients with unreserctable CRC-LM from diagnosis should receive chemotherapy in order to downstage the disease and allow the surgery (Figure 1 and Table 2).

About 70% of patients with CRLM are unresectable at diagnosis[4]. They have a complicated disease, often requiring a combination of loco-regional therapy (chemoembolization, hepatic arterial infusion, ablation or radiation).

Perioperative chemotherapy is widely used also for unresectable CRC-LM, even if there is no proof of OS improvement[50]. Systemic chemotherapy remains the first-line therapy. FOLFOXIRI followed by surgical resection has a 70.4% response rate, and 19% of patients obtain R0. OS at 5 and 8 years are 42% and 33% respectively, and 29% of patients are disease free at 5 years[51].

Downstaging of unresectable CRC-LM ranges from 5% to 38%. This is due to multiple factors including disease extension, type and duration of chemotherapy[51]. The purpose of the "conversion chemotherapy" in unresectable CRC-LM patients is to convert their disease to resectable, and is often the first line treatment. Standard regimens include FOLFIRI or FOLFOX that induce downstaging in 7%-40% of patients[12]. Giacchetti's group reports that FOLFOX reduces the LM dimension by more than 50% in 59% of non-resectable CRC-LM, resulting in 38% of CR[52]. FOLFOXIRI allows 36% of R0 in LM patients[53]. The METHEP trial reports that FOLFIRINOX seems to be better therapy for CRC-LM than the others, bringing to resection 67% of cases with a survival > 48 mo. These results confirm that OS is greater for patients after R0 or

Table 2 Conversion rates in colorectal cancer liver metastases after perioperative chemotherapy

Trial name	Chemotherapy type	Control	*n*	KRAS status	Overall response	Conversion to resection	R0 resection
BEAT[61]	FOLFOX/XELOX/FOLFIRI or fluoropyrimidines + bevacizumab	No	1914	Not selected	NA	11.80%	NA
First BEAT[62]	FOLFOX/XELOX + bevacizumab	Placebo	1914	Not selected	38%	11.80%	6.3% *vs* 4.9%
OPUS[70]	FOLFOX + cetuximab	FOLFOX	233	Wilde type	61% *vs* 37%	9%	4.7% *vs* 2.4%
POCHER[72]	Chr IFLO + cetuximab	No	43	Wild type	79%	60%	25.70%
PRIME[77]	FOLFOX + panitumumab	FOLFOX	591	Wild type	57% *vs* 48%	31% *vs* 22%	29% *vs* 17%
CELIM[11]	FOLFOX6 + cetuximab	FOLFIRI + cetuximab	106	Wild type	68% *vs* 57%	43%	38% *vs* 30%
BOXER[63]	CAPOX + bevacizumab	No	47	Not selected	78%	40%	NA
Loupakis *et al*[55]	FOLFOXIRI + bevacizumab	FOLFIRI + bevacizumab	508	Not selected	65% *vs* 53%	15% *vs* 12%	NA
Ye *et al*[73]	FOLFIRI + cetuximab	FOLFOX + cetuximab	177	Wild type	57% *vs* 29%	26% *vs* 7%	NA
CRYSTAL[71]	FOLFIRI + cetuximab	FOLFIRI	599	Wilde type	47% *vs* 39%	16%	4.8% *vs* 1.7%
OLIVIA[79]	FOLFOXIRI + bevacizumab	FOLFOX + bevacizumab	80	Not selected	81% *vs* 62%	61% *vs* 49%	49% *vs* 23%

CAPOX, XELOX: Capecitabine-oxaliplatin; NA: Not available; Chr IFLO: Chronomodulated irinotecan, 5-fluorouracil, leucovorin, and oxalipaltin; FOLFIRI: 5-fluorouracil, leucovorin and irinotecan; FOLFOX: 5-fluorouracil, leucovorin, and oxalipaltin; FOLFOXIRI: 5-fluorouracil, leucovorin, oxalipaltin and irinotecan.

R1 surgery, 65.2 mo *vs* 18.3 mo of not-operated or R2 patients[54].

The use of bevacizumab is increasing for unresectable CRC-LM[55,56], even if the benefits are extremely limited. A slight gain in response rate is observed when bevacizumab is associated with FOLFOXIRI as first line chemotherapy. The association of bevacizumab to first and second line chemotherapy for CRC-LM improves PFS[57-60] and OS in some studies[59,60]. Available data on the efficacy of bevacizumab associated to perioperative chemotherapy are limited. This may be due to concerns about possible complications in wound healing after resection[61,62]. The Bevacizumab Expanded Access Trial reports good feasibility of LM surgery after first-line chemotherapy associated to bevacizumab, resulting in resection rates of 11.8% and 6% of R0[63]. Bevacizumab association with FOLFOX, however, obtains higher resection rates (16.1%) than with FOLFIRI (9.7%), and higher R0 (6.3%) than FOLFOX plus placebo (4.9%) (P = 0.24)[62]. Neoadjuvant capecitabine and oxaliplatin (CAPOX) plus bevacizumab resulted in 40% of CRC-LM resectability conversion[63]. Loupakis *et al*[55] report 64% of tumor response and 15% of rate of resection of CRC-LM after FOLFOXIRI plus bevacizumab, *vs* 53% and 12% respectively after FOLFIRI/bevacizumab.

Transarterial chemoembolization with irinotecan combined with FOLFOX plus bevacizumab chemotherapy results in a response rate of 78%, and allows resection of 35% of non resectable CRC-LM, offering a new cure option to these patients[64].

A recent report by Stremitzer *et al*[65] shows that mutated BRAF/RAS are correlated to a poor outcome after CRC-LM surgery. This is in agreement with the results of other 3 studies[66-69]. These important evidences support the application of newer methods for the therapy of liver metastases, associating biological molecular aspects (biological resectability) to the other clinical and pathological indexes for the selection of good surgical candidates and the prediction of their outcomes.

Anti-EFGR agents such as cetuximab and panitumumab are effective alone as well as in association with chemotherapy in CRC-LM that are RAS (both *KRAS* and *NRAS*) wild type[69]. Some randomized trials report the effects of cetuximab for the therapy of unresectable CRC-LM. The OPUS trial[70] showed that the association of FOLFOX-4 plus cetuximab as up front therapy doubled R0 (4.7%). The CRYSTAL study[71] showed that the association of FOLFIRI plus cetuximab as up front therapy increased the R0 resection rate from 3.7% to 7.0%. The CELIM trial[11] reported that neoadjuvant treatment with FOLFIRI plus cetuximab or FOLFOX6 resulted in 34% of R0 resections. Other studies also report that chemotherapy containing cetuximab significantly improves R0 in unresectable CRC-LM with *KRAS* wild-type[72,73]. There are differences in resection rates among the above studies. Overall response rate is in the range 60%-79%, however, resection rates after chemotherapy/cetuximab are very variable (Table 2). These discrepancies may be due to the fact that the resection rate is defined and determined by clinical conditions of the patients and not by specialist oncologists in CRYSTAL and OPUS studies. Resection evaluation is done by a multidisciplinary team in the other trials.

The COIN[74] and NORDIC Ⅶ[75] trials report no advantage for the association of oxaliplatin based chemotherapy/cetuximab in first-line treatment of CRC-LM, independently from K-RAS status.

Resection rates of first-line FOLFIRI/panitumumab treatment of CRC-LM are 15% and 7% in the *KRAS* wild type (WT) and mutant groups respectively[76]. FOLFOX4 plus panitumumab results in 32% of R0 resections *vs* 28% of those receiving only FOLFOX4[77]. A post hoc analysis of the PRIME study on RAS WT (*KRAS, NRAS*) shows that panitumumab/FOLFOX can convert to resection 31% of initially unresectable CRC-LM patients and lead to 29% of R0 (Table 2)[78]. A further analysis of PRIME trial also shows that NRAS mutations are indications of non-response to panitumumab[77]. For this reason, it is extremely important to analyze other

types of mutations in the RAS gene to improve patient selection for anti EGFR therapy.

The OLIVIA trial studies FOLFOXIRI + bevacizumab *vs* mFOLFOX-6 + bevacizumab and reports an overall resection rate of 61% *vs* 49%, with R0 resection rates of 49% *vs* 23%[79].

In conclusion "biologically directed" chemotherapy reduces the number and size of unresectable lesions. It also allows rescue of 15%-35% of patients, bringing them to surgery. These therapies are increasingly used worldwide.

EXTRA HEPATIC DISEASE

Extra hepatic disease (EHD) has a poor prognosis[28]. Most common sites of EHD from CRC are lymph nodes, lungs, peritoneum, brain and bone. EHD is currently no longer a contraindication to metastasis resection, and patients after surgery have[5] longer DFS and five-year-survival rates compared to those receiving only chemotherapy[5,6].

OS after lymph node resection is different according to their site and number[80]. Celiac or aorto-caval lymph node resections are associated with a worse outcome when compared to hepatic pedicle nodes, and mediastinal lymph nodes have a worse median survival than intra-thoracic ones[80]. A high number of lymph nodes positive for metastases have also a poor outcome[80].

In conclusion, the treatment of EHD is substantially palliative, aiming to improve the quality of life[81].

LOCO-REGIONAL THERAPIES

Loco-regional therapies (Figure 2) are indicated for patients that are elderly, have a poor performance status, refusing surgery or chemotherapy, or refractory to chemotherapy. They also allow chemo-holidays with suspension of chemotherapy, and prolong the non-treatment period in between different chemotherapy lines. This reduces the treatment costs in respect to systemic chemotherapy.

In the last years new strategies have been developed in order to overcome several problems: High percentage of unresectable CRC-LM at diagnosis, high recurrence rates and presence of extensive disease. These methods increase the number of patients indicated for non surgical procedures.

Ablation techniques include radiofrequency ablation (RFA), Microwave ablation and external beam radiotherapy (EBRT). RFA is widely used and allows the application of extreme temperature to ablate the lesion with minimal toxicity (< 1%) in the surrounding liver tissue. RFA results in mortality and morbidity < 10% independently from the administration route[82]. The "heat sink effect" is however a major disadvantage of RFA and may cause important hepatic or vascular injury. For this reason, RFA is not indicated for unresectable tumors, lesions near blood vessels or the diaphragm because of the high risk of perforation. Another disadvantage of RFA is the recurrence rate that is higher when the tumor is > 3 cm or when treatment is delivered percutaneously[82,83].

Microwave ablation uses high frequency microwave radiation to induce coagulation with necrosis of lesions. This method, however, is not well known and there are several concerns about its feasibility[84]. Available data on this method show a 6% local recurrence rate[85].

Improvements in imaging methods have increased the use of EBRT[86]; that, however, has a low therapeutic window, and toxicity is still a major issue. EBRT is safe (at 60 Gy) and effective for liver tumors in general and in selected patients[87,88].

Intra-arterial therapies: Hepatic artery infusion

Hepatic artery infusion (HAI) is indicated for patients with unresectable lesions when physicians want to associate an intra-arterial with an endovenous treatment.

The advantage of HAI is to minimize the toxicity to normal liver tissue, because the chemotherapeutic agents are injected directly to the tumor[89]. Potential risks of this method are treatable complications related to catheter and pump placement, or life-threatening complications such as biliary sclerosis, hepatotoxicity and systemic toxicity. For this reason it should be performed by experienced hospitals[89-91].

Intravenously 5-FU and intra hepatic artery oxaliplatin are successfully used[92] for unresectable CRC-LM. Best results concerning survival and response rates are obtained with floxuridine based HAI[93].

The comparison of OS between HAI therapy and systemic therapy alone (15.9 mo *vs* 12.4 mo) does not show any difference, however, there was a great response rate in favor of HIA (43% *vs* 18%)[94].

In conclusion, HAI has interesting results; however it is a cumbersome method because it requires the implantation of an infusion pump.

Chemoembolization

Trans-arterial chemoembolization (TACE) is increasingly used for unresectable CRC-LM, improving survival and tumor response[95]. TACE is indicated for unresectable CRC-LM as third line therapy, and allows the attainment of important palliative results.

The use of drug-eluting beads for TACE increases efficacy, while reducing adverse events due to systemic drug leakage or liver toxicity[95-98]. The advantage of these beads is the direct delivery of toxic drugs inside the arterial capillary bed of the tumor, releasing the drug in a controlled manner. In this way the systemic exposure to toxic drugs is reduced, their local concentration is increased and a greater tissue necrosis than classic TACE with lipiodol is obtained[99,100].

The indication for TACE is presence of multinodular LM, absence of extra hepatic disease, refractory to systemic chemotherapy[101].

Recent reports show that TACE with irinotecan (DEBIRI) for the treatment of CRC-LM is effective, feasible and has limited side effects[95-101]. Systemic chemotherapy (FOLFIRI) is compared to DEBIRI for

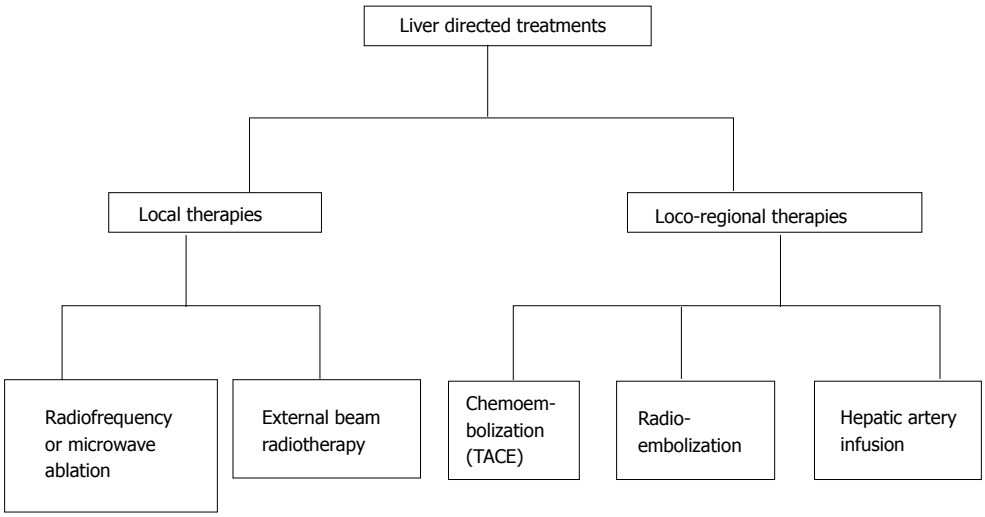

Figure 2 Liver directed treatments. TACE: Trans-arterial chemoembolization.

the therapy of refractory CRC-LM in some studies. This comparison shows that DEBIRI is statistically better than FOLFIRI in terms of OS, PFS, time to extra-hepatic progression, and quality of life[95].

The association of cetuximab and TACE with irinotecan is an improvement in the treatment of CRC-LM, because these drugs are efficacious and have acceptable, and not cumulative, toxicities[102].

The TACE methodology is constantly improving, in particular, the last innovation is the introduction of new embolics for drug delivery. Among the new types of microspheres there are polyethylene glycol (PEG) microspheres (LifePearls, Terumo), that are more resistant to stress and attrition. The advantages of these embolics are increased suspension time, better catheter deliverability and drug retention and release[103].

In a recent study we show the data of TACE with PEG embolics for the treatment of 20 cases of non resectable liver tumors and metastases from colorectal carcinoma, breast cancer and uveal melanoma. Irinotecan and doxorubicin are used for PLC and LM respectively. More than 80% of cases respond to TACE patients. We observe 63% of CR, and 37% PR. The chemoembolization procedure is well tolerated by all the patients with only mild or moderate adverse events. These results indicate that PEG embolics-TACE is effective and tolerable for the therapy of hepatic primary and metastatic cancer[103].

Radioembolization

In the last decade radioembolization (RE) with Yttrium 90 (Y90) has been widely used for the treatment of CRC-LM that are refractory to chemotherapy[104]. Objective tumor response rates of RE are 33%-48% in second line[105,106] and 10%-48% in third line[107-109]. Survival and progression free survival are also improved after RE application as third line[109]. RE with Y90 has, however, a low recommendation in the last ESMO guidelines[110].

The treatment decision is very challenging for CRC-LM patients that are refractory to chemotherapy. Several patients are unfit and have a biologically unfavorable progression often associated to comorbidities. Palliative care with chemo- or radio-embolization is indicated in these cases, in order to avoid too aggressive therapies.

MULTIDISPLINARY TEAM

The involvement of a multidisciplinary approach should be promoted in order to obtain the best CRC-LM management and outcomes, and to reduce peri-operative morbidity and mortality, prolonging OS and rising resection rates[110,111].

For this reason, the multidisciplinary team management of CRC-LM is growing in most Western countries[112]. The team includes different types of specialists including: Liver surgeons; interventional radiologists specialized in hepatobiliary disease; an oncologist; a pathologist; and a case manager nurse. They have to discuss each case to ensure resectability appropriateness and lead to down-staging wherever possible. The team should be consulted about the choice of chemotherapy combination and type of targeted agents and care to be used, timing of chemo-holidays, and follow up.

Medical oncologists select the most active treatment for the shortest time combining chemotherapy to targeted drugs, in order to reduce tumor size without damaging the normal liver. The definition of the acceptable FRL should be performed by a radiologist and a liver surgeon. Repeating the resection is safe and effective, obtaining survival rates close to those after first resection[112,113]. Finally the case manager nurse or the practitioner are important in patient's management, because they provide indications on the follow up and assistance.

CONCLUSION

Recent improvements of CRC-LM treatment allows the down-staging of several patients, resulting in increased number of patients cured or living with longer disease

control. There is currently no agreement about the correct sequence of surgical resection of the primary cancer and metastatic disease, however, the neoadjuvant chemotherapy is widely accepted as up front treatment.

Surgical resection can be performed if the complete removal of cancer is achievable leaving an adequate FRL. The use of adjuvant chemotherapy is highly suggested, even if standardized protocols are still unclear. The use of chemotherapy may lead to disease regression for unresectable CRC-LM, allowing resection and cure.

The application of loco-regional therapies is increasing, resulting in high tumor response, however, they are not recommended as first-line treatment in case of unresectable CRC-LM.

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REVIEW

Evolving role of Sorafenib in the management of hepatocellular carcinoma

Ioannis A Ziogas, Georgios Tsoulfas

Ioannis A Ziogas, Aristotle University of Thessaloniki School of Medicine, 54124 Thessaloniki, Greece

Georgios Tsoulfas, 1st Department of Surgery, Aristotle University of Thessaloniki, 56403 Thessaloniki, Greece

Author contributions: Ziogas IA and Tsoulfas G contributed equally to this work.

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Correspondence to: Georgios Tsoulfas, MD, PhD, FACS, Assistant Professor of Surgery, 1st Department of Surgery, Aristotle University of Thessaloniki, 66 Tsimiski Street, 56403 Thessaloniki, Greece. tsoulfasg@gmail.com
Telephone: +30-6971-895190
Fax: +30-2310-332022

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Abstract

Hepatocellular carcinoma (HCC) is one of the most common malignant diseases worldwide and comes third in cancer-related mortality. Although there is a broad spectrum of treatment options to choose from, only a few patients are eligible candidates to receive a curative therapy according to their stage of disease, and thus palliative treatment is implemented in the majority of the patients suffering from liver cancer. Sorafenib, a multikinase inhibitor, is the only currently approved agent for systemic therapy in patients with advanced stage HCC and early stage liver disease. It has been shown to improve the overall survival, but with various side effects, while its cost is not negligible. Sorafenib has been in the market for a decade and has set the stage for personalized targeted therapy. Its role during this time has ranged from monotherapy to neoadjuvant and adjuvant treatment with surgical resection, liver transplantation and chemoembolization or even in combination with other chemotherapeutic agents. In this review our aim is to highlight in depth the current position of Sorafenib in the armamentarium against HCC and how that has evolved over time in its use either as a single agent or in combination with other therapies.

Key words: Sorafenib; Hepatocellular carcinoma; Liver neoplasm; Multikinase inhibitor; Targeted therapy; Tumor angiogenesis; Signaling pathways; Adjuvant therapy; Liver cancer; Liver transplantation; Liver resection

Core tip: Hepatocellular carcinoma (HCC) is an aggressive and invasive malignancy. Curative options, such as resection and liver transplantation, are limited to only a few patients, who are suitable candidates. Sorafenib is the only approved systemic treatment in HCC, especially for advanced tumor stage and early stage liver disease. Recent findings suggest that it may also be helpful in carefully selected decompensated patients. Its adjuvant role is yet to be proven with more promising results. The combination of Sorafenib with other chemotherapy agents has shown improved efficacy and safety. We aim

to present the evolution of Sorafenib's use over the last decade.

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INTRODUCTION

Hepatocellular carcinoma (HCC), the most common primary malignant neoplasm of the liver (85%-90%)[1], is the sixth most frequent cancer in the world and the third cause of cancer-related[2]. Cirrhosis is the stage of chronic liver disease characterized by disrupted architecture of the liver, therefore resulting in its dysfunction over the time. Regardless of the cause leading to cirrhosis, it is a major condition predisposing to a malignant transformation of the liver eventually leading to HCC[3]. Nowadays, the incidence of HCC is increasing rapidly owing to the large number of people suffering from cirrhosis, mainly caused by hepatitis B and C virus infection, as well as due to longer survival among cirrhotic patients[1].

Equally important to the presence and stage of cirrhosis, is the stage of the HCC, as any treatment that will follow will be in accordance to that. Specifically, surgical resection, ablation and liver transplantation are the only acceptable potentially curative options, but as it turns out, despite screening and frequent follow-ups, only 40%-60% of cirrhotic patients are diagnosed with very early or early stage HCC, therefore being eligible for curative treatment[4,5]. Unfortunately, most patients are diagnosed with more advanced stage HCC, *i.e*., portal vein invasion and/or extrahepatic spread or general symptoms attributed to cancer, unresponsive to such modalities. As a result alternative treatment combinations and algorithms including embolization, chemotherapy, radiotherapy, molecular target therapy or immunotherapy are constantly being generated in order to improve the overall survival (OS) of such patients[4,6,7].

In particular, an aspect of systemic therapy tends to focus on an important characteristic of HCC, its angiogenesis, by developing antiangiogenic drugs that impede the formation of new blood vessels, thus inhibiting the proliferation and growth of the liver tumor[8]. Sorafenib, an antiangiogenic drug, is the first and currently the only chemotherapeutic regimen approved as a palliative type of treatment in advanced stage HCC[9]. This review describes the general characteristics of Sorafenib, its current place in the clinician's therapeutic armamentarium, as well as the clinical results of the evolving role of Sorafenib when combined or compared to other treatment modalities.

GENERAL PRINCIPLES

Molecular mechanisms

As stated above, HCC is a tumor with abundant vasculature and high heterogeneity, especially when it comes to the various signaling pathways involved[10]. One of the key pathways involved in the growth and proliferation of HCC is the Raf/MEK/ERK mitogen-activated protein (MAP) kinase cascade, which shows particularly increased activity[11]. This over-activation is mainly achieved by the combined action of hepatitis virus proteomics and growth factors, with platelet-derived growth factor (PDGF) and vascular endothelial growth factor (VEGF) playing a critical role and highlighting the linkage between angiogenesis and HCC development[12-14]. Sorafenib (Nexavar, BAY 43-9006), a biaryl urea, is an oral multikinase inhibitor of the serine/threonine-kinases (c-RAF and BRAF), therefore blocking the Raf/MEK/ERK pathway, and of the vascular endothelial growth factor receptor 2 (VEGFR2), VEGFR3, platelet-derived growth factor receptor (PDGFR), FLT3, Ret, and c-KIT[15]. Moreover, it has been shown to result in apoptosis in various human tumor cell lines, independently of its involvement in the Raf/MEK/ERK pathway, by: (1) down-regulating an anti-apoptotic protein, the myeloid cell leukemia-1 (Mcl-1), member of the Bcl-2 family; and (2) inhibiting the phosphorylation of eukaryotic translation initiation factor 4E (eIF4E), which normally, when phosphorylated, promotes the expression of oncogenic genes[16]. According to this rationale, Sorafenib is an effective drug against not only the tumor compartment, but also the formation of new vessels[17,18]. It's mechanism of action is illustrated in Figure 1.

Sorafenib's history

This therapeutic action was firstly assessed in an uncontrolled phase 2 clinical trial of 137 patients with advanced and unresectable HCC, not having received any prior systemic therapy and with Child Pugh (CP) A or B cirrhosis[19]. The dosage administered was 400 mg orally twice a day in 4-wk cycles with a partial response of 2.2%, a minor response of 5.8% and a 33.6% of the patients reporting non progressive disease for at least 16 wk. Some other major data reported were the 4.2-mo median time to progression (TTP) and the 9.2-mo OS, while CP A and B patients showed only negligible differences regarding the pharmacokinetics[19].

Such positive results could not but be followed by the international phase 3, randomized, double-blind, placebo-controlled "Sorafenib HCC Assessment Randomized Protocol" (SHARP) clinical trial[9]. For this purpose, 602 patients with advanced stage HCC, Eastern Cooperative Oncology Group (ECOG) performance status from 0 to 2, CP A liver disease and without any preceding systemic treatment, were randomized either for Sorafenib, same dosage as in phase 2, or for placebo. According to the data reported, Sorafenib resulted in a median OS of 10.7 mo *vs* the 7.9 mo of the placebo, as well as in a median

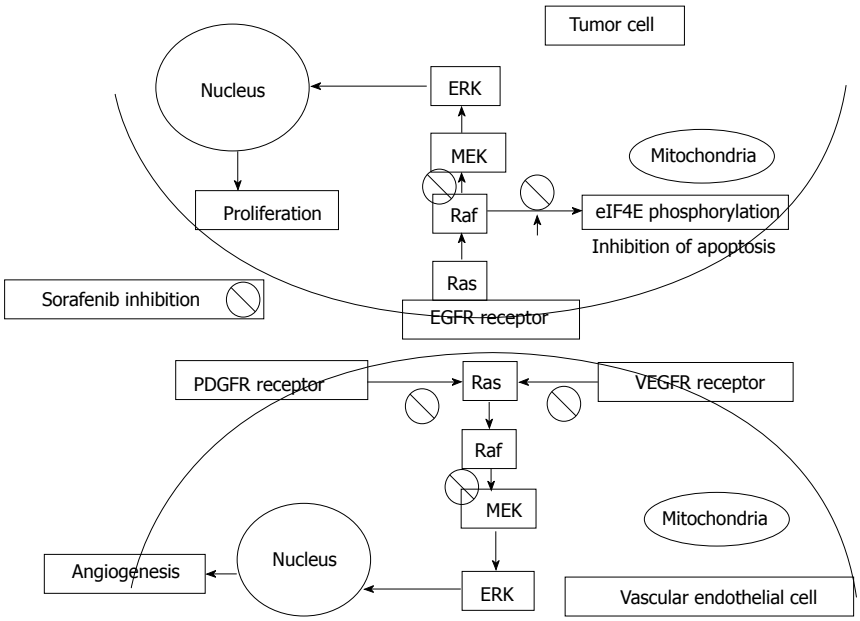

Figure 1 Sorafenib's mechanism of action. In tumor cells sorafenib blocks the Raf/MEK/ERK cascade and can lead to apoptosis through various mechanisms, such as inhibition of eukaryotic translation initiation factor 4E phosphorylation. In vascular endothelial cells, it inhibits receptor tyrosine kinases, such as VEGFR and PDGFR. PDGFR: Platelet-derived growth factor receptor; VEGFR: Vascular endothelial growth factor receptor.

TTP of 24 wk compared to 12 wk of the placebo. Also, although the median TTP based on radiologic findings was 5.5 mo in the Sorafenib arm compared to 2.8 mo in the placebo arm, there was again no complete response, while the partial response was limited[9]. In spite of the positive clinical effects and the improvement in OS, Sorafenib was assessed within the frontiers of advanced stage HCC, but very early stage liver disease. This leads to many questions regarding its potential place in the treatment of patients with both advanced HCC and liver disease.

Adverse effects

On the other hand, nobody claimed that Sorafenib was harmless. The SHARP trial, as a phase 3 study, except for the effectiveness, also reported details about some possible adverse effects, which were more frequent in the Sorafenib group compared to the placebo one (80% *vs* 52%, respectively). The most commonly described toxicities were grade 1 and 2 regarding the severity, *i.e.*, weight loss, anorexia, diarrhea, changes in voice, hand-foot skin reaction, rash or desquamation and hair loss[9]. Some of these toxicities led to drug discontinuation (Sorafenib 11% *vs* placebo 5%)[9]. Another important study, the Sorafenib Italian Assessment (SOFIA) trial, showed that intervening by down-dosing at the appropriate time might be beneficial regarding an improved toxicity-tolerance rate and an increased OS[20].

Moreover, significant findings from the routine clinical practice were presented by Sacco *et al*[21], who stated that when Sorafenib is administered early at a low dose, especially in patients characterized as high-risk, it may be easier to render the patients compliant to the continuation of the therapy and for the drug to be well-tolerated. As a result, Sorafenib may induce some harmful events, mostly minor, which can be better tolerated by adjusting the dosage.

FOOD AND DRUG ADMINISTRATION APPROVAL

According to the European Association for the Study of the Liver (EASL) - European Organisation for Research and Treatment of Cancer (EORTC) guidelines (2012), Sorafenib is currently the only standard systemic treatment for HCC[6]. Its use is approved since 2007 upon the publication of the results of two studies: (1) the SHARP trial[9], conducted in the United States of America and Europe; and (2) the Sorafenib Asia-Pacific (Sorafenib-AP) trial[22], conducted in South Korea, China and Taiwan, which both showed an increased OS and a reduced risk of mortality in patients treated with Sorafenib. However, the aforementioned guidelines[6] highlight that Sorafenib is recommended only in patients with early stage liver disease - Child-Pugh A - and advanced stage HCC - Barcelona - Clinic Liver Cancer (BCLC) stage C - or as an adjuvant therapy combined with loco-regional treatment options. Sorafenib's current place in the treatment algorithm, in accordance with the BCLC staging system for HCC, is presented in Figure 2[4,23].

MONOTHERAPY

As mentioned above, the results of systemic monotherapy with Sorafenib were encouraging according to a phase 2 trial[19] and two phase 3 trials (SHARP[9] and Sorafenib-AP[22]). There was general agreement that Sorafenib has a great impact in increasing the OS, even though in the

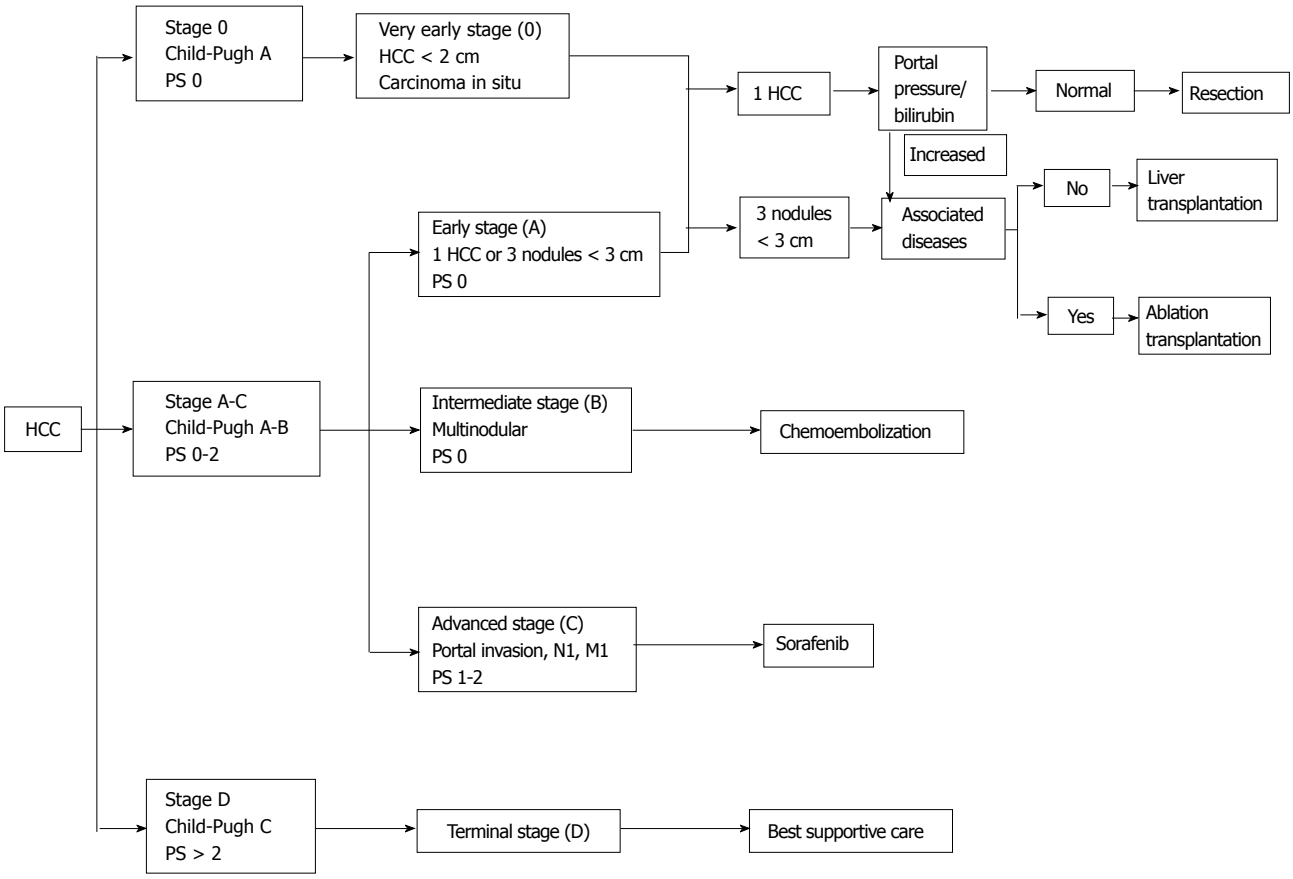

Figure 2 Barcelona clinic liver cancer staging system and treatment algorithm. PS: Performance status; N: Nodules; M: Metastases; HCC: Hepatocellular carcinoma.

phase 2 study 28% of the patients, who had CP B cirrhosis, showed a shorter median OS of 3.2 mo and could tolerate the treatment for only 1.8 mo. Also the incidence of ascites, encephalopathy and advanced hyperbilirubinemia is higher in advanced liver disease[24]. Interestingly, a phase 1 study, assessing the use of Sorafenib in patients with higher Child-Pugh class, underlined its link with the dose-limiting rises in serum bilirubin concentration[25]. Therefore, treatment guidelines[7] recommend taking bilirubin into consideration when adjusting the dose of Sorafenib. In addition, a post-marketing trial (GIDEON)[26] has shown equivalent results regarding safety and dosing strategy regardless of the Child-Pugh score. On the other hand, several studies evaluating the role of Sorafenib among the different stages of liver function reserve, reported a decreased response in advanced CP class, while liver-specific toxicities were independent of the liver cirrhosis stage[27-29].

On the whole, a systematic review has shown that in a male elderly population with advanced HCC and CP A cirrhosis, Sorafenib monotherapy can yield a statistically significant, yet clinically insignificant, increase in OS, time to tumor progression and disease control rate[30]. Besides, the cumulative data underline the decrease response of HBV-infected patients when compared to HCV, while patients with worse level of cirrhosis tend to display a more prominent Sorafenib-driven toxicity[30].

A study published in 2017 analysing the SEER-Medicare database, reported that elderly patients with advanced stage HCC may survive longer if treated with Sorafenib *vs* placebo (150.5 d *vs* 62 d, respectively), while the most remarkable factor associated with increased mortality was treatment taking place in an urban setting, although this survival effect was found to be neither prolonged, nor cost-effective in decompensated patients[31]. Currently, a randomized controlled phase 3 study - the B Child Patient-Optimization of Sorafenib Treatment (BOOST) study - is ongoing so as to evaluate the safety and efficacy of Sorafenib in CP B patients and is going to provide helpful information regarding the treatment of patients with decompensated disease[32]. However, reality is that for most patients Sorafenib is only one of the treatments that they receive, thus rendering it essential to review the adjuvant role of Sorafenib within the spectrum of other therapies.

SORAFENIB AND SURGICAL RESECTION

Currently, surgical resection remains the treatment of choice for HCC, when it is associated with solitary masses and the hepatic remnant can maintain liver function[6]. Recently, there has been great interest concerning the down-staging of advanced HCC in order to make surgical resection even more efficient. One way to accomplish that is by taking advantage of Sorafenib's use as a

neoadjuvant treatment. In fact, a study has reported the incidence of Sorafenib-driven tumor necrosis, when used pre-operatively, therefore making resection an applicable treatment modality for a previously unresectable HCC tumor[33]. Moreover, the use of Sorafenib before surgery was not found to lead to any intra- or post-operative side-effects[34].

However, it is unclear whether Sorafenib could also be efficacious as an adjuvant therapy post-operatively. Specifically, a phase 3 study (STORM) evaluating its use after resection or ablation showed that Sorafenib is not superior to placebo when it comes to OS, recurrence-free survival or time to recurrence[35]. Unfortunately, many patients enrolled in this study could not tolerate the standard dose used[35]. These results are against incorporating Sorafenib in the guidelines as an appropriate adjuvant treatment option after resection[6].

SORAFENIB AND LIVER TRANSPLANTATION

Another curative treatment, especially for patients within the Milan criteria is orthotopic liver transplantation[36]. The challenges involved in liver transplantation, such as graft availability, have led to the increased use of grafts, including split grafts or those from living donors or from marginal donors. However, sometimes the delay between joining the waiting list and actually having a liver transplant may be quite significant, leading to patients dropping off the list[37]. Consequently, those patients with HCC waiting for a liver donor for at least six months are recommended to receive the so called "bridging therapy", which mainly consists of locoregional treatment approaches, such as radiofrequency ablation (RFA) or transarterial chemoembolization (TACE)[6]. The rationale of "bridging" is entirely understandable when trying to prevent tumor progression in cirrhotic patients with HCC, who patiently wait a suitable donor organ to become available. An alternative strategy is down-staging of HCC patients outside the Milan criteria, in an effort to make them eligible for transplantation. A legitimate question is whether Sorafenib has an adjuvant role in this endeavor.

This neoadjuvant use of Sorafenib for down-staging comes with little evidence not demonstrating any significant advantages, even though some cases seem to accomplish reduction in the tumor boarder, down-staging and therefore allowing the patient to be added to the waiting list[38,39]. All-in-all, Sorafenib has shown a safe profile, when used before transplantation, with insignificant post-operative negative events[40,41]. Besides, the Sorafenib-driven hypoxia, because of its antiangiogenic effects, is thought to result in alterations in molecular mechanisms and growth factors, thus allowing the tumor to develop resistance and become more invasive or even metastatic[42]. Until more convincing data is reported from large clinical trials, the use of Sorafenib in this setting should be limited to investigational protocols.

On the other hand, the post-operative adjuvant use of Sorafenib has proven to be inefficient (STORM trial)[35], but when it comes to post-transplantation, results may be different. Specifically, a lot of studies agree with the fact that the use of Sorafenib, either concomitantly with mammalian target of rapamycin (mTOR) inhibitors or without them, can improve the survival when used for recurrent disease after liver transplantation, with the disadvantage of some drug-induced toxicity leading to a decrease in the dosage or even cessation of treatment[43-50]. As a matter of fact, Sorafenib has also resulted in complete remission of recurrent HCC after liver transplantation[51]. In general its use in this setting is thought to be safe[52].

Alltogether, current evidence is not favorable regarding the adjuvant use of sorafenib either pre- or post-transplantation and more research on this particular topic needs to take place, especially in the form of randomized controlled trials[53].

SORAFENIB AND LOCOREGIONAL THERAPIES

Current guidelines suggest the implementation of transarterial TACE in patients with intermediate stage HCC, consisting of multiple nodules, presenting without symptoms, invasion of the vessels or metastases and without advanced liver disease[6]. Although TACE can be helpful and efficient in this particular group of patients by improving survival[4], it is classified as a palliative option because it cannot achieve complete necrosis of the tumor and is associated with increased recurrent disease and tumor proliferation[54]. This tumor growth is also promoted by the ischemic area appearing after treatment with TACE, and owes its existence to the overexpression of certain growth factors, with VEGF playing a major role[55,56]. VEGF's place in this equation lies on the side of tumor progression and metastasis and thus Sorafenib can be the ideal agent to deal with this process and impede angiogenesis, while simultaneously supplementing the promising action of TACE by eliminating the possibilities of future proliferation or recurrence[57].

Some phase 2 studies[58,59] evaluating the concurrent use of TACE and Sorafenib in patients with HCC not amenable to resection have shown a fairly safe profile for this combination with encouraging results regarding the efficacy and toxicity. When this duet was compared to TACE plus placebo in intermediate stage HCC on the background of HCV infection, it greatly improved time to tumor progression, without any unforeseen adverse events[60]. The comparison mentioned above was also assessed in a meta-analysis of six studies (1254 patients) reassuring that TACE plus sorafenib in either intermediate or advanced stage HCC patients can increase OS, time to tumor progression, as well as objective response rate, while the risk of side effects is also high[61]. Other recent meta-analyses, however, evaluating the marriage of Sorafenib and TACE for unresectable HCC showed an improvement in time to tumor progression, but not in

OS[62,63].

It should also be mentioned that Sorafenib has been assessed in combination with drug-eluting beads (DEB)-TACE, an alternative method of delivering regional chemotherapy with minimal systemic exposure, for the management of both intermediate and advanced stage HCC. The results proved the increased efficacy and safety of this strategy[57].

Another important issue is that of the time of TACE and Sorafenib administration, for which three different options have been suggested: (1) TACE is followed by antiangiogenic therapy; (2) continuous antiangiogenic treatment interrupted only for the moment of TACE administration; and (3) continuous antiangiogenic therapy with no interruption at the moment of TACE administration[64]. Although the first two options are superior regarding the risk of bleeding, which is reduced, the third eliminates the possibilities of VEGF increase after TACE.

In general, it appears that Sorafenib plus TACE can lead to improved clinical results, especially regarding the intermediate stage HCC, mainly consisting of a highly heterogeneous group of patients for whom the overall approach is still to be defined based on several ongoing studies[57,65].

SORAFENIB AND OTHER CHEMOTHERAPEUTIC DRUGS

Sorafenib and hypoxia-inducible factor-1a inhibitors

Locoregional treatment modalities can be efficient when it comes to HCC, but up to a point. Radiofrequency and microwave ablation trigger hypoxia and consequently hypoxia-induced angiogenesis, thus increasing the possibility of HCC recurrence. This process is primarily mediated by the hypoxia-inducible factor (HIF)-1a/vascular endothelial growth factor-A (VEGF-A) pathway, which can be impeded by Sorafenib[66]. Therefore, Sorafenib has been shown to limit the tumor's invasive nature *in vitro*, a result of the cobalt chloride's increase of the expression of HIF-1a, and to reduce proliferation and promote apoptosis in HCC cells[66].

2-Methoxyestradiol (2ME2), an inactive end product of estrogen metabolism, has recently been proven to have an antitumor effect by inhibiting proliferation and angiogenesis and by promoting apoptosis in many cancer types and especially in HCC[67]. The most important mechanism 2ME2 acts is through the inhibition of HIF-1 and the down-regulation of the HIF-driven VEGF expression[68]. It has been shown that 2ME2 comes up with synergistic effects in combination with Sorafenib in accordance to HCC suppression and antiangiogenesis, effects mostly driven be HIF-1 and -2 deregulation[69].

Sorafenib and mTOR inhibitors

mTOR, a protein kinase, plays a key role in cell growth, proliferation, angiogenesis and metabolism in several cancers, including HCC[70]. It represents the target of rapamycin and its analogues, as well as Everolimus and Sirolimus, which present with an antitumor profile through the down-regulation of hypoxia-inducible factor, thus resulting in low VEGF and PDGF expression.

Everolimus has been evaluated in a phase 1/2 study in patients with advanced stage HCC, who were previously treated with systemic therapy, and has shown encouraging results in terms of tolerability and efficacy[71]. However, when Everolimus was combined with Sorafenib, again in a phase 1 trial, so that its maximum tolerated dose (MTD) could be determined, the results were disappointing regarding its efficacy in the MTD[72]. In addition, a randomized clinical trial (EVOLVE-1)[73] assessing the use of Everolimus in patients with advanced HCC, who presented with tumor progression during or after taking Sorafenib or who showed limited tolerability towards Sorafenib, reported no increase in OS.

On the other hand, the significant immunosuppressive role of mTOR inhibitors has been used in combination with Sorafenib *vs* Sorafenib alone in cases of post-transplantation late recurrent HCC, thus highlighting the broadening of the horizons in the treatment options against HCC towards the direction of personalized molecular targeted therapy[74]. Besides, cohort studies[46,49] assessing the combination of Sorafenib and mTOR inhibitors in the same disease context showed improved survival, but with some serious adverse events.

As a result, it is suggested that further studies are carried out, so as to evaluate the combination of mTOR inhibitors with Sorafenib in terms of achieving the maximum possible synergy and the minimum possible toxicity overlap.

Sorafenib and PI3K/AKT inhibitors

Despite the blockade of the Raf/MEK/ERK cascade by Sorafenib, HCC has remarkable compensation through the over-expression of several other pro-survival pathways. The phosphoinositide 3-kinase (PI3K)/AKT pathway comes into play here as one of those and data state that it can render the tumor less susceptible to Sorafenib[75]. Thus, synergy may result from the combination of Sorafenib with a PI3K/AKT inhibitor, such as PKI-587 which simultaneously blocks the mTOR pathway, and this significant additive inhibitory effect has been proven in liver cancer stem cell patterns[76].

Sorafenib and WNT/B-catenin inhibitors

The complexity of the molecular mechanisms involved in the multistep process of tumor growth in HCC has been shown to incorporate mutations in the Wnt/β-catenin pathway as well[77]. Therefore, it is possible that the Wnt/β-catenin pathway represents a novel target for systemic treatment in HCC and as such it may also show an additive effect when used concurrently with Sorafenib. Indeed, not only has Sorafenib been able to down-regulate this pathway in different models[78], but also FH535, a Wnt/β-catenin inhibitor, was found to impede tumor growth of HCC and hepatoblastoma[79,80]. Moreover, when Sorafenib was combined with FH535, their synergistic

effect on inhibiting the proliferation of HCC was more significant[81,82].

Sorafenib and MEK inhibitors

The MAPK/ERK kinases (MEK) 1 and 2 can be consequently activated if a Ras mutation shows up, as they are found downstream in the RAS cascade, the activation of which can therefore provide proliferative and anti-apoptotic capabilities to the tumor. This "vertical" type of inhibition totally differs from the "parallel" blockade previously described in the mTOR inhibition, in which two unconnected cascades are concurrently inhibited[83]. Interestingly, MEK inhibitors, such as Refametinib (BAY 869766) which is an allosteric MEK 1/2 inhibitor, have proven their efficacy in preclinical HCC models[84]. When combined with Sorafenib in a phase 2 trial, Refametinib was found efficacious, especially in case of Ras mutations, and was well-tolerated[85].

Sorafenib and JAK/STAT inhibitors

The Janus kinase/signal transducer and activator of transcription (JAK/STAT) pathway plays an important part as a signal transduction cascade, with several proteins of the STAT family participating in cell growth, immunity and survival[86]. The one with the most significant role in oncogenesis is STAT3[87]. This STAT3 protein is key in modulating sensitization of HCC in recombinant tumor necrosis factor-related apoptosis-inducing ligand (TRAIL), an antitumor drug with encouraging efficacy[88]. When Sorafenib was combined with TRAIL, it decreased the expression of STAT3 and proteins involved in its actions, thus rendering, the previously resistant to TRAIL, HCC susceptible to TRAIL-induced apoptosis[88]. Besides, Sorafenib targets STAT3 in a kinase-independent manner in patients with HCC[88,89].

In addition, the SH2 domain-containing tyrosine phosphatases family (SHP-1 and SHP-2), which are included in the family of protein tyrosine phosphatases (PTP), consist of two Src Homology (SH) 2 domains just as their name indicates[90]. These phosphatases dephosphorylate STAT3, leading to a significant decrease in its activation[91] and as a result they represent a potential target for systemic treatment of HCC. In fact, SHP-1 is a target of Sorafenib and through conformational modifications and signaling pathways, in which STAT3 is also involved, Sorafenib can also exhibit its anti-HCC effect[92]. However, we have already experienced the evolution of Sorafenib through its derivatives, such as SC-43 and SC-40, which are potent SHP-1 agonists and have proven to be superior to Sorafenib for the management of HCC[92]. Another novel derivative of Sorafenib, SC-59, when combined with radiotherapy has also shown to be superior to Sorafenib for treating HCC and its actions are mediated through STAT3 inhibition[93]. Last but not least, the synergistic combination of Sorafenib with SC-43, through their SHP-1 agonist effects, has been found efficacious, as it decreased tumor size and improved survival in preclinical models[94].

Sorafenib and phytochemicals

Data from preclinical models indicate that dietary phytochemicals with anti-inflammatory, antioxidant and anti-neoplastic characteristics may reduce the risk of HCC.

Curcumin is a yellow polyphenol derived from turmeric and has been shown to be protective against HCC caused by aflatoxins in mice[95]. Due to its solubility issues, polymeric nanoparticle formations of curcumin (NFC) have been developed and it is reported that the use of NFC alone or in combination with Sorafenib presents with remarkable findings regarding the suppression of tumor proliferation and invasiveness of HCC, as well as that of lung metastases[96].

Resveratrol is also a dietary polyphenol, mostly present in grapes, berries, peanuts and red wine, and has appeared as a promising chemopreventive agent against liver cancer[97]. The combination of Resveratrol and Sorafenib can lead to apoptosis and reduced tumor growth in HCC mice by fighting the diverted metabolic phenotype of aerobic glycolysis[98].

Indole-3-carbinol (I3C), found in cruciferous vegetables, is also one of the phytochemicals that have recently emerged with antineoplastic and antiangiogenic properties[99]. Specifically, its combined use with sorafenib has shown synergy by increasing the latter's cytotoxicity and antiangiogenic properties, by promoting cell cycle arrest and apoptosis, as well as by reducing the expression of p-Akt, HIF-1a, VEGF and EGFR in HCC cells[99].

Regorafenib: A new era

Several antiangiogenic drugs with the same antiangiogenic capabilities as Sorafenib have been developed over time for the management of HCC, mostly as second-line systemic therapy agents.

In case of failure to respond to Sorafenib, patients with HCC can be treated with another multikinase inhibitor, Regorafenib[100]. The addition of a fluorine atom in the central phenyl ring of Sorafenib transforms Regorafenib into an agent with increased potency[101]. A phase 2 study evaluating Regorafenib for intermediate or advanced HCC in patients that had previously received Sorafenib reported encouraging results, such as an OS of 13.8 mo, a safety profile similar to Sorafenib and no deaths attributed to Regorafenib[102]. Recently, in July 2016, at the ESMO World Congress on Gastrointestinal Cancer in Barcelona findings from a phase 3 trial (RESORCE, NCT01774344) assessing Regorafenib in HCC patients, who received prior therapy with Sorafenib, exhibited a remarkable increase in median OS for those treated with Regorafenib *vs* those receiving placebo as a second-line agent after radiologic progression under Sorafenib (10.6 *vs* 7.8)[103].

Almost a decade has passed with numerous clinicians and scientists getting negative results in trials for systemic therapy in HCC patients, while the RESORCE trial is the only one after the SHARP trial to come forward with positive findings. The most important causes of those negative results are: (1) the heterogeneity among the

HCC patients recruited and the lack of selection criteria based on molecular patterns; and (2) the imbalance between adverse events and tolerable dosage *vs* anticancer efficiency and drug potency of the tested agents. Current advances in medicine and biology will improve our knowledge regarding the different and complex molecular mechanisms and driving mutations involved in this vast heterogeneity of this unique and multidimensional type of cancer and will guide us towards the right direction of conducting successful trials in the near future [104].

CONCLUSION

Sorafenib represents a type of medicinal revolution, therefore making antiangiogenesis drugs a feasible choice when it comes to dealing with cancer and opening the road for personalized targeted therapy. Currently, Sorafenib is the only accepted treatment for systemic therapy, as it has shown to increase the OS in patients suffering from advanced HCC, but with liver disease of early stage with tolerable adverse effects. Recently, studies show that Sorafenib is also safe in patients with advanced liver disease as well, but neither adequately efficient, nor cost-effective. Thus, ongoing studies (*i.e.*, BOOST trial) are going to define its role in decompensated population in the future and up until then, patient selection in patients treated with Sorafenib is critical.

All-in-all, Sorafenib has evolved through time by being evaluated in several treatment protocols either as a neoadjuvant or as an adjuvant agent. Its use prior to or after liver transplantation has demonstrated a range of some minor advantages to even complete remission of recurrence, while preserving an acceptable safety profile. Still, a lot of research is needed in this field, as Sorafenib's role post-resection was not that much promising, while its combination with TACE showed encouraging results. Overall, understanding the molecular mechanisms of HCC and Sorafenib, as well as those resulting from the implementation of other treatment methods, will guide us to the future development of combinations involving Sorafenib, agents with higher efficacy that derive from Sorafenib or even second-line agents that will complement the therapeutic role that Sorafenib could not accomplish by itself.

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REVIEW

Magnetic resonance imaging for diagnosis and neoadjuvant treatment evaluation in locally advanced rectal cancer: A pictorial review

Gulgun Engin, Rasul Sharifov

Gulgun Engin, Oncology Institute, Istanbul University, 34390 Capa, Istanbul, Turkey

Rasul Sharifov, Department of Radiology, Faculty of Medicine, Bezmialem Vakif University, 34093 Fatih, Istanbul, Turkey

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Correspondence to: Gulgun Engin, MD, Oncology Institute, Istanbul University, Millet Street, 34390 Capa, Istanbul, Turkey. gengin@istanbul.edu.tr
Telephone: +90-212-4142000
Fax: +90-212-5348078

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Abstract

High-resolution pelvic magnetic resonance imaging (MRI) is the primary method for staging rectal cancer. MRI is highly accurate in the primary staging of rectal cancer; however, it has not proven to be effective in re-staging, especially in complete response evaluation after neoadjuvant therapy. Neoadjuvant chemoradiotherapy produces many changes in rectal tumors and on adjacent area, as a result, local tumor extent may not be accurately determined. However, adding diffusion-weighted sequences to the standard approach can improve diagnostic accuracy. In this pictorial review, an overview of the situation of MRI in the staging and re-staging of rectal cancer is exhibited as a pictorial assay. An experience- and literature-based discussion of limitations and difficulties in interpretation are also presented.

Key words: Rectal cancer; Locally advanced; Magnetic resonance imaging; Staging; Neoadjuvant treatment

Core tip: Accurate staging and circumferential resection margin evaluation significantly impacts determining optimal treatment scheme. Preoperative magnetic resonance imaging (MRI) is highly accurate; however, it has yet to be proved as effective in re-staging. The adding of diffusion-weighted sequences to standard T2-weighted MRI can positively affect its diagnostic accuracy.

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INTRODUCTION

Multimodal treatment of rectal cancer, with the combination

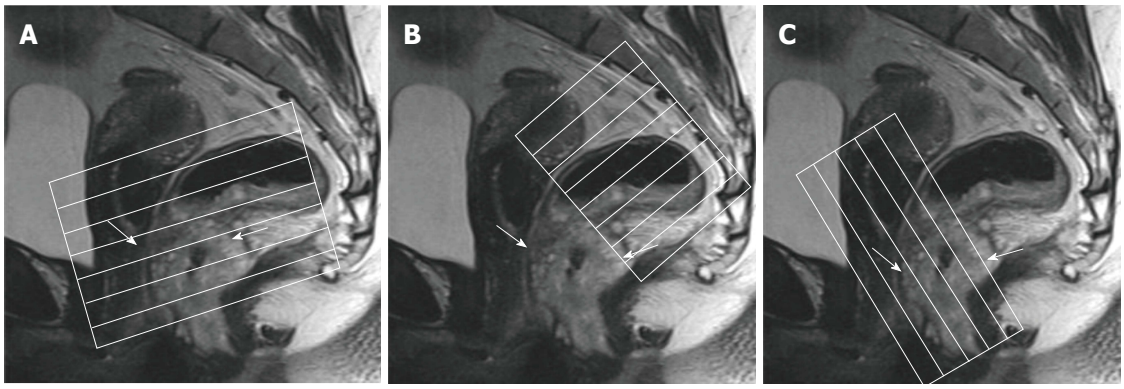

Figure 1 Magnetic resonance imaging planes. T2-weighted sagittal images are used to determine the longitudinal tumor axis in order to angle the axial and coronal planes. A: Oblique axial plane is obtained perpendicular to the rectal wall at the level of the rectal mass; B: Oblique axial plane is angled perpendicular to the pelvic floor, used to cover lymph node drainage territory; C: Coronal plane is angled parallel to the anal canal for imaging of low rectal tumors. Rectal tumor is indicated by arrows.

of preoperative (neoadjuvant) chemoradiotherapy (CRT) followed by surgery increases local control in locally advanced cancers and has become the standard approach to such rectal cancers[1-5].

High-resolution pelvic magnetic resonance imaging (MRI) is the primary method for evaluation in rectal cancer[6-10]. When applied according to the optimal protocols, high-resolution MRI accurately determining patients regarding neoadjuvant CRT requirement[11]. Moreover, assessing treatment response in tumors using MRI also predicts probable survival outcomes, and could be used in the future to further adjust treatment according to the patients' response[12]. In recurrent rectal cancer, MRI enables the depiction of the extent of tumor growth, and can establish the resectability of disease[13,14].

MRI has not met expectations in re-staging, especially in complete response evaluation after neoadjuvant CRT because of post-therapeutic fibrosis and inflammation[15-19]. However, adding functional MR sequences such as dynamic contrast-enhanced and diffusion-weighted sequences to the standard approach can improve diagnostic accuracy of MRI[20-23].

In this pictorial review, we present a synopsis of the current standing of MRI in the staging and re-staging of rectal cancer. We also present an experience- and literature-based discussion of limitations and difficulties in interpretation.

MRI TECHNIQUE

Rectal MRI should be performed with pelvic phased-array coils. Rectal MRI using this technique provides overall assessment of the rectal wall layers with high-spatial-resolution and benefits from a large field of view[15,24].

PATIENT PREPARATION

Routine rectal filling using endoluminal contrast agents such as ultrasonography gel is discouraged[24] because this can distend of the rectum and compress the mesorectal fat, which may result in overestimation of fascial involvement and interfere with assessment of mesorectal nodes[25].

Bowel preparation is generally not necessary before the examination, but spasmolytics can be used when excessive fecal matter is visible on the planning images[15,24]. For this purpose, a dose of 40 mg butylscopolamine is used intramuscularly unless contraindicated, immediately prior to placing the patient on the MRI table.

IMAGING PROTOCOL

Standard MR rectal protocols must at least include 2D T2-weighted sequences in sagittal, axial, and oblique coronal planes with 1-3 mm slice thicknesses. Sagittal sequences are used to identify the longitudinal tumor axis such that axial and coronal planes may be angled as perpendicular and parallel to the tumor axis as possible, respectively. Coronal planes must be angled in line with the anal canal for low tumors in order to evaluate the relation to the anal complex and pelvic floor muscles[15,24,26] (Figure 1). Axial images are useful for evaluation of the tumor and its relationship with the intestinal wall, mesorectal fascia (MRF), and the adjacent pelvic tissue. Sagittal images are useful for the assessment of the tumor height and length and its relationship with peritoneum and other adjacent tissue.

In addition to T2-weighted sequences, diffusion-weighted imaging (DWI) sequences are recommended for inclusion in restaging protocols. DWI provides no additional benefit in primary staging; however, evidence is accumulating suggesting that it increases the diagnostic capability of MRI in the assessment of therapy response (yT-stage) after CRT[24]). DWI also helps T2-weighted fast-spinecho (FSE) sequences to distinguish patients having good *vs* poor response[20-23]. However, there is not adequate proof for supporting the usage of DWI for primary T-staging and lymph node assessment[27].

ANATOMIC LANDMARKS

The rectum is approximately 15 cm in length from the anal verge, which is the lowest part of the anal canal.

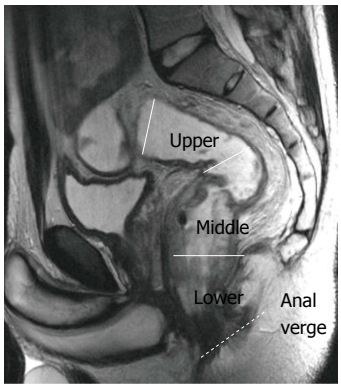

Figure 2 Rectal segments. T2-weighted sagittal image shows rectal segments: Lower, < 5 cm; middle, 5-10 cm; upper, > 10 cm from the anal verge.

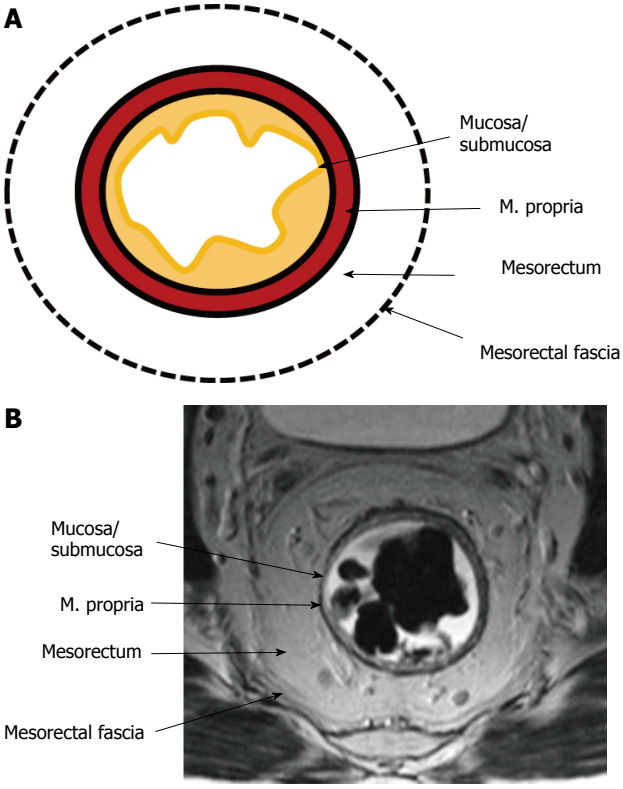

Figure 3 Normal rectal wall anatomy of higher and middle rectum. Schematic (A) and T2-weighted axial magnetic resonance imaging (B) presentation. The internal hyperintense layer represents the mucosa and submucosa (no distinction is possible between in two layers); the medial hypointense layer and external hyperintense area represent the muscularis propria and the mesorectum, respectively. Mesorectal fascia is seen thin hypointense layer enveloping the mesorectum (arrows).

The rectum has traditionally been divided into three segments according to the distance from the anal verge: Upper (> 10 cm), middle (5-10 cm), and lower (< 5 cm)[27,28] (Figure 2).

The upper and middle rectal walls consist of three separate layers that can be distinguished in MRI. T2-weighted MRI sequences are the best for visualizing rectal wall anatomy. The internal hyperintense layer represents the mucosa and submucosa (no distinction is possible between in two layers); the medial hypointense layer and external hyperintense area represent the muscularis propria and the mesorectum, respectively[15,29] (Figure 3).

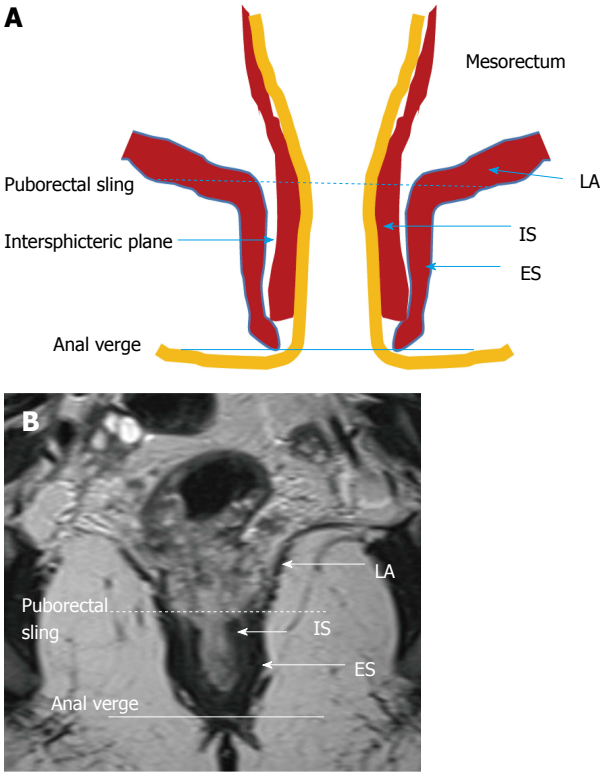

Figure 4 Normal anatomy of lower rectum. Schematic (A) and coronal plane T2-weighted (B) magnetic resonance imaging presentation. Puborectal sling, the upper portion of the puborectal muscle displaying the uppermost portion of the anal canal (intermittent line). Anal verge is the lowermost portion of the anal canal (line). LA: Levator ani muscle; IS: Internal sphincter; ES: External sphincter.

The puborectal sling constitutes the upper limit of the anal canal. The inner muscular wall of the anal canal comprises the internal sphincter, which is the direct continuation of the circular layer of the muscularis propria of the rectum. The outer muscular wall of the anal canal is cranially composed of the puborectal muscle and caudally of the external sphincter[15,26] (Figure 4).

The puborectal sling constitutes the upper limit of the anal canal. The internal sphincter (the internal muscular wall) of the anal canal is consisted of the direct continuity of the muscularis propria circular layer of the rectum. The external muscular wall of the anal canal is formed by the puborectal muscle in cranially and the external sphincter in caudally[15,26] (Figure 4).

The peritoneal reflection covers the anterior wall of the upper rectum; the risk of peritoneal perforation in upper rectal tumors is high[27]. The peritoneal reflection can be easily displayed on sagittal and axial high-resolution T2-weighted images. In sagittal images, it can be depicted whereon upper pole of the seminal vesicles in men and at the uterocervical angle in women[15]. The evaluation of the peritoneal invasion is very important in staging, because rectal tumor is staged as T4a in the presence of peritoneal invasion (Figure 5).

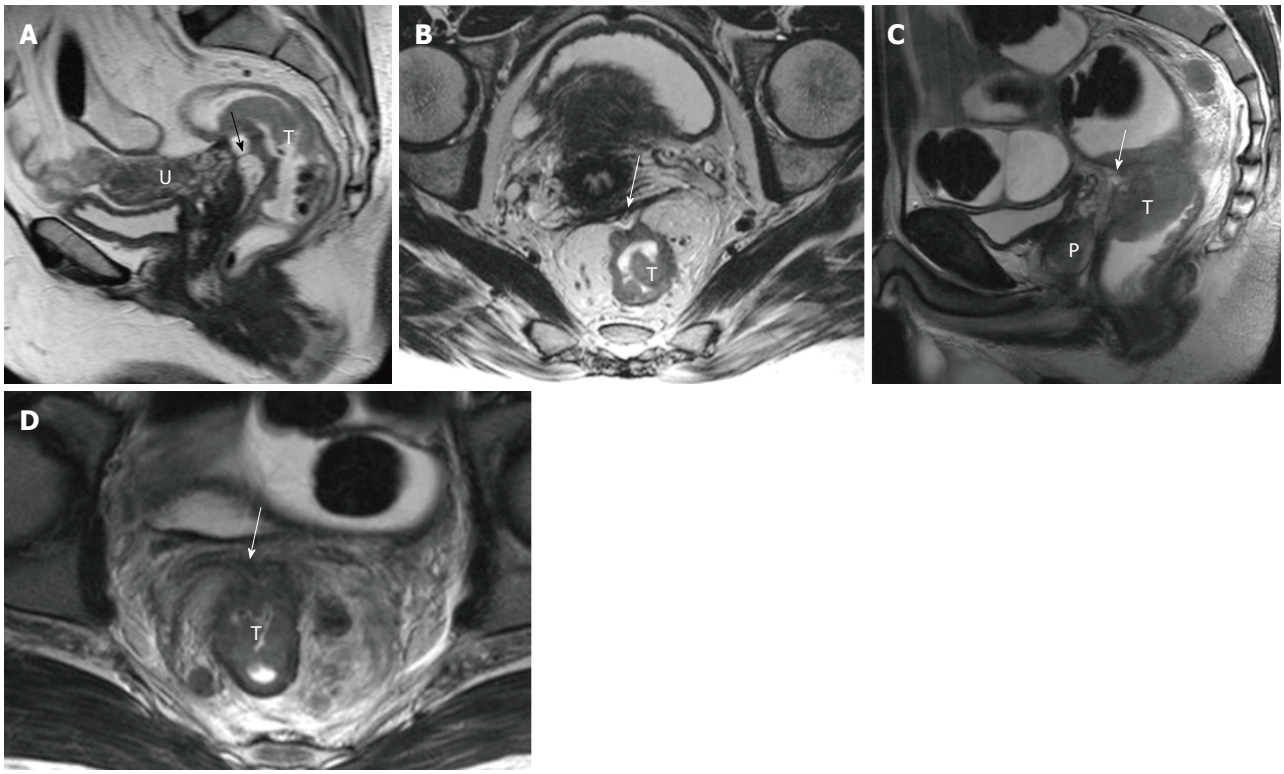

Figure 5 Periton invasion in female (A and B) and male (C and D) patients with T4a rectal tumors. On sagittal T2-weighted images, periton is seen as a hypointense linear structure in front of the tumor (arrows in A, C). On axial T2-weighted images, the peritoneum has a V shape and attaches onto the anterior aspect of the rectal cancer (arrows in B and D). T: Tumor; U: Uterus; P: Prostate.

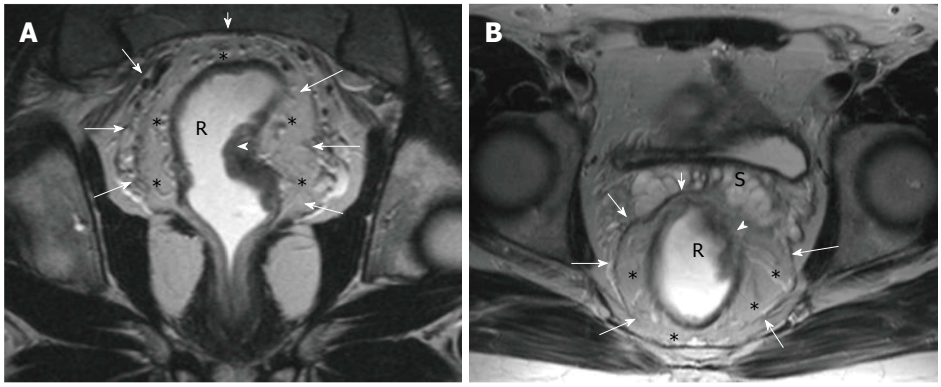

Figure 6 Magnetic resonance imaging anatomy of mesorectum and mesorectal fascia. On T2-weighted (A) axial and (B) coronal plane magnetic resonance images, mesorectal fascia (arrows) is seen as a thin, low-signal intensity layer enveloping the mesorectal fatty tissue (*) and rectum in a male patient with rectal carcinoma.

The middle rectum, which lies below the peritoneal reflection, is completely surrounded by mesorectal fatty tissue which is called the mesorectum. Mesorectum is encircled by the MRF which is constitutes the circumferential resection margin (CRM)[26-29]. The MRF can be seen as a thin, low-signal intensity envelop which surrounds the rectum and mesorectum (Figure 6). MRF tapers downward at the lower rectal level[26]. The MRF is easily seen in posterolateral views, although it is difficult to distinguish it from Denonvilliers' fascia in the anterior wall[30].

PRIMARY STAGING OF RECTAL CANCER

Tumor height and length

Tumor height and length should be routinely reported because outcomes and surgical management are affected by the location of the tumor[24].

The distance and length are measured on a line drawn on the sagittal MR images. For tumor localization, the distance of the lowest portion of the tumor from the anal verge is measured. Rectal tumors are classified as high, middle or low when their most caudal border

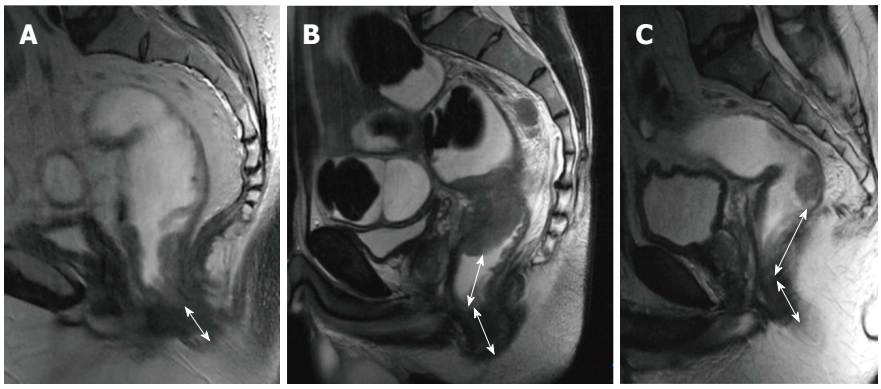

Figure 7 Rectal tumor levels. T2-weighted sagittal images in different patients with rectal carcinoma show distance from the anal verge (double-headed arrows) in (A) low rectal, (B) midrectal, and (C) upper rectal tumors (low rectal tumor, < 5 cm; midrectal, 5-10 cm; upper rectal, > 10 cm).

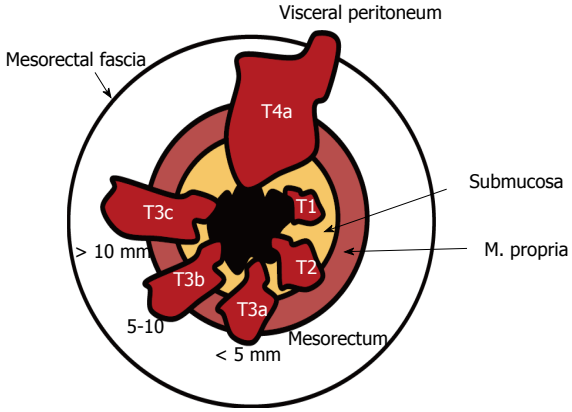

Figure 8 Rectal tumor T staging. The American Joint Committee on Cancer suggested an optional stratification of T3 tumors based on the extramural depth of invasion: Less than 5 mm, T3a; 5-10 mm, T3b; and more than 10 mm, T3c (adapted from ref. [27]: Nougaret S, Reinhold C, Mikhael HW, Rouanet P, Bibeau F, Brown G. The use of MR imaging in treatment planning for patients with rectal carcinoma: have you checked the "DISTANCE"? *Radiology* 2013; **268**: 330-344).

is > 10 cm, 5-10 cm, or < 5 cm from the anal verge, respectively[15] (Figure 7).

T staging for middle and high tumors

On T2-weighted imaging, the muscularis propria is seen as a hypointense line between the hyperintense mesorectal fat and the inner submucosa and mucosa, which show intermediate to mild hyperintensity. The signal intensity of a rectal tumor on T2-weighted images is typically intermediate between the signal intensity of the muscularis propria and mucosa (Figure 8).

T1 tumors are confined to the submucosa; T2 tumors extend into, but not beyond, the muscularis propria. The differentiation of T1 tumors from T2 tumors on MRI is usually not reliable without an endorectal coil or endorectal ultrasound, and tumors should generally be staged as T1/T2[15]. A tumor is staged as T3 when it extends beyond the muscularis propria and strands the mesorectal fat. Disruption of the muscularis propria because of penetrating vessels should not be overstaged as T3 (Figures 8 and 9).

The extramural depth of invasion refers to extension of tumor beyond the muscularis propria[31]. The American Joint Committee on Cancer suggested an optional stratification of T3 tumors based on the extramural depth of invasion: Less than 5 mm, T3a; 5-10 mm, T3b; and more than 10 mm, T3c[32]. An extramural depth of invasion of less than 5 mm presents a significantly higher survival rate, and these early T3 tumors may be adequately managed with surgery alone and have a prognosis comparable to that of tumors characterized as T1/T2[33]. T4 tumors extend onto the surface of the visceral peritoneum or an adjacent structure (Table 1, Figures 8 and 10).

Distance to the mesorectal fascia

For T3 tumors, the shortest distance between the most penetrating parts of the tumor and the MRF should be measured[34,35]. The distance to the MRF is a critical local prognostic factor for rectal cancer[36,37]. A tumor-MRF distance of more than 1 mm is a reliable predictor for negative margins after TME[38]. In the presence of satellite nodules such as tumor deposits, lymph nodes or extramural vascular invasion (EMVI), the shortest distance between the nodules and the MRF should also be reported[15] (Figures 11 and 12).

EMVI

EMVI is associated with local and distant recurrence and poor survival[39]. It is defined as the presence of malignant cells within blood vessels located beyond the muscularis propria in the mesorectal fat. EMVI is suggested when vessels close to the tumor are obviously irregular or expanded by tumoral signal intensity[39] (Figure 13).

The assessment of EMVI is a routine component of MR evaluation for primary staging; however, for restaging, there is no agreement as to whether evaluation of EMVI remains beneficial[24].

T staging for low tumors

A specific T staging system is used to identify tumors and its circumferential resection margin (CRM)[40] (Table 1, Figures 14 and 15).

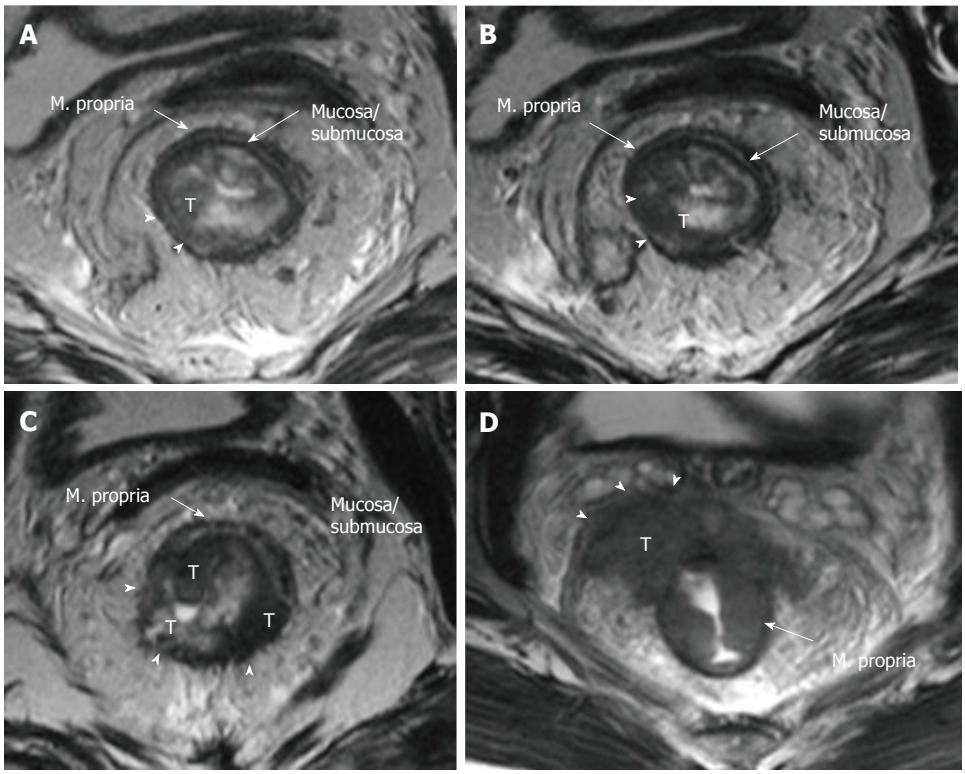

Figure 9 Rectal cancer T staging on magnetic resonance imaging. T2-weighted axial images showing rectal carcinomas with different T stages. A: T1 tumor is confined to the submucosa, has not entered the muscularis propria (arrowheads); B: T2 tumor extends into, but not beyond, the muscularis propria (arrowheads); C: T3 tumor extends beyond the muscularis propria and strands into mesorectal fat (arrowheads); D: T4a tumor invades the visceral peritoneum (arrowheads). T: Tumor.

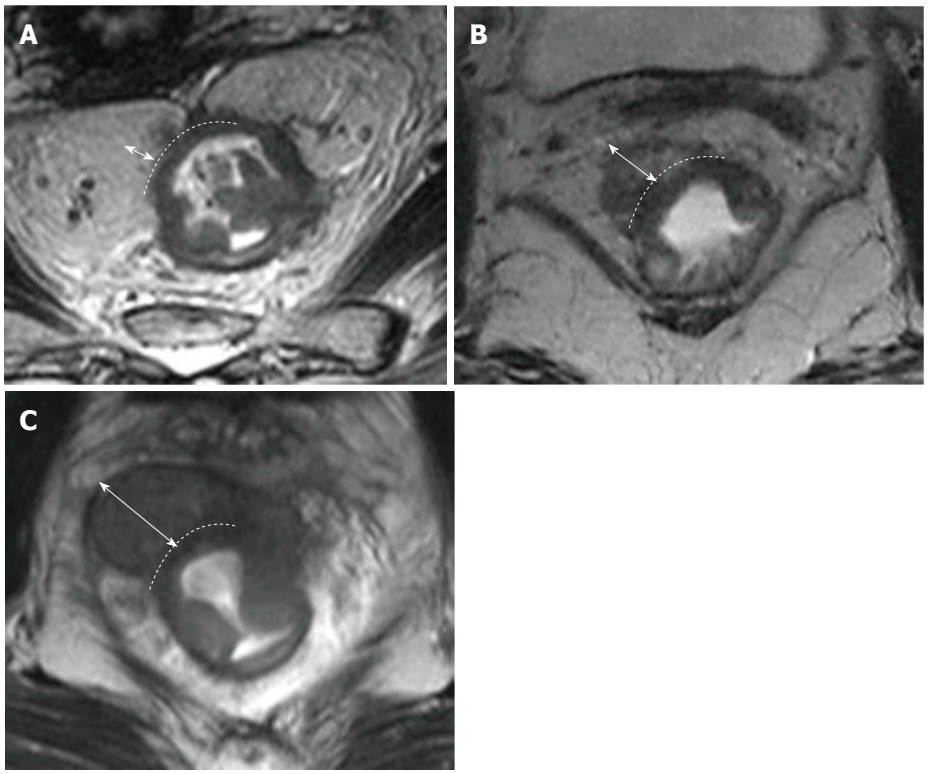

Figure 10 Stratification of T3 tumors on magnetic resonance imaging. T2-weighted axial magnetic resonance images in different patients with T3 rectal carcinoma showing extension of the tumor beyond the muscularis propria (double-headed arrows). The distance A: Less than 5 mm, T3a; B: 5-10 mm, T3b; and C: More than 10 mm, T3c.

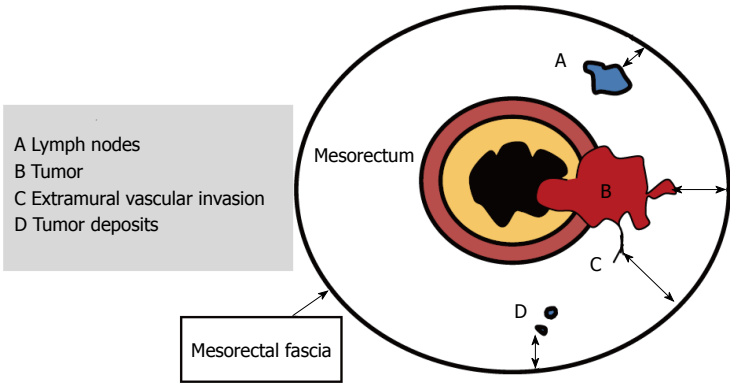

Figure 11 Schematic representation of positive resection margin. For T3 tumors, the shortest distance between the most penetrating parts of the tumor and the MRF is measured (double-headed arrows). A tumor mesorectal fascia distance of more than 1 mm is a reliable predictor for negative margins. In the presence of satellite nodules such as tumor deposits, lymph nodes or EMVI the shortest distance between the nodules and the MRF should also be reported (Adapted from ref. [27]: Nougaret S, Reinhold C, Mikhael HW, Rouanet P, Bibeau F, Brown G. The use of MR imaging in treatment planning for patients with rectal carcinoma: have you checked the "DISTANCE"? *Radiology* 2013; **268**: 330-344). EMVI: Extramural vascular invasion; MRF: Mesorectal fascia.

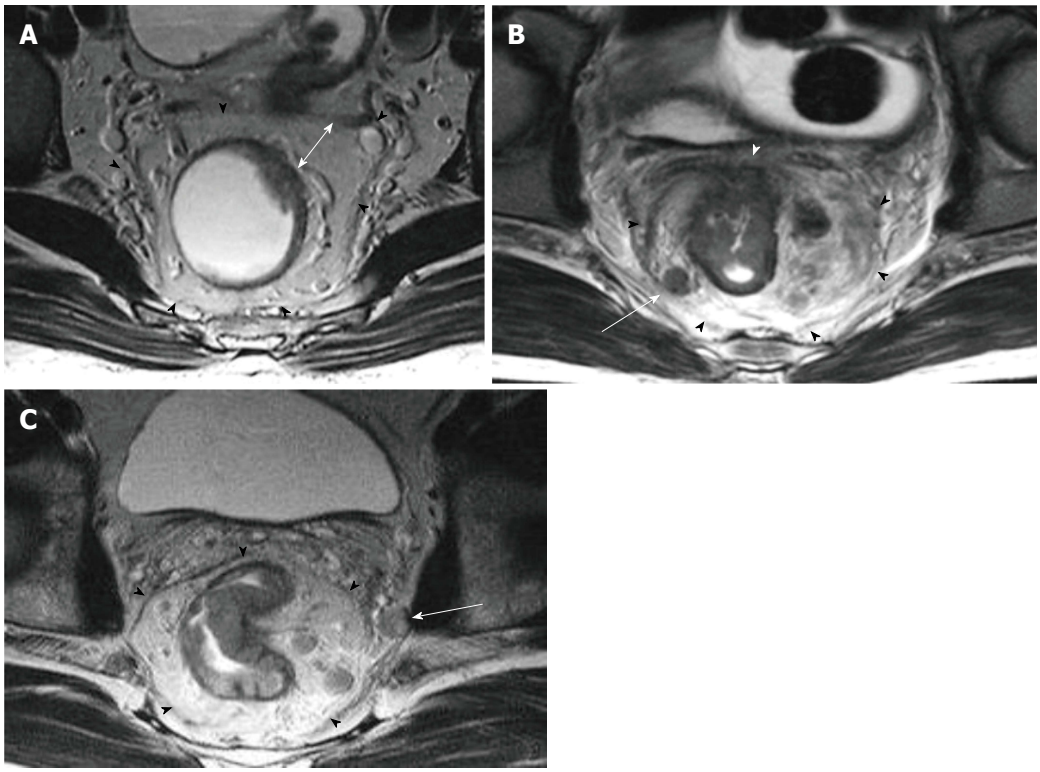

Figure 12 Distance to mesorectal fascia and mesorectal fascia invasion in different patients on T2-weighted axial images. A: T3a tumor is far away from the mesorectal fascia (double-headed arrow); B: T4a tumor (white arrowhead) and a suspicious mesorectal lymph node (arrow) are abutting the mesorectal fascia; C: Rectal tumor is lying > 1 mm from the mesorectal fascia; however, a suspicious lymph node, located out of the mesorectal fascia, is lying within < 1 mm of the mesorectal fascia (arrow). Mesorectal fascia is indicated with black arrowheads.

N-staging

Staging of nodes is very important for planning preoperative treatment[41]. In the TNM system, disease involving only the regional nodes, including the mesorectal and internal iliac nodes, accounts for the N stage (Table 1); involvement of other nodes is regarded as metastasis[38].

Mesorectal nodes are often the first and the most commonly involved group of nodes. Nodal metastases are usually within the proximal 5 cm of the tumor[41].

Extramesorectal nodes (iliac, superior rectal or inferior mesenteric nodes) are generally involved in locally advanced cancers[42]. Low rectal tumors can also spread superficial inguinal nodes and imply poor prognosis[43].

Node size is the usual criterion in nodal staging using MRI. Lymph nodes are usually considered pathologic when their short axis is longer than 0.5 cm; however, no optimal cut-off threshold exists for involved nodes[24]. The inclusion of morphologic features such as round shape, irregular contour, and nonhomogeneous signal intensity to a size cutoff increases the accuracy of MR[44]. Although DW MRI is not accurate enough for characterizing nodes, it may be useful for locating them[45] (Figure 16).

RESTAGING AFTER NEOADJUVANT TREATMENT

Neoadjuvant CRT provides downstaging and downsizing along with improvement in less extensive surgery, decreased local recurrence, and general survival[12,46]. Tumor restaging involves correlating the posttreatment images with the pretreatment images with respect to

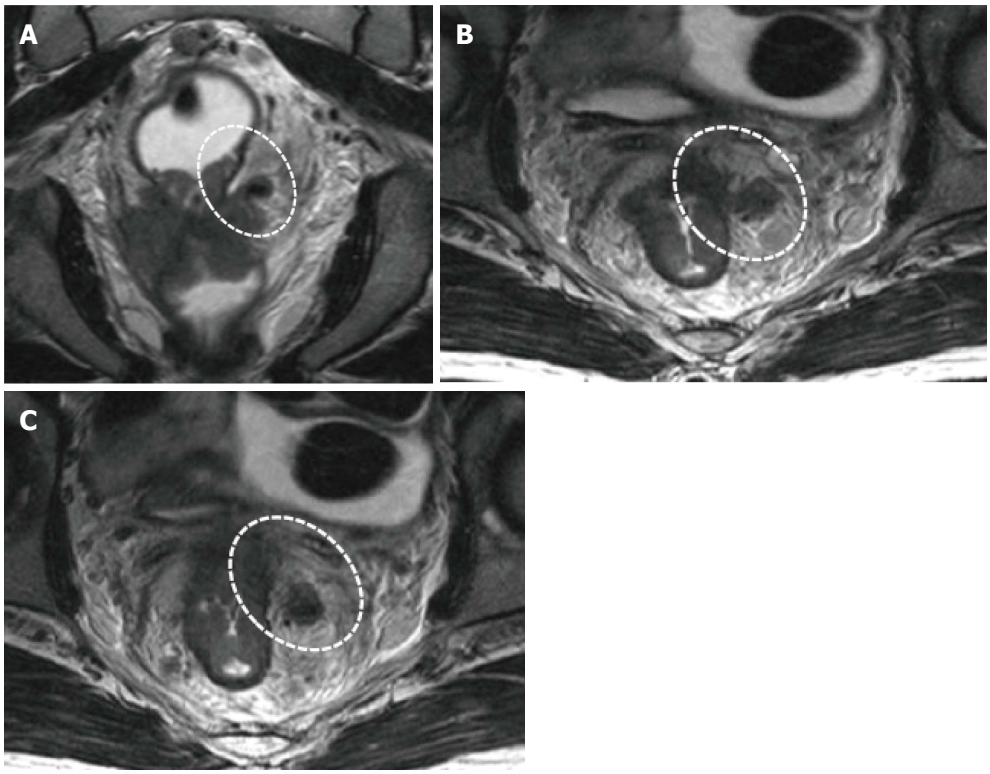

Figure 13 Extramural vascular invasion. T2-weighted (A) coronal and (B and C) serial axial magnetic resonance images in the same patient with T4a rectal cancer showing an irregular and expanded vessel insert to the tumor with tumoral signal intensity (circles).

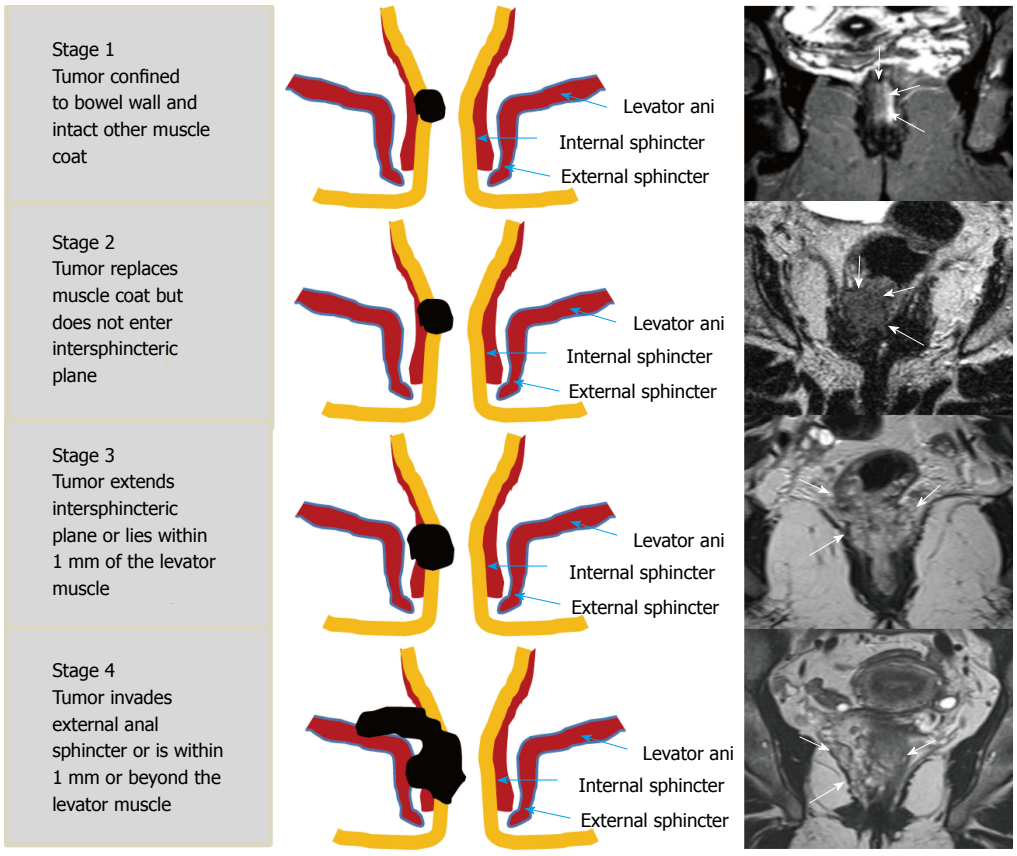

Figure 14 Schematic and high-spatial-resolution coronal T2-weighted magnetic resonance images for each stage according to the low rectal cancer. Rectal tumors in different patients are indicated with arrows on magnetic resonance images (Adapted from ref. [27]: Nougaret S, Reinhold C, Mikhael HW, Rouanet P, Bibeau F, Brown G. The use of MR imaging in treatment planning for patients with rectal carcinoma: have you checked the "DISTANCE"? *Radiology* 2013; **268**: 330-344).

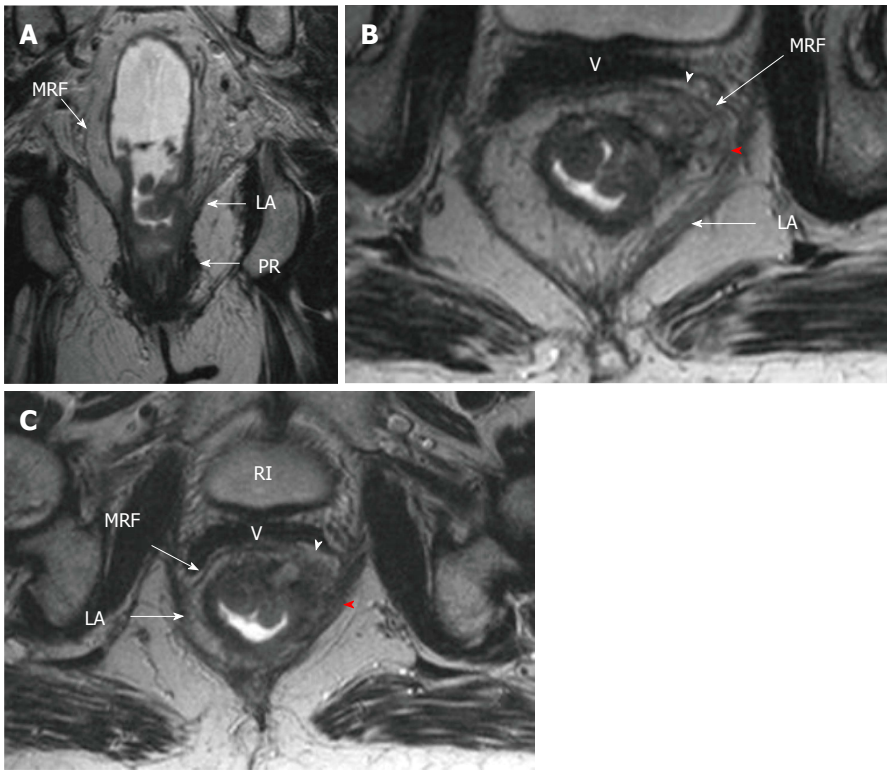

Figure 15 Stage 4 low rectal cancer. On T2-weighted (A) coronal (B, C) serial axial magnetic resonance images, rectal cancer showing invasion of levator ani (red arrowheads) and mesorectal fascia (white arrowhead). LA: Levator ani; PR: Puborectal; MRF: Mesorectal fascia; BL: Bladder; V: Vagina.

Table 1 Staging systems for rectal cancer

Stage	MRI findings
T stage for middle and high tumors[1]	
T1	Tumor signal intensity is confined to the submucosal layer
T2	Tumor signal intensity extends into the muscle layer, with loss of the interface between the submucosa and circular muscle layer
T3	Tumor signal intensity extends through the muscle layer into the perirectal fat, with obliteration of the interface between muscle and perirectal fat
T3a	Tumor < 5 mm into the perirectal fat
T3b	Tumor 5-10 mm into the perirectal fat
T3c	Tumor > 10 mm into the perirectal fat
T4a	Tumor signal intensity extends to surface of visceral peritoneum
T4b	Tumor signal intensity extends into an adjacent structure or viscus
T stage for low tumors[2]	
T1	Tumor signal intensity confined to bowel wall, outer muscle coat intact
T2	Tumor signal intensity replaces muscle coat but does not enter intersphincteric plane
T3	Tumor signal intensity extends intersphincteric plane or lies within 1 mm of levator muscle
T4	Tumor signal intensity extends external anal sphincter or is within1 mm or beyond levator muscle with/ without adjacent organ invasion
N stage	
Nx	Regional lymph nodes cannot be assessed
N0	No regional lymph node metastasis
N1	Metastasis in 1-3 regional lymph nodes
N2	Metastasis in > 3 regional lymph nodes

[1]Adapted from ref. [32]: Edge SB, Byrd DR, Compton CC. AJCC cancer staging handbook: from the AJCC cancer staging manual, 7th ed. New York, NY: Springer, 2010: 718; [2]Adapted from ref. [40]: Taylor FG, Swift RI, Blomqvist L, Brown G. A systematic approach to the interpretation of preoperative staging MRI for rectal cancer. *AJR* 2008; **191**: 1827-1835. MRI: Magnetic resonance imaging.

all the elements assessed in the initial staging, and necessitates image acquisition with almost the same protocol and on the same planes.

T staging

Post-CRT restaging using conventional MR sequences is less accurate than primary staging, especially when confirming complete response (yT0), mostly because it is difficult to distinguish fibrosis, edema and normal mucosa from small foci of residual tumor[46-48]. As such, a normal, two-layered rectal wall after CRT is indicative of complete response, whereas residual fibrosis indicates either residual tumor or complete response (Figure 17). In practice, areas of fibrosis have very low signal intensity

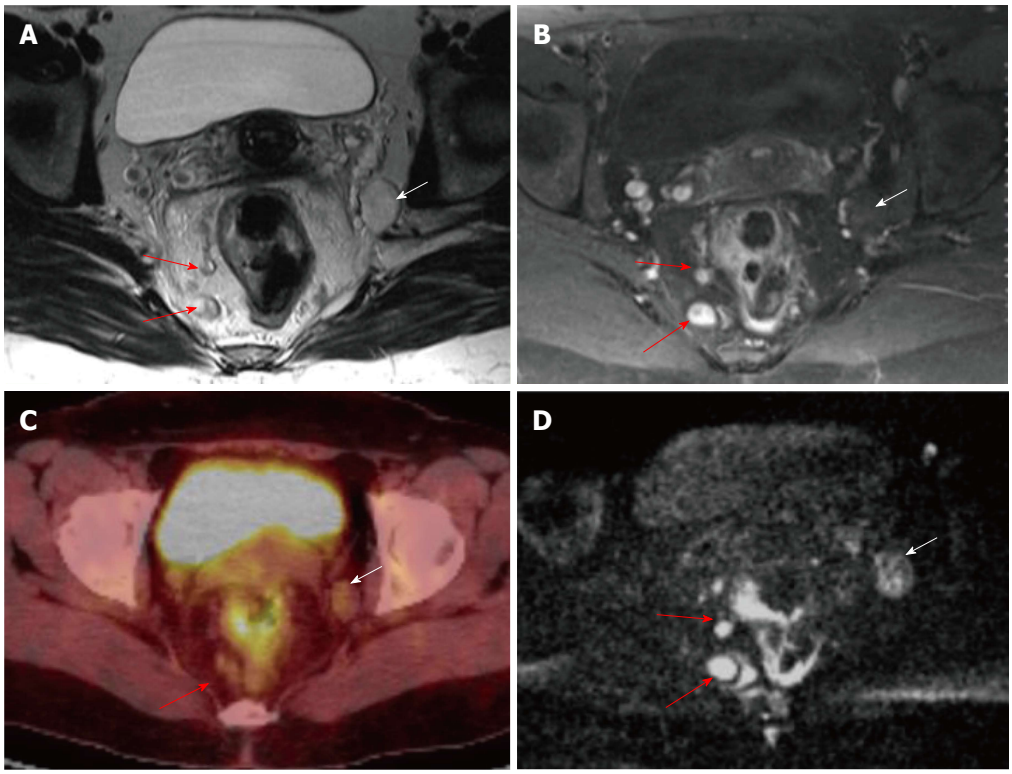

Figure 16 Mesorectal and extramesorectal lymph node involvement in rectal cancer. A: T2-weighted; B: T1-weighted contrast-enhanced axial MR images; C: ^{18}F-FDG PET-CT; D: DWI showing suspicious lymph nodes in mesorectal (red arrows) and extramesorectal areas (white areas). On DWI, extramesorectal lymph node is more remarkable than T2W and contrast-enhanced T1W sequences. DWI: Diffusion-weighted imaging; ^{18}F-FDG PET-CT: ^{18}F-fluorodeoxyglucosepositron emission tomography-computedtomography.

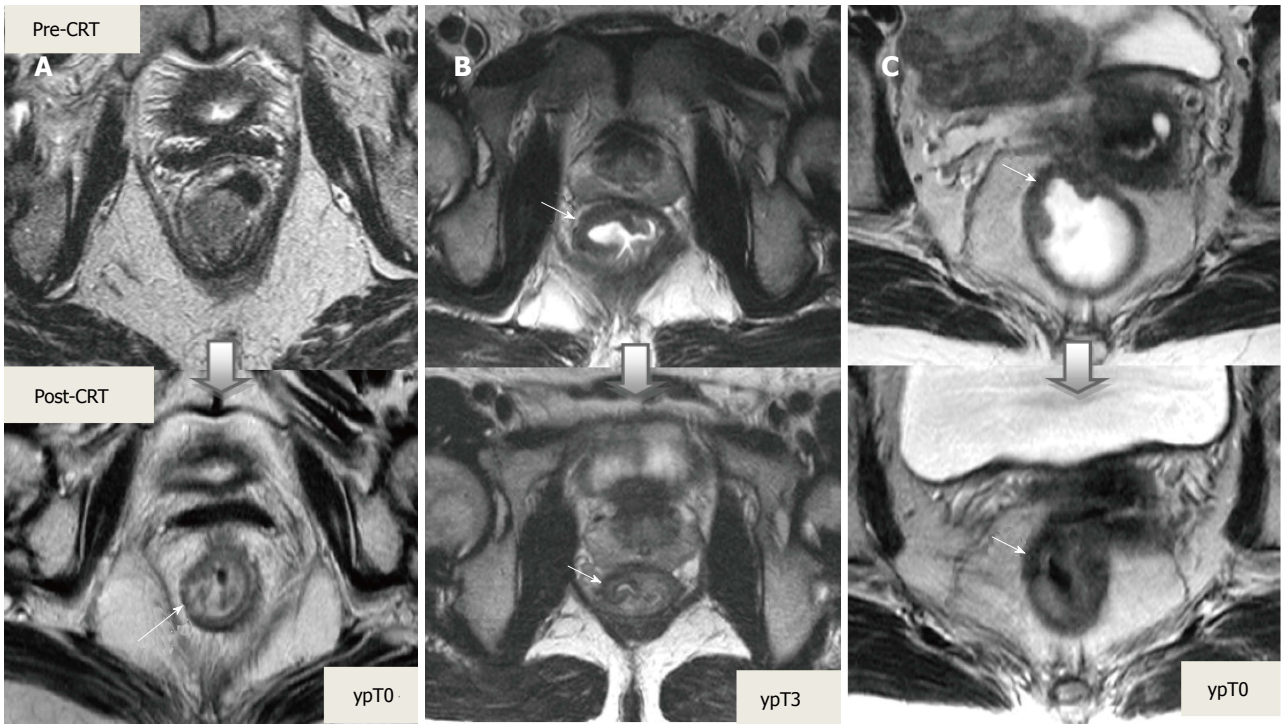

Figure 17 Tumor restaging after neoadjuvant chemoradiotherapy. On T2-weighted MR images in different patients showing baseline and post-CRT images on upper and lower series, respectively. A: In ypT0 rectal tumor, posttreatment axial image shows a normal, two-layered rectal wall (arrow), corresponding to complete response; B: In ypT3 rectal tumor, posttreatment axial image shows normal, two-layered rectal wall (arrow). This is an example for false-negative MR assessment of complete tumor regression; C: In ypT0 rectal tumor, posttreatment axial image shows thick, fibrotic low signal intensity scar (arrow) in pretreatment T3 tumor area. CRT: Chemoradiotherapy.

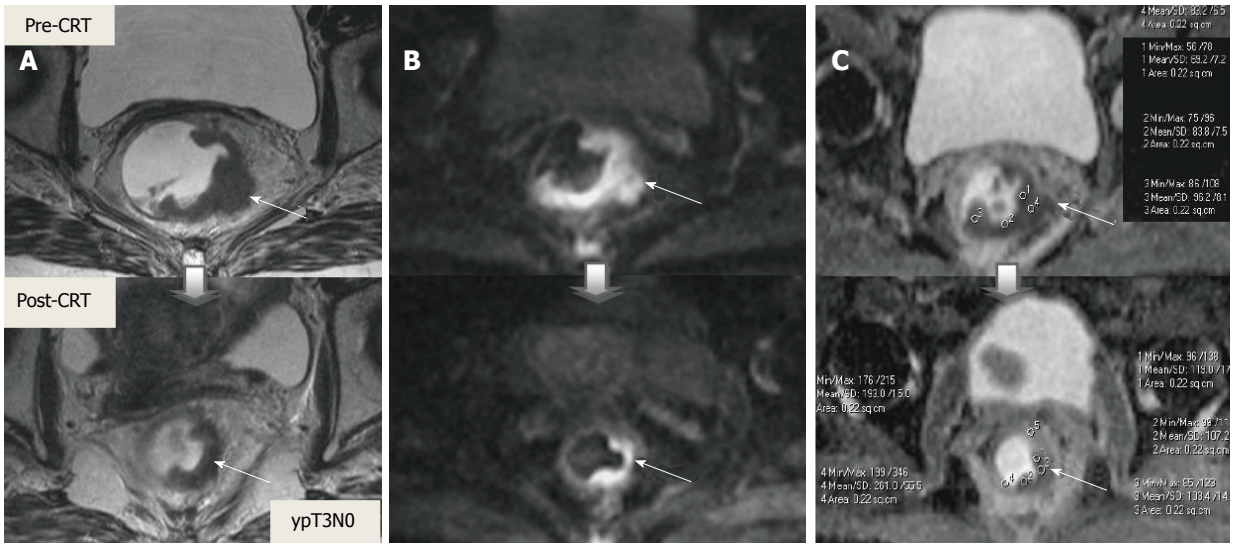

Figure 18 Post-chemoradiotherapy restaging using diffusion-weighted imaging in ypT3 rectal tumor. On T2-weighted (A), DW (B) and ADC (C) images in the same patient, baseline and post-CRT images are shown on upper and lower series, respectively. A: Posttreatment T2-weighted axial image shows semiannular infiltrating tumor, compatible with a residual T3 tumor (arrow); B: Posttreatment DW; C: ADC images delineate high and low signal-intensity corresponding to the tumor, respectively (arrow). Pre- and post-treatment mean ADC values are 0.68-0.72, 1.22-1.44 × 10^{-3} mm²/s, respectively, in the tumor area. Post-therapy ADC increase is compatible with therapy response. CRT: Chemoradiotherapy.

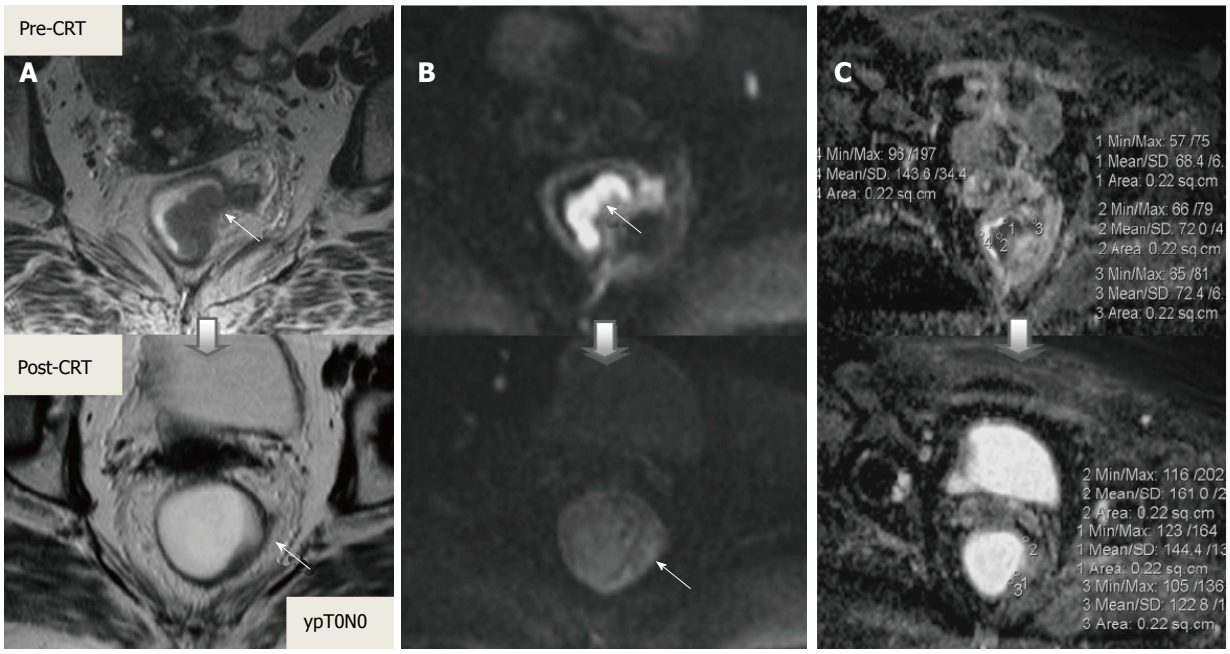

Figure 19 Post-chemoradiotherapy restaging using diffusion-weighted imaging in ypT0 rectal tumor. On T2-weighted (A), DW (B) and ADC (C) images in the same patient, baseline and post-CRT images are shown on upper and lower series, respectively. A: Posttreatment T2-weighted axial image shows a thick wall of low-signal-intensity fibrosis in the previous rectal tumor area (arrow). It is difficult to determine whether this area contains tumor cells or completely devoid of tumor cells (complete response); B: On posttreatment DW image (B-800), there is no diffusion signal in previous tumor area (arrows), compatible with complete response. In this case, DWI allows the correct differentiation of viable tumor from fibrosis; C: ADC images show post-therapy mean ADC increase (0.70 × 10^{-3} mm²/s *vs* 1.40 × 10^{-3} mm²/s) compatible with therapy response, but does not allow prediction of complete response. DWI: Diffusion-weighted imaging; CRT: Chemoradiotherapy.

on post-CRT T2-weighted MRI, in contrast, areas of residual tumor have intermediate signal-intensity[46]. Careful review of high-resolution images and DWI can enable distinction of small residual tumor within fibrosis (Figure 18).

In addition to morphologic findings, DWI can provide functional information that can be correlated with changes at the cellular level in response to treatment. After CRT, the decrease in cellularity and development of fibrosis or necrosis in responders results in an increase in diffusion, which decreases diffusion signal intensity in diffusion-weighted images and increases ADC values

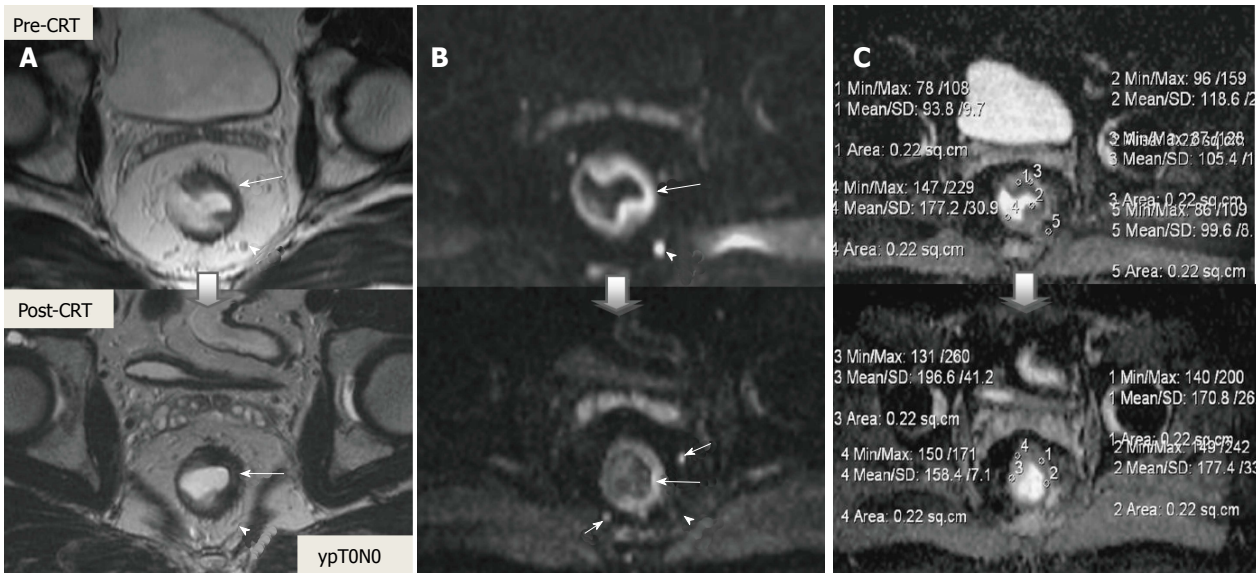

Figure 20 Post-chemoradiotherapy restaging using diffusion-weighted imaging in ypT0 rectal tumor. On T2-weighted (A), DW (B) and ADC (C) images in the same patient, baseline and post-CRT images are shown on upper and lower series, respectively. A: Posttreatment T2-weighted axial image shows a thick wall of low-signal-intensity fibrosis and areas suspicious for residual tumor have intermediate signal-intensity in the previous rectal tumor area (long arrow); B: Posttreatment DW images delinate a small foci of intermediate and low signal-intensity, respectively, compatible with residual tumor (long arrow); C: ADC images show post-therapy mean ADC increase (1.05×10^{-3} mm²/s *vs* 1.80×10^{-3} mm²/s), compatible with therapy response, but not with complete response. The suspicious mesorectal lymph node (arrowheads) is invisible on T2 and DWI after CRT, but the other two are still visible (short arrows). This case is an example for false-positive tumor and lymph node response evaluation of DWI. DWI: Diffusion-weighted imaging; CRT: Chemoradiotherapy.

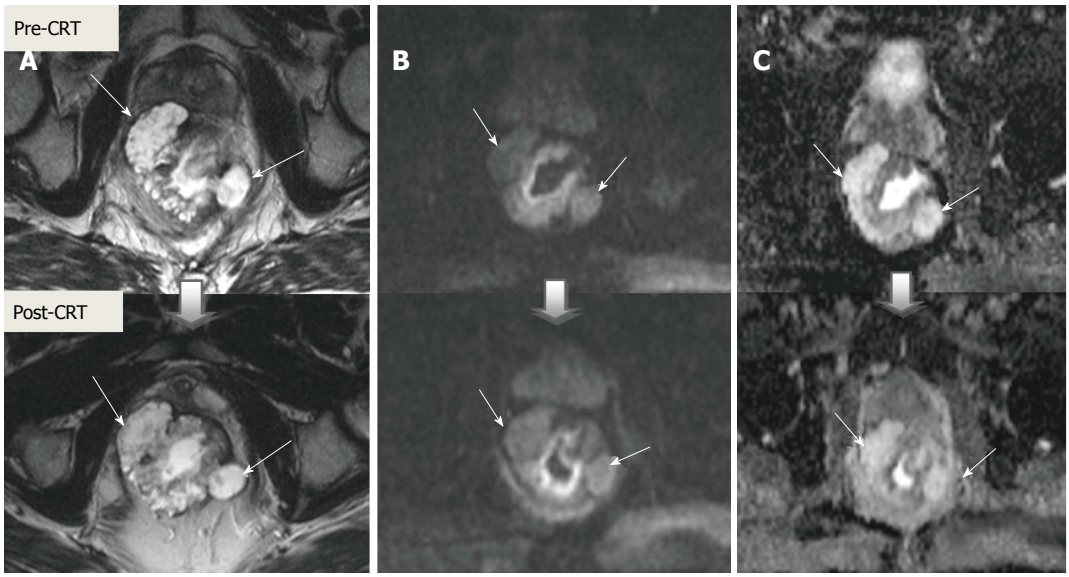

Figure 21 Mucinous adenocarcinoma. A: T2; B: Diffusion-weighted; C: ADC images in the same patient, baseline and post-CRT images are shown on upper and lower series, respectively. The mucinous tumor exhibits hyperintensity on T2, diffusion, and ADC images before and after treatment regardless of their response to treatment. Pre-and post-treatment ADC values are 1.70×10^{-3} mm²/s and 2.10×10^{-3} mm²/s, respectively. Their response to CRT cannot be assessed using diffusion-weighted imaging. CRT: Chemoradiotherapy.

and ADC signal intensity in ADC images[20,23] (Figures 18 and 19). Although DWI can differentiate viable tumor from fibrosis and good and bad response, it does not allow for predicting complete response[19] (Figure 20). Moreover, the response of mucinous tumors to CRT cannot be assessed using DWI because they exhibit ADC hyperintensity even before treatment (Figure 21).

DISTANCE TO THE MESORECTAL FASCIA

CRM is considered uninvolved if a tumor free margin is seen at least 1 mm from MRF after CRT. This finding has strong negative predictive value (98%) of MR imaging for CRM involvement, whereas it has low positive

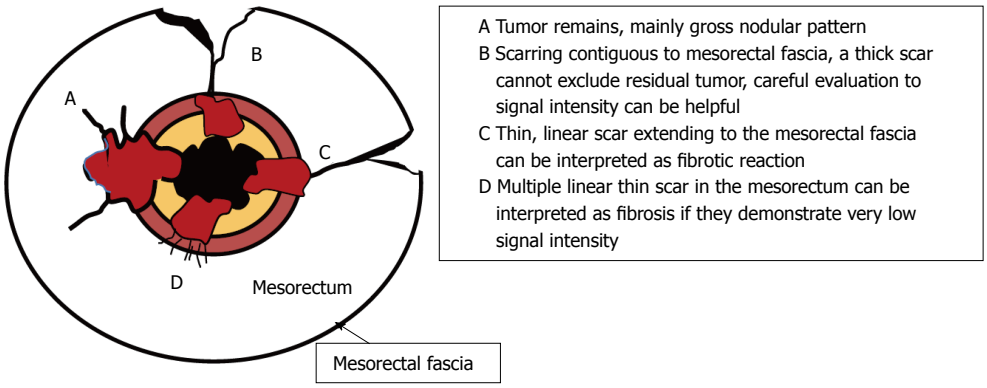

Figure 22 Schematic representation of effects of chemoradiotherapy on a rectal tumor and circumferential resection margins. Adapted from ref. [27]: Nougaret S, Reinhold C, Mikhael HW, Rouanet P, Bibeau F, Brown G. The use of MR imaging in treatment planning for patients with rectal carcinoma: have you checked the "DISTANCE"? *Radiology* 2013; **268**: 330-344.

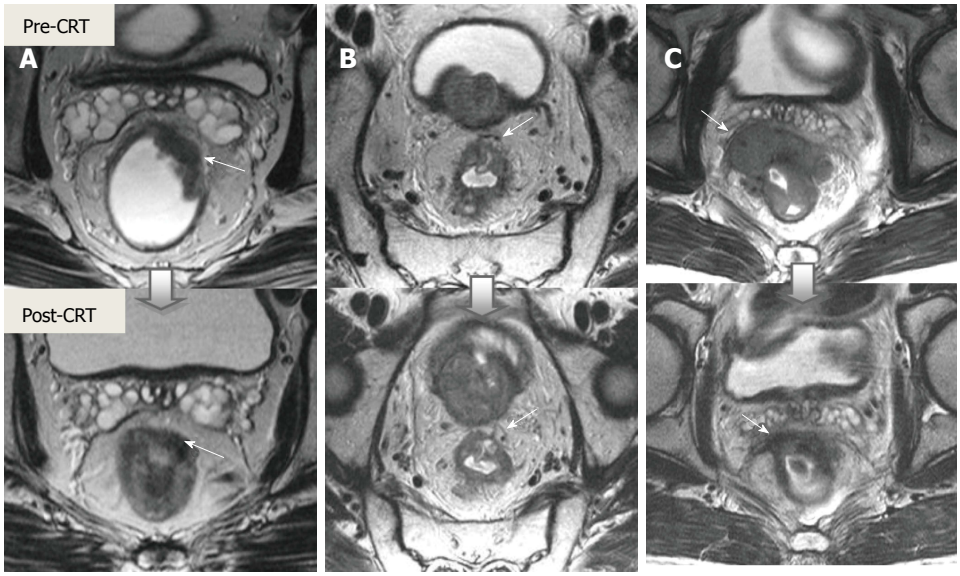

Figure 23 The effects of chemoradiotherapy on a rectal tumor and circumferential resection margins. T2-weighted axial magnetic resonance images in different patients show baseline and post-CRT images on upper and lower series, respectively. A: Overstaging due to thick, hypointense tissue infiltration at the mesorectal fascia (arrow) in ypT2 rectal tumor with no MRF invasion; B: In ypT3 rectal tumor with no MRF invasion, thick fibrous retractions of the tumor, suspicious for CRM positivity (arrow); C: Rectal mass is markedly shrunken with low-signal-intensity tissue infiltration at the mesorectal fascia (arrow). At surgery, there was tumor invasion of the mesorectal fascia. CRM: Circumferential resection margins; MRF: Mesorectal fascia; CRT: Chemoradiotherapy.

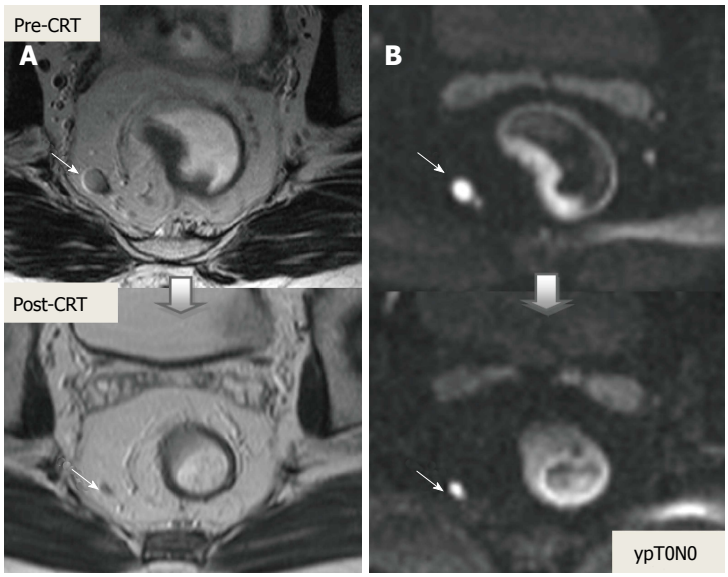

Figure 24 On diffusion-weighted imaging, false-positive mesorectal lymph node evaluation after chemoradiotherapy in ypT0N0 rectal cancer. A: T2-weighted axial magnetic resonance images show significant diminution in nodal size after chemoradiotherapy, compatible with negative lymph node (arrows); B: Diffusion-weighted images, high diffusion signal continues after treatment in the perirectal lymph node, compatible with positive lymph node (arrows). CRT: Chemoradiotherapy.

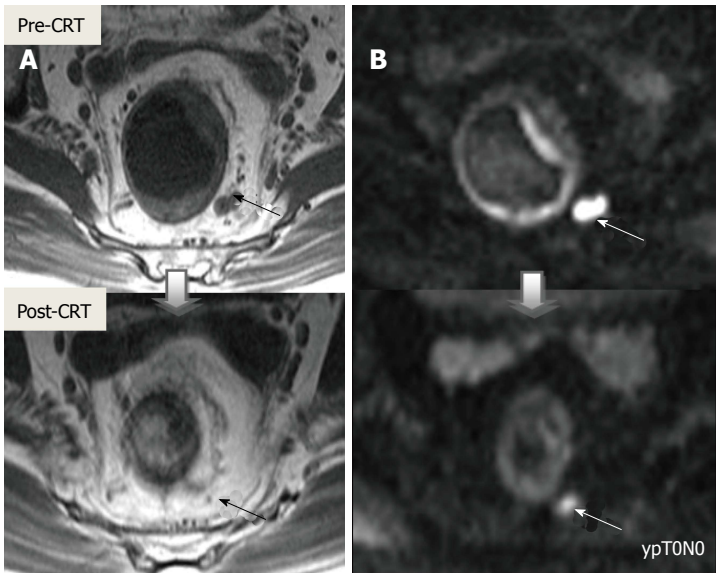

Figure 25 On diffusion-weighted imaging, false-positive mesorectal lymph node after chemoradiotherapy in ypT0N0 rectal cancer. A: T2-weighted axial images show significant diminution in nodal size, compatible with complete response; B: The contuniation of high diffusion signal intensity on residual fibrotic lymph node incorrectly corresponds to a metastatic lymph node (arrows). CRT: Chemoradiotherapy.

predictive value[49]. In some rectal tumor, however, CRT results in a markedly reduction tumor volume, but also in retraction of pre-existing contacts with MRF. It is difficult to determine whether this area contains tumor cells or completely devoid of tumor cells[50] (Figures 22 and 23).

N-staging

After CRT, nodal size (short axis diameter) is more reliable for nodal re-staging. It is difficult to differentiate a metastatic lymph node from a healthy lymph node with irradiation changes using morphologic criteria or DWI; therefore, lymph node restaging often results in overstaging[27,50] (Figures 24 and 25).

The accuracy of MRI for restaging is generally lower than the accuracy of MRI for initial staging, mainly owing to overstaging of nodal disease, failure to differentiate tumoral infiltration or residual tumor from desmoplastic reaction or radiation fibrosis[50]. According to recent meta-analysis results, MRI accuracy was variable for restaging rectal cancer after neoadjuvant treatment; however, significantly better results were achieved when DWI was used or with experienced observers. The authors also reported that MRI could be used for evaluating CRM staging, but nodal staging remained a challenge[51].

CONCLUSION

Using high-resolution MRI, standardizing image acquisition techniques and interpretation of images, comparative evaluation of pre- and post-CRT MR images, adding DWI to the standard approach, and importantly, experience and awareness of the limitations can improve diagnostic accuracy of MRI for re-staging.

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MINIREVIEWS

Immunotherapy in pancreatic cancer: Unleash its potential through novel combinations

Songchuan Guo, Merly Contratto, George Miller, Lawrence Leichman, Jennifer Wu

Songchuan Guo, Merly Contratto, George Miller, Lawrence Leichman, Jennifer Wu, Division of Hematology and Oncology, Perlmutter Cancer Center, New York University School of Medicine, New York, NY 10016, United States

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Correspondence to: Jennifer Wu, MD, Associate Professor, Division of Hematology and Oncology, Perlmutter Cancer Center, New York University School of Medicine, 462 First Ave, BCD556, New York, NY 10016,
United States. jennifer.wu@nyumc.org
Telephone: +1-212-2636530
Fax: +1-212-2638210

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Abstract

Pancreatic cancer is the third leading cause of cancer mortality in both men and women in the United States, with poor response to current standard of care, short progression-free and overall survival. Immunotherapies that target cytotoxic T lymphocyte antigen-4, programmed cell death protein-1, and programmed death-ligand 1 checkpoints have shown remarkable activities in several cancers such as melanoma, renal cell carcinoma, and non-small cell lung cancer due to high numbers of somatic mutations, combined with cytotoxic T-cell responses. However, single checkpoint blockade was ineffective in pancreatic cancer, highlighting the challenges including the poor antigenicity, a dense desmoplastic stroma, and a largely immunosuppressive microenvironment. In this review, we will summarize available clinical results and ongoing efforts of combining immune checkpoint therapies with other treatment modalities such as chemotherapy, radiotherapy, and targeted therapy. These combination therapies hold promise in unleashing the potential of immunotherapy in pancreatic cancer to achieve better and more durable clinical responses by enhancing cytotoxic T-cell responses.

Key words: Immunotherapy; Pancreatic cancer; Anti-programmed cell death protein-1; Anti-programmed cell death protein-ligand1; Anti-cytotoxic T lymphocyte antigen-4; Single therapy; Combination therapies; Radiation therapy; GVAX; CRS-207; CD40 agonist

Core tip: Pancreatic cancer is the third leading cause of cancer mortality in both men and women in the United States. Pancreatic cancer is one of nonimmunogenic

cancers that lacks of optimal treatments especially from immunotherapy prospective. Therefore, combining immune checkpoint therapies with other treatment modalities in pancreatic cancer will be the best strategy to achieve better and more durable clinical responses by enhancing cytotoxic T-cell responses.

Guo S, Contratto M, Miller G, Leichman L, Wu J. Immunotherapy in pancreatic cancer: Unleash its potential through novel combinations. *World J Clin Oncol* 2017; 8(3): 230-240 Available from: URL: http://www.wjgnet.com/2218-4333/full/v8/i3/230.htm DOI: http://dx.doi.org/10.5306/wjco.v8.i3.230

INTRODUCTION

Pancreatic cancer is the third leading cause of cancer mortality in both men and women in the United States[1]. The vast majority of patients with pancreatic cancer are diagnosed with advanced disease, and there has been a lack of optimal treatment option as the cancer is highly refractory to standard chemotherapy. Recently, two chemotherapy regimens, FOLFIRINOX and gemcitabine plus albumin-bound paclitaxel (nab-paclitaxel), have emerged as the standard of care for metastatic pancreatic cancer. These two regimens showed improved overall and progression-free survival (PFS) compared to gemcitabine alone in two phase Ⅲ randomized controlled trials[2,3]. Nevertheless, only up to 30% of patients showed response to either of these two regimens. The median PFS and overall survival (OS) remain poor, under 6 and 12 mo, respectively. Thus, there is still an urgent need to develop therapies that deliver more effective and durable clinical responses.

RELEVANCE OF IMMUNITY TO PANCREATIC CANCER

Observations in human disease and murine modeling has suggested that pancreatic cancer is almost invariably associated with a robust inflammatory infiltrate which can have divergent influences on disease progression by either combating cancer growth *via* antigen-restricted tumoricidal immune responses or by promoting tumor progression *via* induction of immune suppression (Figure 1)[4-6]. For example, cluster of differentiation 8 ($CD8^+$) and T-helper type 1 cells (Th1)-polarized cluster of differentiation 4 ($CD4^+$) T cells mediate antitumor effects in murine models of pancreatic cancer and are associated with increased survival in patients with pancreatic cancer[7-10]. Conversely, we recently reported that T- helper type 2 cells (Th2)-polarized $CD4^+$ T cells promote pancreatic cancer progression in mice and intra-tumoral $CD4^+$ Th2 cells infiltrates correlate with reduced survival in human disease[7-9,11-13]. Similarly, $Foxp3^+$ T-regulatory cells (Tregs) facilitate tumor immune escape in pancreatic cancer[14]. Myeloid cells can influence T cells differentiation and cytotoxicity in pancreatic cancer. We reported that tumor-infiltrating myeloid-derived suppressor cells (MDSCs) negate cytotoxic $CD8^+$ T cells anti-tumor responses, accelerates pancreatic cancer growth and metastasis[8,15-17]. Similar to T cells, macrophages also have cell types with different properties such as classically activated (M1) macrophages induce immunogenic responses, whereas alternatively activated (M2) macrophages have permissive influences on tumor growth by recruiting Tregs and Th2 cells[18]. However, the drivers of immunosuppressive cell differentiation in pancreatic cancer are based on comprehensive understanding of regulation of the balance between immunogenic and immune-suppressive T cell populations.

THE EMERGENCE OF CHECKPOINT IMMUNOTHERAPY

The last few years witnessed a paradigm shift in cancer treatment strategy incorporating immunotherapy. Unprecedented clinical success has been observed for therapies targeting two major checkpoints of T cell response (Figure 2): Cytotoxic T lymphocyte antigen-4 (CTLA-4) and programmed cell death protein-1 (PD-1). Both checkpoints are expressed on activated T cells, but they act in distinct pathways. CTLA-4 blocks the essential cluster differentiation 28 (CD28) costimulation by competing and depleting the ligand of CD28 (B7-1 and B7-2) on antigen presenting cells (APCs). On the other hand, PD-1 interferes with the signaling pathways mediated by the T cell receptor and serves as a more distal block of T cell response by binding to its ligands (programmed death-ligand 1 (PD-L1) and programmed death-ligand 2 (PD-L2) which are present on many cell types including tumors cells[19].

Monoclonal antibodies targeting CTLA-4 or PD-1 have shown durable clinical responses and prolonged OS in patients with melanoma, a highly immunogenic cancer. While single agent PD-1/PD-L1 inhibitors demonstrate impressive clinical benefits in many cancers such as non small cell lung cancer (NSCLC), renal cell carcinoma, bladder cancer, and Hodgkin's lymphoma[20-29]. These results have led to FDA approval of Ipilimumab (anti-CTLA-4) in 2011 in melanoma[30]. PD-1 inhibitors such as pembrolizumab and nivolumab were approved later in melanoma as well[23,28,29]. PD-1 inhibitors (nivolumab and pembrolizumab), along with PD-L1 inhibitors such as atezolizumab have been approved in NSCLC, another example of immunogenic cancer[21,22,24,29]. The activity of CTLA-4 and PD-L1 inhibitors are being explored in pancreatic cancer as well[22,31].

EVIDENCE OF MINIMAL ACTIVITY OF SINGLE AGENT CHECKPOINT IMMUNOTHERAPY IN PANCREATIC CANCER

In early clinical trials single agent therapy with anti-CTLA-4

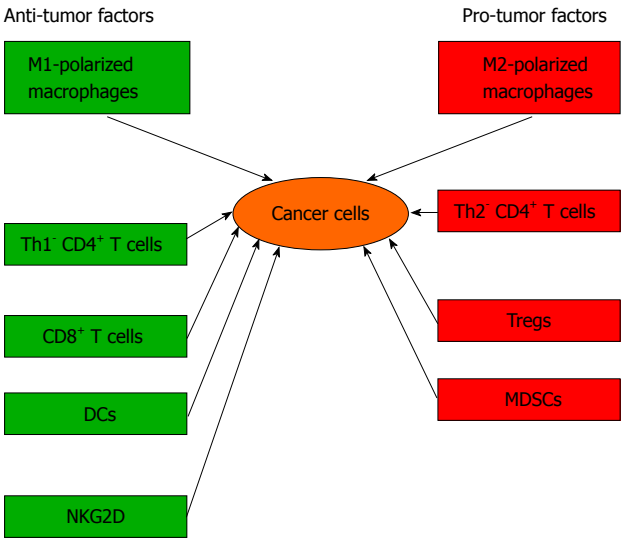

Figure 1 Anti-tumor and pro-tumor factors. Anti-tumor factors: M1 (classically activated macrophages), $Th1^{-}CD4^{+}$ T cells (T-helper type 1-cluster differentiation 4 T cells), $CD8^{+}$ T cells, DC (dendritic cells), NKG2D (natural killer group 2 member). Pro-tumor factors: M2 (alternatively activated macrophages), $Th2^{-}CD4^{+}$ T cells (T-helper type 2-cluster differentiation 4 T cells) Th2, Tregs (T-regulatory cells), and MDSCs (myeloid-derived suppressor cells).

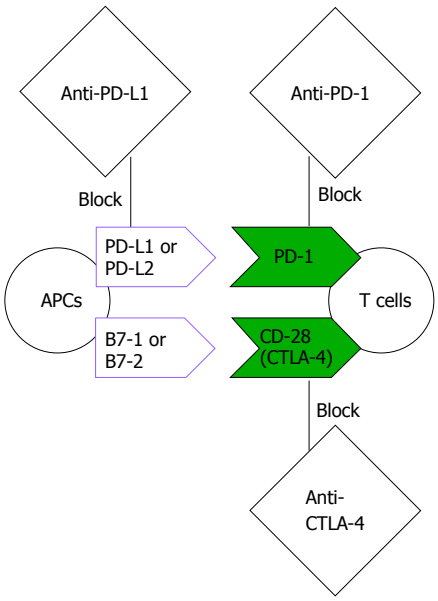

Figure 2 Immunotherapy basics. Anti-PD-L1 inhibit PD-L1 (programmed cell death-ligand 1) binding to PD-1 (Programmed cell death protein-1). Anti-PD-1 inhibit PD-1 on T-cell that binds to PD-L1 or PD-L2 (programmed cell death ligand-2) on APC (antigen presenting cell). Anti-CTLA-4 (anti-cytotoxic T lymphocyte antigen 4) inhibit CD28 (cluster differentiation 28) on T cell that binds to B7-1 or B7-2 (ligand of CD28) on APC.

or anti-PD-1/anti-PD-1 pathway (anti-PD-L1) alone were largely ineffective in pancreatic cancer[22,31,32]. In a single-arm phase Ⅱ study, Ipilimumab failed to induce tumor response in patients with advanced pancreatic cancer[32]. Similarly, single agent BMS-936559, an anti-PD-L1 monoclonal antibody, did not show any activity in 14 patients with advanced pancreatic cancer in a phase Ⅰ study[22].

POTENTIAL BARRIERS THAT HINDER EFFICACY OF IMMUNOTHERAPY

The efficacy of immunotherapy in pancreatic cancer is handicapped by small number of cumulative mutational load that can lead to expression of non-self-antigens, or "neoantigens" which are recognized by the immune system as foreign. Cancers with higher number of mutational load are associated with more neoantigens that are easier to be recognized by the immune system, compared to cancer with lower number of mutational load[33-35]. There are 3 major barriers for the utility of immunotherapy in pancreatic cancer. First, the mutational load in pancreatic cancer is very low as compared with melanoma and lung cancers[36,37]. Second, pancreatic cancer features a largely immunosuppressive microenvironment, characterized by a dense desmoplastic reaction with prominent infiltration of tumorigenic macrophages and myeloid derived suppressor cells (MDSCs)[38]. Third, there are very few infiltrating T cells in the microenvironment of pancreatic cancer, therefore could not provide sufficient T cell responses. Pancreatic cancer creates a nonimmunogenic (or "cold") tumor microenvironment, limiting the activity of immune checkpoint therapies[31].

EVIDENCE OF T CELL IMMUNITY

On the other hand, there is still evidence of T cell-mediated immunity in pancreatic cancer. An analysis of resected surgical samples of pancreatic cancer patients has shown that higher levels of $CD4^{+}$ and $CD8^{+}$ tumor infiltrating T cells are associated with better prognosis[10]. In addition, since immunosuppression occurs early during tumorigenesis as shown in $Pdx1^{Cre}$;$Kras^{G12D}$;$Tp53^{R172H}$ (KPC) mouse model, the tumor cells may have been shielded from immune pressure, thus preserving their sensitivity to T cell attack[38].

In addition, downstream signals are also critical in the T cell immune responses. Interferon-gamma (IFN-γ) promotes inhibition of melanoma cell growth and induces apoptosis of tumor cells by regulating T-cells responses[39-44]. Immune checkpoint inhibitors increase production of IFN-γ from T-cell[45-50]. However its effect will be suboptimal if there is a defect in the IFN-γ pathway[51]. Studies in patients with melanoma showed that a defect in the IFN-γ pathway can lead to resistance to anti-CTLA4 and anti-PD-1 therapies[51,52]. Several genomic biomarkers of IFN-γ pathways such as interferon gamma receptor 1, janus kinase 1 (JAK1), and JAK2 have been identified in melanoma patients with good response to immune checkpoint therapies[41-43,51,52]. On the other hand, genes such as suppressor of cytokine signaling 1 (SOCS1) and protein inhibitor of activated signal transducer and activator of transcription 4 (PIAS4) have

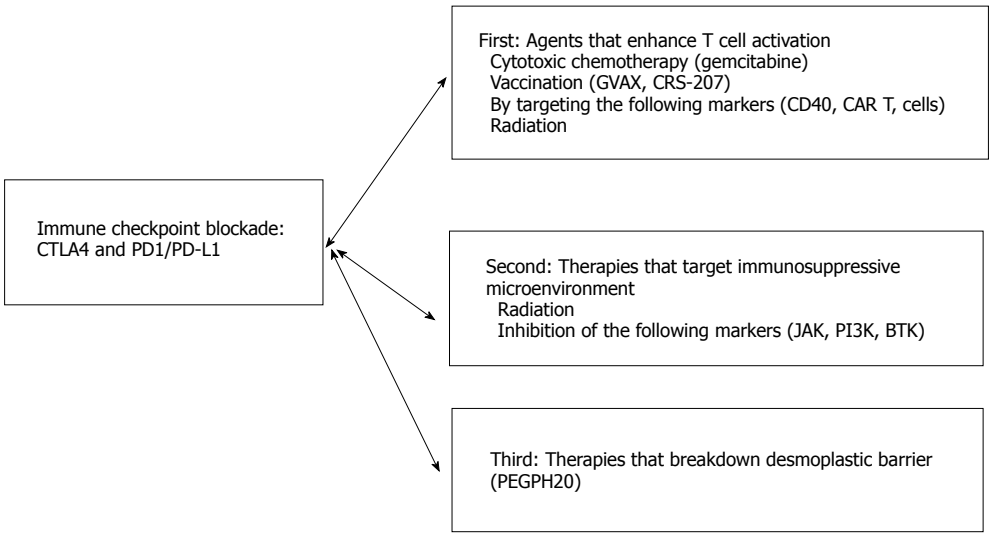

Figure 3 Searching for the optimal combination to maximize the potential of immune checkpoint blockade for the treatment of pancreatic cancer. CTLA-4: Cytotoxic T lymphocyte antigen-4; PD-1: Programmed cell death protein-1; PD-L1: Programmed death ligand-1; CD40: Cluster differentiation 40; CAR T cells: Chimeric antigen receptor T cells; PI3K: Phosphoinositide-3-kinase; BTK: Bruton tyrosine kinase; JAK: Janus kinase; PEGPH20: Pegylated hyaluronidase.

demonstrated the opposite effects by inhibiting IFN-γ signaling pathway[51,53,54].

STRATEGIES OF TURNING ON THE ACTIVITY OF IMMUNOTHERAPY

Thus, the incorporation of additional therapies that can turn a "cold" tumor microenvironment into a "hot" one presents an important strategy to elicit clinical activity of immune checkpoint therapies. These additional therapies mainly fall into three categories (Figure 3): First, therapies that enhance tumor antigen presentation to help T cell priming/activation; second, therapies that modulate tumor microenvironment to relieve immunosuppression. Third, therapies which breakdown the desmoplastic barrier surrounding pancreatic cancer to bring infiltrating T cells. Below we will summarize the combination therapies that have already been assessed clinically and provide future directions of new combinations that may hold promise.

FIRST (ENHANCE T CELL ACTIVATION)

Immune checkpoint therapy + chemotherapy

Gemcitabine is one of the backbone chemotherapy agents for the treatment of pancreatic cancer. It has been suggested that gemcitabine is not immunosuppressive in pancreatic cancer patients and may be able to enhance naïve T cells activation[55]. Combination of gemcitabine and immune checkpoint blockade has been evaluated for their potential synergistic activity.

Gemcitabine plus CTLA-4 blockade: A phase Ⅰ clinical study evaluated the combination of gemcitabine and an anti-CTLA-4 antibody (tremelimumab) in treatment naive patients with metastatic pancreatic cancer. This combination showed a tolerable side effect. Among 28 out of 34 evaluable patients, 2 achieved partial response (PR) and 7 showed stable disease (SD) for > 10 wk[4]. In another ongoing phase Ib study of unresectable pancreatic cancer, preliminary results showed that, among 11 evaluable patients (out of 13 enrolled), ipilimumab and gemcitabine resulted in 2 PR and 5 SD[56,57].

Gemcitabine plus PD-1/PD-L1 blockade: An immunohistochemistry analysis has shown that positive PD-L1 expression in resected pancreatic cancer was correlated with worse OS[58]. In a mouse model of pancreatic cancer, combining gemcitabine with either anti-PD-1 or anti-PD-L1 antibody enhanced tumor infiltration of $CD8^+$ T cells and resulted in complete responses in treated mice[58]. A clinical pilot study of combination of gemcitabine and anti-PD-1 antibody has closed to enrollment (NCT01313416).

Immune checkpoint therapy + cancer vaccines

The most extensively studied pancreatic cancer vaccine is GVAX. GVAX is a whole cell vaccine composed of irradiated, allogeneic pancreatic tumor cells genetically engineered to secret granulocyte macrophage-colony stimulating factor (GM-CSF), a cytokine that stimulates dendritic cell activation and T cell priming. When used as part of adjuvant therapy in the post-resection setting, GVAX was able to induce pancreatic cancer specific $CD8^+$ T cell expansion as shown in a phase Ⅱ study[59]. Also, when used as neoadjuvant and adjuvant therapy, GVAX and low dose cyclophosphamide (an alkylating agent with an ability to deplete Tregs) resulted in formation of intratumoral tertiary lymphoid aggregates and T cell infiltration, suggesting the ability of GVAX in the conversion of pancreatic cancer from a "non-immunogenic" into an "immunogenic" state[60].

GVAX plus CTLA-4 blockade: In a small phase Ib

study, GVAX in combination with anti-CTLA-4 antibody ipilimumab was evaluated in 30 patients with advanced, refractory pancreatic cancer that were previously treated with gemcitabine-based chemotherapy. Compared to ipilimumab alone, the combination therapy resulted in improved survival (27% *vs* 7% at 1 year). Also, a longer survival was associated with an increase in peak mesothelin-specific T cells and a larger T cell repertoire (the percentage of mesothelin peptides for which enhanced T-cell responses were measured), indicating a positive role of T cell response[61].

GVAX plus PD-1/PD-L1 blockade: Detailed analysis of lymphoid aggregates formed after GVAX therapy revealed elevated expression of PD-L1 on monocytes/macrophages[60,62], suggesting the potential benefit of targeting PD-1/PD-L1 checkpoint. This concept was supported by experiments in a pancreatic cancer mouse model, where the combination of GVAX and an anti-PD-1 antibody resulted in better survival than anti-PD-1 antibody alone, and this activity was correlated with increased $CD8^+$ T cells and elevated IFN-γ production in the tumor microenvironment[62]. Currently, a randomized clinical study (NCT02451982) is ongoing to evaluating GVAX with or without anti-PD-1 antibody (nivolumab) as neoadjuvant and adjuvant treatment in patients with resectable pancreatic cancer.

GVAX and CRS-207 plus PD-1/PD-L1 blockade: CRS-207 is a bacterial vaccine composed of live-attenuated, double deleted Listeria monocytogenes expressing human mesothelin, an antigen commonly overexpressed in pancreatic cancer cells. CRS-207 can induce robust innate as well as mesothelin-specific adaptive immune response, therefore allowing for a "boost" to the immune response initiated by GVAX. In a randomized, phase Ⅱ study, GVAX prime followed by CRS-207 boost resulted in prolonged OS compared to GVAX alone in patients with metastatic, refractory pancreatic cancer. This study also showed that mesothelin-specific $CD8^+$ T cell response was correlated with better survival[63,64]. On the basis of these findings, a randomized phase Ⅱ study (NCT02243371) was to evaluate whether adding anti-PD-1 therapy (nivolumab) will further enhance the activity of this prime-boost strategy[65]. This study has closed to enrollment.

In a phase IIb study (NCT02004262) in refractory and metastatic pancreatic cancer, 303 patients were randomized between GVAX and CRS-207 (arm A), only CRS-207 (arm B), and single agent chemotherapy (arm C)[66]. No OS advantage was seen in arm A when compared to arm C[66]. A large number of patient drop out prior to treatment was observed in both arm A and C (40% versus 60%, respectively), indicating the challenge of therapeutic benefit in refractory pancreatic cancer. It also hints that these patients in the refractory setting may be too sick to benefit from immunotherapy due to rapid deterioration of disease.

Immune checkpoint therapy + agents enhancing T cell immunity

CD40 agonist: CD40 is a member of the tumor necrosis factor receptor family. Ligation of CD40 can occur on dendritic or B cells, or at CD40 ligand (CD154) on activated T cells, such effect can enhance T cell immunity[67]. In a 22 patients series with unresectable pancreatic cancer, a CD40 agonist (CP-870, 893) and gemcitabine led to an encouraging clinical response[7,11]. Rather unexpectedly, it showed that tumor infiltration by macrophages played a larger role for depletion of tumor stroma and killing of tumor cells[7]. In a more recent study in the KPC mouse model, however, the use of CD40 agonist monoclonal antibody (mAb) with gemcitabine and nab-paclitaxel induced macrophage-independent T cell immunity. This study also found that CD40 agonist in addition to chemotherapy was able to sensitize the tumors to anti-CTLA-4 and/or anti-PD-1 therapies, leading to tumor regression and improved survival[31]. A recent study using an orthotopic pancreatic cancer mouse model also demonstrated tumor regression and enhanced immune response with the combination of CD40 agonist antibody with gemcitabine/Nab-paclitaxel[68]. It is yet to be seen whether these pre-clinical results can translate into clinical benefits.

CAR T cells: Autologous T cells genetically engineered to express a chimeric antigen receptor (CAR) have been developed to trigger cancer-specific T cell immunity and have shown impressive activity in acute lymphoblastic leukemia[69]. For the treatment of pancreatic cancer, the CARs are engineered to recognize mesothelin, a specific membrane protein antigen overexpressed on pancreatic cancer cells. Mesothelin-specific CAR T cells are currently under phase Ⅰ clinical evaluation, with preliminary results suggesting acceptable safety profiles and potential clinical activity against advanced pancreatic cancer. This study demonstrated that 2 out of 6 patients achieved SD and one patient with liver metastasis at baseline showed no fluorodeoxyglucose (FDG) uptake within 1 mo of treatment[12,70,71]. Therefore, CAR T cells represent another treatment modality to combine with immune checkpoint therapies.

Immune checkpoint therapy + radiotherapy

The effects of radiotherapy (RT) on the immunology of pancreatic cancer have not been intensively studied. However, work in other cancers has suggested that RT should be considered an immune adjuvant as evidenced by radiotherapy (RT) induced enhancement of both innate and adaptive immunity. For example, the immunogenicity of dendritic cells (DCs) is reportedly improved by RT-induced necrotic tumor cell release of high mobility group box 1 protein (HMGB1) which ligates toll-like receptor 4 (TLR4) and toll-like receptor 9 (TLR9) on DCs. Such events promote DCs' cellular maturation and enhance their antigen processing capabilities[72]. Another consequence of RT-induced necrotic cell death is the translocation of calreticulin from the endoplasmic reticulum to the

plasma membrane which facilitates assembly of major histocompatibility-1 (MHC I)-peptide complexes. Calreticulin also enhances DCs cross presentation of antigens to cytotoxic T lymphocytes. In addition to upregulating the antigen-presentation machinery in DCs, RT can reportedly enhance immunogenicity by inducing the release of tumor antigens, upregulating the expression of T-cell co-activating ligands, and sensitizing tumor cells to antigen-independent cell death *via* the Fas receptor[72]. RT is further thought to augment diverse aspects of T cell immunity *via* adenosine triphosphate release from apoptotic cells which induces secretion of Interleukin-1-beta (IL-1β). A consequence of this cascade is T helper1 (Th1) polarization of antigen-restricted $CD4^+$ T cell responses and activation of cytotoxic T cells. Additionally, activation of cytotoxic T cells can be further activated by irradiation, *via* natural killer group 2 member D (NKG2D) receptor on cytotoxic T cells. NKG2D receptor can be induced in a stress event such as DNA damage which can be achieved by RT[72]. Therefore, ionizing radiation can result in "immunogenic cell death', in which the dying tumor cells trigger "danger signals" (a signal of releasing HMGB1 and binding to TLR4 and TLR9 on DCs to process the antigen) to boost T cell activation[72,73].

SECOND (TARGETING IMMUNO-SUPPRESSIVE MICROENVIRONMENT)

As described earlier, an important barrier to the success of immunotherapy in pancreatic cancer is an immunosuppressive tumor microenvironment, enriched with immunosuppressive cells such as tumor associated macrophages (TAMs) and MDSCs. In animal models of pancreatic cancer, blockade of immunosuppressive MDSCs could promote antitumor T-cell responses and block protumor macrophage responses[6,74-76]. Therefore, drugs that block these immunosuppressive cells in the tumor microenvironment represent attractive strategies to sensitize pancreatic cancer to immune checkpoint therapies.

Immune checkpoint therapy + radiotherapy

RT's theoretical potential ability to convert the tumor microenvironment from a "cold" to a "hot" state suggests the opportunity of RT combination with immune checkpoint therapy. In the KPC pancreatic mouse model, any combination of immune checkpoint inhibitor with RT substantially increased OS, when compared to anti-CTLA-4 antibody or anti-PD-L1 antibody alone without RT. In particular, the triple therapy (RT + CTLA-4 antibody + PD-1 antibody) resulted in the highest response rate and longest OS among any of the immunotherapy group as single therapy or in combinations[77].

However, our recent preclinical studies on RT in pancreatic cancer suggest caution as we found that RT induced the programming and recruitment of immunosuppressive M2-like macrophages which lead to the expansion of tumor promoting Th2-polarized CD4+ T cells and Tregs. We also found that combining RT with either macrophage neutralization or M-CSF blockade resulted in synergistic efficacy in mice model, suggesting another treatment strategy for pancreatic cancer utilizing RT combining with colony stimulating factor-1 receptor inhibitor[76,78].

So far there have been no published clinical results on RT plus checkpoint blockade for the treatment of pancreatic cancer. Currently, an open-label, three-cohort, multi-institutional phase Ib study is ongoing at New York University (NCT02868632) to assess stereotactic body radiation therapy (SBRT) in combination with either MEDI4736 (an anti-PD-L1 antibody) alone, tremelimumab (an anti-CTLA4 antibody) alone, or the combination of MEDI4736 and tremelimumab in patients with unresectable/locally advanced previously untreated pancreatic cancer. A study with similar design that tests the combination of radiation with checkpoint blockade in second line setting is also ongoing (NCT02311361).

Immune checkpoint therapy + therapies targeting immunosuppressive microenvironment

JAK inhibitors: The Janus kinase (JAK) and its downstream factor signal transducer and activator of transcription (STAT) are important mediators of signaling pathways initiated from cytokine and growth factor receptors. Excessive JAK/STAT signaling can lead to production and release of inflammatory cytokines, promote recruitment, expansion of MDSCs and Tregs which induce an immunosuppressive tumor microenvironment[79]. Also, JAK/STAT pathway has been shown to induce the expression of PD-L1 on cells in the tumor microenvironment[14,80]. In preclinical studies, JAK inhibitors led to decreased numbers of Tregs, TAMs and MDSCs, with enhanced number of activity of $CD4^+$ and $CD8^+$ T cells[18]. The study of JAK inhibitor Ruxolitinib and capecitabine for the treatment of advanced pancreatic cancer has closed to enrollment (JANUS study; NCT02117479)[81].

PI3K inhibitors: Phosphoinositide-3-kinase (PI3K) is a family of lipid kinases that catalyze the production of second messenger phosphatidylinositol-3,4,5-triphosphate (PIP3), which leads to activation of downstream kinases. PI3K was known to play an important role in signaling pathways in B cells, which were found to contribute to an immunosuppressive microenvironment that dampens T cell immunity[82]. Inactivation of PI3K was associated with a decrease in Tregs and MDSCs and an increase in $CD8^+$ cytotoxic T cell activity, indicating a role of PI3K in regulating tumor microenvironment[5]. PI3K inhibitors could shift immunosuppressive microenvironment in pancreatic cancer into a more immunogenic one. Therefore PI3K inhibitors could help potentiate the activity of immune checkpoint inhibitors.

BTK inhibitors: BTK is a cytoplasmic, Tec family tyrosine kinase important in B-lymphocyte development, differentiation, and signaling. In pancreatic cancer, the BTK

inhibitor (ibrutinib) was shown to inhibit mast cells, and as a result, to reduce fibrosis in the tumor microenvironment both in a KPC mouse model and patient-derived xenograft[83]. Ibrutinib was also known to inhibit interleukin-2-inducible T-cell kinase (ITK), an important enzyme for the survival of Th2 cells; thus ibrutinib may be able to shift the balance away from the Th2 cells protumor response and toward the Th1 cells antitumor immune responses. A phase I/II clinical study assessing ibrutinib in combination with anti-PD-L1 antibody MEDI4736 in relapsed or refractory solid tumors, including pancreatic cancer has closed to enrollment (NCT02403271)[84].

THIRD APPROACH (BREAKDOWN DESMOPLASTIC BARRIER)

Strategy that targets the desmoplastic stroma

PEGPH20: In pancreatic cancer, high levels of hyaluronan in the extracellular matrix contribute to a high interstitial pressure in the tumor stroma, leading to vascular compression and hypoperfusion. Pegylated hyaluronidase PEGPH20 is an enzyme that can degrade hyaluronan, and has been shown in a KPC mouse model to deplete hyaluronan in the tumor stroma and enhance the activity of gemcitabine[85]. In a phase Ⅰ (28 patients) and a phase Ⅱ (135 patients) studies, patients with previously untreated advanced pancreatic cancer, PEGPH20 along with chemotherapy (gemcitabine, or gemcitabine/nab-paclitaxel) resulted in good tumor response and PFS, but only in patients with high levels of hyaluronan[15,86]. Therefore, in pancreatic cancers with high levels of hyaluoran, PEGPH20 therapy may allow more effective T cell infiltration and enhance the activity of immune checkpoint therapies.

CONCLUSION

Both challenges and opportunities exist for the development of effective immunotherapy for pancreatic cancer. Given that single agent therapies against CLTA-4 or PD-1 or PD-L1 immune checkpoint were largely ineffective in pancreatic cancer, ongoing investigations and future directions lie in the field of combination therapies, where additional treatment modalities may unleash durable anti-tumor immune responses by enhancing tumor-specific T cell activation and antagonizing the immunosuppressive microenvironment in pancreatic cancer.

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MINIREVIEWS

Current state and controversies in fertility preservation in women with breast cancer

Enes Taylan, Kutluk H Oktay

Enes Taylan, Kutluk H Oktay, Innovation Institute for Fertility Preservation and In Vitro Fertilization, New York, NY 10019, United States

Enes Taylan, Kutluk H Oktay, Laboratory of Molecular Reproduction and Fertility Preservation, Department of Obstetrics and Gynecology, New York Medical College, Valhalla, NY 10595, United States

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Correspondence to: Kutluk H Oktay, MD, PhD, Laboratory of Molecular Reproduction and Fertility Preservation, Department of Obstetrics and Gynecology, New York Medical College, 40 Sunshine Cottage Rd, Valhalla, NY 10595, United States. koktay@fertilitypreservation.org
Telephone: +1-877-4923666

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Abstract

On average, over 25000 women are diagnosed with breast cancer under the age of 45 annually in the United States. Because an increasing number of young women delay childbearing to later life for various reasons, a growing population of women experience breast cancer before completing childbearing. In this context, preservation of fertility potential of breast cancer survivors has become an essential concept in modern cancer care. In this review, we will outline the currently available fertility preservation options for women with breast cancer of reproductive age, discuss the controversy behind hormonal suppression for gonadal protection against chemotherapy and highlight the importance of timely referral by cancer care providers.

Key words: Fertility preservation; Female breast cancer; Cryopreservation; Oocyte; Embryo; Ovarian suppression; Gonadotropin-releasing hormone agonist; Letrozole; Ovarian tissue cryopreservation

Core tip: Field of fertility preservation has experienced remarkable advances within the last 20 years. As a result, young cancer survivors have numerous options to maintain an important aspect of their quality of life, fertility. In this article we review the current state and controversies in fertility preservation. The article should be an important resource for professionals who take care of young women with breast cancer and other malignancies.

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INTRODUCTION

Breast cancer is the most common malignancy in women

and on average more than 25000 women are diagnosed with breast cancer before reaching the age of 45 years, each year in the United States[1]. Early diagnosis by virtue of significant advances in detection, and newly developed treatment strategies have remarkably improved the course of breast malignancies. According to the National Cancer Institute, 5-year-survival rate for the women under age 45 was estimated to be as high as 88%-98.5% in 2011[2].

While survivorship rates have dramatically increased in women with breast cancer, an important issue related to reproductive function has emerged. Most women with breast cancer are likely to undergo systemic adjuvant or neo-adjuvant chemotherapy with gonadotoxic side effects. As a consequence, preserving fertility potential has become an essential concept in the management of young cancer survivors. Fertility preservation has emerged from this concept as a new and dynamic discipline where oncology and reproductive medicine intersect.

In this review, we aimed to highlight the importance of fertility preservation as a part of routine care for breast cancer patients of childbearing age and outline the key fertility preservation options along with still experimental but promising therapeutic procedures.

COUNSELING FOR FERTILITY PRESERVATION

Because of the trend for having children in later reproductive ages, the number of women who experience breast cancer before completing childbearing is growing. Coupled with the increased survival rates and the growing healthy survivor population, fertility preservation has become an important component of cancer care and the maintenance of quality of life for survivors[3].

American Society of Clinical Oncology (ASCO) and American Society of Reproductive Medicine (ASRM) guidelines for fertility preservation in cancer patients strongly recommend that oncologist should inform their patients about the potential negative effects of chemotherapy on fertility before the initiation of the planned treatment and promptly refer patients to reproductive specialist to discuss the risk of ovarian damage and currently available fertility preservation options[4,5]. However, less than half of the oncologists in the United States always or often refer their cancer patients with fertility-related questions to fertility preservation specialist[6].

It should be stressed that providing timely and accurate information for women of reproductive age with breast cancer is critical for the preservation of future fertility chances before complete loss of the limited and irreplaceable ovarian reserve due to chemotherapy. We have previously shown that early referral of breast cancer patients, especially before breast surgery results in larger number of oocytes and embryos being cryopreserved and less time to the initiation of chemotherapy[7].

IMPACT OF CANCER TREATMENT ON OVARIAN RESERVE

Modern chemotherapeutic agents that are in use for breast cancer treatment can have a spectrum of ovarian toxicity, depending on the class of the agent, age of the patient, and the cumulative dose[8]. We have shown that the most gonadotoxic agents are those that mainly target oocyte genome causing DNA double strand breaks (DBSs)[9]. Under normal circumstances, DNA repair mechanisms are capable of maintaining genomic integrity, however, at the level of severe DNA damage due to genotoxic agents, those repair mechanisms remain insufficient. The severe DNA damage consequently leads to apoptotic death[9]. Ovarian reserve is made up of about 1 million primordial follicle oocytes at birth, and this number is reduced to approximately 500000 at the onset of puberty. These numbers are reduced to about 25000 at age 37 and nearly exhausted at menopause. Because primordial follicles cannot be regenerated, any chemotherapeutic agent that induces DNA breaks in primordial follicle oocyte will result in apoptotic death and cause irreversible reduction in ovarian reserve[9].

Among all gonadotoxic agents, those belong to the alkylating category such as cyclophosphamide, are the most gonadotoxic agents[10]. Because alkylating agents are non cell-cycle specific chemical compounds and hence can target and damage resting primordial follicles that constitute ovarian reserve[9,10].

The risk of chemotherapy-induced ovarian damage has been investigated in numerous clinical studies. Unfortunately, menstruation was used as the surrogate for ovarian function and fertility in the majority of the past studies[11]. However, return of menses is a poor surrogate for reproductive potential, and ovarian reserve might be severely diminished despite the resumption of regular menses[12,13]. In this context, it is reported that after treatment with CMF protocol (cyclophosphamide/methotrexate/5-fluorouracil) 20%-70% of women younger than age 40 experienced amenorrhea[14]. Comparing CMF protocol to the AC protocol (doxorubicin/cyclophosphamide), significantly lower rates of amenorrhea (69% *vs* 34%, respectively) have been reported with the AC protocol[15]. This finding is most likely related to a lower cumulative dose of cyclophosphamide reached with AC regimen. When a taxane administered in combination with AC (AC-T), it did not significantly increase the risk of amenorrhea compared with standard AC regimen[16]. Tables 1 and 2 summarize chemotherapeutic agents that are commonly used in breast cancer treatment and their potential impact on ovarian function[15-19].

Patient age at the time of chemotherapy inversely correlates with the likelihood of post-chemotherapy amenorrhea. In women with breast cancer, while the incidence of chemotherapy-induced amenorrhea was

Table 1 The risk of infertility and mechanism of damage associated with chemotherapeutic agents that are commonly used in breast cancer treatment

Chemotherapeutic agent	Class	Mechanism of action	Cell cycle effect	Risk of infertility
Cyclophosphamide	Alkylating agent	DNA cross-link formation and double strand breaks that result in inhibition of DNA function and synthesis leading to cellular apoptosis	Cell cycle non-specific	High risk
Doxorubicin Epirubicin	Anthracyclines	Inhibition of DNA synthesis and function due to inactivation of DNA topoisomerase II, free oxygen radical formation and induction of DNA double-strand breaks	Cell cycle non-specific	Medium risk
Carboplatin	Platinum analog	Inhibition of DNA synthesis and function *via* intra- and interstrand DNA cross-link formation by covalent binding to genome	Cell cycle non-specific	Medium risk
Paclitaxel Docetaxel	Taxanes	Inhibition of mitotic division by binding to microtubules with enhancement of tubulin polymerization	M phase	Low risk
Methotrexate	Antimetabolites	Inhibition de novo purine nucleotide synthesis by inactivation of dihydrofolate reductase	S phase	Low risk
5-fluorouracil		Inhibition of DNA synthesis and function via inactivation of Thymidylate synthase and alteration in RNA processing	S phase	Low risk
Trastuzumab	Monoclonal antibodies	Blockage of Human epidermal growth factor receptor 2 subdomain IV, antibody dependent cellular toxicity	NA	Low or no risk
Pertuzumab		Blockage of Human epidermal growth factor receptor 2 subdomain II, antibody dependent cellular toxicity		

Table 2 Common adjuvant chemotherapy regimens for breast cancer and their impact of fertility

Chemotherapy regimen	Risk of amenorrhea or infertility	
	Age ⩽ 35 yr	Age >35 yr
CMF	4%-40%	80%-100%
CEF	47%	80%-100%
CAF	No data	30%
AC	13.90%	68.20%
AC-T	9%-13%	65%-67%
AC-TH	0-14%	56%-67%

A: Doxorubicin; C: Cyclophosphamide; E: Epirubicin; F: 5-Fluorouracil; H: Trastuzumab; M: Methotrexate; T: Paclitaxel.

reported as 15%-40% under the age of 30, this incidence dramatically increases to 49%-100% for women older than 40 years of age[20]. The reason for this age-related difference is the fact that younger women have a larger ovarian reserve. Our previous studies indicated that on average, gonadotoxic chemotherapy regimens result in the loss of approximately 10 years worth of ovarian reserve[21]. Though both younger and older women would lose follicles, gonadotoxic chemotherapy is more likely to push older women over the threshold for menopause as they have lower reserve to begin with. However, regardless of age, females of all ages, including children, are expected to experience early menopause after exposure to gonadotoxic chemotherapy agents. Therefore fertility preservation and completion of family building as early as possible, is critical regardless of the age at chemotherapy exposure in most instances[22].

GONADOTROPIN-RELEASING HORMONE ANALOGS AND OVARIAN PROTECTION

There has been an ongoing controversy regarding the role of ovarian suppression in cancer patients using gonadotropin-releasing hormone (GnRH) analogs in order to protect ovaries from chemotherapy-induced damage[23].

The biggest concern regarding the effectiveness of ovarian suppression is that primordial follicles that constitute the ovarian reserve are quiescent and do not express gonadotropin or GnRH receptors[24,25]. Thus, any change in gonadotropin or GnRH serum levels has no plausible direct or indirect effect on primordial follicles (Figure 1). Furthermore, we have shown that gonadotoxic agents induce primordial follicle death *via* inducing DNA double strand breaks in oocytes in a non-cell cycle dependent fashion, hence there is no mechanism for ovarian suppression by GnRHa to prevent chemotherapy-induced DNA damage[9,26]. It should be recognized that GnRHa induces a hormonal state similar to prepubertal stage, and if ovarian suppression were to be protective, children of prepubertal age would be resistant to gonadotoxic effects of chemotherapy, which is shown to be not to be the case[27].

While some studies in women with breast cancer, which used menstruation as a marker, suggested some benefit in restoration of menstruation post-chemotherapy, these studies were marred by numerous weaknesses[28-30]. These include the utility of self-reported menstrual status, a highly unreliable surrogate for fertility, lack of placebo control (instead of GnRHa) or blinding, and lack of correction for the difference in desire to conceive between study and control groups[31].

Use of amenorrhea as the sign of ovarian failure is also key weakness in trials of GnRHa for ovarian protection. Especially for breast cancer patients, chemotherapy often induces occult ovarian insufficiency that most frequently presents as irregular or even normal appearing periods rather than amenorrhea. When the serum anti-Müllerian Hormone (AMH), which is the most reliable quantitative

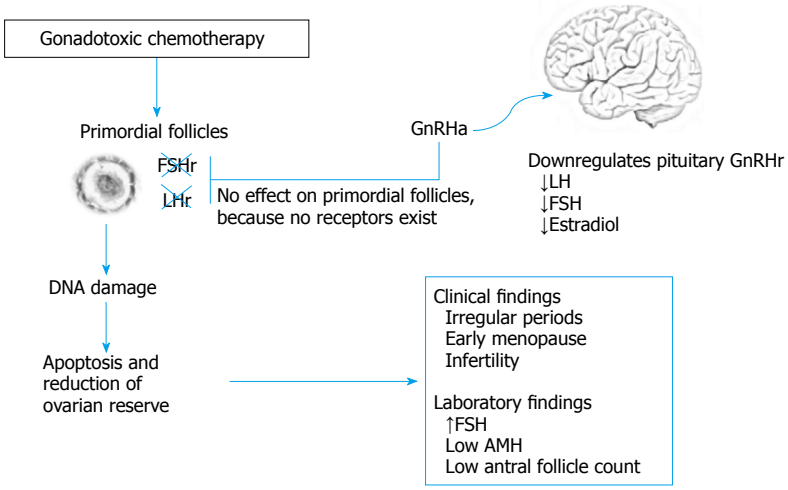

Figure 1 Impact of gonadotoxic chemotherapy and gonadotropin-releasing hormone analog on ovarian reserve and function. Gonadotoxic chemotherapy reduces ovarian reserve, which is made up of resting and hormone-insensitive primordial follicles, by induction of DNA damage and apoptotic death. GnRHa reduces pituitary GnRH production and, as a result, blocks the release of FSH and LH from the pituitary, which in turn results in the cessation of late-stage follicle development. Because primordial follicles do not have FSH, LH, or GnRH receptors, GnRHa cannot have a direct influence on ovarian reserve. AMH: Anti-Müllerian hormone; FSH: Follicle-stimulating hormone; FSHr: FSH receptor; LH: Luteinizing hormone; LHr: LH receptor; GnRH: Gonadotropin-releasing hormone; GnRHr: GnRH receptor. Oktay *et al*. *J Clin Oncol* 2016; **34**: 2563-2565, used with permission.

biomarker for ovarian reserve or appropriate criteria with serum FSH levels for defining ovarian failure was used, none of the studies showed fertility preservation benefit from GnRHa treatment[32-34].

Given the contradictory results and ovarian biological facts, the use of GnRHa for the prevention of ovaries from chemotherapy damage is still controversial and cannot be recommended as an effective method of fertility preservation.

OVARIAN RESERVE IN WOMEN WITH BRCA MUTATIONS

Most hereditary breast cancers are associated with germline mutations in *BRCA1* and *BRCA2* genes. BRCA genes are members of the ataxia-telangiectasia-mutated (ATM)-mediated DNA damage signaling pathway and are essential for DNA double-strand break (DSB) repair[35]. In addition to the increased risk for multiple malignancies, several clinical and experimental studies showed an association between BRCA mutations and diminished ovarian reserve[26,36-41]. While performing ovarian stimulation in women with breast cancer by using aromatase inhibitors for fertility preservation, we found significantly lower ovarian response rates in BRCA mutation carries particularly, among those with BRCA1 mutations[36]. In another important study, authors reported that unaffected women with BRCA mutation experience menopause 3-4 years earlier than healthy controls[38]. Recently, our laboratory showed that in BRCA1 mutant mice there is increased age-related accumulation of DNA double strand breaks in primordial follicle oocytes and the ovarian reserve is significantly lower. These BRCA1 mutant mice also showed reduced litter size and poor embryo development. These findings clearly indicate a biological connection between BRCA mutations, DNA repair and reproductive function. In the same study, we also showed that affected women with BRCA1 mutations had lower serum AMH levels compared to controls. Interestingly we did not find these differences in either BRCA2 mutant mice or affected women with BRCA mutations[26]. Confirming our findings in a prospective study, Philips *et al*[41] found 25% lower AMH concentrations on average in BRCA1 carriers compared to non-carriers. There was no significant association between the BRCA2 mutation status and the AMH levels.

Given the accumulating evidence that the ovarian reserve may be lower in women with BRCA mutations, it is possible that these women are more prone to chemotherapy-induced loss of ovarian reserve and ovarian insufficiency. However this is yet to be shown in prospective clinical trials. Nevertheless, while counseling women with BRCA mutations on fertility preservation, the possibility of higher risk of chemo-induced infertility should not be omitted.

FERTILITY PRESERVATION OPTIONS FOR BREAST CANCER PATIENTS

Embryo cryopreservation after *in vitro* fertilization (IVF) is currently considered as an established fertility preservation option, which offers the best chance of livebirth for women with a partner or single women who elect to use donor sperm. Numerous studies have demonstrated up to 60% clinical pregnancy rates and around 34% livebirth rates after transfer of frozen-thawed embryos in infertility patients with mean age of 35.1 ± 4.03, which is comparable to fresh embryo transfer[42,43]. When preimplantation genetic screening utilized, the livebirth rates can increase up to 77% after transfer of euploid frozen-thawed embryos[44]. In women with breast cancer with the mean age of 35.8 ± 4.1, we have shown a livebirth rate of 45%, which appeared to be superior to those undergoing frozen embryo transfer for infertility[45].

Cryopreservation of mature or immature oocytes is another fertility preservation option for women without a partner and those not wishing to use donor sperm due to legal, ethical or religious considerations. Mature oocytes can be effectively cryopreserved using a vitrification method and the success rates of post-thaw fertilization and pregnancy rates have approached those with

Table 3 Fertility Preservation options for reproductive age women with breast cancer

Fertility preservation option	Current status	Advantages	Disadvantages
Embryo Cryopreservation	Established	Highest cumulative pregnancy rates	Requires about two weeks delay in the initiation of cancer treatment Requires hormonal stimulation for oocyte retrieval Requires *in vitro* fertilization with male partner or donor sperm
Oocyte Cryopreservation	Established	No need for male partner or sperm donor	Requires about two weeks delay in the initiation of cancer treatment Requires hormonal stimulation for oocyte retrieval
Ovarian Tissue Cryopreservation and Transplantation	Currently experimental, may change as success rates are rising	No need for hormonal stimulation No need to significantly delay in the initiation of chemotherapy No need for male partner or sperm donor	Requires outpatient laparoscopic surgery for ovarian tissue harvesting and subsequent transplantation

fresh oocytes in young patients, though success rates with frozen embryos may still be better[46,47]. Oocyte cryopreservation success rates vary depending on age, number of oocytes frozen and the freezing protocol. In a recent individual patient data meta-analysis we calculated these success rates[48] (An interactive online success rate estimator can be found online at http://fertilitypreservation.org/index.php/probability-calc).

Based on an individual patient meta-analysis encompassing thaw cycles with frozen oocytes, we have calculated the age-based success rates for oocyte cryopreservation. An interactive online egg freezing success rate estimator can be found at this link: http://fertilitypreservation.org/index.php/probability-calc, and can be useful in patient counseling.

Immature oocytes can be obtained from patients without undergoing ovarian stimulation due to dearth of time and also at the time of ovarian tissue harvesting for fertility preservation. After retrieval, immature oocyte may be cryopreserved before or after undergoing *in vitro* maturation (IVM) process[49]. Lee *et al*[50] suggested performing IVM for immature oocytes before cryopreservation rather than post-thaw as they observed significantly higher maturation and survival rates with that approach. Although IVM is still an experimental fertility preservation method and limited to a number of fertility centers, this method has recently resulted in live births[51].

Embryo and oocyte cryopreservation methods are widely used and currently considered as established methods of fertility preservation. However, typically 10-14 d of controlled ovarian stimulation is needed to obtain mature oocytes (Table 3).

When there is insufficient time for ovarian stimulation, the only available strategy other than immature oocyte retrieval and IVM for women with breast cancer is ovarian tissue harvesting and cryopreservation for future transplantation. Since the first report of ovarian transplantation with cryopreserved tissue by our group, there have been more than 80 livebirths with over 30% of livebirth rate after ovarian transplantation[52,53]. Some have raised the concern of reintroducing malignant cells back into the body along with ovarian tissue. However, studies showed no evidence of malignant cells in cryopreserved ovarian tissues from non-metastatic breast cancer patients and those with bone and soft tissue tumors[54-56].

CONTROLLED OVARIAN STIMULATION PROTOCOLS

The major issue associated with the conventional ovarian stimulation protocols is elevated circulating estradiol levels due to the development of large number of follicle at once. Therefore, conventional stimulation protocols are considered unsafe in women with estrogen-sensitive breast cancer.

Although oocytes can be retrieved from ovaries without performing ovarian stimulation (natural cycle IVF), this strategy typically does not provide more than one oocyte per cycle and yield an embryo in only 60% of cycles[57]. On the other hand, use of tamoxifen alone for ovulation induction showed better results in mature oocyte and embryo yield compared to natural cycle IVF[58]. Tamoxifen may also be used in combination with low dose gonadotropins for IVF, resulting in increase multiple mature oocytes and embryos[59].

While reducing the circulating estrogen levels, aromatase inhibitors induce the secretion of endogenous FSH by releasing the hypothalamic-pituitary axis from estrogenic negative feedback[60]. We showed that letrozole in combination with gonadotropins can produce comparable outcomes to conventional IVF while providing significantly lower estradiol levels and decreased gonadotropin requirements[45]. We also showed that pregnancy outcomes after ovarian stimulation with letrozole protocol in premenopausal breast cancer patients before adjuvant chemotherapy were similar to a non-cancer population[60]. Moreover, after short and mid-term follow up letrozole-gonadotropin protocol was associated with disease free survival rates[61].

One of the concerns related with ovarian stimulation

before adjuvant or neo-adjuvant chemotherapy is the delay in the initiation of breast cancer treatment. However, studies have shown that initiation of chemotherapy can be delayed up to 12 wk after breast surgery without any adverse effect on survival and recurrence rates[62,63].

Another concern is that letrozole protocol is that it is a teratogenic agent if used during pregnancy. However, in the setting of fertility preservation, embryos are never exposed to letrozole as the fertilization takes place *in vitro* and the resultant embryos are cryopreserved for later use. Additionally, it has been reported that there was no difference in congenital malformation and chromosomal abnormality rates among children born after ovarian stimulation with clomiphene or letrozole for infertility[64].

PREGNANCY AFTER BREAST CANCER

Patients in the decision process for fertility preservation treatments frequently question the safety of pregnancy after completion of cancer treatment. Based on the current evidence, pregnancy after breast cancer is not associated with increased risk of adverse outcomes[65]. In general, patients are advised to delay pregnancy at least 2 years after diagnosis, as the risk of recurrence is highest in this time frame. In the case of ER-positive breast cancer, pregnancy is contraindicated during tamoxifen treatment because of teratogenicity. For breast cancer survivors who do not want to delay childbearing for the completion of tamoxifen treatment or for those with other medical contraindications, gestational surrogacy may be a suitable option to utilize their frozen eggs or embryos in the future[10,65].

CONCLUSION

Fertility preservation has become a crucial part of survivorship and an important aspect of comprehensive cancer care. Fortunately, there are several well-established treatment options including embryo and oocyte cryopreservation and safer ovarian stimulation protocols. Moreover, there are emerging experimental methods such as ovarian tissue cryopreservation and transplantation and IVM, which are showing promise. To maximize the utility of these available options and avoid significant delays in the initiation of chemotherapy, timely referral to fertility preservation counseling should be an integral part of the care of young women with breast cancer.

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MINIREVIEWS

Biological mesh reconstruction of the pelvic floor following abdominoperineal excision for cancer: A review

Boris Schiltz, Nicolas Christian Buchs, Marta Penna, Cosimo Riccardo Scarpa, Emilie Liot, Philippe Morel, Frederic Ris

Boris Schiltz, Nicolas Christian Buchs, Cosimo Riccardo Scarpa, Emilie Liot, Philippe Morel, Frederic Ris, Division of Visceral Surgery, Department of Surgery, University Hospitals of Geneva, 1205 Geneva, Switzerland

Marta Penna, Department of Colorectal Surgery, Churchill Hospital, Oxford University Hospitals, Headington, Oxford OX3 7LE, United Kingdom

Author contributions: Schiltz B, Buchs NC and Morel P designed the research; Schiltz B, Buchs NC, Scarpa CR and Liot E performed the research (local data and literature review); Schiltz B, Buchs NC, Penna M, Liot E and Ris F interpreted the data; Morel P and Ris F revised the article; all authors gave their final approval.

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Correspondence to: Boris Schiltz, MD, Division of Visceral Surgery, Department of Surgery, University Hospitals of Geneva, Rue Gabrielle-Perret-Gentil 4, 1205 Geneva, Switzerland. boris.schiltz@hcuge.ch
Telephone: +41-79-5534161
Fax: +41-22-3727707

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Abstract

Extralevator abdominoperineal excision and pelvic exenteration are mutilating operations that leave wide perineal wounds. Such large wounds are prone to infection and perineal herniation, and their closure is a major concern to most surgeons. Different approaches to the perineal repair exist, varying from primary or mesh closure to myocutaneous flaps. Each technique has its own associated advantages and potential complications and the ideal approach is still debated. In the present study, we reviewed the current literature and our own local data regarding the use of biological mesh for perineal wound closure. Current evidence suggests that the use of biological mesh carries an acceptable risk of wound complications compared to primary closure and is similar to flap reconstruction. In addition, the rate of perineal hernia is lower in early follow-up, while long-term hernia occurrence appears to be similar between the different techniques. Finally, it is an easy and quick reconstruction method. Although more expensive than primary closure, the cost associated with the use of a biological mesh is at least equal, if not less, than flap reconstruction.

Key words: Biological mesh; Rectal cancer; Pelvic exenteration; Abdominoperineal resection; Primary perineal wound closure; Perineal wound infection; Perineal hernia

Core tip: Current literature regarding the use of biological mesh reconstruction after pelvic exenteration and extralevator abdominoperineal excision is scarce. However, it does suggest that the use of biological mesh has a lower short-term perineal hernia rate, but is probably not superior to other approaches with regards to perineal wound complications.

Schiltz B, Buchs NC, Penna M, Scarpa CR, Liot E, Morel P, Ris

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INTRODUCTION

Pelvic exenteration (PE) and extralevator abdominoperineal excision (ELAPE) are mutilating operations, leaving a large perineal incision. ELAPE for low rectal cancer was introduced to decrease the rate of positive resection margins and specimen perforation occurring during conventional abdominoperineal resection (cAPR)[1,2]. In a recent retrospective study, Stelzner *et al*[3] showed that the 5-year recurrence rate was 5.9% in the ELAPE group *vs* 18.2% in the cAPR group (P = 0.153). However, other units have not been able to reproduce such results[4], nor could they demonstrate a statistically significant superiority of ELAPE in terms of CRM positivity and bowel perforation. Furthermore, they reported comparable perineal complication rates for the two APR approaches.

Vivid discussions continue to fuel the debate regarding the pros and cons of ELAPE. Overall, it is well accepted that larger wounds are independent risk factors for perineal wound complications. The combination of neoadjuvant chemoradiotherapy and ELAPE almost doubles the rate of perineal wound complications (31% for ELAPE *vs* 18% for cAPR)[5]. While new techniques and approaches have attempted to reduce the size of the perineal incision (and therefore reduce the risk of wound complications)[6], optimal management of perineal defects is still under investigation. The options include primary closure, myocutaneous flaps, and mesh reconstruction, including the use of a biological mesh.

We aimed to evaluate the outcomes of perineal reconstruction with biological mesh following ELAPE and PE in our center and to review the current literature.

CURRENT STATUS

Perineal wound complications are a major concern following PE and ELAPE leading to increased morbidity, longer hospital stay, and delayed chemotherapy. Different reconstruction methods are currently used in practice with the aim of reducing the rates of wound complications and avoiding perineal herniation.

Risk factors for major perineal wound complications following APR are well known: Preoperative radiotherapy, patients with anal cancer, flap reconstruction, tumor size, obesity, and diabetes[7]. Minor wound complications appear more commonly in patients with inflammatory bowel disease or anal cancer than in those with rectal cancer[8].

Most patients with locally advanced rectal cancer, recurrent rectal cancer, and recurrent or persistent squamous cell carcinoma receive neoadjuvant radiochemotherapy or radiotherapy alone[9,10]. The poor healing ability of irradiated wounds has been attributed to local endarteritis and damaged fibroblasts[11]. It has been clearly demonstrated that preoperative radiotherapy increases the rate of major wound complications[5,12]. For example, Aldulaymi *et al*[13] reported a significantly increased risk of major perineal wound complications in patients undergoing APR for rectal cancer with primary closure of the perineum (26% in non-irradiated *vs* 71% in irradiated patients). Chadwick *et al*[14] found that the risk of developing a wound complication was 10 times higher after previous irradiation. This substantial problem with wound healing calls for the need to consider alternative closure techniques of the perineum.

Different methods have been described ranging from direct/primary closure to mesh reconstruction, gluteal and rectus abdominis flaps or combinations of these techniques. Currently, there is no consensus on which is the most ideal technique[15]. The vertical rectus abdominis flap (VRAM) is indicated to bring non-irradiated tissue into the perineal defect[16]. After VRAM, perineal wound complications have been reported to range from 0% to 28%[17-20]. The use of laparoscopy for the abdominal part of the resection is almost impossible because of the donor site. In addition, in cases of PE (with a right sided urostomy and left sided end colostomy), VRAM is often contra-indicated. A potential solution is the use of a wet double-barreled colostomy[21].

Other myocutaneous flaps can potentially be used, such as the gracilis flap and the gluteus maximus flap, which have a perineal wound complication rate of 12%[22] and 10%[2] respectively. However, these flaps are typically smaller than the VRAM flap and unlikely to provide adequate cover of large defects.

In addition, authors argue that myocutaneous flaps carry significant risks of donor site morbidity, flap necrosis, prolonged operative time, and usually require co-ordination with plastic surgeons[2,23,24]. Mesh reconstruction is another technique, which has attracted a lot of interest in the last few years, especially with the adoption of ELAPE. Briefly, the biological mesh is sutured directly to the pelvic side wall (Figures 1-3). The size of the mesh is adapted to the size of the defect. A perineal drain is routinely left at the end of the procedure, in order to avoid a perineal collection.

Both allogenic and xenogenic biological meshes are available for the reconstruction of the perineum. These types of meshes were initially used for abdominal wall reconstructions[25,26]. The allogenic mesh is predominantly made of human acellular dermis (*e.g*., HADM® Ruinuo, Qingyuanweiye Bio-Tissue Engineering Ltd, Beijing, China) as used by Han *et al*[27,28]. The xenogenic mesh consists of bovine pericardium or porcine dermis and intestinal mucosa. Similar to Musters *et al*[29] in the BIOPEX-study, we used the Strattice® mesh (LifeCell, Acelity Company, Branchburg, NJ) which is composed of non-reticulated porcine dermis. Jensen *et al*[30] and Christensen *et al*[24] used the Permacol® mesh (Tissue Science Laboratories plc, Covington, GA, United States) derived from reticulated

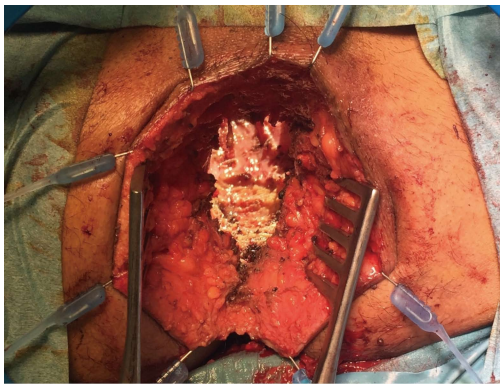

Figure 1 Perineal view before reconstruction in pelvic exenteration patient.

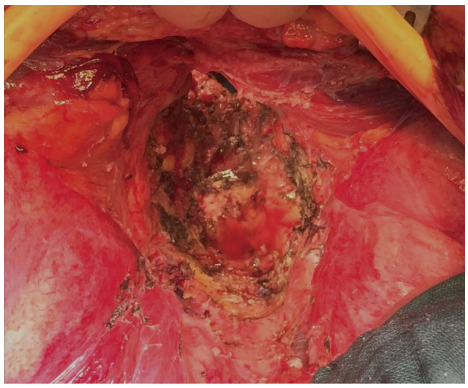

Figure 2 Abdominal view before reconstruction in pelvic exenteration patient.

porcine dermis[24,30]. Surgisis® Biodesign™ (Cook Medical, Bloomington, IN, United States) created using porcine intestinal mucosa was used by Peacock *et al*[31] for their pelvic reconstruction.

Reconstruction using a mesh is relatively simpler and faster compared to flap reconstruction[24]. When considering cost, meshes are expensive, especially if biological. However, with a potentially shorter operative time and length of hospital stay, overall costs can be controlled and even reduced in comparison to VRAM-flaps[32]. Biological meshes also have the advantage of being absorbable and can be used in infected environments[33].

On the other hand, perineal mesh reconstruction is not without its risks. Internal hernias following mesh repair have been reported. Melich *et al*[34] described resecting ischemic small bowel loops incarcerated in a pelvic hernia along the mesh in three patients. Jensen *et al*[30] reported a hole in the biological mesh in a patient with an infected perineal wound, who subsequently required mesh removal. These reports clearly raise concerns and highlight the risk of small bowel incarceration and necrosis associated with the use of a perineal mesh.

Table 1 summarizes the largest studies focusing on the use of biological mesh for perineal reconstruction. Interestingly, only one mesh was removed[30]. The overall safety profile appears to be good.

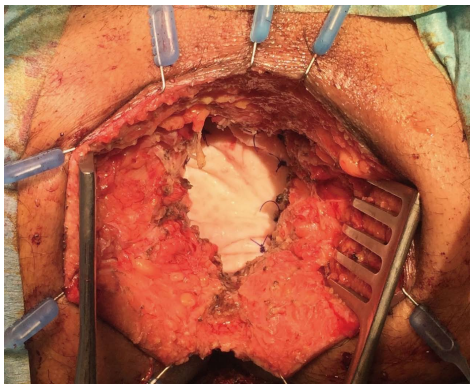

Figure 3 Perineal view after reconstruction using a biological mesh.

Perineal wound complications

The clinical consequences of perineal wound complications are wide and range from a simple redness of the skin to a persistent perineal fistula, and perineal sepsis. Perineal wound complications are often subdivided into two subgroups: Early and delayed wound dehiscence. The delayed (> 4 wk) perineal healing can occur in approximately 25% of cases. Importantly, up to 50% of these cases will develop long-term and persistent perineal symptoms such as pain, chronic sinus, sitting disability or tension between buttocks. All of which can seriously impact the patient's quality of life. Delayed perineal healing may therefore be a risk factor for persistent symptoms providing yet another reason why surgeons must strive to identify the best repair method possible[35,36].

Primary closure leads to perineal wound complications in 18%-34%[27,29,37]. Moreover, one third of patients after PE will develop perineal wound dehiscence[38]. As a corollary, persistent presacral sinus was found in 10% of the patients following APR[39].

As mentioned in Table 1, 17%-37% of patients with biological mesh presented some degree of perineal wound dehiscence/infection. A Danish retrospective study reported that 15% of patients with biological mesh had a surgical re-intervention for perineal infection. In addition, 21% of the patients had a perineal fistula with 9% requiring surgical excision[30]. Similarly, Peacock *et al*[31] reported an overall perineal wound complication rate of 32%. Vacuum assisted wound therapy and surgical debridement were needed in up to 9% of cases.

Christensen *et al*[24] compared gluteal flap reconstruction with biological mesh repair. Seventeen percent of patients in the mesh group had a wound infection compared to 6% in the flap group ($P = 0.26$). At 3 mo, all wounds healed with one persistent sinus in each group[24].

Han *et al*[28] found similar results and subsequently conducted a randomized controlled trial evaluating ELAPE *vs* cAPR. Interestingly, in the ELAPE group, patients had biological mesh reconstruction. Overall, the perineal wound infection rate (11.4%) after ELAPE was lower than in the cAPR group where 18.8% of patients developed a perineal complication. However, seromas were more frequent in the mesh group (11.4% *vs* 0%)[27].

Table 1 Perineal reconstruction with biological mesh

Ref.	Study type	Operation	No.of patients	Average age (median years)	Perineal complications (%)	Surgical perineal debridement n	Perineal hernias	Follow up	Comments
Musters BIOPEX-study 2016[29]	RCT	ELAPE	50	65	37% overall perineal wound complications	4% surgical drainage of perineal abscess, 6% percutaneous drainage of perineal abscess	13% at 1 yr	12 mo	
Jensen *et al*[30], 2014	Cohort, prospective	ELAPE	53	NR	21% perineal fistula, 7.5% superficial perineal abscess, 7.5% deep perineal abscess	5 (9%) fistulectomy, 8 (15%) surgical debridements	5.60%	Median 36 mo	1 mesh removed (infection), 1 mesh failure (hole) replacement of a new mesh
Christensen *et al*[24], 2011	Cohort, retrospective	ELAPE	24	69.7	17%, with one fistula after 3 mo	0	0	Median 1.7 yr	-
Han *et al*[28], 2010	Cohort, retrospective	ELAPE	12	68	16% infection, 8% seroma	0	NR	Median 8 mo	-
Han *et al*[27], 2012	Derived from RCT	ELAPE	32	68	11.4% wound infections 11% seroma	NR	14%	NR	-
Peacock *et al*[31], 2014	Cohort, prospective	ELAPE	34	62	32% overall; 9% superficial wound infections, 14% perineal fistula; 9% perineal abscess	3 (9%) surgical debridement/ VAC therapy	0	Median 21 mo	-
Schiltz present study	Cohort, retrospective	ELAPE + PE	11	63	Overall 27% wound infections with 1 superficial	2 (18%) surgical debridement	0	Mean 18 mo	-

NR: Not reported; ELAPE: Extralevator abdominoperineal excision; PE: Pelvic exenteration.

Seroma formation can be problematic, pushing most of the authors to recommend the routine use of a perineal drain.

Adding to the present literature, we conducted a retrospective study of our local data. From January 2012 to December 2015, all patients undergoing ELAPE or PE with biological mesh reconstruction were analyzed. Eleven patients were found; all of whom had preoperative radiochemotherapy. Overall, perineal complications were found in 3 (27%) of the patients. In 2 (18%) patients, perineal abscesses were surgically drained and treated with a vacuum assisted wound closure system. One superficial wound infection was treated conservatively. No meshes were removed.

The relatively poor quality of the available studies in the literature remains an issue. These are mainly retrospective or simple cohort studies designed to analyze oncological outcomes. Very few of them focus specifically on perineal complications. Additionally, the severity and grading system of wound complications can differ between reports, and thus it is difficult to draw definitive conclusions.

The only multicenter randomized controlled trial focusing on perineal reconstruction using biological mesh after ELAPE, the BIOPEX study[29], was recently published. Patients were randomized into two groups, one with perineal mesh reconstruction and the other with primary closure only (control group). Regular blinded wound follow-up, using the Southampton wound healing score, did not show a significant difference between the two groups at 30 d. In the control group, 34% of perineal wound complications occurred *vs* 37% in the mesh group (P = 0.7177). At 12-mo follow-up, the healing rates did not differ between groups (52% *vs* 54%). Omentoplasty or use of perineal drains did not affect the results in this study[29].

In summary, current evidence suggests that biological mesh reconstruction does not appear to reduce the risk of perineal wound complications. Results are similar between primary closure, flap and biological mesh.

Perineal hernia

The incidence of perineal hernia after APR ranges from 0.6% to 27% in the literature[5,29,40], occurring on average 8 to 22 mo after surgery[41,42] (Table 1). Such a wide range can partly be explained by the definition of a perineal hernia itself. Indeed, a clinical hernia is quite different from an asymptomatic radiologically identified perineal hernia. Smoking and chemoradiotherapy are well reported risk factors[42].

Given that recurrence rates following perineal hernia repair are high (up to 37%), prevention is certainly the best strategy[15]. Perineal hernia occurs significantly less often after biological mesh reconstruction (0%) than

following gluteal flap surgery (21%) ($P < 0.01$)[24]. Thus suggesting that biological mesh repair can be a good option in order to avoid herniation.

The BIOPEX-study showed that 13% of perineal hernias (diagnosed on CT scan) occurred after biological mesh repair *vs* 27% in the primary closure group at one-year follow-up ($P = 0.036$)[29]. The hernias occurred nearer the end of the 12-mo follow-up in the mesh group. The long-term follow-up results are still pending. Interestingly, this delay in the hernia presentation is also described in patients without mesh reconstruction. However, this seems to occur after a median of 8 mo[41]. A possible explanation is that perineal hernias occur later in the mesh group due to the slower degradation of the biological mesh[43].

In our own data, no perineal hernia was found, neither clinically or radiologically, even after a mean follow-up of 18 mo.

Overall, biological mesh seems to protect, at least in early follow up, from the occurrence of perineal hernias in comparison to flap reconstruction or primary closure.

CONCLUSION

Perineal reconstruction following ELAPE, APE or PE remains a major problem and challenge. No ideal solution currently exists but various approaches have been attempted with more or less success. Primary closure remains the most frequent technique, carrying a significant risk of perineal hernia formation. On the other hand, the use of flap or mesh reconstruction could help reduce the risk of herniation. Biological mesh appears to be a valid option, at least in terms of hernia prevention, which can be reduced by up to 50%.

Yet, the role of mesh reconstruction in reducing wound infections is less clear. Whilst perineal infection is frequent in irradiated patients, the use of biological mesh seems logical, even if the evidence is scarce to draw definitive recommendations. On the other hand, perineal wound infection remains frequent and a perineal drain should be routinely used.

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MINIREVIEWS

Potential prognostic biomarkers in pancreatic juice of resectable pancreatic ductal adenocarcinoma

Shefali Agrawal

Shefali Agrawal, Hepatobiliary and Pancreatic Surgery, Department of Surgical Oncology, Indraprastha Apollo Hospitals, New Delhi 110076, India

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Correspondence to: Shefali Agrawal, MD, MS, FACS, Senior Consultant, Hepatobiliary and Pancreatic Surgery, Department of Surgical Oncology, Indraprastha Apollo Hospitals, Sarita Vihar, New Delhi 110076, India. shefali_a@apollohospitals.com
Telephone: +91-81-30009660

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Abstract

Despite potentially curative surgery pancreatic cancer has a dismal prognosis. Serum cancer antigen 19-9 (CA 19-9) correlates with tumor burden, resectability and survival in patients with pancreatic ductal adenocarcinoma. Identification of novel biomarkers may facilitate early diagnosis of pancreatic cancer and improve survival. Pancreatic juice is a rich source of cancer-specific proteins rendering it a promising tool for identifying biomarkers. Recent proteomic and microRNA expression analyses have identified several biomarkers of potential diagnostic and prognostic value. Tumor markers CA 19-9 and carcinoembryonic antigen (CEA) are widely used in the characterization of premalignant and malignant lesions of the pancreas. Elevated level of CEA in bile is a marker for malignancy and a predictor of hepatic recurrence. The potential value of CA 19-9, CEA and lactate dehydrogenase as prognostic biomarkers in pancreatic juice and bile is unknown. Specimens of pancreatic juice and bile can be readily collected during surgical resection of the tumor. Profiling of pancreatic juice and bile to identify novel prognostic biomarkers may improve selection of patients for adjuvant therapy with a favorable impact on overall survival in patients diagnosed with pancreatic cancer.

Key words: Prognostic biomarkers; Pancreatic juice; Bile; Pancreatic adenocarcinoma; Surgery

Core tip: Pancreatic juice is a rich source of cancer-specific proteins rendering it a promising tool for identifying novel biomarkers in pancreatic ductal adenocarcinoma. Recent proteomic and microRNA expression analyses have identified several diagnostic and prognostic biomarkers. Elevated carcinoembryonic antigen (CEA) in bile is a marker of malignancy and a predictor of hepatic recurrence. The potential of cancer antigen 19-9, CEA and lactate dehydrogenase as prognostic biomarkers in pancreatic juice and bile is unknown. Specimens of pancreatic juice and bile can be readily collected during pancreatic resection. Profiling of pancreatic juice and bile to identify novel biomarkers may facilitate early diagnosis and improve selection of patients for adjuvant therapy.

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INTRODUCTION

Pancreatic cancer is the fourth leading cause of cancer-related mortality in the United States. Despite improvements in adjuvant therapy and identification of novel biomarkers, pancreatic cancer continues to have a dismal prognosis[1]. Pancreatic ductal adenocarcinoma (PDAC) is one of the few cancers for which incidence and mortality rates have changed very little over the past three decades. Surgery is the only potentially curative treatment for prolongation of survival and the use of adjuvant therapy following curative surgery significantly improves 5-year survival[2-4].

Pathologic stage of the tumor is the major determinant of survival after curative resection for PDAC[1]. Serum levels of cancer antigen 19-9 (CA 19-9), carcinoembryonic antigen (CEA) and lactate dehydrogenase (LDH) correlate with the extent of disease and are predictive of survival[5-13]. Serum CA 19-9 correlates with tumor burden, resectability and overall survival. Low preoperative serum CA 19-9, postoperative decline and level < 200 U/mL are independent predictors of survival[5]. Identification of novel diagnostic and prognostic biomarkers in the serum, tissue, bile and pancreatic juice of patients with PDAC may improve early diagnosis and selection of patients for adjuvant therapy.

BIOMARKERS OF PANCREATIC ADENOCARCINOMA

CA 19-9 and CEA in PDAC are well-characterized serum and tissue biomarkers of diagnostic and prognostic value. Recent proteomic and microRNA (miRNA) expression analyses have identified several biomarkers of potential value in the early diagnosis of PDAC and improvement in patient selection for aggressive treatment protocols. Comparative proteomic profiling of tumor and nontumor pancreas samples in patients with PDAC identified a new prognostic biomarker prolargin (PRELP)[14]. Survival analysis demonstrated a significant correlation of protein abundance of PRELP with postoperative survival confirming its value as a candidate prognostic biomarker. Pancreatic juice is a rich source of cancer-specific proteins rendering it a promising tool for identifying novel biomarkers. Additional sources of biomarkers including serum, tumor tissue, pancreatic juice, bile and other body fluids have revealed distinct biomarker patterns in PDAC. These data suggest that analysis of pancreatic juice and bile samples collected at the time of a surgical resection may identify prognostic biomarkers of value in PDAC. Biomarkers may be used to stratify patients based on prognosis and those who will benefit from intensive neoadjuvant protocols or adjuvant hepatic artery infusion therapy.

BIOMARKERS IN PANCREATIC JUICE

Diagnostic biomarkers

During the development of PDAC, malignant ductal cells preferentially shed into the ductal lumen, making pancreatic juice a rich source of cancer-specific proteins. CA 19-9 expression is demonstrated in 90% patients with pancreatic head adenocarcinoma compared to 11%-62% periampullary cancers of duodenal, ampullary or distal bile duct origin[15]. Overexpression of CA 19-9 and CEA in PDAC is shown in Figures 1-4 and it correlates with a higher histologic grade[15,16]. Elevation of CEA level and presence of *K-ras* mutation in pancreatic juice is a strong predictor of PDAC[17]. Increased levels of CA 19-9 and CEA in pancreatic juice are predictive of malignant transformation in benign intraductal papillary mucinous neoplasm (IPMN)[18-21]. Immunohistochemical staining of CEA is strongly positive in invasive IPMN and correlates with the grade of cellular atypia[21,22].

In a comparison of the levels of CA 19-9 in the serum and pancreatic juice of patients with PDAC, the authors reported elevated levels in the pancreatic juice of all patients with normal levels in the sera of several patients[23]. Tumor marker levels are predictive of tumor burden with the level in pancreatic juice correlating with the local tumor and serum level with the systemic burden of disease. This may explain the elevation of tumor markers in pancreatic juice with normal serum levels in patients with malignant IPMN.

Genetic and epigenetic markers such as mutant *K-ras*, *p53* mutations, DNA methylation alterations, mitochondrial DNA mutations and miRNAs in pancreatic juice are under evaluation for their role in distinguishing benign pancreatic pathology or chronic pancreatitis from preinvasive pancreatic neoplasia, IPMN and pancreatic intraepithelial neoplasia (PanIN)[24-33]. Mass spectrometry proteomics of pancreatic juice collected at the time of surgical resection of the tumor suggested distinct proteomic signatures for PDAC[34]. CEA and S100 calcium-binding protein P (S100P) concentrations in duodenal juice were significantly higher in PDAC than the benign conditions and may serve as a useful screening test for the detection of PDAC[35,36]. Immunohistochemical expression of human telomerase reverse transcriptase (hTERT) in preoperative pancreatic juice samples was detectable in 84% PDAC and 88% malignant IPMN and the accuracy of diagnosing PDAC improved when combined with cytology[30,37]. Proteomic analysis of pancreatic juice from patients with PDAC demonstrated three up-regulated proteins, matrix metallo proteinase-9 (MMP-9), oncogene DJ1 (DJ-1) and alpha-1B-glycoprotein precursor (AIBG) indicative of their potential as diagnostic biomarkers in PDAC[38]. Accurate peripheral markers of PDAC are lacking and select miRNAs identified in plasma and bile demonstrated excellent

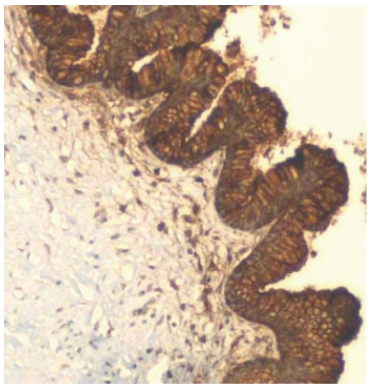

Figure 1 Pancreatic ductal adenocarcinoma with overexpression of carcinoembryonic antigen. The neoplastic cells demonstrate strong cytoplasmic and membranous staining (100 ×). Courtesy, Department of Pathology, Temple University Hospital, Philadelphia, PA, United States.

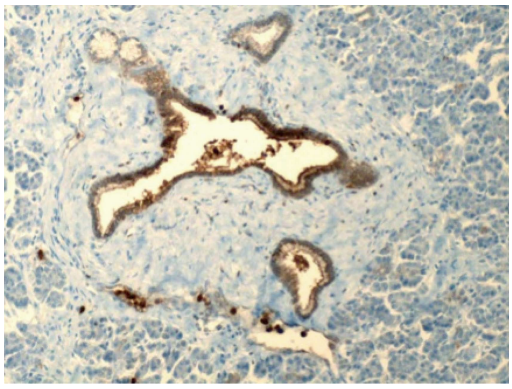

Figure 2 Benign pancreatic ducts and acini with weak staining of the ductal cells for carcinoembryonic antigen (200 ×). Courtesy, Department of Pathology, Temple University Hospital, Philadelphia, PA, United States.

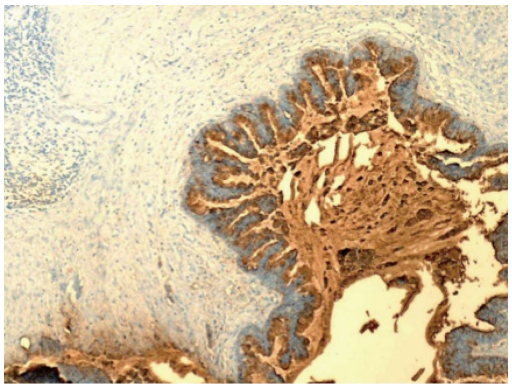

Figure 3 Pancreatic ductal adenocarcinoma with overexpression of cancer antigen 19-9. The neoplastic cells demonstrate strong cytoplasmic staining (100 ×). Courtesy, Department of Pathology, Temple University Hospital, Philadelphia, PA, United States.

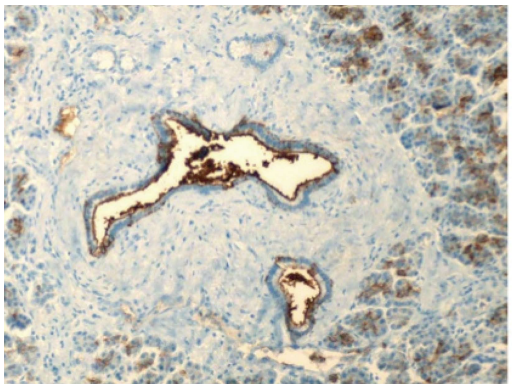

Figure 4 Benign pancreatic ducts and acini with weak staining of the ductal cells for cancer antigen 19-9 (200 ×). Courtesy, Department of Pathology, Temple University Hospital, Philadelphia, PA, United States.

accuracy in distinguishing PDAC from benign conditions[33]. These data highlight the potential value of biomarkers from various biological sources in the early diagnosis of pancreatic cancer.

Prognostic biomarkers

Normal pancreatic juice contains multiple proteins and administration of secretin alters the concentration but not the spectrum of these proteins[39]. The proteome of pancreatic juice in patients with PDAC is markedly altered[39,40]. The proteome of the pancreas after surgical resection contains regenerative and immunomodulatory factors which vary depending on neoadjuvant therapy, history of smoking and vary over time to stimulate restoration of organ function[41]. Profiling of miRNAs in pancreatic juice of patients with PDAC demonstrated higher contents of miR-205 and miR-210 correlating with lymph node metastasis and diminished survival demonstrating their potential value as candidate biomarkers of disease progression and prognosis[42]. Assay of Adnab-9 in pancreaticobiliary secretions and PDAC tumor demonstrated its potential value as a candidate biomarker for diagnosis and prognostication[43]. Elevated level of S100A8 or A9 in pancreatic ductal fluid, a near absence of pancreatic enzymes and high level of mucins (MUC1, 2, 5AC, 5B, 6 and 13) were predictors of poor survival suggesting that pancreatic ductal fluid is a promising tool for identifying prognostic biomarkers[34].

BIOMARKERS IN BILE

Diagnostic and prognostic value

Intraoperative samples of bile from gallbladder in patients with pancreaticobiliary diseases demonstrated significantly higher levels of CA 19-9 in malignancy and correlated with the tumor burden[44]. Biliary CEA > 10 ng/mL in patients undergoing a curative surgery for colorectal cancer is a strong predictor of hepatic recurrence suggesting that it is a marker for occult liver metastases[45,46]. Liver is the site of first recurrence in 50% patients following curative surgery for PDAC[47]. The use of adjuvant liver-directed therapy including hepatic artery infusion chemotherapy (HAI) significantly decreases the incidence of liver metastases with a trend towards improvement in cumulative survival[48,49]. Prediction of the site of early recurrence can impact choice of the optimal modality for adjuvant therapy

Table 1 Potential biomarkers in the pancreatic juice and bile of patients with pancreatic adenocarcinoma

Body fluid	Biomarker
Pancreatic ductal fluid	CA 19-9
	CEA
	K-ras
	p53 mutations
	DNA methylation alterations
	Mitochondrial DNA mutations
	S100 calcium-binding protein P (S100P)
	Human telomerase reverse transcriptase
	Matrix metalloproteinase-9
	Oncogene DJ1
	Alpha-1B-glycoprotein precursor
	MicroRNA- miR-205, miR-210
	Adnab-9
	S100A8 or A9
	Mucins MUC1, 2, 5AC, 5B, 6 and 13
Bile	CEA
	Mac-2-binding protein
	MUC4
	Vascular endothelial growth factor

CA 19-9: Cancer antigen 19-9; CEA: Carcinoembryonic antigen.

following curative surgery for PDAC.

Novel diagnostic biliary biomarkers for biliary tract cancer include Mac-2-binding protein (Mac-2BP) identified in bile using tandem mass spectrometry[50]. Alterations in epithelial mucin expression has identified MUC4 in pancreatic juice as a diagnostic and prognostic marker for pancreatic cancer, biliary MUC4 as a diagnostic biomarker and serum MUC5A as a sensitive diagnostic marker correlating negatively with survival in biliary tract cancer[34,51]. Elevated levels of biliary vascular endothelial growth factor (VEGF-1) distinguishes patients with pancreatic cancer from other etiologies of biliary stricture[52]. Potential biomarkers in the pancreatic juice and bile of patients with pancreatic adenocarcinoma are shown in Table 1. These preliminary data demonstrating the diagnostic and prognostic value of biliary markers in cancer require prospective evaluation and validation in large scale multicenter studies.

SELECTION OF BIOMARKERS

Choice of the optimal biomarker

Biomarkers obtained from readily accessible biological materials *via* non-invasive procedures minimize down-stream investigations and costs[53]. Pancreatic juice and/or bile is readily collected during the course of a pancreatic resection for PDAC. In contrast to the recently identified biomarkers requiring further investigation prior to recommendation for clinical use, CA 19-9 and CEA are widely used and validated markers of diagnostic and prognostic value in PDAC and pre-neoplastic lesions of the pancreas[54]. However, the prognostic value of CA 19-9, CEA and LDH levels in the pancreatic juice and bile of patients with PDAC has not been evaluated. Standardized laboratory protocols are available for the assay of CA 19-9 and CEA in body fluids rendering them optimal biomarkers in the evaluation of patients with PDAC.

CONCLUSION

Pancreatic juice is a rich source of cancer-specific proteins rendering it a promising tool for identifying novel prognostic biomarkers in PDAC. Elevated level of CEA in bile is a marker for malignancy and a predictor of hepatic recurrence. CA 19-9, CEA and LDH are widely used in clinical practice as diagnostic markers of pancreatic cancer however, the prognostic value of their levels in pancreatic juice and bile is unknown. Specimens of pancreatic juice and bile can be readily obtained during surgical resection of the tumor and analyzed according to well-established laboratory protocols for assays of CA 19-9, CEA and LDH to evaluate their prognostic value. Profiling of pancreatic juice and bile to identify biomarkers may improve early diagnosis and selection of patients for the optimal adjuvant therapeutic modality.

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ORIGINAL ARTICLE

Case Control Study

Levels of neutrophil gelatinase-assosciated lipocalin in patients with head and neck squamous cell carcinoma in Indian population from Haryana state

Monica Verma, Kiran Dahiya, Abhishek Soni, Rakesh Dhankhar, Veena S Ghalaut, Aastha Bansal, Vivek Kaushal

Monica Verma, Kiran Dahiya, Veena S Ghalaut, Aastha Bansal, Department of Biochemistry, Pt. B.D. Sharma, University of Health Sciences, Rohtak, Haryana 124001, India

Abhishek Soni, Rakesh Dhankhar, Vivek Kaushal, Department of Radiotherapy, Pt. B.D. Sharma, University of Health Sciences, Rohtak, Haryana124001, India

Author contributions: Verma M and Dahiya K designed the research, interpreted the results and drafted the manuscript; Verma M and Bansal A analyzed the samples; Soni A, Dhankhar R and Bansal A helped in statistical analysis, critical revision and final approval of the article; Ghalaut VS and Kaushal V helped in designing the results, making critical revisions and final approval of the article.

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Correspondence to: Dr. Kiran Dahiya, Department of Biochemistry, Pt. B.D. Sharma, University of Health Sciences, 778/28, Bharat colony, Rohtak, Haryana 124001, India. biochemistry.pgims@hry.nic.in
Telephone: +91-098-96111985

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Abstract

AIM

To study the levels of neutrophil gelatinase associated lipocalin (NGAL) in head and neck squamous cell carcinoma (HNSCC).

METHODS

This was a non randomized case control study conducted at Department of Biochemistry, in collaboration with Regional Cancer Center over a period of one year. The study population included 50 adult newly diagnosed HNSCC patients reporting in outpatient department at Regional Cancer Center and compared with 50 healthy controls. NGAL was estimated by ELISA technique. Student *t* test and χ^2 test were applied for comparison of means of study groups. Correlations between groups were analyzed using Pearson correlation coefficient (*r*) formula.

RESULTS

Patients with HNSCC exhibited significantly increased levels of NGAL ($P < 0.05$) as compared to healthy controls

(978.88 ± 261.39 ng/mL *vs* 34.83 ± 7.59 ng/mL). Out of 50, 26 patients (52%) were in stage Ⅳ, 21 (42%) in stage Ⅲ, 1 (2%) patient in stage Ⅱ and 2 (4%) patients were in stage Ⅰ. Metastasis was absent in 98% patients and mean NGAL levels were highest in these patients but *P* value was not significant. Mean NGAL levels were highest in stage Ⅳ [1041.54 ± 222.15 ng/mL (stage Ⅳ) *vs* 1040 ± 0.00 ng/mL (stage Ⅰ); 900 ± 0.00 ng/mL (stage Ⅱ) and 1031.90 ± 202.55 ng/mL (stage Ⅲ)] and χ^2 test was highly significant ($P < 0.001$). Thirty-six patients (72%) were having moderately differentiated HNSCC and mean NGAL levels were maximum in patients with well differentiated HNSCC (1164 ± 315.64 ng/mL *vs* 1013.33 ± 161.19 ng/mL in moderately differentiated and 890 ± 11.55 ng/mL in poorly differentiated) and the results were also highly significant ($P < 0.001$, χ^2 test).

CONCLUSION

The present work demonstrates a potential role of NGAL as cancer biomarker and its use in monitoring the HNSCC progression.

Key words: Neutrophil gelatinase associated lipocalin; Head and neck squamous cell carcinoma; Metastasis; Biomarker; Lipocalin 2

Core tip: Neutrophil gelatinase associated lipocalin might play a significant role as a biomarker in head and neck squamous cell carcinoma.

Verma M, Dahiya K, Soni A, Dhankhar R, Ghalaut VS, Bansal A, Kaushal V. Levels of neutrophil gelatinase-assosciated lipocalin in patients with head and neck squamous cell carcinoma in Indian population from Haryana state. *World J Clin Oncol* 2017; 8(3): 261-265 Available from: URL: http://www.wjgnet.com/2218-4333/full/v8/i3/261.htm DOI: http://dx.doi.org/10.5306/wjco.v8.i3.261

INTRODUCTION

Head and neck squamous cell carcinoma (HNSCC) is the sixth most common cancer worldwide, affecting 600000 new patients each year. The molecular alterations observed in HNSCC are mainly due to oncogene activation and tumor suppressor gene inactivation, leading to dysregulation of cell proliferation. These alterations include gene amplification and over expression of oncogenes such as ras, myc, epidermal growth factor receptor (EGFR) and cyclin D1 and mutations and deletions leading to p16 and TP53 tumor suppressor genes inactivation[1].

Neutrophil gelatinase associated lipocalin (NGAL) is a small molecule of 178 amino acids that belongs to the superfamily of lipocalins, which are proteins specialized in binding and transporting small hydrophobic molecules. It is also known as lipocalin 2, protumorigenic protein. Increased expression of NGAL was first identified in SV 40 tumour virus infected quiescent mouse primary kidney cells. It plays a significant role in inducing tumour progression and chemoresistance in cancer cells. It acts mainly as a biomarker of kidney injury but now a day it has emerged as a biomarker for several benign and malignant diseases[2]. Lipocalin 2 is thought to be an acute phase protein[3], the expression of which is upregulated in epithelial cells under diverse inflammatory conditions including appendicitis, inflammatory bowel disease and diverticulitis[4]. Lipocalins affect cellular proliferation and differentiation and may be involved in the development of carcinomas[5].

Levels of lipocalin 2 is upregulated or downregulated in different cancers, *i.e.*, lung, colon, pancreas, breast, prostate, *etc*[4,6-8]. But studies are scarce in head and neck carcinoma particularly with respect to its involvement in invasion and metastasis. Five year survival rate of HNSCC is only 50%[9]. Early diagnosis can improve the outcome. So, there is an urgent need for the development of novel biomarkers for timely diagnosis of this fatal disease. Therefore this study was planned to estimate NGAL levels in HNSCC.

MATERIALS AND METHODS

This was a non randomized case control study conducted at Department of Biochemistry, in collaboration with Regional Cancer Center over a period of one year from September 2013 to September 2014. A total of 50 newly diagnosed patients were selected for the study. Group A consisted of 50 HNSCC patients and 50 apparently healthy age and sex matched volunteers acted as controls (Group B).

After taking care of all ethical issues, 12 h fasting venous blood samples without application of tourniquet were collected aseptically from median antecubital vein. Serum was separated and stored at -20 ℃ till analysis. Serum-NGAL was measured using ELISA (Bioporto, Gentofte, Denmark)[10].

Data was subjected to appropriate statistical analysis. Values are shown in the text, tables and figures as mean ± SD. Student *t* test and χ^2 test were applied for comparison of means of study groups. A value of $P < 0.05$ was considered significant. Correlations between groups were analyzed using Pearson's correlation coefficient (*r*) formula.

RESULTS

The mean age of the patients in group A was 57.40 ± 11.33 years (35-95 years), while in group B was 40.01 ± 10.8 years (37-90 years). Out of 50 patients, 40 were males and 10 were females in group A while there were 38 males and 12 females in group B. Both the groups were statistically comparable in age and gender distribution. The biochemical parameters are shown in Table 1. Serum NGAL levels were significantly raised

Table 1 Biochemical parameters in head and neck squamous cell carcinoma patients and healthy controls

Parameter	HNSCC patients	Healthy controls
n	50	50
Age (yr)	57.40 ± 12.61	40.01 ± 10.8
Gender (M:F)	40:10	38:12
Smoker:non-smoker	40:10	12:38
Serum NGAL (ng/mL)	[a]978.88 ± 261.39	34.83 ± 7.59

[a]Significant; all values are in mean ± SD. HNSCC: Head and neck squamous cell carcinoma; NGAL: Neutrophil gelatinase-associated lipocalin.

in HNSCC patients ($P < 0.05$) as compared to controls (978.88 ± 261.39 ng/mL *vs* 34.83 ± 7.59 ng/mL) (Figure 1).

Out of 50, 26 patients (52%) were in stage Ⅳ, 21(42%) in stage Ⅲ, 1 (2%) patient in stage Ⅱ and 2 (4%) patients were in stage Ⅰ. Relation between NGAL and various study variables is shown in Table 2. Mean NGAL levels were highest in stage Ⅳ and χ^2 test was highly significant ($P < 0.001$). Metastasis was absent in 49 patients (98%). Only 1 patient (2%) was having metastasis of unknown origin in head and neck area with occult primary. Results were not significant but mean NGAL levels were highest in patients with no metastasis. Ratio of smokers to non smokers in HNSCC patients was 40:10 while in controls it was 12:38. Smoking history seemed to have no effect on systemic levels of NGAL as $P = 0.097$. Thirty six patients (72%) were having moderately differentiated HNSCC and mean NGAL levels were maximum in patients with well differentiated HNSCC and the results were also highly significant ($P < 0.001$, χ^2 test).

In our study larynx was the most common site involved (38%), other sites being base of tongue (36%), tonsils (22%) and cheek mucosa (4%). Serum NGAL values were significantly higher in patients with base of tongue involvement ($P < 0.001$, χ^2 test).

DISCUSSION

Various studies have shown different levels of NGAL levels in many carcinomas, *i.e.*, lung, colon, pancreas, breast, prostate, *etc*. But very few reports are available for head and neck cancer. A significant increase was observed in serum NGAL levels in HNSCC patients as compared to healthy controls ($P < 0.05$). Moreover, levels were significantly raised in stage Ⅳ ($P < 0.001$) and well differentiated HNSCC carcinoma patients ($P < 0.001$). Metastasis was absent in 98% patients and mean NGAL levels were highest in these patients showing anti-metastatic effect of NGAL. Majority of patients in our set up usually present in advanced stage of the disease due to lack of awareness or resources or both.

Previous studies have shown increased levels of NGAL in adenocarcinoma of lung, colon, pancreas and decreased levels in renal cell carcinomas and prostate

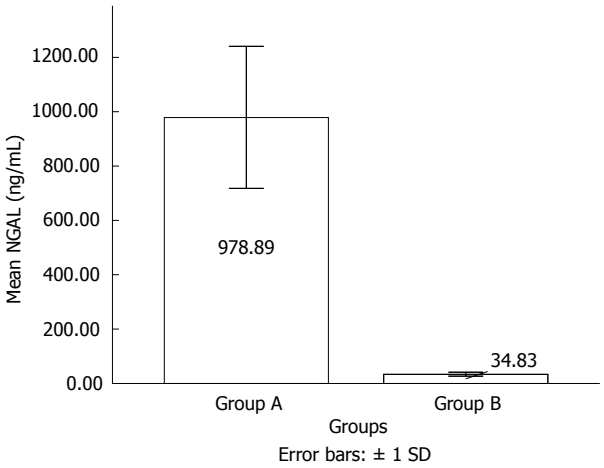

Figure 1 Mean serum neutrophil gelatinase-associated lipocalin levels in head and neck squamous cell carcinoma patients (group A) and healthy controls (group B). NGAL: Neutrophil gelatinase-associated lipocalin.

cancers[11]. Role of NGAL in cancer is controversial. NGAL has been shown to have a pro-tumoral effect in breast, stomach and esophagus cancer. Some studies show that NGAL exert an anti-tumoral and anti-metastatic effect in ovarian and pancreatic cancers similar to present study[12]. These increased levels may be due to increased synthesis induced by factors promoting the development of carcinoma. NGAL and pro-matrix metalloprotein-9 (MMP-9) bind to integral membrane proteins on tumour cells and leads to pro or anti tumour effect on growth, migration, invasion, survival and angiogenesis depending on the type of cancer[13]. *In vitro* studies in human breast and lung cancer cells have revealed that NGAL expression is significantly up regulated in response to multiple apoptosis inducing agents in an attempt to survive the apoptotic stimulus rather than a pro-apoptotic response[14].

Metastasis was absent in 49 patients and an over expression of NGAL, as obvious by increased levels in all these. Only 1 patient was having metastasis of unknown origin in head and neck area. We assumed the primary center being head and neck site. So, it is assumed that over expressed NGAL levels are responsible for reduction in distant metastasis of HNSCC cells as seen in many other cancers also though nothing can be concluded as sample size being very small[15]. The mechanisms by which NGAL regulates tumor metastasis are still unclear. Down-regulation of epithelial proteins and the induction of mesenchymal proteins (EMT) enhance the metastatic potential of epithelial tumors.

Lipocalin 2 is an epithelial inducer, which stimulates the epithelial phenotype in ras transformed cells and reverses their metastatic potential[16]. NGAL has siderophore chelating capacity. It binds to intracellular iron and this complex then interacts with NGAL-R (NGAL receptor) leading to internalization of complex. After entering cytoplasm, it releases iron leading to iron accumulation and regulating specific iron-dependent genetic pathways. These events induce proliferation and

Table 2 Correlation between neutrophil gelatinase-associated lipocalin levels and various study variables

	n	Serum NGAL (mean ± SD)
Differentiation		
Well differentiated	10	[1]1164 ± 315.64
Moderately differentiated	36	1013.33 ± 161.19
Poorly differentiated	4	890 ± 11.55
Site		
Base of tongue	18	[1]1072.22 ± 251.39
Larynx	19	1037.89 ± 217.35
Tonsil	11	947.27 ± 50.02
Cheeks	2	1120 ± 0.00
Metastasis		
No	49	1036.73 ± 206.64
Yes	1	880 ± 0.00
Staging		
I	2	1040 ± 0.00
II	1	900 ± 0.00
III	21	1031.90 ± 202.55
IV	26	[1]1041.54 ± 222.15
Smoking		
Smoker	40	1051 ± 223.57
Non-smoker	10	964 ± 84.22

[1]Highly significant. All values are in mean ± SD. NGAL: Neutrophil gelatinase-associated lipocalin.

epithelial transformation[17]. It inhibits focal adhesion kinase phosphorylation and vascular endothelial growth factor synthesis leading to decreased cell adhesion, invasion and angiogenesis[12]. NGAL also suppresses c-Jun N-terminal kinase (JNK) and phosphoinositide 3-kinase (PI3)/AKT signalling pathways, thereby, decreasing metastasis in HNSCC[13].

Lipocalin 2 levels were significantly higher in well differentiated carcinoma as compared to moderately and poorly differentiated HNSCC ($P < 0.001$). Similar results have been reported by another study in which it was shown by immuno-histochemical examinations that NGAL expression was strongly up-regulated in well-differentiated oral squamous cell carcinoma (OSCC) tissues and moderately to weakly up-regulated in moderately to poorly differentiated OSCC tissues as compared to normal mucosa and leukoplakia showing very weak expressions. Western blot analysis showed positive correlation of NGAL expression levels with cell morphology patterns and loss of E-cadherin[18]. So, NGAL levels may be raised as an effect or cause of tumor. NGAL may act as biomarker for diagnosis of HNSCC and for assessment of its severity.

Some limitations of our study include small sample size, lack of follow up and monitoring of the effect of treatment on NGAL outcome that will make part of our future project.

Our analysis demonstrates a potential role of NGAL as cancer biomarker which may be useful in monitoring the HNSCC progression. Many drugs which induce lipocalin 2 can be of therapeutic benefit in HNSCC to prevent metastasis and further spread.

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COMMENTS

Background

Neutrophil gelatinase-associated lipocalin (NGAL), plays a significant role in generating innate immune response and safeguards against bacterial infections by sequestering iron. Recently, it has emerged as a biomarker for several benign and malignant diseases with its differential expression pattern.

Research frontiers

As NGAL has anti-tumoral and anti-metastatic effect, it could be a new and effective biomarker for monitoring the progression of disease in head and neck squamous cell carcinoma (HNSCC).

Innovations and breakthroughs

NGAL was initially defined as a useful bacteriostatic agent actively against bacteria. Later on it was found that it acts as a biomarker of kidney injury but now a day it has emerged as a biomarker for several benign and malignant diseases. This study describes for the first time the increased levels of NGAL and its association with anti-metastatic effect in HNSCC.

Applications

The goal of treatment in HNSCC is mainly surgery, radiotherapy and chemotherapy. Biomarkers like NGAL having significant diagnostic and prognostic significance and with specific molecular target are demand of newer treatment modalities to increase the survival of patients.

Peer-review

In this study, the authors examined the levels of NGAL in patients with HNSCC in an Indian population. Overall, the methodology of the study is adequate, and the findings are clinically and scientifically relevant.

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SYSTEMATIC REVIEWS

Recurrence-free survival as a putative surrogate for overall survival in phase Ⅲ trials of curative-intent treatment of colorectal liver metastases: Systematic review

Raphael LC Araujo, Paulo Herman, Rachel P Riechelmann

Raphael LC Araujo, Department of Upper Gastrointestinal and Hepato-Pancreato-Bliliary Sugery, Barretos Cancer Hospital, Barretos, SP 14784-400, Brazil

Raphael LC Araujo, Paulo Herman, Department of Gastroenterology, University of São Paulo, São Paulo, SP 05403-900, Brazil

Rachel P Riechelmann, Instituto do Câncer do Estado de São Paulo, Universidade de São Paulo, SP 01246-000, Brazil

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Correspondence to: Raphael LC Araujo, MD, PhD, Department of Upper Gastrointestinal and Hepato-Pancreato-Bliliary Sugery, Barretos Cancer Hospital, Rua Antenor Duarte Villela, 1331, Barretos, SP 14784-400, Brazil. raphael.araujo@hcancerbarretos.com.br
Telephone: +55-17-33216600
Fax: +55-17-33216600

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Abstract

AIM

To verify whether recurrence-free survival (RFS) surrogates overall survival (OS) in phase Ⅲ trials for resectable colorectal liver metastases (CRLM).

METHODS

MEDLINE, EMBASE, and Scopus databases were consulted. Eligible studies were phase Ⅲ trials testing any type of systemic therapy (neoadjuvant, adjuvant or perioperative) added to surgery in patients with resectable CRLM. A linear regression model based on hazard ratios (HR) of OS and RFS was performed.

RESULTS

Of 3059 studies, 5 phase Ⅲ trials (1162 patients) were included for analyses. A linear regression weighted by each trial was used to estimate the association between each HR and RFS. The originated formula was: OS HR = (0.93 × RFS HR) + 0.14; with RFS 95%CI (0.48-1.38), with $P = 0.007$.

CONCLUSION

This association suggests that RFS could work as a putative surrogate endpoint of OS in this population, avoiding bigger, longer and more resource-consuming trials. The OS could be assumed based on RFS and our model could be useful to better estimate sample size calculations of phase Ⅲ trials of CRLM aiming for OS.

Key words: Colorectal liver metastases; Surgery; Chemotherapy; Clinical trial; Long-term outcomes; Surrogate endpoints

Core tip: This study addresses a systematic review of curative-intent treatment of colorectal liver metastasis looking for oncologic outcomes. We describe the association between overall survival (OS) and recurrence free survival in the setting of resectable colorectal liver metastases (CRLM). It suggests that recurrence free survival could work as a putative surrogate of OS in this population, avoiding bigger, longer and more resource-consuming trials. We do believe that our model can be useful to better estimate sample size calculations of superiority phase Ⅲ trials of CRLM aiming for OS.

Araujo RLC, Herman P, Riechelmann RP. Recurrence-free survival as a putative surrogate for overall survival in phase Ⅲ trials of curative-intent treatment of colorectal liver metastases: Systematic review. *World J Clin Oncol* 2017; 8(3): 266-272 Available from: URL: http://www.wjgnet.com/2218-4333/full/v8/i3/266.htm DOI: http://dx.doi.org/10.5306/wjco.v8.i3.266

INTRODUCTION

Randomized clinical trials (RCT) represent a high level of evidence and a mainstream analysis of oncologic outcomes. However, they involve a time-consuming methodology with inherent high costs. Moreover, trials using overall survival (OS) as their primary endpoint in patients with slow progressive malignancies, such as colorectal cancer, must have longer follow-up for events to arise and thus properly evaluate potential differences in OS. In turn, long-term follow up increases cost associated with image and laboratory tests, salaries of research coordinators, pharmacists and research nurses, investigators fees, medications, *etc.* Therefore, such trials claim for fundings that are not always provided by governmental agencies or by pharmaceutical companies. For example, Emanuel *et al*[1] reviewed the cost of conducting clinical trials and demonstrated that monitoring and treating 20 patients in a 12-mo randomized placebo-controlled trial of a new chemotherapeutic agent cost more than United States $ 6900 per enrolled subject in an industry-sponsored trial.

In order to reduce the cost and time to conduct RCT, investigators have looked at surrogate endpoints of OS, such as progression free survival (PFS) and recurrence free survival (RFS), as measures of clinical benefit in cancer trials. Gains in RFS associating chemotherapy to surgery *vs* surgery alone for initially resectable colorectal liver metastases (CRLM) have been demonstrated by phase Ⅲ trials[2-4]. While surgery plus chemotherapy has not been associated with improvements in OS in phase Ⅲ trials, it has been suggested by a meta-analysis of published data[5]. However, it is unknown whether RFS can substitute, and if so to which extent, OS in RCT of CRLM. In this regard we hypothesized that if gains in RFS predicted gains in OS, trials of new drugs in the setting of CRLM could use RFS as a surrogate endpoint, and thus expedite drug development. The objectives of this systematic review were to evaluate RCT of curative-intent treatment to resectable CRLM and to verify whether RFS surrogates OS in phase Ⅲ trials for this population.

MATERIALS AND METHODS

Eligibility criteria

Type of studies: All published RCTs with curative-intent treatment for initially resectable CRLM were evaluated; curative-intent therapies were surgery alone *vs* associated systemic cancer-directed therapy. Two considerations were made to assume curative-intent treatment: Patients were not treated for conversion therapy because they were already resectable at the time of study enrollment and removal of all macroscopic disease (no residual disease). No language restriction was applied. Studies with extra-hepatic disease were generally excluded, but when extra-hepatic disease was present in no more than 5% they were accepted. Studies using regional chemotherapy or presenting initially unresectable disease were also excluded. For situations in which two studies from the same institution were identified, the most recent or the most informative study was selected unless different periods were evaluated or the data of overlapping patients could be subtracted.

Type of interventions: Only treatments with curative-intent treatment for initially resectable hepatic lesions were evaluated. However, any standardized description of resectable disease was used to define this group of patients, as they were defined according to clinic-radiological evaluation of each tumor board of their respective authors' institutions. Any additional systemic treatment was considered as the following: Adjuvant chemotherapy (surgery followed by systemic therapy), neoadjuvant chemotherapy (preoperative chemotherapy followed by surgery), perioperative chemotherapy (preoperative chemotherapy followed by surgery and postoperative chemotherapy), and targeted agents at any perioperative period. This study did not discriminate between type of liver resection or surgical techniques because all of them were procedures with curative-intent. The study also did not aim to compare types of systemic therapies or times of its administration.

Type of outcome measure: The primary end point of the study was to describe the association between OS and RFS in the setting of resectable CRLM. Calculation of OS was based on survivorship status (deceased or alive) at the last follow-up visit as reported by RCT. Calculation of RFS was based on the first detected recurrence or the last follow-up visit without recurrence. Start time

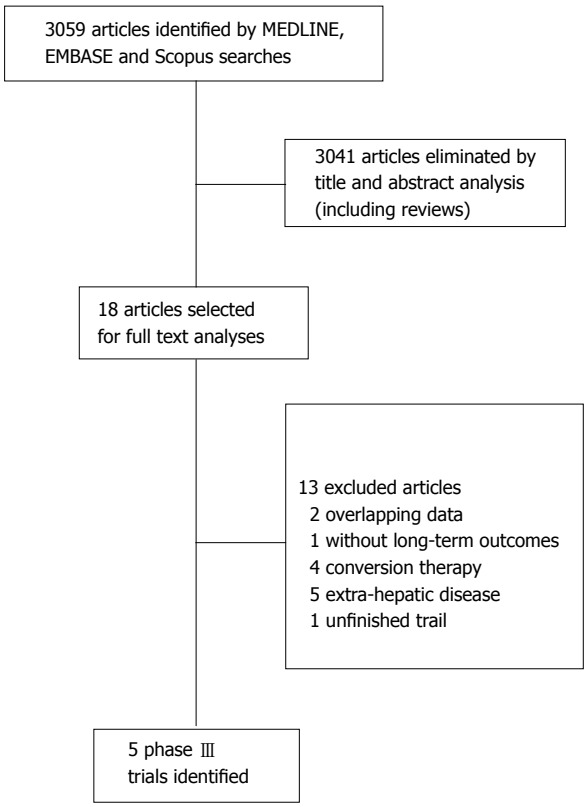

Figure 1 Flowchart of search and article selection process.

was counted as defined by each study. Imaging tests were mostly performed at 3-mo intervals until disease recurrence, as defined by RCT. The terminology chosen was RFS since all patients did not present any residual macroscopic disease after curative-intent treatment. We counted reappearance of distance and/or liver-only disease as recurrence.

Search: The MEDLINE, EMBASE, and Scopus databases were searched using the mesh terms ("colorectal liver metastases" or "colorectal liver metastasis") and (surgery or surgical or chemotherapy or "drug therapy" or "Antineoplastic Agents") and (Clinical Trial or Comparative Study or Randomized Controlled Trial). They were filtered from January 1990 to February 2015 and only for studies in humans.

Data collection process: Relevant data were extracted independently from all the studies by two reviewers (Raphael LC Araujo and Rachel P Riechelmann) and included study features, population characteristics, and data needed for quality assessment. For the purpose of this study, only OS and RFS were extracted according to the description provided by the authors.

Quality assessment: The RCTs were evaluated by individual components based on the Cochrane Risk-of-Bias Tool (version 5.1.0). The qualitative evaluation was performed and discriminated for each RCT. This study was performed according to the recommendations of the preferred reporting items for systematic reviews and meta-analysis (PRISMA) statement. We used Begg's funnel plot as an analytic tool to detect publication bias[6,7].

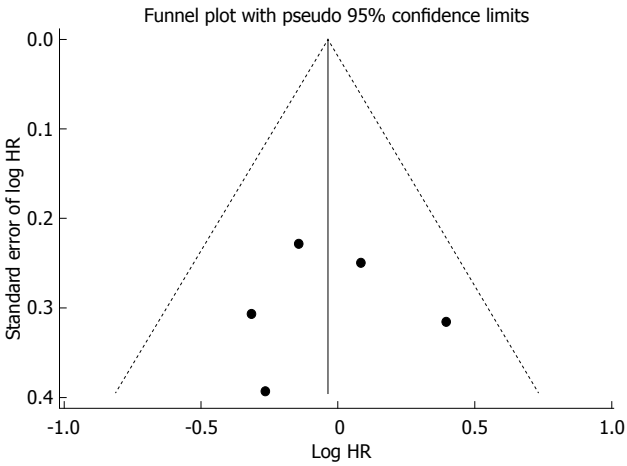

Figure 2 Funnel plots of randomized clinical trials comparing surgery alone or with additional chemotherapy for the treatment of patients with potentially resectable colorectal liver metastases. The HR was fit for overall survival. HR: Hazard ratio.

Statistical analysis

Linear regression was performed to examine the association between HR for both outcomes. Demographics were demonstrated as percentages as appropriate. Survival probabilities were estimated by cited hazard ratios (HR) accordingly to each published study. The graph of linear regression was based on linear prediction of OS HR according to RFS HR, along with a 95%CI based on the mean. For all tests, statistical significance was defined by a two-sided *P* value lower than 0.05. All analyses were performed by STATA 13 statistical software (StataCorp, College Station, TX, United States).

RESULTS

We identified five RCT addressing curative-intent treatment with surgery alone or with systemic therapy for initially resectable CRLM. They were selected among 3059 articles. The flowchart of selection process is summarized in a flow diagram in Figure 1.

This systematic review was made properly to the *PRISMA* Statement (Preferred Reporting Items for Systematic Reviews and Meta-Analysis). Additionally, the Cochrane Risk-of-Bias Tool was used to qualitative evaluation of RCTs, and it is described in Table 1. As frequently seen in surgical trials, the difficulty concealing the allocation of patients and blinding in the randomization between chemotherapy and surgery first could not be granted. However, it was not considered as a drawback neither affecting outcomes directly nor compromising the primary endpoint of our review. No publication bias was demonstrated using Begg's funnel plot as depicted in Figure 2.

Only five phase Ⅲ trials were accepted for this review, and all of them were looking for initially resectable CRLM[2-4,8,9]. Comparative distributions of accessible

Table 1 Quality assessment of selected randomized clinical trials evaluated by Cochrane Risk-of-Bias Tool (Risk of bias per study)

Criteria	Langer	Portier	Nordlinger	Ychou	Primrose
Random sequence generation	Unclear	Low	Low	Low	Low
Allocation concealments	Low	Low	Low	Low	Low
Blinding of participants and personnel[1]	Low	Low	Unclear	Low	Low
Blinding of outcome assessment[1,2]	Low	Low	Low	Low	Low
Incomplete outcome data	Unclear	Low	Low	Unclear	Low
Selective reporting	Low	Low	Low	Low	Low
Other bias	Unclear	Low	Low	Low	Low

[1]Blinding is not possible; [2]Implementation of a protocol for postoperative management was considered the best alternative.

Table 2 Comparative distribution of accessible baseline characteristics of patients among studies included in the systematic review

Characteristics	Langer *n* = 107 (%)		Portier *n* = 171 (%)		Nordlinger *n* = 364 (%)		Ychou *n* = 306 (%)		Primrose[1] *n* = 257 (%)	
	Surg *n* = 55	S + C *n* = 52	Surg *n* = 85	S + C *n* = 86	Surg *n* = 182	S + C *n* = 182	S + 5-FU *n* = 153	S + FOLFIRI *n* = 153	S + C *n* = 128	S + C + Cetuximab *n* = 129
Median age	60	63.5	63	63	62	64	61	63	64	63
Gender (male)	65.4	65.4	62.4	53.5	63	70	65.4	58.8	63	71
Primary site (rectum)	30.9	26.9	40	40.7	37	46	26.1	28.8	-	-
DFI ⩽ 12 mo	38.2	34.6	74.1	74.4	24	27	62.3	61.4	-	-
Node-positive primary	45.4	50	50.6	44.3	57	55	-	-	-	-
No. of lesions > 1	32.7	36.5	30.1	31.4	52	51	35.9	36	-	-
Largest met ⩾ 5 cm	-	-	-	-	-	-	-	-	-	-
Chemotherapy	5-FU		5-FU		FOLFOX		5 –FU	FOLFIRI	5-FU + OX or 5-FU + Cap or FOLFIRI	5-FU + OX or 5-FU + Cap or FOLFIRI + Cetuximab

[1]*n* of eligible patients = 257, although only 234 patients had response rates analyzed; –: Represents data not assessable by authors; Surg: Surgery only; S + C: Surgery with additional chemotherapy; 5-FU: 5-fluorouracil based; FOLFOX: Fluorouracil, leucovorin, and oxaliplatin; FOLFIRI: Fluorouracil, leucovorin, and irinotecan.

baseline characteristics in the studies are depicted in Table 2. Three of them compared surgery alone *vs* surgery plus chemotherapy, and two RCT compared surgery plus chemotherapy on both arms, with one of them testing the addition of cetuximab, a monoclonal antibody against epidermal growth factors receptor (EGFR). Four studies had RFS as their primary endpoint. Only Langer *et al*[2] pursued OS as primary endpoint, but they failed to show a significant difference with postoperative chemotherapy. A total of 1162 patients (per protocol) were included in this pooled analysis. Most of them were male, the median age ranged from 60 to 64 years old, most presented colon as their primary site and with a single hepatic lesion. Comparisons of original planned and analyzed design of RCT are demonstrated in Table 3.

A linear regression was used to fit a predict model for OS based on RFS and using HR values. The assumption of linearity was based on this formula: OS HR = (0.93 × RFS HR) + 0.14; with RFS 95%CI (0.48-1.38); standard error of 0.14, and $P = 0.007$. HR for RFS and OS, the originals and those assumed by the formula above are described in Table 4 and depicted in Figure 3 (the intention-to-treat analysis from Nordlinger *et al*[3] was used).

DISCUSSION

This systematic review and pooled analyses of published RCT of resectable CRLM demonstrates that RFS can be considered a surrogate endpoint for OS in this setting. We found a linear association between RFS gains, as measured by HR, and OS increments. This finding has numerous implications for future trial designs of new cancer-directed therapies added to curative-intent hepatic resection of CRLM.

The practice of evidence-based medicine (EBM) has been in vogue in the last 30 years. Sacket *et al*[10] categorized levels of evidence according to quality of study designs, ranging from expert opinion (level V - the lowest level) to RCT (level I - highest level). While RCT represent the best way to deliver evidence-based medicine, over the last decades, their costs have skyrocketed, what may limit national fundings and consequently, demand for-profit sponsorship[11]. This is particular relevant for clinical cancer research[12]. The cost of RCT, including cancer trials, can be split in fixed (trial administration, hospital facilities, personnel training, equipment, infrastructure, *etc.*), variable (randomization, recruitment cost, patient

Table 3 Comparison of original planned and analyzed design of randomized clinical trials with patients who underwent surgery and additional chemotherapy for initially resectable colorectal liver metastases

Studies	Initial design			No. of patients				Chemotherapy			%	Median FU		RFS		OS	
by author	Primary endpoint	Planned HR	Type of analyses	Planned	Accrued	ITT enrolled	PP (weight)	Regimen	Std Arm	Exp Arm	Resected	Std Arm	Exp Arm	Std Arm	Exp Arm	Std Arm	Exp Arm
Langer	OS	NR	PP	NR	129	129	107 (9)	Adj	0	5-FU × 6	100%	NR	NR	20	39	43 median	53 median
Portier	RFS	20% abs dif 2 yr[1]	ITT	200	173	171	171 (15)	Adj	0	5-FU × 6	100%	87.4	87.4	17.6	24.4	46.4 median	62.1 median
Nordlinger	RFS	0.714	Both	NR	364	364	342 (29)	Periop	0	FOLFOX × 12	93%	8.7 yr	8.7 yr	20	12.5	54.3	61.3
Ychou	RFS	NR	PP	420	321	321	306 (26)	Adj	1 5FU	FOLFIRI × 6	100%	42.4	41.7	21.6	24.7	72% at 3-yr	73% at 3-yr
Primrose	RFS	0.68	ITT	268	272	257	236 (20)	Periop	FOLFOX	Cetux + FOLFOX (70%)	85% (Chemo) 82% (Cetux)	21.1	19.8	14.1	20.5	39.1	NR

[1]Absence of difference at 2-year. HR: Hazard ratio; OS: Overall survival; RFS: Recurrence free survival; ITT: Intention to treat; PP: Per protocol; Std: Standard; Exp: Exposed; Adj: Adjuvant; Periop: Perioperative; 5-FU: 5-Fluorouracil; FOLFOX: 5-FU + Leucovorin + Oxaliplatin; FOLFIRI: 5-FU + Leucovorin + irirnotecan; Cetux: Cetuximab; Chemo: Chemotherapy; NR: Non-reported.

Table 4 Comparison of original hazard ratio and those from linear regression formula

Studies (by author)	*n* total (weight %)	RFS			OS			Assumption OS HR
		HR	95%CI		HR	95%CI		
Langer	107 (9)	0.78	0.46	1.31	0.77	0.42	1.4	0.87
Portier	171 (15)	0.66	0.45	0.96	0.73	0.48	1.1	0.75
Nordlinger[1]	342 (29)	0.78	0.61	0.99	0.87	0.66	1.14	0.87
Ychou	306 (26)	0.89	0.66	1.19	1.09	0.72	1.64	0.97
Primrose	236 (20)	1.48	1.04	2.12	1.49	0.86	2.6	1.52

[1]Intention-to-treat analysis. HR: Hazard ratio; OS: Overall survival; RFS: Recurrence free survival.

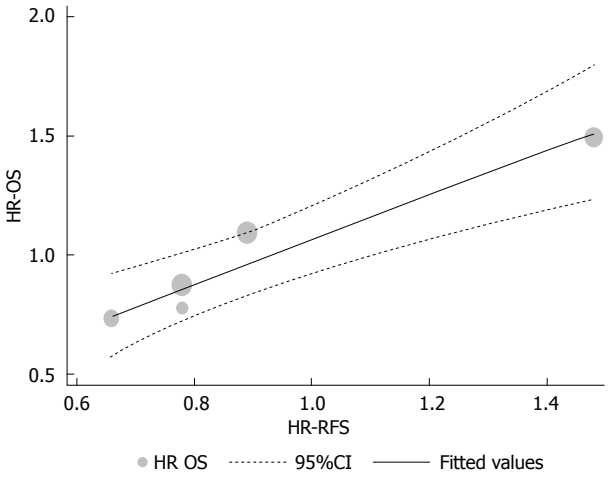

Figure 3 Linear prediction of overall survival according to recurrence free survival. The sizes of dots are proportional to weight of each study. The linear regression was based on linear prediction of OS HR according to RFS HR, along with a 95%CI based on the mean. RFS: Recurrence-free survival; OS: Overall survival; HR: Hazard ratio.

track and follow-up) and indirect costs (hospital overhead, public relations and networking, and legal consultancy)[11]. In this context, expensive new cancer drugs can cost to society in two different ways: Firstly, it costs directly to payers; and secondly, their high prices preclude new trials to compare their effectiveness against effective but cheaper alternatives[13]. Looking for oncologic outcomes in resectable CRLM, RCT can be even more expensive since OS is usually a required primary endpoint. For example, it is clear that slow-progressive tumors demand longer follow-up (more than 5 years to reach median OS, *e.g.*,) in phase Ⅲ trials than those just looking for RFS. Therefore, there are several pros and cons of utilizing OS as a primary endpoint for a cancer RCT. The first issue with the measurement of OS is that the curative-intent treatment works as just first-line therapy; when disease progresses, the patient can still undergo further lines of systematic therapy or R0 surgery, what contaminates and dilutes eventual OS gains from first line. This phenomenon can sometimes be overcome by planning trials with large samples aimed to look for small statistical OS benefits. This is turn, increases the cost of conducting RCT in oncology. The argument in favor of using only OS as the primary endpoint in cancer RCT is that survival is a hard endpoint, not subjected to measurement biases. On the other hand, those in favor of surrogate endpoints for OS, such as PFS and RFS, highlight benefits in terms of faster trial results, less cost and perceived clinical benefit by patients and physicians. We argue that while both OS and surrogates endpoints can be used depending

on the scenario, surrogates endpoints, if mathematically demonstrated, are useful tools to expedite clinical research and avoid unrealistically large and expensive trials, and also to early identify and stop enrollment into futile trials.

PFS has been demonstrated to be a surrogate endpoint for OS in treatments for metastatic[14,15] as well as for early stage colorectal cancer. In the adjuvant, *i.e.*, curative setting, Sargent *et al*[16] pooled individual data of 18 RCT (20898 patients) for early stage colorectal cancer, showing that gains in disease free survival predicted for gains in OS. Our study resembles the results of the Sargent *et al*[16] because we selected studies with a population more inclined to be cured since patients presented potentially resectable CRLM and underwent curative-intent treatment. In RFS, likewise OS, recurrence is a hard endpoint that is not subject to measurement bias, although it is dependent on the intervals of radiological evaluation.

In this review four studies were powered to RFS but not OS[3,4,8,9]. Although Langer *et al*[2] investigated OS as the primary endpoint, it failed to show any benefits of adjuvant chemotherapy compared to surgery alone. Nordlinger *et al*[17] reported the long-term outcomes with median follow-up of 8.5 years, which also did not find differences in OS. We recently published a systematic review and meta-analysis concerning also surgery alone *vs* surgery plus chemotherapy, and we found a relative increasing of 23% in OS at 5 years[5]. However, it was only possible using published data from both randomized and non-randomized trials. One may think that all these negative trials for OS suggest that larger trials would still be necessary to detect small differences in OS. We argue that this is unrealistic and that surrogates endpoints such as RFS should replace OS in RCT of CRLM.

Our study has some limitations. Despite our extensive search, only five studies were suitable to our analysis and it was conducted based on published instead of individual data. As most patients presented low volume disease, our model likely reflect CRLM patients with better prognosis and might not be generalizable to settings of bulky or conversion CRLM. Moreover, as expected, part of those patients will recur but they will still be candidate to rescue treatments (chemotherapy with or without surgery or radiofrequency ablation)[18,19]. For theses reasons, we do not consider our formula useful for individual estimative of OS in clinical practice. The patterns of recurrence are heterogeneous and our correlations could not address such questions since they were not addressed in the original trials. The limitations of our study are those inherent of systematic reviews. And because of that, we think predictive value of RFS demonstrated by our model should be externally tested in future studies. However, we attempted to search as wide as possible, and moreover, we did not detect publication bias. Another limitation to our study and to all others in the field of CRLM is that colorectal cancer is a heterogeneos disease, with patients presenting variable outcomes even when following similar treatments.

Recently, colorectal cancer has been molecularly classified as four distinct prognostic subgroups: CMS1 (microsatellite instability and immune activation features, better prognosis), CMS2 (epithelial, with marked WNT and MYC signaling activation), CMS3 (metabolic dysregulation) and CMS4 (mesenchymal features, worse outcomes)[20]. It is clear that while perioperative benefits patients with resectable CRLM, many relapse and are not cured. Hence is crucial to properly identify the patients who are more likely to be cured or not by hepatectomy. Once this is done, the surrogacy of RFS on OS will have to be revaluated according to treatments tailored to each of these molecular subgroups.

Based on RCT, it seems that chemotherapy should always be offered as additional treatment to curative-intention liver resections, increasing RFS, and likely OS[5]. However, given the lack of evidence on OS gain by RCT, we foresee that surgical trials of systemic treatment for CRLM may prefer OS as their main endpoint. We think such approach should be revisited since larger sample than those already used would be necessary. Based on this systematic review and pooled analysis, we suggest RFS as surrogate of OS for phase Ⅲ trials comprising patients with resectable CRLM could be used.

In summary, this study demonstrates a linear prediction of OS based on RFS of RCT of patients with resectable CRLM who were managed by curative-intent surgery and systemic therapy. This association suggests that RFS could work as a putative surrogate of OS in this population, avoiding bigger, longer and more resource-consuming trials. Our model can be useful to better estimate sample size calculations of superiority phase Ⅲ trials of CRLM aiming for OS. However, future RCT should test this model to externally validate its efficiency.

ACKNOWLEDGMENTS

The statistical methods of this study were reviewed by Marcos Alves Lima, biostatistician from Epidemiology and Biostatistics Center, Institute of Learning and Research, at Barretos Cancer Hospital.

COMMENTS

Background

Gains in recurrence-free survival (RFS) for resectable colorectal liver metastases (CRLM) have been demonstrated by phase III trials, but have not been associated with improvements in overall survival (OS). This systematic review verified whether RFS surrogates OS in phase Ⅲ trials for resectable CRLM.

Research frontiers

Although OS is considered the most appropriate outcome sought in oncology clinical trials, its use is not always feasible in trials of curative-intent treatment of CRLM. Most studies have evaluated RFS as their primary endpoint and none of them had demonstrated benefit in OS, except for a meta-analysis of published randomized trials. The study hypothesized a linear correlation between RFS and OS for this population after a systematic review of literature.

Application

This study addresses an alternative option for analyses of oncologic outcomes in patients who have undergone a curative-intent treatment for CRLM. The linearity identified suggests a corresponding comportment between RFS and

OS. The authors' model could be useful to calculate the sample size for new trials in this field.

Innovations and breakthroughs

The use of modern chemotherapies and surgical resection for CRLM has made the comportment of this disease change into a more indolent profile, with many patients achieving long-term survival. Therefore clinical trials looking for OS as their primary endpoint are associated with long follow up times and high cost. The present study proposes a paradigm change in oncology clinical research because it sought to investigate another outcome which associated with patient benefit, RFS, that may be used in future research to avoid resource-consuming trials.

Peer-review

The paper is well written, properly designed, and comprehensive.

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META-ANALYSIS

Robot-assisted laparoscopic *vs* open gastrectomy for gastric cancer: Systematic review and meta-analysis

Stefano Caruso, Alberto Patriti, Franco Roviello, Lorenzo De Franco, Franco Franceschini, Graziano Ceccarelli, Andrea Coratti

Stefano Caruso, Franco Franceschini, Department of General Surgery and Surgical Specialties, Unit of General Surgery, "Santa Maria Annunziata" Hospital, Local Health Unit Center Tuscany Company, 50012 Florence, Italy

Alberto Patriti, Department of Surgery, Division of General Surgery, Hospitals of Pesaro and Fano, "Ospedali Riuniti Marche Nord" Hospital Company, 61032 Marche, Italy

Franco Roviello, Lorenzo De Franco, Department of Medical, Surgical and Neuroscience, Unit of General and Minimally Invasive Surgery, University of Siena, 53100 Siena, Italy

Graziano Ceccarelli, Department of Medicine and General Surgery, Unit of Minimally Invasive and General Surgery, Local health Unit South-Est Tuscany Company, "San Donato" Hospital, 52100 Arezzo, Italy

Andrea Coratti, Division of Oncological and Robotic General Surgery, "Careggi" University Hospital, 50134 Florence, Italy

Author contributions: Caruso S conceived the design of the study, directed the acquisition of data, the analysis and interpretation of data; De Franco L and Franceschini F performed the literature search, the acquisition of data, the analysis and interpretation of data; Patriti A, Roviello F, Ceccarelli G and Coratti A contributed to the critical appraisal of the work, revising the article critically for important intellectual content and supervising the interpretation of data for final approval.

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Correspondence to: Stefano Caruso, MD, Department of General Surgery and Surgical Specialties, Unit of General Surgery, "Santa Maria Annunziata" Hospital, Local Health Unit Center Tuscany Company, ASL Firenze, Via dell'Antella 58, Bagno a Ripoli, 50012 Florence, Italy. stefano.caruso@teletu.it
Telephone: +39-55-9508373

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Abstract

AIM

To evaluate the potential effectiveness of robot-assisted gastrectomy (RAG) in comparison to open gastrectomy (OG) for gastric cancer patients.

METHODS

A comprehensive systematic literature search using PubMed, EMBASE, and the Cochrane Library was carried out to identify studies comparing RAG and OG in gastric cancer. Participants of any age and sex were considered for inclusion in comparative studies of the two techniques independently from type of gastrectomy. A meta-analysis of short-term perioperative outcomes was performed to evaluate whether RAG is equivalent to OG. The primary outcome measures were set for estimated blood loss, operative time, conversion rate, morbidity, and hospital stay. Secondary among postoperative complications, wound infection, bleeding and anastomotic leakage were also analysed.

RESULTS

A total of 6 articles, 5 retrospective and 1 randomized controlled study, involving 6123 patients overall, with 689 (11.3%) cases submitted to RAG and 5434 (88.7%) to OG, satisfied the eligibility criteria and were included in the meta-analysis. RAG was associated with longer operation time than OG (weighted mean difference 72.20 min; $P < 0.001$), but with reduction in blood loss and shorter hospital stay (weighted mean difference -166.83 mL and -1.97 d respectively; $P < 0.001$). No differences were found with respect to overall postoperative complications ($P = 0.65$), wound infection ($P = 0.35$), bleeding ($P = 0.65$), and anastomotic leakage ($P = 0.06$). The postoperative mortality rates were similar between the two groups. With respect to oncological outcomes, no statistical differences among the number of harvested lymph nodes were found (weighted mean difference -1.12; $P = 0.10$).

CONCLUSION

RAG seems to be a technically valid alternative to OG for performing radical gastrectomy in gastric cancer resulting in safe complications.

Key words: Robot-assisted gastrectomy; Gastric resection; Open gastrectomy; Gastric cancer

Core tip: We took into consideration how safe and efficient robot-assisted gastrectomy (RAG) is compared to open gastrectomy (OG) for gastric cancer *via* systematic review and meta-analysis. The available studies to date and the analysis of pooled data extracted from these showed that RAG is safe and feasible, making it possible to obtain lower blood loss related to surgery and a more rapid patient recovery. At the same time similar lymph node dissection between the two techniques were revealed. We can reasonably expect that the innovative robotic technique could represent a valid alternative with potential benefit to equal oncological adequacy with respect to OG.

Caruso S, Patriti A, Roviello F, De Franco L, Franceschini F, Ceccarelli G, Coratti A. Robot-assisted laparoscopic *vs* open gastrectomy for gastric cancer: Systematic review and meta-analysis. *World J Clin Oncol* 2017; 8(3): 273-284 Available from: URL: http://www.wjgnet.com/2218-4333/full/v8/i3/273.htm DOI: http://dx.doi.org/10.5306/wjco.v8.i3.273

INTRODUCTION

Since its introduction by Kitano *et al*[1] in 1994, laparoscopy has been increasingly used for the treatment of gastric cancer. During this period of time, a number of works have shown laparoscopic gastrectomy (LG) to be a feasible option in treating gastric cancer and level Ⅲ studies provided the evidence that laparoscopic assisted distal gastrectomy (LADG) leads to better results in the short-term than conventionally performed open gastrectomy (OG) for early gastric cancer[2-5]. Due to the high incidence of gastric cancer and the extremely high levels of expertise achieved by Asian surgeons, LG is now a routine procedure for early gastric cancer in eastern states[6]. On the other hand, laparoscopic surgery did not meet the same widespread requirements for the management of advanced gastric cancer, mainly due to the technical difficulties posed by the D2 lymphadenectomy and the intestinal reconstruction after total gastrectomy. Concerns regarding oncological adequacy, in particular for potential inadequate lymphadenectomy and long-term outcomes[6,7], make LG for advanced gastric cancer still questionable. Therefore, a significant proportion of patients with advanced stage disease are still treated with OG, especially in Western countries. In an effort to overcome the technical disadvantages of laparoscopic technique, robotic surgery has been introduced and it has gradually spread worldwide. Robotic systems have three-dimensional (3D) high-resolution imaging, tremor filter, and internal articulated endoscopic wrist (EndoWrist™ System), which lead to significant improvements in visibility and manipulation with respect to conventional laparoscopy. With this advanced equipment, robot-assisted gastrectomy (RAG) has been advocated to give a global advantage over the traditional laparoscopic approach, particularly in performing the D2 lymphadenectomy and facilitating complex reconstruction after gastrectomy[8-10]. A variety of reports have demonstrated the safety and feasibility of this technique[6]. However, so far most of the reports derives from not large, retrospective, non-randomized studies.

In order to achieve a confirmed acceptance, an innovative technique with minimum invasiveness absolutely has to show that it is not disadvantageous to oncologic result. As LG still has not reached a comprehensive validation for the treatment of all (advanced) gastric cancer, the introduction of robotic surgery can represent a fair cue of advancement potentially able to make the laparoscopic technique more oncologically adequate, and so to increase its use as alternative procedure to the conventional open approach. Yet, to date a mere handful of trials have shown high quality comparative analysis of RAG *vs* OG in the treatment of gastric cancer, and most of these studies have been limited to small sample size and a single institution design. To overcome these limitations, we performed a systematic review and meta-analysis which can increase the statistical power of short-term results available so far on these two techniques. Thus, relevant trials comparing the safety and efficacy of RAG *vs* OG in treating gastric cancer were analyzed, to verify if at present there is actual evidence of an advantage to the introduction of this new minimally invasive technique with respect to the validated open procedure. Positive results could represent the preliminary impulse to potentiate the robotic tool which, by overcoming some intrinsic limits of the conventional laparoscopic method, might increase the use and acceptance of the minimally invasive procedure for gastric cancer in the future.

MATERIALS AND METHODS

Literature search

A comprehensive systematic literature search was conducted using PubMed, EMBASE, and the Cochrane Library to identify relevant articles comparing RAG *vs* OG for the treatment of gastric cancer published up to December 2016. The Preferred Reporting Items for Systematic Reviews and Meta-Analysis (PRISMA) statement guidelines were adopted for performing and reporting meta-analysis data[11]. No restriction was set for type and date of publication, and for age and sex of participants. Article language was limited to English. The following terms were used for the search strategy: "Robot" or "robotic" or "robot-assisted" or "robotic-assisted" and "open" in combination with "gastrectomy" or "gastric resection" or "gastric cancer" or "stomach cancer" or "gastric carcinoma". Either free-text and medical subject heading (MeSH) searches were used for keywords. The search was further broadened by extensive cross-checking of all reference in the retrieved articles fulfilling the inclusion criteria in order to identify eventual additional non indexed literature. Discrepancies in the search were resolved by consensus discussion among the entire author group. All relevant texts, tables and figures were reviewed for data extraction.

Study selection

Two authors (FF and LDF) independently screened the primary data from the studies identified in the electronic search. The initial assessed data included authors, titles and abstract. Then, the following inclusion criteria were set for inclusion the studies in the meta-analysis: (1) trials comparing robotic and OG for gastric adenocarcinoma, independently from the type of gastrectomy (distal gastrectomy, proximal gastrectomy and total gastrectomy) and tumour stage (early or advanced gastric cancer); and (2) Studies reporting at least one of the perioperative outcome measures among the following: Operative blood loss, operative time, numbers of harvested lymph nodes, postoperative complication rate, postoperative mortality, and hospital stay (interval from operation to discharge).

The following exclusion criteria were set: (1) duplicate studies; (2) non-comparative studies; (3) if publications are reviews, conference abstracts, letters, comments or case reports; (4) non-relevant topic papers or when all the reported appropriate outcomes were not included; (5) studies where it was not possible to extract or calculate data of interest from the published results; and (6) if more than one study was reported by the same institute, the most recent work or that containing more complete data was selected.

Primary relevant data from the original included studies were independently extracted and summarized by the same two authors. In addition, in terms of postoperative complications, anastomotic leakage, bleeding, as well as wound infection were also analyzed when reported. Any disagreement was resolved by consensus among the author group.

Quality assessment

The modified Newcastle-Ottawa Scale was used to assess the methodological quality of retrospective non randomized studies[12]. The scale consists of eight multiple-choice questions assessed essentially on three major categories: Patient selection, comparability (of cases and controls in case-control studies, of cohorts in cohort studies), and the assessment of the outcome (in case-control studies) or exposure (in cohort studies)[12]. The number of possible answers per question ranges from 2 to 5. High-quality responses earn a star, totaling up to nine stars (the comparability question earns up to two stars).

The quality of randomized clinical trials was assessed using Jadad's scoring system[13]. The Jadad's scale, widely validated for reporting randomized controlled trials quality, assess a score (ranging 0 to 6) on the base of three major elements: randomization (0-2 points), blinding (0-2 points) and patients withdrawal (0-1 point).

Risk of bias

Assessment for potential publication bias was analysed through drawing of funnel plots which were inspected for asymmetry for all outcome measures and evaluated by the Begg's[14] and Egger's tests[15].

Statistical analysis

The statistical analysis was performed using Review Manager software version 5.3 (RevMan 5.3, Cochrane Collaboration, Oxford, United Kingdom). Weighted mean differences (WMD) and odds ratios (OR) were used as a summary measure of efficacy for continuous and dichotomous variables respectively. A 95%CI was reported.

Statistical heterogeneity among the studies was evaluated using the χ^2 test and according to the Higgins' I^2 statistic[16]. I^2 values of 0-25%, 25%-50% and > 50% were considered as indicative of homogeneity, moderate heterogeneity and high heterogeneity, respectively[17]. To estimate the pooled WMD or OR, the inverse variance method with fixed-effects model was applied when no or moderate heterogeneity was detected among studies (I^2 < 50%) according to Mantel-Haenszel method[18], whereas the random-effect model was used for analysis when I^2 was greater than 50% (DerSimonian and Laird method)[19]. WMD was pooled by using the inverse variance model. The Z test was used to determine the pooled WMD or OR. Sensitivity analyses and funnel plots were assumed to investigate potential publication bias.

Funnel plot asymmetry, which reflects the presence of publication bias in the studies, was assessed using Begg's and Egger's tests. Begg and Mazumdar's rank correlation tests the rank correlation (Kendall's tau) between the standardized effect size and the variances (or standard errors) of these effects[14]; the Egger's linear

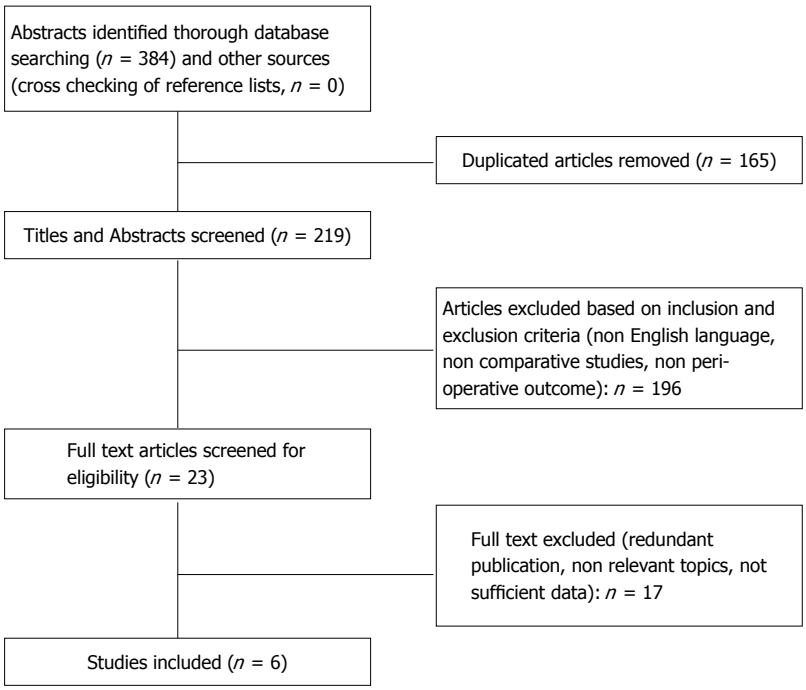


Figure 1 Flow chart of study selection.

regression method[20] quantify the bias captured by the funnel plot. *P* value < 0.05 were considered to indicate statistical significance.

RESULTS

Study selection

The literature search yielded a total of 384 articles (Figure 1). After elimination of duplicates (*n* = 165), the remaining 219 titles and abstracts were reviewed. Based on the methodological inclusion and exclusion criteria, 196 studies were excluded: 115 did not compare techniques, 21 were non English studies, 60 were review articles, letters, case reports or comment. The full text of the remaining 23 articles were reviewed; of these, 1 was excluded because it was a redundant and lower level series, 11 contained non relevant topics, 5 because it was impossible to retrieve or calculate data of interest. Finally, a total of 6 articles[21-26] (South Korea 2, China 2, Italy 1, Romania 1) were considered eligible for inclusion in the meta-analysis (Figure 1).

Only one of these studies was a randomized controlled trial[26], while the others were retrospective non-randomized trials. The same two authors extracted the number and characteristics of patients of both the RAG and OG groups, which globally included 6.123 patients.

Huang *et al*[23] did not provide in their original papers data regarding means and standard deviations of peri-operative outcome, which instead were expressed as medians and ranges. This additional initially unpublished information was retrieved from a previous meta-analysis[27], in which the data was obtained by contacting the authors.

The baseline characteristic, quality assessment and main perioperative data of the included studies were listed in Tables 1 and 2.

Operative time

All included studies[21-26] reported a significantly longer operation time of the RAG group than OG (Table 2). The meta-analysis of pooled data (Figure 2A) confirmed the result showing a significantly lower operative time in the group of OG compared to RAG group (WMD: 72.20 min, 95%CI: 48.82 to 105.13 min, *P* < 0.001). There was significant heterogeneity (I^2 = 85%) (Figure 2A).

Estimated blood loss

All the included studies reported the mean intra-operative related to surgery estimated blood loss. A concordant result of statistical significantly lower blood loss volume in the RAG group than in the OG group (Table 2) was reported. The pool meta-analyzed data confirmed that blood loss was notably less in the RAG group as opposed to OG (WMD: -166.83 mL, 95%CI: -205.18 to -65.80 mL, *P* < 0.001) with a significant heterogeneity between studies (I^2 = 82%) (Figure 2B).

Harvested lymph nodes

The mean number of harvested lymph nodes was reported in all studies (Table 2). The pooled data from the included studies showed that the two groups did not differ significantly in the number of harvested lymph nodes (WMD = -1.12; 95%CI: -2.31 to 0.58; *P* = 0.10), with low heterogeneity between studies (I^2 = 25%) (Figure 2C).

Postoperative hospital stay: All the 6 included studies reported the length of hospital stay, showing in agreement a statistically significant reduction in favour of the RAG group compared to OG (Table 2). The meta-analysis of combined data confirmed the result, showing shorter postoperative hospital stay in the RAG group

Table 1 Baseline characteristics of include studies and quality assessment

Ref.	Year	Country	Type of study	Total patients (*n*)	Group	*n*	Sex (M/F)	*P* value	Age (mean ± SD)	*P* value	BMI (mean ± SD)	*P* value	Quality assessment
Kim *et al*[21]	2010	South Korea	Retrospective clinical trial	28	RAG	16	10/6	NS	53.8 ± 15.6	NS	21.3 ± 3.4	> 0.05	6 stars[1]
					OG	12	9/3		56.0 ± 12.4		25.2 ± 1.9		
Caruso *et al*[22]	2011	Italy	Retrospective clinical trial	149	RAG	29	18/11	NS	64.8 ± 12.4	NS	27 ± 3	NS	6 stars[1]
					OG	120	65/55		65.1 ± 11		28 ± 4		
Huang *et al*[23]	2012	China	Retrospective clinical trial	625	RAG	39	19/20	< 0.05	65.1 ± 15.9	NS	24.2 ± 3.7	NS	5 stars[1]
					OG	586	406/180		67.9 ± 30.1		23.7 ± 3.6		
Kim *et al*[24]	2012	South Korea	Retrospective clinical trial	4978	RAG	436	265/171	NS	54.2 ± 12.5	< 0.05	23.6 ± 3.1	NS	5 stars[1]
					OG	4542	3008/1534		57.7 ± 11.8		23.8 ± 8.0		
Procopiuc *et al*[25]	2015	Romania	Retrospective clinical trial	47	RAG	18	13/5	NS	59.1 ± 13.7	NS	26.0 ± 3.24	NS	6 Stars[1]
					OG	29	21/8		60.1 ± 12.4		24.8 ± 4.58		
Wang *et al*[26]	2016	China	Randomized clinical trial	296	RAG	151	109/42	NS	57.5 ± 12.7	NS	22.1 ± 2.9	NS	3 points[2]
					OG	145	89/56		55.9 ± 13.1		21.3 ± 2.5		

[1]According to the NOS (Newcastle-Ottawa Scale) classification; [2]According to Jadad's scale for reporting randomized controlled trials. RAG: Robot-assisted gastrectomy; OG: Open gastrectomy; NS: Not statistically significant.

Table 2 Main perioperative data of the included studies

Ref.	Open conversion (%)	Group	Operation time (min ± SD)[1]	*P* value	Blood loss (mL ± SD)[1]	*P* value	Harvested nodes (*n* ± SD)[1]	*P* value	Morbidity (%)	*P* value	Mortality (%)	*P* value	Hospital stay (d ± SD)[1]	*P* value
Kim *et al*[21]	0	RAG	259.2 ± 38.9	< 0.05	30.3 ± 15.1	< 0.05	41.1 ± 10.9	NS	0	NS	0	NS	5.1 ± 0.3	< 0.05
		OG	126.7 ± 24.1		78.8 ± 74.1		43.3 ± 10.4		20		0		6.7 ± 1.4	
Caruso *et al*[22]	0	RAG	290 ± 67	< 0.05	197.6 ± 202.1	< 0.05	28.0 ± 11.2	NS	10.3[2]	NS	0	NS	9.6 ± 2.8	< 0.05
		OG	222 ± 94		386.1 ± 95.5		31.7 ± 15.6		10.0[2]		3.3		13.4 ± 8.5	
Huang *et al*[23]	NR	RAG	415.9 ± 101.2	< 0.05	93.9 ± 89	< 0.05	32 ± 13.7	NS	15.4	NS	1.4	NS	11.3 ± 14.4	< 0.05
		OG	331.8 ± 92.9		192 ± 193		34 ± 14.8		14.7		2.6		16.5 ± 13.6	
Kim *et al*[24]	NR	RAG	226 ± 54	< 0.05	85 ± 160	< 0.05	40.2 ± 15.5	NS	10.1	NS	0.5	NS	7.5	< 0.05
		OG	158 ± 52		192 ± 193		40.5 ± 16.6		10.7		0.5		10.2	
Procopiuc *et al*[25]	0	RAG	320.8 ± 85.1	< 0.05	208.2 ± 139.8	< 0.05	22.0 ± 8.9	NS	11.1[2]	NS	0	NS	8.1 ± 2.0	< 0.05
		OG	243.3 ± 57.9		564.6 ± 468.4		25.2 ± 9.0		20.7[2]		0		11.4 ± 2.9	
Wang *et al*[26]	1.9[3]	RAG	242.7 ± 43.8	< 0.05	94.2 ± 51.5	< 0.05	29.1 ± 6.7	NS	9.3	NS	0	NS	5.7 ± 2.3	< 0.05
		OG	192.4 ± 31.5		152.8 ± 94.2		30.1 ± 7.2		10.3		0		6.4 ± 2.5	

[1]Mean value; [2]Major complications rate base on Clavien-Dindo classification ⩾ 3, such as anastomotic and duodenal lekeage; [3]Rate of patients excluded from the study analysis. RAG: Robot-assisted gastrectomy; OG: Open gastrectomy; NS: Not statistically significant difference.

compared to OG (Figure 2D). The robotic approach reduced the postoperative stay by a mean of 1.97 d (WMD = -1.97; 95%CI: -2.47 to -1.18 d; $P < 0.001$). Although there was a significant heterogeneity among the studies (I^2 = 55%) (Figure 2D).

Postoperative complications: Short-term postoperative complications were recorded in all analyzed studies. The meta-analysis did not significantly differ in the overall postoperative complication rate of the two groups (OR = 0.95, 95%CI: 0.60-1.34, P = 0.65) with low heterogeneity (I^2=12%) (Figure 3A).

Five out of 6 studies[22-26] reported the incidence by group of the following subtype of early postoperative complications: wound infection, bleeding and anastomotic leakage. The meta-analysis of pooled data regarding these complications showed no difference between the two groups (respectively: Wound infection, OR = 1.48, 95%CI: 0.86-3.12, P = 0.35, I^2 = 10%; bleeding, OR = 1.10, 95%CI: 0.40-4.49, P = 0.65, I^2 = 0%; anastomotic leakage OR = 1.74, 95%CI: 0.99-3.05, P = 0.06, I^2 = 0%) (Figure 3B-D).

Three studies out of 6[22-24] reported postoperative mortality rate value ranging from 0.5% to 3.3%, without statistically significant differences between the robotic and open procedures, while the rest of the studies[21,25,26] did not detect any case of mortality related to both surgical techniques (Table 2). A meta-analysis of pooled data was therefore considered unnecessary, as 50% of studies did not report any event of mortality in both groups and the data are insufficient to calculate an objective OR, thus the combined data reflected the evident equality of mortality rates among RAG and OG groups.

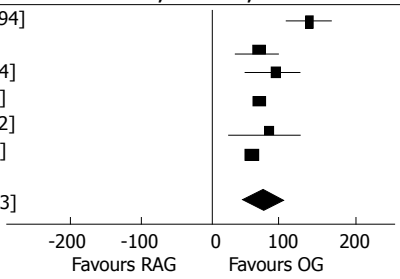
Publication bias

A standard-error based funnel plot using fix effect size between RAG and OG was constructed for morbidity (Figure 4). The overall postoperative complication rate of all the studies lay within the limits of 95%CIs with just a slight asymmetry, indicating no serious publication

A Operation time

Study or subgroup	RAG Mean	RAG SD	RAG Total	OG Mean	OG SD	OG Total	Weight	Mean difference IV, Random, 95%CI
Kim MC 2010	259.2	38.9	16	126.7	24.1	12	16.3%	132.50 [109.06, 155.94]
Caruso S 2011	290.0	67.0	29	222.0	94.0	120	15.5%	68.00 [38.8, 97.62]
Huang KH 2012	415.9	101.2	39	331.8	92.9	586	14.5%	84.10 [51.46, 116.74]
Kim KM 2012	226.0	54.0	436	158.0	52.0	4542	19.6%	68.00 [62.71, 73.29]
Procopiuc L 2015	320.8	85.1	18	243.3	57.9	29	11.5%	77.47 [32.81, 122.12]
Wang G 2016	242.7	43.8	151	192.4	31.5	145	21.6%	50.30 [41.64, 58.96]
Total (95%CI)			689			5434	100.0%	72.20 [48.82, 105.13]

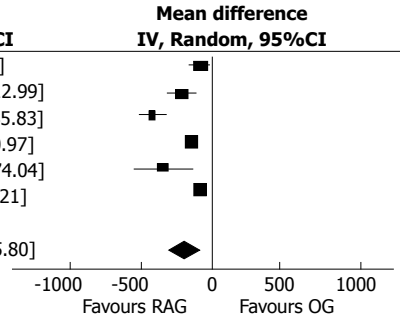
Heterogeneity: Tau^2 = 1340.42; χ^2 = 58.09, 3 d.f., P < 0.001; I^2 = 85%
Test for overall effect: Z = 5.63, P < 0.001



B Blood loss

Study or subgroup	RAG Mean	RAG SD	RAG Total	OG Mean	OG SD	OG Total	Weight	Mean difference IV, Random, 95%CI
Kim MC 2010	30.3	15.1	16	78.8	74.1	12	18.3%	-48.50 [-91.7, -5.93]
Caruso S 2011	197.6	202.1	29	386.1	95.5	120	15.4%	-188.50 [-264.04, -112.99]
Huang KH 2012	93.9	89.0	39	534.2	577.6	586	14.1%	-440.30 [-494.77, -385.83]
Kim KM 2012	85.0	160.0	436	192.0	193.0	4542	19.6%	-107.00 [-123.03, -90.97]
Procopiuc L 2015	208.2	139.8	18	564.6	468.4	29	12.1%	-356.40 [-538.69, -174.04]
Wang G 2016	94.2	51.5	151	152.8	94.2	145	21.5%	-58.60 [-75.99, -41.21]
Total (95%CI)			689			5434	100.0%	-166.83 [-205.18, -65.80]

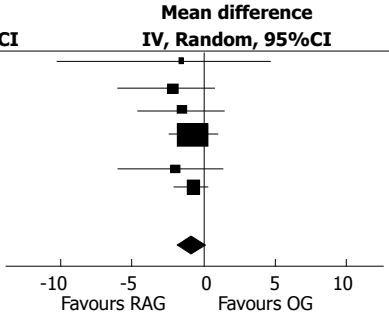
Heterogeneity: Tau^2 = 3468.72; χ^2 = 27.35, 3 d.f., P < 0.001; I^2 = 82%
Test for overall effect: Z = 4.53, P < 0.001



C Harvested lymph nodes

Study or subgroup	RAG Mean	RAG SD	RAG Total	OG Mean	OG SD	OG Total	Weight	Mean difference IV, Random, 95%CI
Kim MC 2010	41.1	10.9	16	43.3	10.4	12	1.1%	-2.20 [-10.15, 5.75]
Caruso S 2011	28.0	11.1	29	31.7	15.6	120	5.8%	-3.70 [-8.64, 1.24]
Huang KH 2012	32.0	13.7	39	34.0	14.8	586	6.2%	-2.00 [-6.46, 2.46]
Kim KM 2012	40.2	15.5	436	40.5	16.6	4542	75.3%	-0.30 [-1.83, 1.23]
Procopiuc L 2015	22.0	8.9	18	25.2	9.0	29	2.1%	-3.20 [-8.46, 2.10]
Wang G 2016	29.1	6.7	151	30.1	7.2	145	9.5%	-1.00 [-2.28, 0.28]
Total (95%CI)			689			5434	100.0%	-1.12 [-2.31, 0.58]

Heterogeneity: χ^2 = 2.35, 3 d.f., P = 0.20; I^2 = 25%
Test for overall effect: Z = 1.42, P = 0.10



D Postoperative hospital stay

Study or subgroup	RAG Mean	RAG SD	RAG Total	OG Mean	OG SD	OG Total	Weight	Mean difference IV, Random, 95%CI
Kim MC 2010	5.1	0.3	16	6.7	1.4	12	27.5%	-1.60 [-2.41, -0.79]
Caruso S 2011	9.6	2.8	29	13.4	8.5	120	12.6%	-3.80 [-5.63, -1.97]
Huang KH 2012	11.3	14.4	39	16.5	13.6	586	5.6%	-5.20 [-9.85, -0.55]
Kim KM 2012	7.5	13.7	436	10.2	8.5	4542	22.3%	-2.70 [-4.01, -1.39]
Procopiuc L 2015	8.1	2.0	18	11.4	2.9	29	11.4%	-3.30 [-4.36, -1.52]
Wang G 2016	5.7	2.3	151	6.4	2.5	145	20.6%	-0.80 [-1.79, 0.19]
Total (95%CI)			689			5434	100.0%	-1.97 [-2.47, -1.18]

Heterogeneity: Tau^2 = 1.40; χ^2 = 8.18, 3 d.f., P = 0.03; I^2 = 55%
Test for overall effect: Z = 5.42, P < 0.001

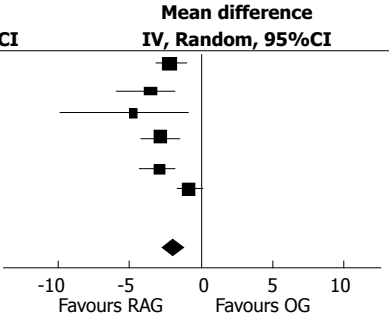


Figure 2 Forest plot showing the meta-analysis of pooled data on robot-assisted gastrectomy *vs* open gastrectomy. A: Operation time; B: Estimated blood loss; C: Harvested lymph nodes; D: Postoperative hospital stay. RAG: Robot-assisted gastrectomy; OG: Open gastrectomy.

biases. No evidence of publication bias was revealed among the studies from statistical tests for any primary outcomes (Begg's test all P > 0.10; Egger's test all P > 0.10).

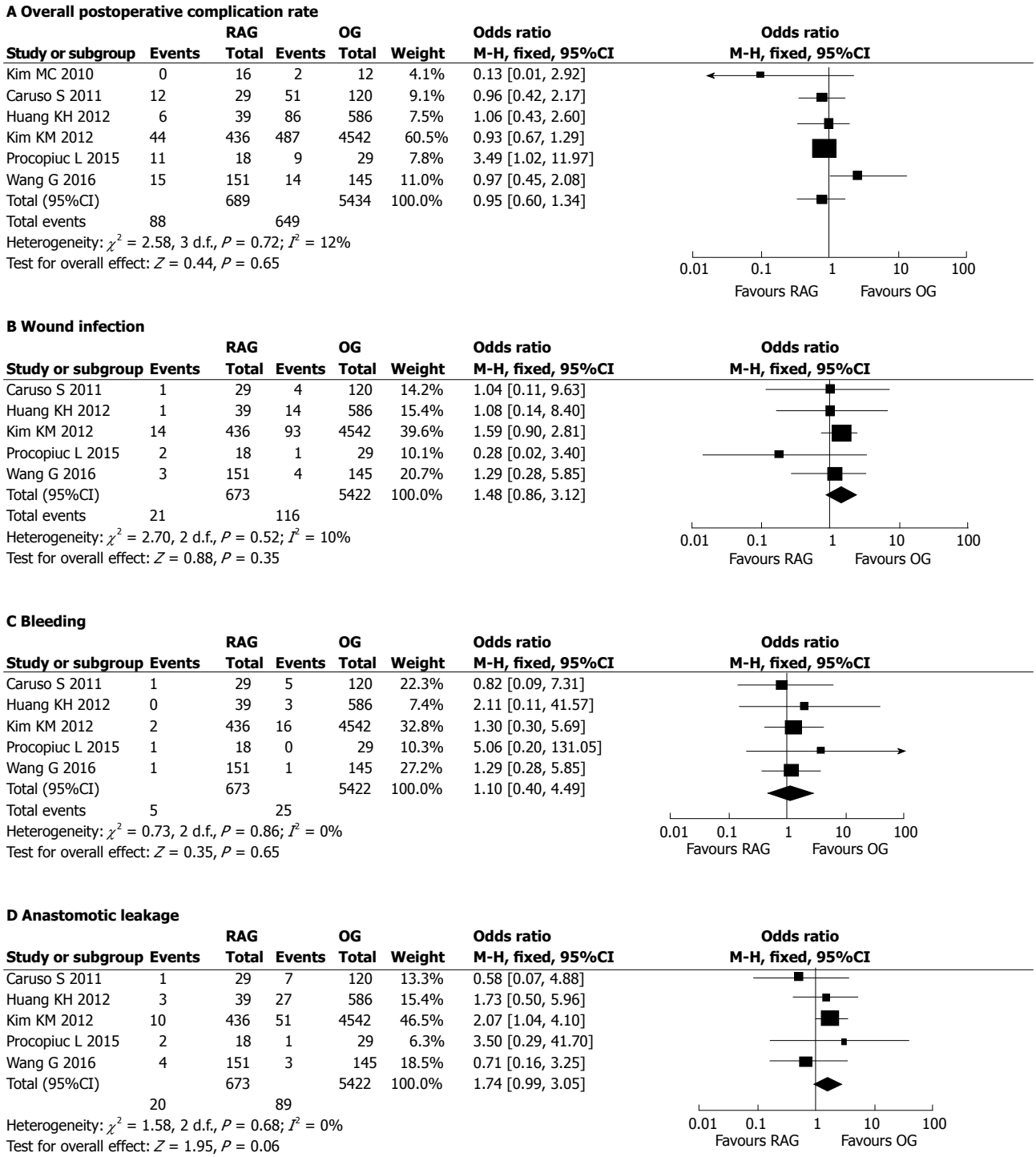


Figure 3 Forest plot showing the meta-analysis of postoperative complication between robot-assisted gastrectomy and open gastrectomy. A: Overall postoperative complications; B: Wound infection; C: Bleeding; D: Anastomotic leakage. RAG: Robot-assisted gastrectomy; OG: Open gastrectomy.

Sensitivity analysis: Sensitivity analysis was performed by excluding the study with the lowest quality score and the smallest sample size[21]. All variables were conducted for sensitivity analysis. The results were not affected by sensitivity analysis as shown in Table 3.

DISCUSSION

Procedures which offer minimum invasiveness would present a perfectly acceptable alternative to open surgery, with better short-term results, if it were possible to respect oncologic criteria to the same degree as the open approach, and if there were no compromising effect on long-term survival. Even though long-term survival is one of the major oncologically prominent issue, lymph node metastasis has long been seen as the element which most significantly predicts recurrence and therefore survival in patients suffering from gastric

Table 3 Sensitivity analysis of outcomes

Outcomes	No. of studies	Patients		WMD/OR	Analysis model	95%CI	*P* value	Heterogeneity	
		RAG	OG					I^2 (%)	*P* value
Operative time (min)	5[22-26]	673	5422	60.12	Random	41.31, 98.06	< 0.00001	80	0.41
Estimated blood loss (mL)	5[22-26]	673	5422	-193.78	Random	-215.77, -72.13	< 0.0001	72	0.007
Harvested lymph nodes	5[22-26]	673	5422	-1.05	Random	-2.01, 0.39	0.35	0	0.12
Overall postoperative complication	5[22-26]	673	5422	0.92	Fixed	0.61, 1.36	0.6	12	0.72
Postoperative hospital stay	5[22-26]	673	5422	-2.57	135.8 ± 133.9	-2.68, -1.56	< 0.001	0	0.54

RAG: Robot-assisted gastrectomy; OG: Open gastrectomy; WMD: Weighted mean difference; OR: Odds ratio.

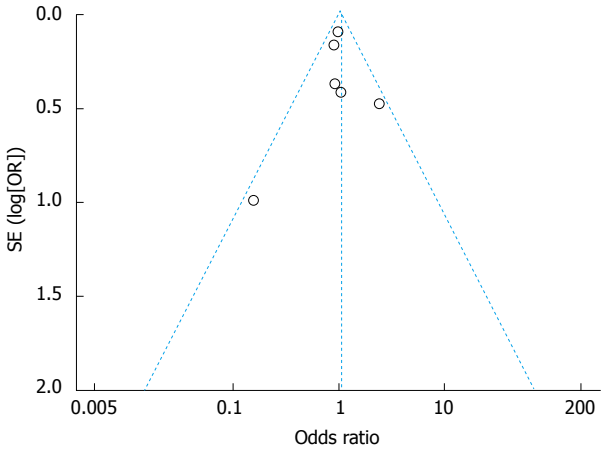


Figure 4 Funnel plot for results from each study comparing overall morbidity between robot-assisted gastrectomy and open gastrectomy. OR: Odds ratio; SE: Standard error.

cancer[28]. Thus, the amount of harvested lymph nodes is an accurate reflection of whether gastric resection for adenocarcinoma is an adequate option, and can be used as indicator of oncological adequacy when no long follow-up times are available.

Total and distal gastrectomy with D2 lymphadenectomy node is the recommended surgical procedure for most resectable gastric cancer patients[29]. LG with lymph node dissection has developed as a minimally invasive surgery for gastric cancer over the last two decades and it has been utilized principally for early gastric cancer. Some randomized studies and meta-analysis showed that LG with limited lymph node dissection for patients with early-stage gastric lesion provided oncologic results which were not inferior compared to OG, with however improved short-term outcomes[2-5].

In contrast, a handful of trials, which all contained not large cohort of patients, outline the safety of laparoscopic assisted distal and total gastrectomy with D2 lymph node dissection in advanced-stage of gastric cancer. Several meta-analysis regarding this issue have been recently published. However, the outcomes were contradictory, especially regarding postoperative complications and the amount of harvested lymph nodes[30-32].

Thus, although LADG has been widely developed for early gastric cancer, the global effectiveness in therapeutic terms of LG still has not been extensively looked into with regards to the treatment of advanced-stage of gastric cancer. Although a totally LG with extended D2 lymphadenectomy has been demonstrated to be feasible by several authors[33-36], owing to the intrinsic difficulty of execution, oncologic concerns still exist regarding the possibility of performing a D2 lymphadenectomy radically and suitably. Indeed, the meta-analysis of the randomized controlled trials (RCTs) demonstrates that whenever results on LADG is gathered from advanced gastric cancers together with the early stage the same extent of lymph node dissection as in traditional surgery could not be guaranteed[37,38].

Although laparoscopic sub-D2 lymphadenectomy may be seen as suitable for nearly all early gastric cancer in which lymph node metastases rarely occur (2%-20% of cases)[37], and so far is routine in Asia[39], the same cannot be said about advanced gastric cancer and so LG cannot be advised as a standard approach for all patients with gastric cancer.

With the development of technology, the introduction of a robotic tool as a useful adjunctive method to assist laparoscopy has gradually increased the use of minimally invasive procedures in several fields of surgery. For the treatment of gastric cancer, RAG has been widely demonstrated to be feasible and safe in many studies[8,40-49]. Robotic surgery is progressively becoming an attractive option for surgeons, in particular because it may overcome some intrinsic limitations of conventional laparoscopy, in particular for the D2 lymphadenectomy, expanding the application of minimally invasive procedures. In fact, this technique has certain indisputable advantages, such as high definition 3D imaging, improved dexterity enabled by the endowristed movements, tremors filtration, motion scaling, stereoscopic visualization, which are particular useful when precise dissection is needed, such as during the lymphadenectomy along major abdominal vessels (gastric, gastroepiploic, common hepatic, and celiac artery lymph nodes). Thus, as long as drawbacks of the LG technique exist, the introduction of new innovative technologies, such as robotic gastrectomy, are desirable. In fact, the median number of retrieved nodes, reported by many authors through the use of robotic system for D2 lymphadenectomy, is not dissimilar to that of traditional open technique, and in several instances even superior to laparoscopy[27,40,41,50-57].

However, significant limitations exist in the inter-

pretation of data available so far regarding the comparison of RAG with respect to OG, as a result of the shortage of randomized trials, the restricted amount of observational and comparative studies of high quality, the small sample sizes so far, and the shortened length of follow-up. Therefore, there has been difficulty in drawing final conclusions regarding the superiority of one approach over another.

A meta-analysis is a suitable way to widen the source of evidence. Evaluating pooled data among the most relevant studies is a quantitative method that may increase the statistical power of otherwise poorly consistent results and may resolve some controversy of evidence.

Robotic surgery is a technical innovation which improves the effectiveness of laparoscopic technique, which is used through the same laparoscopic way as a non independent adjunctive tool. Thus, we strictly limit the research by focusing exclusively on RAG with the intent to evaluate the real merit of the addition of robotic assistance to laparoscopy over the traditional OG for gastric cancer, performing a comprehensive systematic review and meta-analysis. Such a way of conducting the trial will provide a more objective appraisal of the effectiveness of RAG in gastric cancer patients, in order to confirm the single-institute promising results in favour of this innovative technique to date reported. This could represent the preliminary cue in support of the increasingly widespread view which considers robotics to be a completion of laparoscopy, making it possible to fill the existing performance gap with respect to OG.

Six studies, of which 5 retrospective clinical trials and 1 RCT, involving 6123 patients with 689 (11.3%) cases of RAG and 5434 (88.7%) of OG, were considered eligible for inclusion in this meta-analysis.

The results show globally that RAG provided short-term results which can be compared to OG, with outcomes which can be considered as satisfactory with regards to perioperative results and oncological effectiveness.

The operation time was significantly longer with RAG than OG ($P < 0.001$). The greater length of robotic surgery is principally due to the additional time for set-up and docking of the robotic system[58]. Nevertheless, it should be noted that the time of operation notably diminished as surgical experience increased and the robotic procedure was standardized[8,47,59].

An advantageous lower blood loss and shorter hospital stay were revealed in favor of RAG, that can be principally due to globally lesser surgical damage than OG. The robotic system enables a meticulous and precise dissection in a magnified vision, which minimizes the risk of bleeding. Moreover, the technical advancement of the robotic device, which is provided by a high definition 3D stereoscopic vision, enabling a better detection of vascular structures and allowing to easier inspect the bleeding occurring intra abdominally with tremor filtration and stable haemostatic strain provided with the robotic instrument.

No statistical difference was observed between RAG and OG in terms of postoperative complication rate ($P = 0.65$), and also specifically referring to subcategories of complications, such as wound infection, bleeding, anastomotic leakage. In particular regarding the most feared adverse event after gastric cancer, the rate of anastomotic leakage is comparable to that reported by previous studies[60,61], ranging from 1% to 10%, and the rate among pooled data was 2.97% (20/673) for RAG and 1.64% (89/5422) for OG ($P = 0.06$).

Analysis of the pooled data revealed that the number of harvested lymph nodes was similar between RAG and OG. The feeling is that the technically advantageous properties of robotic surgery can easily and safely execute an effective, and oncologically adequate lymphadenectomy[62,63]. In particular, the meticulous dissection, together with the high 3D definition image and dexterity provided by the robotic system, seems to make the lymph node dissection safely feasible in difficult lymphatic stations around major vessels or in difficult area[6], with less blood loss[6,38].

The main limitation of this meta-analysis is that it does not resolve certain heterogeneity of the included studies, such as in terms of baseline characteristics of patients, type of gastrectomy, stage of disease, details of surgery, difference in reporting perioperative outcomes. For example, in the study of Kim *et al*[21] the body mass index (BMI) of the RAG group was significantly lower than that of the open ($P = 0.0004$). Huang *et al*[23] included patients in the robotic group which were associated with female predominance and were reconstructed mainly by Roux-en-Y anastomosis. In the study of Kim *et al*[24], the patients of RAG group were significantly younger than OG. Kim *et al*[24] and Huang *et al*[23] reported in their series a significantly higher proportion ($P < 0.001$) of advanced gastric cancers in the OG gastrectomy group with respect to the RAG group, that would suggest a corresponding higher number of lymph nodes retrieved in advanced stages than in early stages. Effectively, that reflects a trend of a higher amount of lymph nodes dissected with the open procedure than with the robotic technique, both in the single institute reports and in the pooling data meta-analysis, however this difference did not reach a statistical significance. Globally, this result suggests that RAG, even if applied in a greater proportion of early gastric cancer than OG, guarantees an adequate removal of lymph nodes, similar to that of OG in a larger amount of advanced gastric cancer. Since it was difficult to match baseline characters in all selected studies, the meta-analytic method planned the use of a random effected model to evaluate these parameters. However, high heterogeneity still existed in terms of operation time, blood loss and postoperative hospital stay, which the meta-analysis cannot completely resolve.

However, the meta-analytic method can represent a valid preliminary analysis of the global framework of these data, eventually susceptible to a sub-set analysis of more homogeneous groups. Two previous meta-analysis[27,57], comparing RAG with conventional laparoscopy and OG, conducted a subgroup analysis

matched for some of these parameters, such as the extent of lymphadenectomy, type of gastrectomy (total or subtotal), and blood loss. However, the final results were substantially equal to the pooled data here presented in our meta-analysis. Moreover, although sensitivity analysis using matched data should reduce some of these potential bias, it cannot eliminate all of them and essentially it was impossible to match patient characteristics in all studies. For example, robotic procedures included the initial learning period, which may have resulted in an unequal surgical quality comparison. Moreover, most of the studies had small sample sizes with fewer than 50 RAG procedures and one single high-volume centre (Kim *et al*[24]) contributed more than half of the total number of RAG; this uneven distribution in the number of patients contributed to heterogeneity.

An advantage of our meta-analysis with respect to previous ones is that it included, even if only one, RCT and presently it is the most up to date work with the largest sample size comparing RAG and OG.

In conclusion, RAG seems to offer a viable option to OG in treating gastric cancer patients. It allows the reduction of the estimated blood loss and the length of postoperative stay with respect to OG with, at the same time, a comparable oncologically adequate lymphadenectomy. The longer operative time did not seem to affect the patient's recovery, with equal postoperative complications rate, risk of bleeding, wound infection and anastomotic leakage compared to open procedure.

Moreover, by overcoming some of the intrinsic limits of conventional laparoscopy, robotic gastrectomy probably represents the most promising technological innovation able to fill the gap still existing between laparoscopy and traditional open approach, particularly in the performance of D2 lymphadenectomy.

That could make LG when assisted with the robotic tool more oncologically adequate and then more widespread, so as to maintain and expand the well-known advantages of a minimally invasive surgery with respect to the open procedure.

Future research should be directed towards comparing RAG to OG, to delineating significantly quantifiable advantages between the two techniques, also in terms of cost analysis, especially in well-designed prospective randomized controlled trials. Finally, as a result of a lacking adequate follow-up and a small amount of high quality studies, it is too soon to formulate certain conclusive opinions.

COMMENTS

Background

Robot-assisted gastrectomy (RAG) is an innovative technique which improves the effectiveness of traditional laparoscopy, making it possible to overcome some of its typical limits. Several reports have demonstrated that this new procedure is technically feasible and safe, but no consensus is available in literature yet about the potential benefit of this technique with respect to the traditional open procedure.

Research frontiers

Minimally invasive surgery has progressively improved and spread, because it offers a number of patient benefits compared to open surgery. Future research will be directed towards innovative techniques which could further minimize the surgical invasiveness for patients, so as to improve postoperative outcomes. From this point of view, RAG appears to be a promising advancement of minimally invasive surgery, and will probably continue to be increasingly used in the treatment of gastric cancer.

Innovations and breakthroughs

Here the authors presented the meta-analysis of pooled data originating from the systematic review of relevant studies which compared short-term outcomes between RAG and open gastrectomy. Presently, this is the most up to date and largest clinical work comparing the effectiveness of these two techniques, and the only one that included a randomized controlled trial.

Applications

The present work elucidates the current scientific evidence concerning the hypothesized beneficial application of RAG in gastric cancer patients.

Peer-review

This paper is a meta-analysis of 6 reports comparing the outcomes of robot-assisted laparoscopic gastrectomy for early gastric cancer with open gastrectomy, with favourable results for the former group. The information is important and needs to be made known. It is well written.

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CASE REPORT

Bilateral diffuse grade 5 radiation pneumonitis after intensity modulated radiation therapy for localized lung cancer

Virginia W Osborn, Andrea Leaf, Anna Lee, Elizabeth Garay, Joseph Safdieh, David Schwartz, David Schreiber

Virginia W Osborn, Andrea Leaf, Anna Lee, Elizabeth Garay, Joseph Safdieh, David Schwartz, David Schreiber, Veterans Affairs New York Harbor Healthcare System, Brooklyn, NY 11209, United States

Virginia W Osborn, Anna Lee, Elizabeth Garay, Joseph Safdieh, David Schwartz, David Schreiber, SUNY Downstate Medical Center, Brooklyn, NY 11203, United States

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Correspondence to: David Schreiber, MD, Attending Physician, Veterans Affairs New York Harbor Healthcare System, 800 Poly Place, 114A, Brooklyn, NY 11209, United States. david.schreiber@va.gov
Telephone: +1-718-6303605
Fax: +1-718-6302857

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Abstract

We are reporting a case of fatal radiation pneumonitis that developed six months following chemoradiation for limited stage small cell lung cancer. The patient was a 67-year-old man with a past medical history of Hashimoto's thyroiditis and remote suspicion for CREST, neither of which were active in the years leading up to treatment. He received 6600 cGy delivered in 200 cGy daily fractions *via* intensity modulated radiation therapy with concurrent cisplatin/etoposide followed by additional chemotherapy with dose-reduced cisplatin/etoposide and carboplatin/etoposide and then received prophylactic cranial irradiation. The subsequent months were notable for progressively worsening episodes of respiratory compromise despite administration of prolonged steroids and he ultimately expired. Imaging demonstrated bilateral interstitial and airspace opacities. Autopsy findings were consistent with pneumonitis secondary to chemoradiation as well as lymphangitic spread of small cell carcinoma. The process was diffuse bilaterally although his radiation was delivered focally to the right lung and mediastinum.

Key words: Radiation; Pneumonitis; Small cell lung cancer; Intensity modulated radiation therapy

Core tip: Radiation pneumonitis is an uncommon but serious complication from radiation therapy which can on rare occasions be fatal. This report not only documents the details of such a case but also includes pathologic confirmation and computed tomography images. Although the radiation field was limited to the right lung and mediastinum, the process was also noted to be bilateral and diffuse.

Osborn VW, Leaf A, Lee A, Garay E, Safdieh J, Schwartz D,

Schreiber D. Bilateral diffuse grade 5 radiation pneumonitis after intensity modulated radiation therapy for localized lung cancer. *World J Clin Oncol* 2017; 8(3): 285-288 Available from: URL: http://www.wjgnet.com/2218-4333/full/v8/i3/285.htm DOI: http://dx.doi.org/10.5306/wjco.v8.i3.285

INTRODUCTION

Pneumonitis is an inflammatory lung reaction marked by dyspnea, cough, and occasional fever. It can occur following radiation therapy as a result of cytokine production[1,2], and patients are at increased risk of developing pneumonitis if they have a history of chronic lung disease or smoking[3], or if they received concurrent chemotherapy[3,4]. Rarely, it can be fatal. In the following case report we examine a patient who developed fatal pneumonitis six months after receiving concurrent chemoradiation for small cell lung cancer (SCLC).

CASE REPORT

A 67-year-old man with a 40 pack-year smoking history initially presented with chills and a productive cough and was given antibiotics for presumed pneumonia. When his condition did not improve, a computed tomography (CT) of the chest was performed and revealed a large right hilar mass with extensive mediastinal adenopathy as well as surrounding infiltrate and atelectasis. Bronchial brushings and a right hilar node FNA were consistent with SCLC. The remainder of the workup, including brain magnetic resonance imaging (MRI), bone scan and positron emission tomography (PET)-CT, was negative for distant metastatic disease, establishing a diagnosis of limited stage (LS) disease. His medical history was significant for numerous coexisting medical conditions including a remote history of suspected but unconfirmed connective tissue disorder (CREST), colitis, esophagitis, duodenitis, livedo reticularis, Hashimoto's thyroiditis, multinodular goiter, arthritis, glaucoma, hypertension, multifocal motor neuropathy and atrioventricular (AV) nodal reentry tract for which he had undergone AV nodal ablation. Of note, neither the Hashimoto's nor CREST were active for multiple years leading up to his diagnosis of SCLC. The latter diagnosis had been suspected by the Rheumatology Service but after a negative workup, he was discharged from their clinic.

After completion of staging, he was advised to undergo definitive chemoradiation. He was also advised to re-establish follow up with the Rheumatology Service, but declined. After a detailed discussion of the potential for increased risk of complications from radiation with an underlying connective tissue disorder, he elected to proceed. He was treated with intensity modulated radiation therapy to the right lung and mediastinum in 33 daily fractions of 200 cGy to a total dose of 6600 cGy with two cycles of concurrent cisplatin (cis) and etoposide. After 3000 cGy, another CT was performed to allow for decrease in treatment field after initial response. RT was completed in 8 wk and 1 d. A representative image from his intensity modulated radiation therapy (IMRT) radiation plan is presented in (Figure 1). His treatment course was complicated by pancytopenia (for which he received filgrastim and one unit of packed red blood cells), as well as dysphagia and odynophagia. He received two cycles of chemotherapy during the radiation and two cycles in the adjuvant setting after concurrent chemotherapy and radiation therapy, though the last three cycles were dose-reduced because of hematologic toxicities. During chemotherapy he was treated for clostridium difficile colitis and was briefly admitted for generalized weakness. Approximately three months after completion of thoracic RT, he received prophylactic cranial irradiation (PCI) which was given as 10 fractions of 250 cGy.

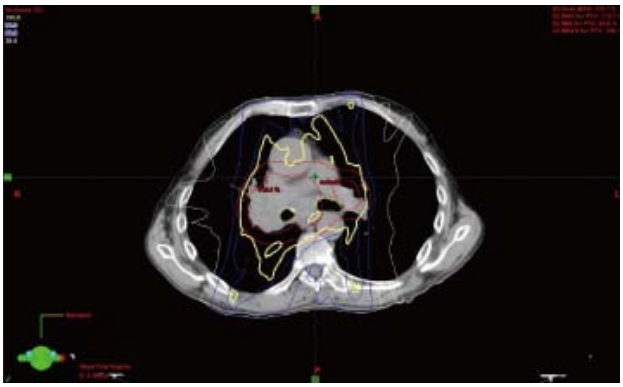

Figure 1 Intensity modulated radiation therapy radiation plan. The yellow line represents the 100% isodose line, blue lines represent the 90% and 50% isodose lines, and the white line represents the 20% isodose line. The red lines represent the gross tumor and planning treatment volumes (GTV and PTV).

During PCI, he required admission due to inability to tolerate daily travel. Shortly after completion of PCI he developed recurrent clostridium difficile colitis and within weeks of completion of PCI he was readmitted and remained hospitalized for two months. While admitted, he experienced episodes of hypoxemic respiratory failure requiring repeated use of a nonrebreather and for which he underwent intubation twice. Chest imaging demonstrated development of worsening bilateral interstitial and airspace opacities (Figure 2). He was aggressively treated with broad spectrum antibiotics and high dose steroids. Eventually he developed tachycardia, respiratory distress, hypotension and suspected disseminated intravascular coagulation. In accordance with his family's wishes he underwent palliate extubation and expired shortly thereafter.

An autopsy was performed and the report described extensive, diffuse, bilateral alveolar damage consistent with post-radiation changes, as well as small cell carcinoma in multiple foci within septal capillaries and contiguous alveolar spaces.

DISCUSSION

Radiation pneumonitis is an uncommon complication of

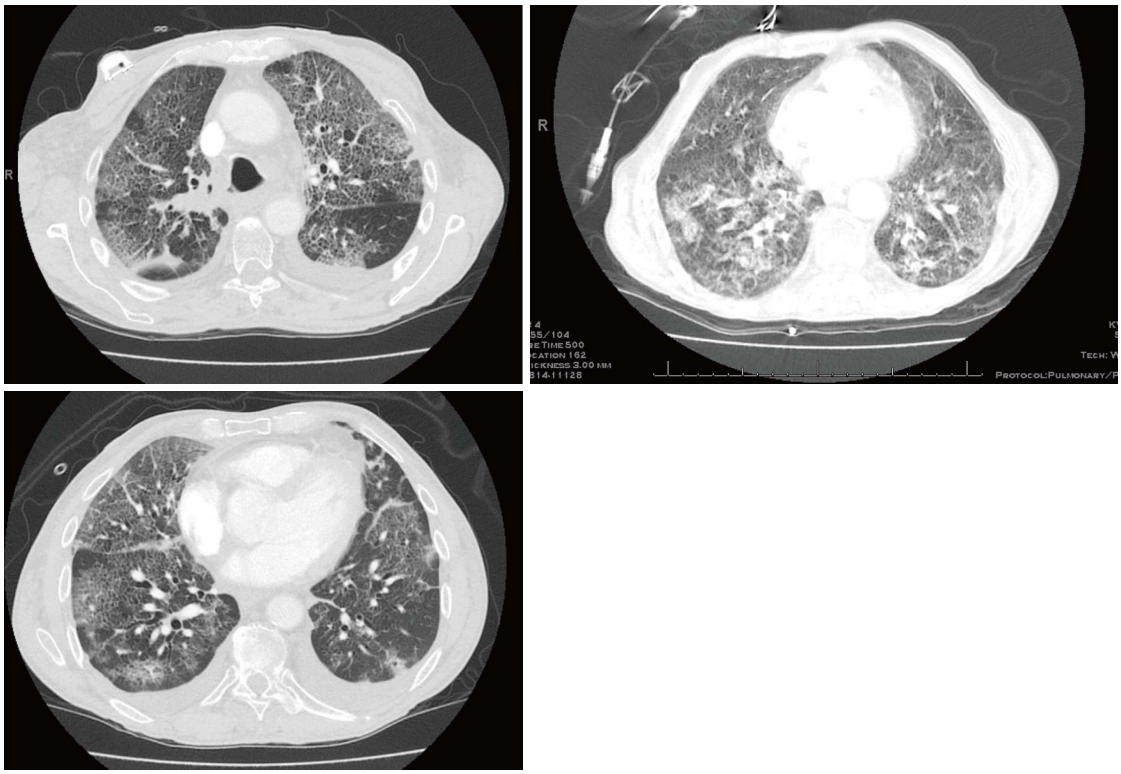

Figure 2 Chest computed tomography scan images demonstrating bilateral interstitial and airspace opacities.

chemoradiation for lung cancer but one which can be fatal in almost 2% of patients[4]. It has been previously been described as having two types of presentations: "Classical" *vs* "sporadic". The former is attributed to local cytokine production within the radiated field, while the latter is likened to a hypersensitivity reaction and can be out of proportion to volume irradiated or manifest its effects outside of the treated field. It has even been proposed that the majority of patients develop subclinical lymphocytic alveolitis following lung radiation, but that acute pneumonitis only develops in the fraction that have some genetic or environmental predisposition[5]. Our literature search did not reveal any specific associations between connective tissue disorders and pneumonitis, however in the event that our patient did have a true diagnosis of a connective tissue disorder, one could postulate that it could have served as such a predisposing factor for him.

Although certain radiation dose parameters have also been found to be associated with increased risk for radiation pneumonitis, including mean lung dose (MLD), volume of lung receiving 20 Gy (V20) and possibly 5 Gy (V5), this patient's parameters were within recommendations. His MLD was 1822 cGy, V20 28%, and V5 69.5%. Qualitative Analysis of Normal Tissue Effects in the Clinic (QUANTEC) guidelines, as well as others, indicate that mean lung dose of 13 Gy results in a 10% rate of symptomatic pneumonitis, MLD of 20 Gy results in 20% risk, and V20 of $\leqslant$ 30%-31% keeps the risk below 20%[6,7]. The current Radiation Therapy Oncology Group protocols recommend V20 not to exceed 40% and MLD of no more than 20 Gy[8]. Not only did our patient's plan meet all of the recommended criteria, it was essentially unilateral, targeted at the right hilar mass and mediastinum. His presentation is therefore more consistent with the development of "sporadic" radiation pneumonitis, given that his ultimate condition was spatially diffuse and out of proportion to what would have been expected from the doses received by his normal tissues.

Further complicating this patient's condition was the presence of lymphangitic spread of tumor which may have contributed to compromise of the patient's lung function. Additionally, he had a history of both a possible CREST and autoimmune disease (Hashimoto's Thyroiditis). Connective tissue disorders have been described as potential predisposing factors for increased toxicity from radiation therapy, and the mechanism of sporadic radiation pneumonitis itself is in some ways analogous to an autoimmune reaction with cytokine-mediated destruction[9]. However in this case the autoimmune diseases had not been active for years and the collagen vascular disease, though suspected, had not been officially diagnosed, so it is difficult to evaluate whether the patient's toxicity could be attributed to these medical issues.

This case is notable for striking imaging findings of diffuse interstitial and alveolar processes (Figure 2) as well as pathologic confirmation of diagnosis of a rare complication from radiation for lung cancer. Limitations are akin to those of any case report, in that it is anecdotal. The patient had multiple processes occurring in the lungs as determined by autopsy, including lymphangitic spread of tumor as well as pneumonia so the fatal respiratory

failure may not be entirely attributable to radiation pneumonitis. Furthermore, the patient received concurrent chemotherapy and additional cycles of chemotherapy after radiation which may have resulted in its own toxicity.

This is a case report of grade 5 radiation pneumonitis in a patient with a potential history of connective tissue disease and/or autoimmune disease who also developed lymphangitic spread of tumor. Standard of care chemo-radiation was provided to this patient and all of the radiation dose parameters were well within commonly accepted ranges. Furthermore, connective tissue disorder diagnosis was in question and autoimmune disorder was not active. Despite appropriate precautions, he still developed fatal pneumonitis. Further research is needed to develop a better understanding of the interplay of all of these factors.

COMMENTS

Case characteristics

This is a case report of grade 5 radiation pneumonitis in a patient with a potential history of connective tissue disease and/or autoimmune disease who also developed lymphangitic spread of tumor after receiving chemoradiation with intensity modulated radiation therapy (IMRT) technique for limited stage small cell lung cancer.

Clinical diagnosis

Grade 5 radiation pneumonitis and lymphangitic spread of tumor developed after chemoradiation for small cell lung cancer.

Differential diagnosis

Differential included pneumonitis, lymphangitic spread of tumor, pneumonia, or other interstitial and/or airspace disease.

Imaging diagnosis

Chest X-ray and computed tomography showed worsening bilateral interstitial and airspace opacities.

Pathological diagnosis

Autopsy examination of lung tissue demonstrated extensive, diffuse, bilateral alveolar damage consistent with post-radiation changes, as well as small cell carcinoma in multiple foci within septal capillaries and contiguous alveolar spaces.

Treatment

Initial therapy consisted of IMRT radiation therapy with concurrent and adjuvant chemotherapy. For his pneumonitis, he was treated with steroids, antibiotics, non-invasive and later mechanical ventilation.

Experiences and lessons

Standard of care chemoradiation was provided to this patient and all of the radiation dose parameters were well within commonly accepted ranges. Furthermore connective tissue disorder diagnosis was in question and autoimmune disorder was not active. Despite appropriate precautions, he still developed fatal pneumonitis in addition to lymphangic tumor spread. Further research is needed to develop a better understanding of the interplay of all of these factors.

Peer-review

The authors present a case report showing a patient with a fatal radiation pneumonitis 6 mo after radiation for limited stage of small cell lung cancer. The article is well explained and implemented.

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CASE REPORT

Prostatic adenocarcinoma oncocytic variant: Case report and literature review

Matthew M Klairmont, Nadeem Zafar

Matthew M Klairmont, Nadeem Zafar, Department of Pathology, University of Tennessee Health Science Center, Memphis, TN 38163, United States

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Correspondence to: Nadeem Zafar, MD, Associate Professor, Department of Pathology, University of Tennessee Health Science Center, 930 Madison 5th Floor, Memphis, TN 38163, United States. nzafar@uthsc.edu
Telephone: +1-901-4486436
Fax: +1-901-4486979

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Abstract

The oncocytic variant of prostatic adenocarcinoma is exceptionally rare with only 4 cases reported in the English literature. Little is known about the clinical behavior of this variant of prostatic adenocarcinoma, because of the exceptionally low number of reported cases. The 2016 World Health Organization Classification of Tumors of Prostate does not recognize the oncocytic variant, again likely related to the exceptional paucity of reported cases. Here, we report the fifth case of the oncocytic variant of acinar type prostatic adenocarcinoma in an asymptomatic 64-year-old Caucasian American male with elevated serum prostate specific antigen (7.33 ng/mL; normal range 0-4.00 ng/mL) during routine blood screening for diabetes mellitus. At subsequent transrectal prostate biopsy, the right side of prostate was infiltrated by adenocarcinoma with tumor cells forming variably differentiated glands, including some poorly differentiated. Tumor cell nuclear: cytoplasmic ratio was low, with small to intermediate sized vesicular nuclei and only rare discernable small nucleoli. Cellular cytoplasm was characteristically granular pink with sharply defined cell membranes. Positive AMACR (P504S) epithelial immunohistochemical staining and absence of staining for prostatic basal cells confirmed the tumor to be primary prostatic adenocarcinoma. AMACR immunohistochemical staining was also helpful with accurate grading of the tumor due to the difficulty of differentiating tumor cells from residual prostate myocytes at routine hematoxylin and eosin (HE) staining. This new case adds to the exceptionally small number of previously reported cases of the oncocytic variant of primary prostatic adenocarcinoma. It also highlights the difficulty associated with Gleason scoring of the oncocytic variant by routine HE evaluation and the usefulness of AMACR (P504S) immunostaining for accurate grading of prostatic adenocarcinoma in the oncocytic variant.

Key words: Prostate; Adenocarcinoma; Clinical behavior; Oncocytic; Gleason; Prognosis

Core tip: The oncocytic variant of prostatic adenocarcinoma is exceptionally rare with only 4 cases reported so far. Through reporting this new case, the oncocytic variant is being highlighted and challenges associated with its accurate diagnosis and staging discussed. The use of immunohistochemistry to confirm prostatic origin of this tumor for accurate grading of this lesion is also highlighted. It is also postulated that the tumor cells may be difficult to locate for their presence and organization at hematoxylin and eosin evaluation, potentially resulting in inaccurate grading of the tumor, the tumor likely behaves no different from the usual/typical variant of acinar-type adenocarcinoma if appropriately graded.

Klairmont MM, Zafar N. Prostatic adenocarcinoma, oncocytic variant: Case report and literature review. *World J Clin Oncol* 2017; 8(3): 289-292 Available from: URL: http://www.wjgnet.com/2218-4333/full/v8/i3/289.htm DOI: http://dx.doi.org/10.5306/wjco.v8.i3.289

INTRODUCTION

Prostatic adenocarcinoma is a common malignancy, however, the 2016 World Health Organization (WHO) Classification of Prostatic Tumors[1] does not mention the oncocytic variant of acinar-type adenocarcinoma, likely due to the very small number of reported cases in the literature. There is a paucity of data concerning the clinical behavior of this variant compared to the traditionally established varieties of acinar-type prostate adenocarcinoma. Accordingly, there is a critical need for more cases of the oncocytic variant to be reported, for it to be added to a future WHO classification, and to identify a variable clinical behavior from the usual variant, if that is indeed the case.

CASE REPORT

The 64-year-old Caucasian male with past medical history of hypertension, hyperlipidemia, type-2 diabetes, and otherwise asymptomatic, was also found at routine screening to have an elevated total serum prostate-specific antigen (PSA) of 7.33 ng/mL (range 0-4.0 ng/mL). Review of systems was unremarkable. He denied tobacco use and reported occasional alcohol use. Family history was unremarkable for genitourinary malignancy. Digital rectal exam indicated irregular prostate borders with a single indurated nodule on the right. The patient subsequently underwent transrectal prostate biopsy which revealed a right-sided prostate adenocarcinoma, the left-side being unremarkable. Patient was discharged after biopsy and elected to undergo targeted cryoablation of the prostate at an outside institution.

Histology

At routine hematoxylin and eosin (HE) histology, the right side of the prostate contained a poorly delineated malignancy with tumor cells arranged in vague glandular forms, as well as apparent cords, and possibly some single cells (Figure 1A). The cells had a low nuclear:cytoplasmic ratio, small to intermediate sized vesicular nuclei with only very rare prominent nucleoli, granular amphophilic to acidophilic cytoplasm, and sharp cell membranes (Figure 1B). It was difficult to reliably differentiate tumor cells from residual prostatic myocytes because of overlapping cytomorphology and staining quality (Figure 1B). Routine ABC immunohistochemistry with AMACR (P504S) and prostate basal cell markers (PIN4) was very helpful to confirm this cancer to be primary to prostate, and for accurate Gleason scoring of the tumor, as it clearly demonstrated the absence of single tumor cells and extensive gland formation, mostly discrete, with some tumor cells merging into more solid structures (1C and 1D). The tumor was assigned Gleason score 3 + 4 (20%) = 7, present in all 6/6 cores, 25% of total biopsy, with perineural invasion, but no vascular invasion. Biopsies of the left prostate were negative for malignancy.

DISCUSSION

Oncocytic tumors of prostate are exceedingly rare. The first reported case was an oncocytoma in an 87-year-old man who underwent transurethral resection for prostatic hypertrophy[2]. While the tumor cells were immunoreactive with cytochrome-oxidase, they were not reactive with PSA.

In 1992, Ordóñez *et al*[3] reported the first case of prostatic carcinoma with oncocytic features in a 63-year-old patient who presented with inguinal lymph node metastasis and an unknown primary, with a normal serum PSA. The tumor cells had finely granular cytoplasm, which ultrastructural examination showed to contain numerous mitochondria. The cells were immunoreactive with PSA and prostatic acid phosphatase. Subsequent prostatectomy confirmed primary oncocytic adenocarcinoma of the prostate. The authors postulated that the reason for oncocytic transformation may involve possible mitochondrial dysfunction in the cancer cell of origin, resulting in the proliferation of an oncocytic cancer cell type.

Pinto *et al*[4] reported the 2nd case of primary carcinoma of prostate with diffuse oncocytic changes in a 66-year-old patient, who presented with a retro-ocular tumor and a PSA level of 100 ng/mL. Digital rectal exam indicated prostatic enlargement, which was subsequently biopsied, and the retro-ocular metastasis was also resected. Both the tumor sites contained identical poorly differentiated oncocytic tumor cells with strong immunoreactivity for PSA. This patient also had hyperdense metastatic lesions in various bony sites. The authors postulated that the prognosis of these very rare oncocytic tumors is no different from the usual prostatic acinar carcinoma and is more related to the tumor differentiation (Gleason

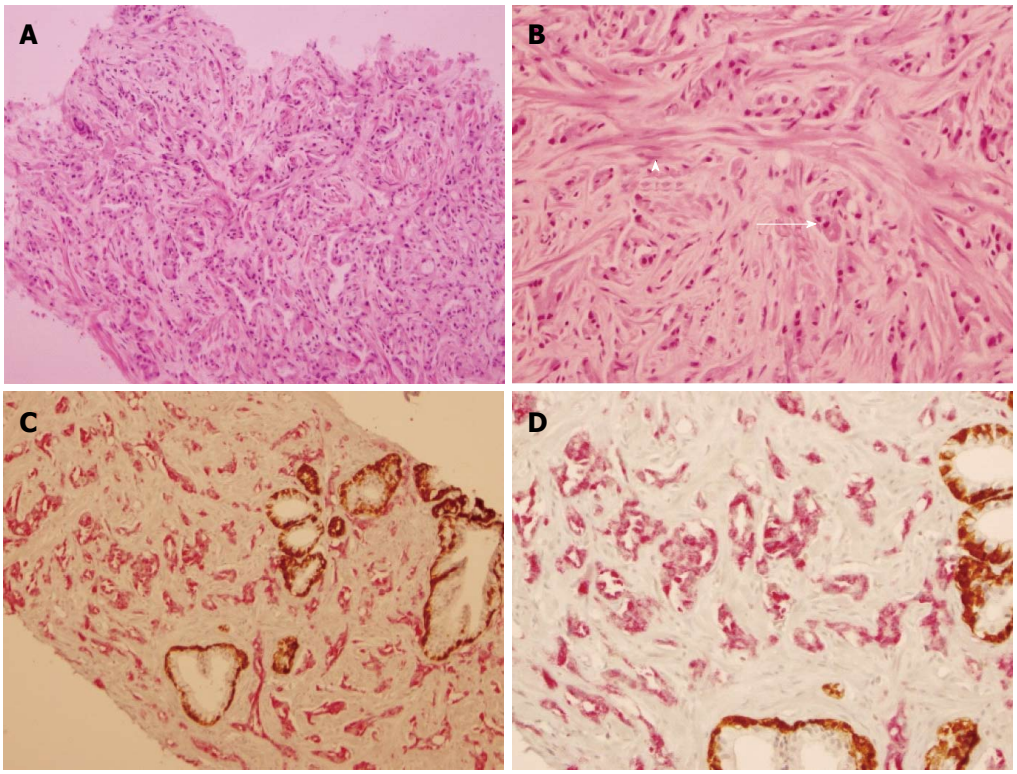

Figure 1 Oncocytic variant of prostatic adenocarcinoma: Hematoxylin and eosin and Immunohistochemical evaluation. A: Low power view of tumor, showing the tumor cells arranged in glandular and loose epithelial clusters (100 ×); B: High power view of tumor in glandular formations (arrow) and spindled residual prostate myocytes (arrowhead) (200 ×); C: PIN4 immunohistochemical staining identifies tumor (in red) and benign prostatic glands with residual basal cells (in brown) (100 ×); D: PIN4 immunohistochemical staining high power (200 ×) view with tumor (in red) and benign prostatic glands with residual basal cells (in brown).

scoring). We agree with this opinion, though we feel this is still anecdotal because of insufficient experience with this tumor variant.

Fiandrino *et al*[5] reported the third case of prostatic adenocarcinoma with oncocytic features in a 72-year-old patient who presented with dysuria and prostate enlargement. The patient underwent prostatectomy which revealed prostatic adenocarcinoma with oncocytic features involving the entire tumor mass. Capsular infiltration and perineural invasion were also present. Based on extensive gland fusion, it was assigned a Gleason score of 8 (4 + 4) involving 60% of the prostate (both lobes). At immunohistochemistry, the oncocytic tumor cells were strongly positive for PSA and prostatic acid phosphatase (PSAP). Cells also stained positive for antimitochondrial antibody which demonstrated granular cytoplasmic reactivity in tumor cells but not normal glands. Ultrastructural evaluation was performed and similarly demonstrated a high mitochondrial density in tumor cells compared to the adjacent parenchyma.

Khadim *et al*[6] reported the most recent case of oncocytic variant of prostatic adenocarcinoma in a 57-year-old, who presented with urinary urgency, hesitancy, increased frequency, poor stream, enlarged firm prostate at digital rectal examination and a markedly elevated PSA level of 40 ng/mL. At transurethral resection, the entire prostatic tumor comprised of oncocytic cells, arranged in solid sheets, with round to ovoid hyperchromatic nuclei and granular eosinophic cytoplasm and PSA immunoreactivity. Gleason score of 5 + 4 = 9 was assigned, involving 80% of the tissue sampled and without perineural or lymphovascular invasion. No follow-up was provided.

Gilloteaux *et al*[7] have reported a peculiar, rare oncocyte-like cell in prostatic carcinoma (DU145) cell line, with a small nucleus and with cytoplasm almost entirely filled with often distorted mitochondria. It is enticing to speculate if this might be the cell-type which gives rise to the oncocytic variant of prostatic adenocarcinoma.

In summary, the reasons for presenting this case are multiple, foremost to add to the very limited literature on this variant of prostatic adenocarcinoma and to highlight the challenge of optimal Gleason scoring at HE assessment only. Our calculated Gleason score prior to AMACR (P504) staining was 4 + 5 = 9 because of the presence of poorly differentiated glands and perceived numerous single eosinophilic cells, with only rare well-formed glands. Our final Gleason score was 3 + 4 (20%) = 7, as AMACR (P504S) staining confirmed the absence of single tumor cells in the biopsy and the presence of numerous glands, mostly well-formed, with rare additional distorted and merged tumor glands. We believe the overestimation of Gleason score at HE is related to the difficulty of differentiating residual benign myocytes from tumor cells because of the overlapping cytomorphology and staining characteristics. We feel that AMACR (P504S) staining is critically important for optimal assessment of tumor differentiation and Gleason scoring of the oncocytic variant of prostatic adenocarcinoma. The non-recognition

of this cancer variant in the 2016 WHO classification of tumors of prostate, among other known variants of classic acinar type prostatic adenocarcinoma[8], is likely related to the exceptionally low number of reported cases, most likely related to very low incidence. Variants of conventional prostate cancer (pseudohyperplastic, foamy gland, hypernephroid, atrophic, microcystic, with Paneth cell-like changes, with collagenous micronodules, with glomeruloid formations, and oncocytic) do not have any known prognostic significance and are graded according to the Gleason system. The prognosis and clinical behavior of the oncocytic variant, therefore, is also likely to be related to the degree of tumor differentiation and clinical staging, and not morphologic variation.

COMMENTS

Case characteristics

A 64-year-old Caucasian male with a serum prostate-specific antigen level of 7.33 ng/mL (range 0-4.0 ng/mL).

Clinical diagnosis

Prostatic enlargement.

Differential diagnosis

Prostatic carcinoma, prostatitis, prostatic hypertrophy.

Pathologic diagnosis

Primary adenocarcinoma of prostate, oncocytic variant, Gleason score 3 + 4 (20%) = 7, 6/6 cores, 20% of total tissue involved on the right, with peri-neural invasion.

Treatment

The patient opted for cryoablation of the prostate at another facility. No further follow-up is available at this point.

Peer-review

This is an interesting case report of a very rare tumor.

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CASE REPORT

Pancreatic neuroendocrine tumor Grade 1 patients followed up without surgery: Case series

Mitsuru Sugimoto, Tadayuki Takagi, Rei Suzuki, Naoki Konno, Hiroyuki Asama, Ko Watanabe, Jun Nakamura, Hitomi Kikuchi, Yuichi Waragai, Mika Takasumi, Satoshi Kawana, Yuko Hashimoto, Takuto Hikichi, Hiromasa Ohira

Mitsuru Sugimoto, Tadayuki Takagi, Rei Suzuki, Naoki Konno, Hiroyuki Asama, Ko Watanabe, Jun Nakamura, Hitomi Kikuchi, Yuichi Waragai, Mika Takasumi, Hiromasa Ohira, Department of Gastroenterology, Fukushima Medical University, School of Medicine, Fukushima City, Fukushima Prefecture 960-1295, Japan

Naoki Konno, Ko Watanabe, Jun Nakamura, Hitomi Kikuchi, Takuto Hikichi, Department of Endoscopy, Fukushima Medical University Hospital, Fukushima City, Fukushima Prefecture 960-1295, Japan

Satoshi Kawana, Yuko Hashimoto, Department of Diagnostic Pathology, Fukushima Medical University, School of Medicine, Fukushima City, Fukushima Prefecture 960-1295, Japan

Author contributions: Sugimoto M designed and performed the research and wrote the paper; Takagi T and Suzuki R designed the research and supervised the report; Konno N designed the research and contributed to the analysis; Asama H, Watanabe K, Nakamura J, Kikuchi H, Waragai Y and Takasumi M provided clinical advice; Kawana S and Hashimoto Y provided histopathological advice; Hikichi T and Ohira H supervised the report.

Institutional review board statement: This study was reviewed and approved by the Ethics Committee of the Fukushima Medical University Hospital.

Informed consent statement: Patients were not required to give informed consent for the study because the analysis used anonymous clinical data that were obtained after each patient agreed to treatment by written consent. For full disclosure, the details of the study are published on the home page of Fukushima Medical University.

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Correspondence to: Tadayuki Takagi, MD, Associate Professor, Department of Gastroenterology, Fukushima Medical University, School of Medicine, 1 Hikarigaoka, Fukushima City, Fukushima Prefecture 960-1295, Japan. daccho@fmu.ac.jp
Telephone: +81-24-5471202
Fax: +81-24-5472055

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Abstract

Among the three grades of neuroendocrine tumors (NETs), the prognosis for Grade 1 (G1) with surgery is very good. Therefore, we evaluated the prognoses of pancreatic NET (PNET) G1 patients without surgery. A total of 8 patients who were diagnosed with NET G1, with an observation period of more than 6 mo until surgery or without surgery, were recruited. The patients who underwent surgery were ultimately diagnosed using specimens obtained during the surgery, whereas the patients who did not undergo surgery were diagnosed using specimens obtained by endoscopic ultrasonography-guided fine needle aspiration. Overall, we mainly evaluated the observation period and tumor growth. The observation period for the five cases

with surgery ranged from 6-80 mo, and tumor growth was observed in one case. In contrast, the observation period for the three cases without surgery ranged from 17-54 mo, and tumor growth was not observed. Therefore, although the first-choice treatment for NETs is surgery, our experience includes certain NET G1 patients who were followed up without surgery.

Key words: Pancreatic neuroendocrine tumors; Metastasis; Neuroendocrine tumors Grade 1; Follow-up; Surgery

Core tip: We evaluated the prognoses of pancreatic neuroendocrine tumor Grade 1 (NET G1) patients without surgery. A total of 8 patients who were diagnosed with NET G1, with an observation period of more than 6 mo until surgery or without surgery, were recruited. The observation period for the five cases with surgery ranged from 6-80 mo, and tumor growth was observed in one case. In contrast, the observation period for the three cases without surgery ranged from 17-54 mo, and tumor growth was not observed. Our experience thus includes certain NET G1 patients who were followed up without surgery.

Sugimoto M, Takagi T, Suzuki R, Konno N, Asama H, Watanabe K, Nakamura J, Kikuchi H, Waragai Y, Takasumi M, Kawana S, Hashimoto Y, Hikichi T, Ohira H. Pancreatic neuroendocrine tumor Grade 1 patients followed up without surgery: Case series. *World J Clin Oncol* 2017; 8(3): 293-299 Available from: URL: http://www.wjgnet.com/2218-4333/full/v8/i3/293.htm DOI: http://dx.doi.org/10.5306/wjco.v8.i3.293

INTRODUCTION

Neuroendocrine tumors (NETs) of the digestive organs are classified as Grade 1 (G1) or Grade 2 (G2) or as neuroendocrine carcinoma (NEC) by the World Health Organization (WHO) 2010 classification, which is based on cellular proliferative potential (Ki-67 index and the mitotic count)[1]. Generally speaking, pancreatic NETs (PNETs) are a rare condition, accounting for only 2%-5% of pancreatic tumors[2]. However, reports about PNETs have been increasing in direct proportion to more detailed diagnostic imaging.

Among the three grades of NETs, the prognosis for G1 is very good. It has been reported that the two-year progression-free survival rate for NET G1 is 92%[3] and that the two-year survival rate is 100%[4]. Five-year survival was reported to be 55.7% by Zeng *et al*[5] and 82.6% by Yang *et al*[6]. In other reports, however, the five-year survival rate was 90% or more[4,7-10].

Regarding PNET treatment, the National Comprehensive Cancer Network[11], the North American Neuroendocrine Tumor Society[12], and the European Neuroendocrine Tumor Society[13] have established guidelines. The first-choice treatment is surgery for all grades of PNETs if the lesions are resectable.

Regarding diagnosing NETs before surgery, the efficacy of endoscopic ultrasonography-guided fine needle aspiration (EUS-FNA) has been reported[14-17]. As mentioned above, the first-choice treatment for resectable PNETs is surgery. However, if a patient is diagnosed with NET G1 based on the Ki-67 index of an EUS-FNA specimen, there is a possibility that the patient will not agree to surgery because of a good prognosis.

Accordingly, we examined the following two topics in this report: (1) the prognoses of NET G1 diagnosed by EUS-FNA without surgery; and (2) the tumor growth of NET G1 from diagnosis until surgery.

CASE REPORT

A total of 34 patients were diagnosed with PNETs from February 2001 to December 2015. Among these patients, 21 underwent measurement of the Ki-67 index using specimens obtained by EUS-FNA or surgery (Figure 1). Thirteen patients were diagnosed with NET G1, seven patients were diagnosed with NET G2, and one patient was diagnosed with NEC. We recommended surgery for the NET patients, regardless of their WHO 2010 classification. However, if a patient did not agree to surgery, we only performed a follow-up. We focused on eight NET G1 patients who waited for surgery for no less than six months or who were followed up for no less than six months without surgery. The observation period was defined as no less than 6 mo based on a report on everolimus by Yao *et al*[18]. In that report, the length of progression-free survival of the placebo group was 5.4 mo.

The patients who underwent surgery were ultimately diagnosed using specimens obtained during surgery, and the patients who did not undergo surgery were diagnosed using specimens obtained by EUS-FNA. UCT260, GF-UCT240-AL5, or GF-UC240P (Olympus Medical Systems, Tokyo, Japan), was used as the echoendoscope, and EU-ME1 or EU-ME2 (Olympus Medical Systems, Tokyo, Japan) was used as the ultrasonography diagnostic device. EchoTip 19 or 22 or 25G (Cook Medical Inc., NC, United States), and EZ Shot 22G (Olympus Medical Systems) and Expect 22G (Boston Scientific, MA, United States) were used as the aspiration needles.

All patients underwent echoendoscope insertion under sedation with midazolam. After we drew the target on the monitor and checked that no blood flow was present in the aspiration line, we punctured the target, passing through the gastric or duodenal wall. We excluded the stylet of the needle and connected a syringe with 10-20 mL negative pressure to the edge of the needle. We then moved the needle back and forth 20 times within the lesion. In particular, we moved the needle to multiple locations within the target (this has been reported as the "fanning method")[19]. After we terminated the negative pressure, we removed the needle. The EUS-FNA sample was then placed on a glass slide, and the specimen was

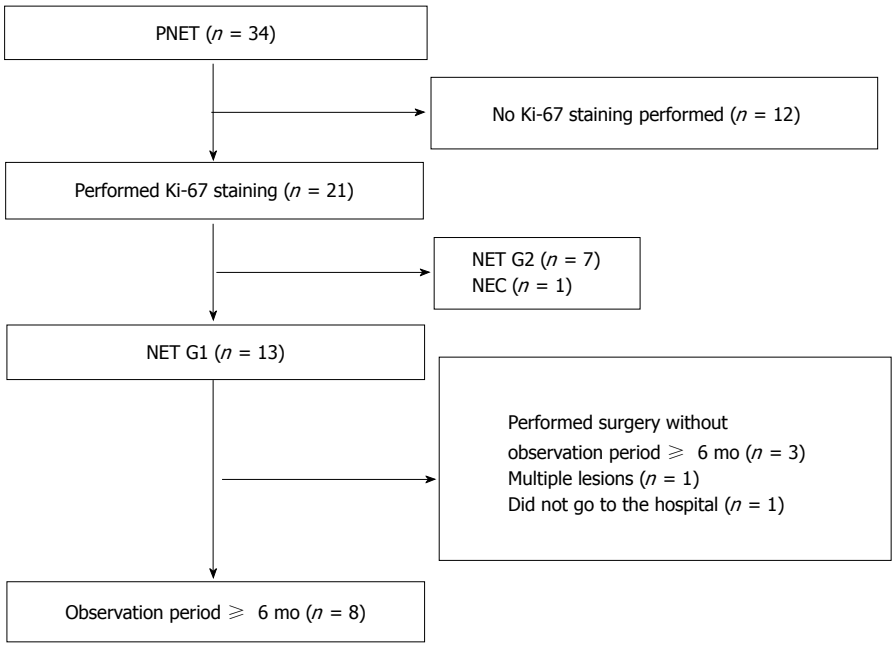

Figure 1 The characteristics of pancreatic neuroendocrine tumor patients at our hospital. A total of 34 patients were diagnosed with PNETs. Among these patients, 21 had specimens that underwent Ki-67 immunostaining. There were 13 PNET G1 patients, and the observation period was more than six months long for eight PNET G1 patients. PNET: Pancreatic neuroendocrine tumor; G1: Grade 1.

preserved in 15% formalin for histological diagnosis. All other samples were stained using Cyto-Quick. We observed the samples to assess whether a sufficient number of cells were sampled (rapid on-site cytological evaluation, or ROSE)[20]. If a sample was sufficient, we halted the EUS-FNA; if a sample was not sufficient, we performed another aspiration. The samples obtained for histological diagnosis were stained with hematoxylin and eosin and were also immunostained for the following: Ki-67, chromogranin, synaptophysin (DAKO, Glostrup, Denmark), and CD56 (ZYMED, Carlsbad, CA, United States). The grades of the PNET cases were determined based on the Ki-67 index outlined in the WHO 2010 classification. The grades of the specimens obtained during surgery were also determined based on the Ki-67 index and the mitotic count, as defined in the WHO 2010 classification.

We reviewed each patient's characteristics (sex, age, initial tumor size, and location of the tumor), the method of diagnosis (EUS-FNA or surgery), the Ki-67 index, the mitotic count, whether the patient was functional or not, tumor marker levels, observation period, and tumor growth. The observation period was determined as the number of months from tumor discrimination by abdominal echo or computed tomography (CT) until the tumors were resected. For the patients without surgery, the observation period was determined as the number of months from tumor discrimination by abdominal echo or CT until the tumors were recognized by a final abdominal echo or CT. The patients specifically underwent dynamic CT or abdominal echo approximately 2 times per year, performed by an attending physician.

The age range of the patients was 41-81 years, and the patient group included two males and six females (Table 1). The initial major tumor axes ranged from 3-40 mm. The locations of the tumors were the pancreatic head (*n* = 3), pancreatic body (*n* = 3), and pancreatic tail (*n* = 2). Five patients underwent surgery, and three patients did not but did undergo EUS-FNA. The Ki-67 index ranged from 0.4%-1.3% (five patients did not undergo precise measurement, but their index was < 2.0%). The mitotic count of the specimens obtained during surgery was 0-2/10 HPFs. Three patients were functional (1 with a growth hormone-producing tumors, 1 with a glucagonoma, and 1 with an insulinoma). AFP, NSE, CEA or CA19-9 was also measured, but these tumor markers were not elevated in any of the patients.

The observation periods ranged from 6-80 mo for patients 1-4. Only patient 2 was observed to exhibit tumor growth (Figure 2). In contrast, the observation periods for the three cases without surgery ranged from 17-54 mo, and all three cases did not show tumor growth. Among these three cases, one case is shown in Figure 3.

DISCUSSION

In this report, we examined whether we could follow up NET G1 without surgery. Among eight patients who were observed before surgery for no less than six months or who did not undergo surgery for at least six months, tumor growth was observed in one patient.

As described above, the prognoses of the NET G1 were very good. However, the data were relevant to prognoses only after surgery. Sadot *et al*[21] reported the prognoses of 104 PNET patients who were diagnosed pathologically

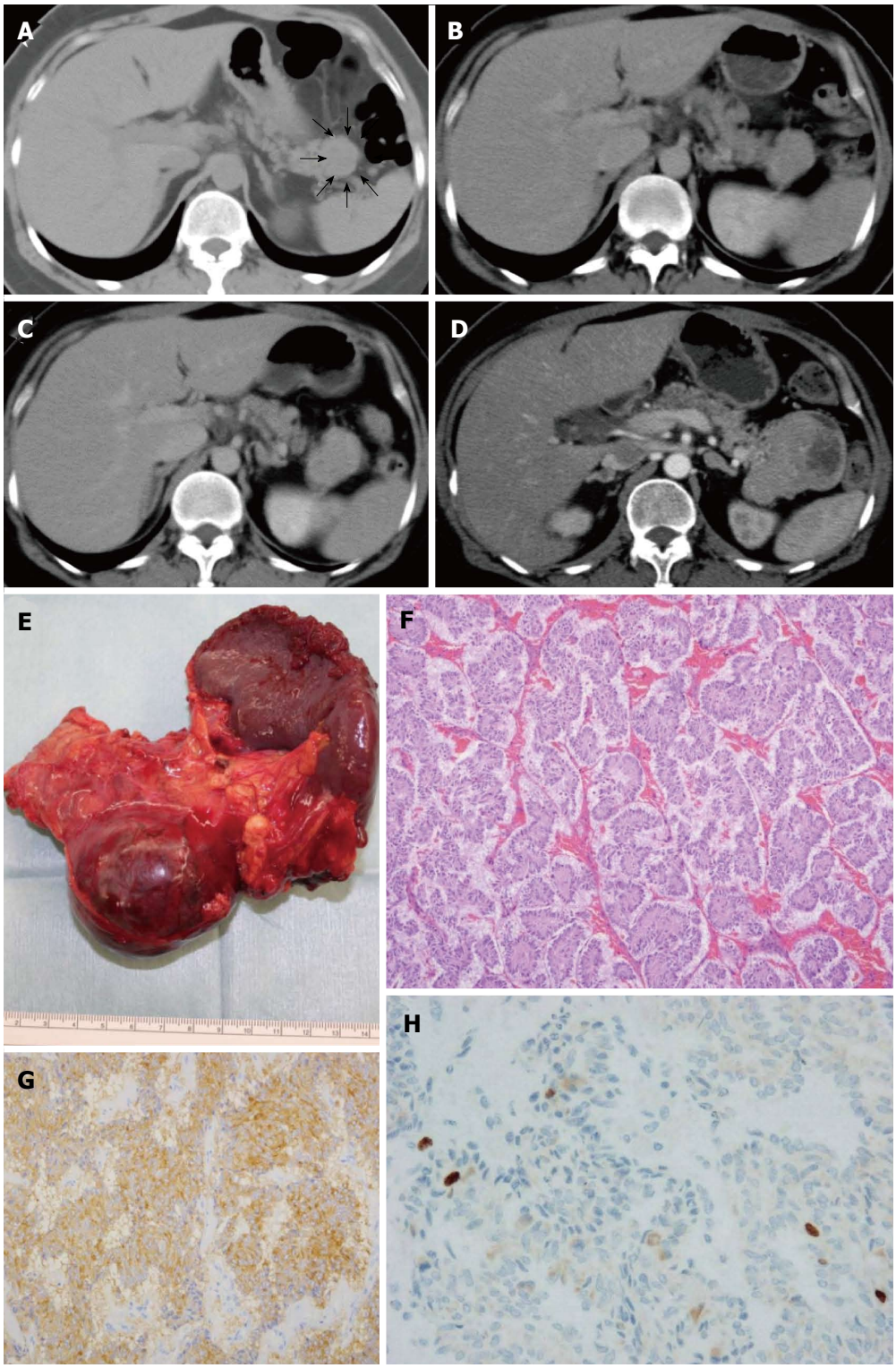

Figure 2 The patient who exhibited growth of the pancreatic neuroendocrine tumor. A: Abdominal CT. Initial CT indicated a PNET. The lesion was identified in the pancreatic tail. The diameter of the PNET was 34 mm (arrow); B: The lesion grew slightly after 11 mo; C: The lesion grew further after 29 mo; D: The diameter of the tumor became larger than 70 mm after 79 mo; E: The patient underwent distal pancreatectomy after 80 mo; F: Hematoxylin and eosin stain (× 100). Tumor cells formed ribbon-like lines; G: Chromogranin A staining (× 200). Tumor cells were chromogranin A positive; H: The Ki-67 index was 0.9%, with tumor grade G1 (× 200). PNET: Pancreatic neuroendocrine tumor; CT: Computed tomography.

or by imaging. In that report, the diameters of all PNET lesions were smaller than 3.0 cm. Among the patients, 26 did not undergo surgery; those without surgery who were only followed up did not exhibit tumor growth or metastases to other organs. Though cases diagnosed by only imaging were included in that report, certain PNET patients could be followed up without surgery. Additionally, Shin *et al*[22] reported 72 gastroenteropancreatic NET cases

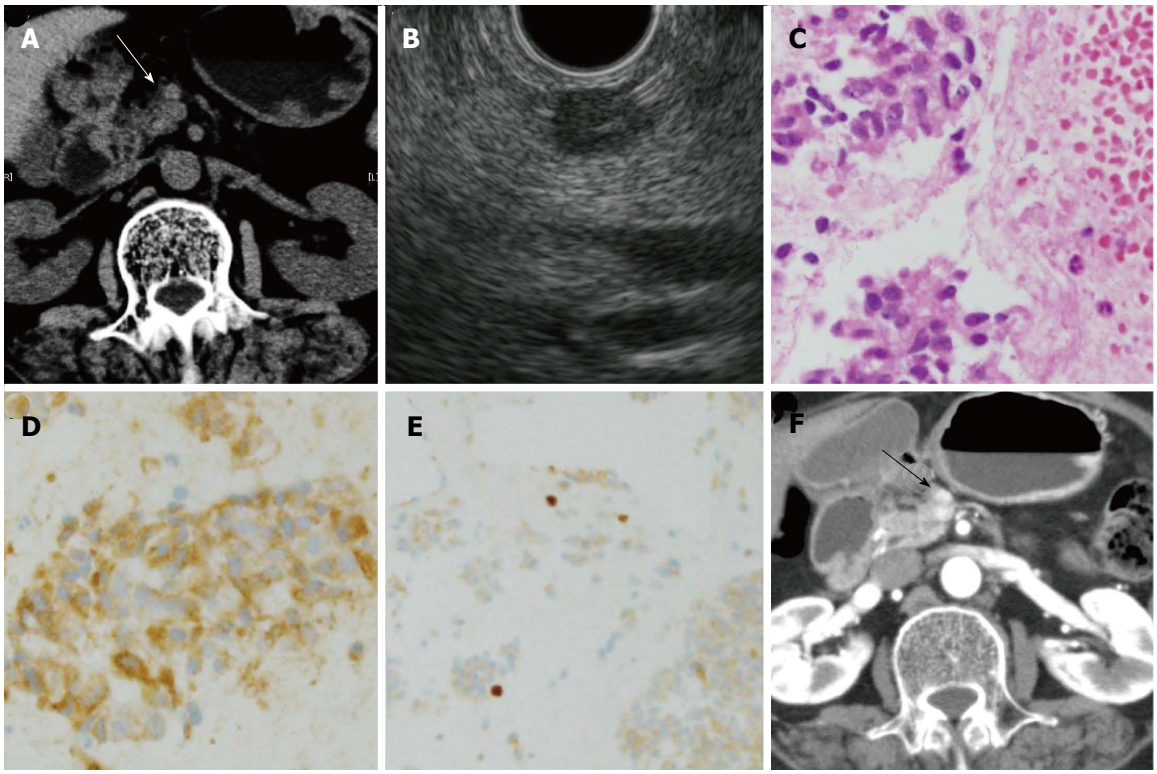

Figure 3 Pancreatic neuroendocrine tumor case followed up without surgery. A: Abdominal CT. A tumor was recognized in the pancreatic body. The diameter of the lesion was 8 mm; B: Endoscopic ultrasonography. The tumor was recognized as a low echoic lesion. A 22G needle was inserted into the tumor; C: Hematoxylin and eosin stain (× 400). Spindle-shaped tumor cells with ellipse nuclei formed funicular lines; D: Chromogranin A staining (× 400). Tumor cells were chromogranin A-positive; E: The Ki-67 index was < 1.0% (× 200), with tumor grade G1; F: Abdominal CT. The tumor did not grow after 54 mo. PNET: Pancreatic neuroendocrine tumor; CT: Computed tomography.

with liver metastases. Among these cases, 12 were NET G1 (17%). Zerbi *et al*[23] reported that 16.1% of NET G1 showed metastases to the lymph nodes and that 12.6% of NET G1 showed liver metastases. In addition, Gaujoux *et al*[24] reported 20 PNET G1 cases with liver metastases. In the present report, one case exhibited tumor growth in the observation period. Therefore, we have to follow up NET G1 while taking the risk factors for metastases and tumor growth into consideration.

What are the specific risk factors for NETs? In the past reports, nonfunction and symptoms such as abdominal pain, weight loss, and jaundice were reported to be risk factors for liver metastases. Moreover, Tao *et al*[25] reported that elevated tumor markers (AFP, CEA, CA125, CA19-9) were predictive factors for liver metastases or lymph node metastases, and Jiang *et al*[26] reported that a tumor diameter larger than 25 mm was a risk factor for lymph node metastases. In the present report, the lesion diameters of 3 cases were larger than 25 mm; the patients were numbered 2, 4, and 5 (Table 1). Though patients 4 and 5 underwent surgery six months after diagnosis, the lesion of patient 2 grew from 34 to 76 mm in diameter. Though past studies involved not only NET G1 but also other grades of NETs, the risk factors cited in these past reports were considered to be important to determine follow-up without surgery.

In this report, there were certain limitations. First, the research was retrospectively performed at a single institution, and a small number of patients were included. More patients will be needed for more conclusive research. Second, the followed-up patients were diagnosed only by EUS-FNA. However, a high accordance rate between specimens obtained during surgery and specimens obtained by EUS-FNA was reported in past studies[14-17], and for NET G1, the accordance rate between specimens obtained during surgery and specimens obtained by EUS-FNA was 92.3% (36/39)[14-17] (Larghi, 2012 #59). We believe that we relatively correctly judged the grading based on the Ki-67 index of NET G1. Third, mitotic counts were not measured in EUS-FNA specimens. Therefore, surgery is desirable as a treatment for NETs. Fourth, we did not measure several of the tumor markers described above. Rossi *et al*[27] reported the efficacy of plasma chromogranin A as a predictive factor for NET progression; this should be studied further in the future.

The first-choice treatment for NETs is absolutely surgery. However, our experience includes certain patients who were followed up without surgery because of a lack of consent for surgery.

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Table 1 Prognoses of pancreatic neuroendocrine tumor Grade 1 patients

	Sex	Age (yr)	Initial size (mm)	Location of tumor	Method of final diagnosis	Ki-67 index (%)	Mitotic count (/10 HPFs)	Function	Elevated tumor markers	Observation period (mo)	Tumor growth (mm)
1	F	79	19	Body	Surgery	< 2.0	0	No	No	6	No
2	F	41	34	Tail	Surgery	0.9	2	Yes	No	80	76
3	M	69	3	Body	Surgery	< 2.0	0	Yes	No	15	No
4	M	55	40	Head	Surgery	< 1.0	0	Yes	No	6	No
5	F	73	32	Head	Surgery	1.3	0	No	No	9	No
6	F	81	4	Head	EUS-FNA	< 1.0	Difficult	No	No	22	No
7	F	64	8	Tail	EUS-FNA	0.4	Difficult	No	No	17	No
8	F	70	8	Body	EUS-FNA	< 1.0	Difficult	No	No	54	No

M: Male; F: Female; EUS-FNA: Endoscopic ultrasonography-guided fine needle aspiration.

University Hospital as well as American Journal Experts, an English-language proofreading company.

COMMENTS

Case characteristics

Pancreatic neuroendocrine tumor Grade 1 (PNET G1) patients who were followed for more than six months before surgery or who were followed up without surgery for more than six months.

Clinical diagnosis

PNETs were diagnosed using specimens obtained by endoscopic ultrasonography-guided fine needle aspiration (EUS-FNA) or obtained during surgery.

Differential diagnosis

Metastatic pancreatic tumors, accessory spleen, acinar cell carcinoma, paraganglioma.

Laboratory diagnosis

All tumor markers were not elevated.

Imaging diagnosis

PNETs are pancreatic tumors that are strongly enhanced on contrast-enhanced computed tomography.

Pathological diagnosis

Spindle-shaped tumor cells were observed. The tumor cells formed funicular lines and were positive for immunostaining of chromogranin A.

Treatment

Surgery or follow-up.

Related reports

The prognosis of PNET G1 is very good. However, certain PNET G1 patients exhibit metastases. Therefore, the first-choice treatment for resectable NETs is surgery.

Term explanation

EUS: A technique in which an echoendoscope is used to enable observation of the chest and abdominal organs, namely, the esophagus, stomach or duodenum; EUS-FNA: A technique used to obtain specimens from chest and abdominal lesions by aspiration under EUS guidance.

Experiences and lessons

The gold standard of treatment for NET G1 is surgery. However, if patients are diagnosed with NET G1 by EUS-FNA, there is a possibility that the patients will not agree to surgery. In fact, certain NET G1 patients did not agree to surgery in the current case series, so the authors only followed up these patients. If we only follow up PNET G1 patients, the authors have to be careful about certain risk factors for metastasis of the PNETs.

Peer-review

This is an interesting paper on whether patients with G1 pancreatic NET can be followed without surgery using a case series of patients.

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CASE REPORT

Target migration from re-inflation of adjacent atelectasis during lung stereotactic body radiotherapy

Bijing Mao, Vivek Verma, Dandan Zheng, Xiaofeng Zhu, Nathan R Bennion, Abhijeet R Bhirud, Maria A Poole, Weining Zhen

Bijing Mao, Department of Oncology, Guizhou Provincial People's Hospital, Guiyang, Guizhou 550002, China

Vivek Verma, Dandan Zheng, Xiaofeng Zhu, Nathan R Bennion, Abhijeet R Bhirud, Maria A Poole, Weining Zhen, Department of Radiation Oncology, University of Nebraska Medical Center, Omaha, NE 68198, United States

Author contributions: Zhen W conceived of the project; Mao B, Verma V, Zheng D and Poole MA collected data; all authors analyzed data, wrote, and reviewed the paper and provided final approval.

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Correspondence to: Weining Zhen, MD, Department of Radiation Oncology, University of Nebraska Medical Center, Ground Floor, Clarkson Tower, Omaha, NE 68198, United States. wzhen@unmc.edu
Telephone: +1-402-5523844
Fax: +1-402-5523013

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Abstract

Stereotactic body radiotherapy (SBRT) is a widely accepted option for the treatment of medically inoperable early-stage non-small cell lung cancer (NSCLC). Herein, we highlight the importance of interfraction image guidance during SBRT. We describe a case of early-stage NSCLC associated with segmental atelectasis that translocated 15 mm anteroinferiorly due to re-expansion of the adjacent segmental atelectasis following the first fraction. The case exemplifies the importance of cross-sectional image-guided radiotherapy that shows the intended target, as opposed to aligning based on rigid anatomy alone, especially in cases associated with potentially "volatile" anatomic areas.

Key words: Radiation therapy; Stereotactic body radiation therapy; Non-small cell lung cancer; Image-guided radiation therapy; Stereotactic ablative radiation therapy

Core tip: This is a case of early-stage non-small cell lung cancer associated with segmental atelectasis that translocated owing to re-expansion of the adjacent segmental atelectasis following the first fraction. There are image-guidance systems that register solely based on rigid (bony) anatomy and others that also show soft tissue; if the former would have been used, the translocated target would have been missed. The case exemplifies the importance of cross-sectional image-guided radiotherapy that shows the intended target, as opposed to aligning based on rigid anatomy alone, in cases associated with

potentially "volatile" anatomic areas.

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INTRODUCTION

Among other indications, stereotactic body radiotherapy (SBRT) plays an important role in the treatment of early-stage non-small cell lung cancer (NSCLC), chiefly in medically inoperable candidates, or if patients refuse surgery[1-7]. It is well-known that target and respiratory motion management is critical, and that spatial uncertainty in SBRT can be caused by both internal motion with respiration and set-up errors. Therefore, image-guided radiotherapy (IGRT) before each treatment is strongly recommended for SBRT; IGRT can confirm that the gross tumor is consistently located within the pre-defined treatment volume.

SBRT may utilize one of two IGRT subtypes: Systems that rely on rigid bony anatomy, and those that provide soft tissue discrimination. For the first type, two in-room systems are specifically designed for stereotactic treatments and are widely used: CyberKnife (Accuray Inc., Sunnyvale, CA, United States) and ExacTrac (BrainLabAG, Feldkirchen, Germany). These systems use orthogonal kilovoltage (kV) electronic 2-D radiographs, generated by X-ray tubes combined with flat panel detectors, to align and verify the patient treatment position - usually according to bony anatomy. Though providing better soft-tissue contrast than its MV counterparts, 2D kV IGRT systems largely consider bony landmarks for registration instead of the internal target alignment, except in cases with bulky thoracic tumors or implanted fiducial markers. For the second type, good soft tissue cross-sectional visibility is provided by 3D images. Some of these systems use kV cone-beam computed tomography (CBCT) generated by linear-accelerator (LINAC)-integrated systems such as On-Board-Imager (Varian Medical Systems, Palo Alto, CA, United States) and X-ray-Volume-Imaging (Elekta Oncology Systems, Crawley, United Kingdom). Alternatively, kV computed tomography (CT) can also be implemented by CT-on-Rails (Siemens, Erlangen, Germany). These systems offer 3D images and soft-tissue-based target verification without fiducials for small lung lesions treated with SBRT[8]. The most common application of this IGRT scheme is a two-step verification process with an initial bony registration followed by a soft-tissue target alignment[9,10].

Though most studies report localization accuracy improvements of 3D-*vs*-2D on the order of a few millimeters for lung SBRT[11-13], we describe a patient with an early-stage NSCLC which translocated after the first fraction of SBRT owing to re-expansion of segmental atelectasis. We further discuss the role of IGRT systems that align to bone *vs* soft-tissue in detection and management of the resulting misalignment.

CASE REPORT

A 72-year-old man presented with a nonproductive cough; computed tomography (CT) scan showed a 2.5-cm right lower lobe nodule. He had a 50-year history of smoking and used 4 L of nighttime oxygen (ECOG performance status 3). On auscultation, he had diminished breath sounds; pulmonary function tests showed an FEV1 (forced expiratory volume, 1 s) 41% of the predicted value and a DLCO (diffusion capacity of carbon-monoxide) 29% of the predicted value. Subsequent positron emission tomography (PET) scan showed no other hypermetabolic foci. Needle biopsy revealed poorly-differentiated lung adenocarcinoma.

He was not a surgical candidate owing to poor pulmonary function, and was appropriate for SBRT. On CT simulation including a four-dimensional (4D) and free-breathing CT, new distal segmental atelectasis was noted near the nodule (Figure 1A). The target was delineated as an internal target volume (ITV) based on maximum intensity projection (MIP) generated by 4DCT simulation, with an additional 5 mm expansion to create the planning target volume (PTV). He then started Volumetric Modulated Arc Therapy to the right lower lobe PTV at a dose of 5000 cGy in 5 daily fractions. Daily kV CBCT was used for IGRT on a TrueBeamSTx LINAC. After the first dose, the tumor was found to have translocated 15mm anteroinferiorly due to re-expansion of segmental atelectasis as demonstrated by the kV CBCT prior to the second fraction. Of note, the movement was significant enough that it was only partially covered by the PTV. He underwent re-simulation, which confirmed the geometric target migration (Figure 1B and C). SBRT was re-adjusted for the new target location after migration. For subsequent fractions, daily kV CBCTs validated tumor position within the ITV; the remainder of therapy was completed uneventfully. Post-treatment CT showed resolution of disease at 4 mo post-SBRT.

DISCUSSION

Although surgery is currently the principal option for early-stage NSCLC patients that are medically operable, stereotactic body radiotherapy (SBRT) has emerged as the option of choice for patients who are medically inoperable. SBRT is a technique that administers high doses of radiation to the target while minimizing the dose to surrounding normal tissues. Reports show high local tumor control rates upwards of 90%, with severe toxicities well under 10%[14]. This has made it a favorable option for medically inoperable patients with stage I NSCLC, endorsed by both the National Comprehensive Cancer Network (NCCN) Guidelines

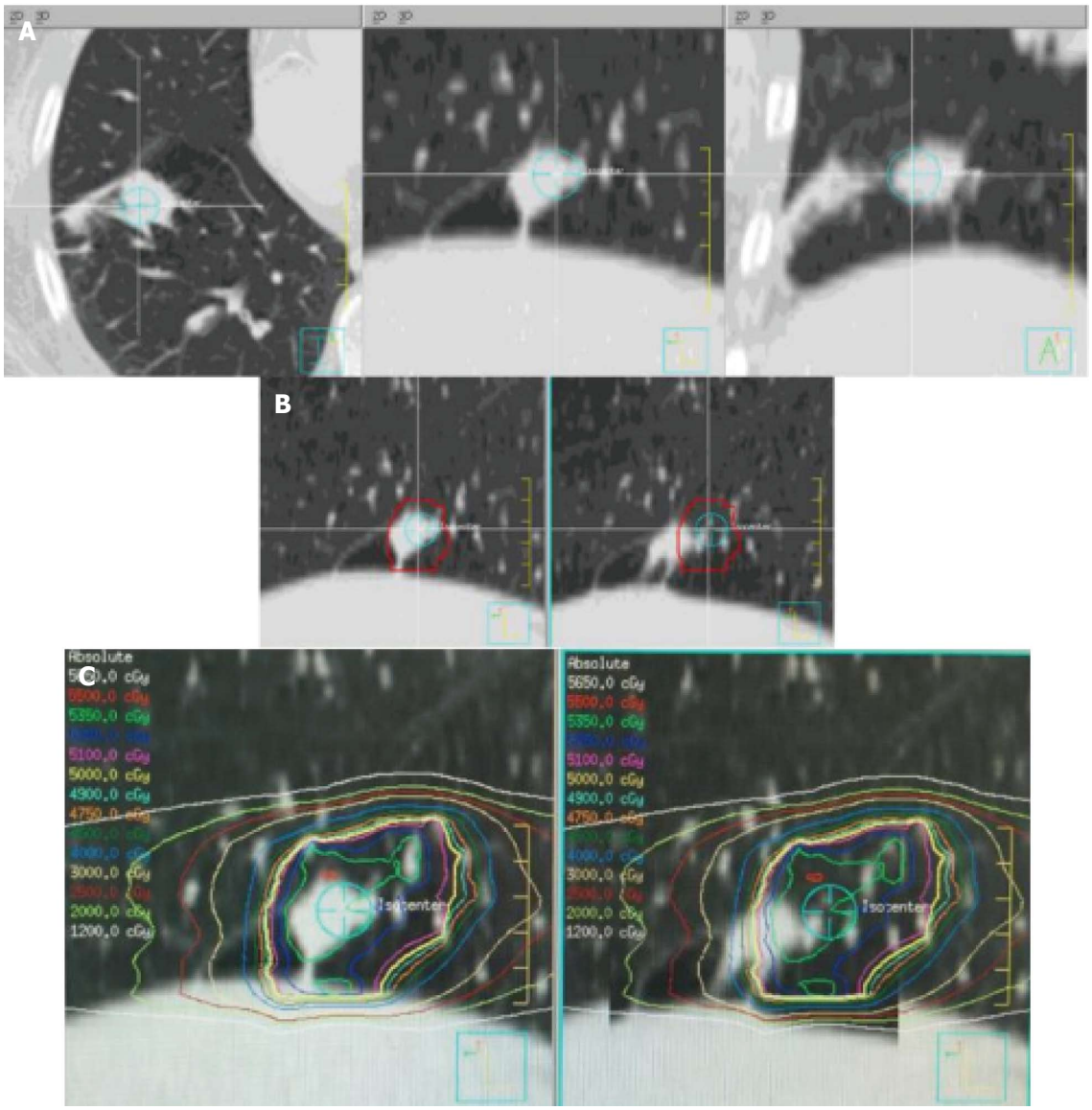

Figure 1 Computed tomography simulation including a four-dimensional and free-breathing computed tomography. A: Computed tomography (CT) images at simulation (left to right; axial, sagittal, and coronal views) showing the tumor, treatment isocenter, and the adjacent segmental atelectasis (best pictured on coronal image, lateral to isocenter); B: Left panel shows the tumor enclosed within the planning target volume (PTV) on the initial simulation CT; right panel demonstrates the translocation as compared with the original PTV on the re-CT after the first fraction. Sagittal views are shown in both panels; C: Dose distribution of initial (left) and translocated (right) tumor with isodose line values provided on left. Sagittal views are shown in both panels.

and the European Society of Medical Oncology (ESMO) Clinical Practice Guidelines[15].

However, accounting for target and respiratory motion presents a challenge to proper delivery. Hence, in order to verify target location with high accuracy, high-fidelity IGRT is considered essential to SBRT. In our case report, the target translocated 15 mm anteriorly and inferiorly due to re-expansion of adjacent atelectasis after the first fraction. Without soft tissue discrimination in cross-sectional imaging, 2D IGRT based on bony anatomy only would have resulted in systematically missing the tumor in the remaining 4 fractions. These advantages of 3D IGRT are highlighted in anatomic areas liable to changes in morphology.

There have been several studies describing the utility of IGRT in SBRT. A study was carried out to evaluate the potential of image guidance, gating and real-time tumor tracking to improve accuracy in pulmonary SBRT. It illustrated that CBCT-based IGRT for pre-treatment verification of the target position and online correction of errors reduced safety margins most effectively in pulmonary SBRT[16]. Another recent study illustrated that application of continuous monitoring and intra-fraction target position correction during treatment improved the target coverage for patients in prostate SBRT. Without these IGRT techniques, intra-fractional motion would have significantly altered coverage in about 10% of patients[17]. These studies have demonstrated that inter-fractional, and possibly even intra-fractional IGRT, can improve SBRT delivery. We advocate for increased use of cross-sectional imaging IGRT with soft tissue definition, especially in cases of tumors near potentially "anatomically volatile" areas.

While such large translocations such as reported here may be unlikely, intra-fractional real-time tumor tracking may provide additional benefit. An ionization radiation-free system using thoracic transducers and radiofrequency tracking using the Calypso system is under development (Varian Medical Systems, Palo Alto, CA, United States).

In summary, image guidance is a prerequisite for SBRT delivery, but 2D IGRT systems that solely align patients based on rigid bony anatomy may be notably inadequate in some cases. Instead, the use of imaging that provides cross-sectional soft tissue anatomical information to verify the target may prevent systematic misses from changes in target position.

COMMENTS

Case characteristics

A 72-year-old man of stage I non-small cell lung cancer associated with segmental atelectasis that translocated owing to re-expansion of the adjacent segmental atelectasis following the first dose of stereotactic body radiation therapy (SBRT).

Clinical diagnosis

Lung re-expansion of segmental atelectasis.

Differential diagnosis

Diminished breath sounds, pulmonary function, positron emission tomography scan and needle biopsy.

Laboratory diagnosis

The patient was not a surgical candidate owing to poor pulmonary function, and was appropriate for SBRT.

Imaging diagnosis

Re-simulation with high resolution computed tomography (CT) and image comparison using ridged image registration of primary CT simulation images confirmed geographic moves of the tumor due to re-expansion of an adjacent pulmonary atelectasis.

Pathological diagnosis

Needle biopsy showed poorly-differentiated lung adenocarcinoma.

Treatment

Daily kV cone-beam computed tomography was used for IGRT during SBRT.

Related reports

Related reports have demonstrated that inter-fractional, and possibly even intra-fractional IGRT, can improve SBRT delivery.

Term explanation

Non-small cell lung cancer is a deadly disease that may threaten people's life.

Experiences and lessons

Image guidance is extremely important for SBRT delivery.

Peer-review

This is an interesting case report worthy for publication. The authors reported on an early-stage lung tumor undergoing SBRT, translocating outside of the PTV after re-inflation of nearby atelectasis. The case herein presented highlight the risks of relying on IGRT system based on rigid anatomy alone. The manuscript is original, well-written and summarized in very explanatory figures.

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Contents

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Contents

World Journal of Clinical Oncology
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REVIEW

Magnetic resonance imaging for prostate cancer before radical and salvage radiotherapy: What radiation oncologists need to know

Felipe Couñago, Gemma Sancho, Violeta Catalá, Diana Hernández, Manuel Recio, Sara Montemuiño, Jhonathan Alejandro Hernández, Antonio Maldonado, Elia del Cerro

Felipe Couñago, Elia del Cerro, Department of Radiation Oncology, Hospital Universitario Quirónsalud Madrid, 28223 Madrid, Spain

Felipe Couñago, Elia del Cerro, Clinical Department, Faculty of Biomedicine, Universidad Europea, 28670 Madrid, Spain

Gemma Sancho, Department of Radiation Oncology, Hospital de la Santa Creu i Sant Pau, 08026 Barcelona, Spain

Violeta Catalá, Jhonathan Alejandro Hernández, Department of Radiology, Fundació Puigvert, 08025 Barcelona, Spain

Diana Hernández, Department of Radiology, Hospital de la Santa Creu i Sant Pau, 08026 Barcelona, Spain

Manuel Recio, Department of Radiology, Hospital Universitario Quirónsalud Madrid, 28223 Madrid, Spain

Sara Montemuiño, Department of Radiation Oncology, Hospital Universitario de Fuenlabrada, 28942 Madrid, Spain

Antonio Maldonado, Department of Nuclear Medicine, Hospital Universitario Quirónsalud Madrid, 28223 Madrid, Spain

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Correspondence to: Felipe Couñago, MD, Department of Radiation Oncology, Hospital Universitario Quirónsalud Madrid, Calle Diego de Velázquez 1, Pozuelo de Alarcón, 28223 Madrid, Spain. felipe.counago@quironsalud.es
Telephone: +34-676-839746

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Abstract

External beam radiotherapy (EBRT) is one of the principal curative treatments for patients with prostate cancer (PCa). Risk group classification is based on prostate-specific antigen (PSA) level, Gleason score, and T-stage. After risk group determination, the treatment volume and dose are defined and androgen deprivation therapy is prescribed, if appropriate. Traditionally, imaging has played only a minor role in T-staging due to the low diagnostic accuracy of conventional imaging strategies such as transrectal ultrasound, computed tomography, and morphologic magnetic resonance imaging (MRI). As a result, a notable percentage of tumours are understaged, leading to inappropriate and imprecise EBRT. The development of multiparametric MRI (mpMRI), an imaging technique that combines morphologic studies with functional diffusion-weighted sequences and dynamic contrast-enhanced imaging, has revolutionized the diagnosis and management of PCa. As a result, mpMRI is now used in staging PCa prior to EBRT, with possible implications for both risk group classification and treatment decision-making for EBRT. mpMRI is also being used in salvage

radiotherapy (SRT), the treatment of choice for patients who develop biochemical recurrence after radical prostatectomy. In the clinical context of biochemical relapse, it is essential to accurately determine the site of recurrence - pelvic (local, nodal, or bone) or distant - in order to select the optimal therapeutic management approach. Studies have demonstrated the value of mpMRI in detecting local recurrences - even in patients with low PSA levels (0.3-0.5 ng/mL) - and in diagnosing bone and nodal metastasis. The main objective of this review is to update the role of mpMRI prior to radical EBRT or SRT. We also consider future directions for the use and development of MRI in the field of radiation oncology.

Key words: Prostate cancer; Staging; Radical radiotherapy; Multiparametric magnetic resonance imaging; Biochemical failure; Radical prostatectomy; Salvage radiotherapy

Core tip: Multiparametric magnetic resonance imaging (mpMRI) has revolutionized the management of prostate cancer, including external beam radiotherapy (EBRT). mpMRI has also improved local staging and recurrence detection after radical prostatectomy, even in patients with low prostate-specific antigen levels, and it has increased the accuracy of EBRT, potentially improving survival outcomes while reducing side effects. For these reasons, mpMRI is an essential tool in the evaluation and treatment of prostate cancer.

Couñago F, Sancho G, Catalá V, Hernández D, Recio M, Montemuiño S, Hernández JA, Maldonado A, del Cerro E. Magnetic resonance imaging for prostate cancer before radical and salvage radiotherapy: What radiation oncologists need to know. *World J Clin Oncol* 2017; 8(4): 305-319 Available from: URL: http://www.wjgnet.com/2218-4333/full/v8/i4/305.htm DOI: http://dx.doi.org/10.5306/wjco.v8.i4.305

INTRODUCTION

In the last decade, the growing use of multiparametric magnetic resonance imaging (mpMRI) in the diagnosis and treatment of prostate cancer (PCa) has revolutionized patient management. Numerous studies confirm the emerging and increasingly important role of mpMRI in PCa in a wide range of clinical contexts, including: Tumour screening and detection[1]; prostate biopsy guidance[2]; staging[3]; assessment of tumour aggressiveness[4]; active surveillance protocols[5]; treatment planning (surgery, radiotherapy, and focal therapies)[6-8]; and detection of recurrence after radical prostatectomy (RP) or external beam radiotherapy (EBRT)[9,10].

There are two main indications for radiotherapy in PCa: (1) the initial treatment of patients with a recent diagnosis of Pca; and (2) salvage treatment in patients with recurrent disease after RP. In both of these clinical scenarios, conventional diagnostic strategies [digital rectal examination (DRE), transrectal ultrasound (TRUS) with "blind" biopsies, computed tomography (CT), and bone scintigraphy] all have a low yield for establishing the T stage and in detecting recurrences post RP, all of which could result in undertreatment. In this context, the objective of this review is to update the role of mpMRI in the radical treatment of PCa with EBRT and in salvage radiotherapy (SRT) after RP. In addition, we discuss future directions for the use and development of MRI in the field of radiation oncology.

WHAT IS PROSTATE MPMRI?

mpMRI is an imaging technique that allows for the non-invasive assessment of the prostate gland. It is called multiparametric because various pulse sequences (*i.e.*, multiple parameters) are used to help detect and characterize the prostate lesions. Currently, mpMRI includes both morphologic (T1 and T2) and functional sequences [diffusion-weighted imaging (DWI) and dynamic contrast-enhanced (DCE) imaging with gadolinium]. Spectroscopy no longer plays an important role and is thus not included in current MRI guidelines[11,12].

MORPHOLOGIC IMAGING

T1-weighted pulse sequence

The T1 sequence consists of a T1-weighted (T1W) Fast Spin Eco (FSE) from the aortic bifurcation to the symphysis pubis, assessed on the axial plane (Figure 1). The T1W sequence cannot discriminate various prostate gland zones and therefore its main utility is in detecting the presence of post-biopsy bleeding (a common cause of false positives in PCa diagnosis) (Figure 2), nodal disease, and bone metastasis[13].

T2-weighted pulse sequence

The T2 sequence consists of a T2-weighted (T2W) FSE sequence that includes the prostate gland and seminal vesicles (Figure 1B-D). The T2W sequence is normally performed in three spatial planes (axial, coronal, and sagittal). T2W sequence is capable of discriminating various anatomic zones of the prostate, including the peripheral, central, and transitional zones, as well as the anterior fibromuscular stroma, neurovascular plexus, surgical pseudocapsule, and the prostate capsule. In normal prostates, the peripheral zone is homogeneous and hyperintense on MRI (Figure 1B). In adults, the transitional zone is larger, with a heterogenous signal and hyperplastic nodules of varying appearance, thus this zone can sometimes present diagnostic difficulties[13].

Cancerous prostate lesions usually appear as nodules or hypointense areas on T2W images, with less well-defined margins at the transitional zone. A

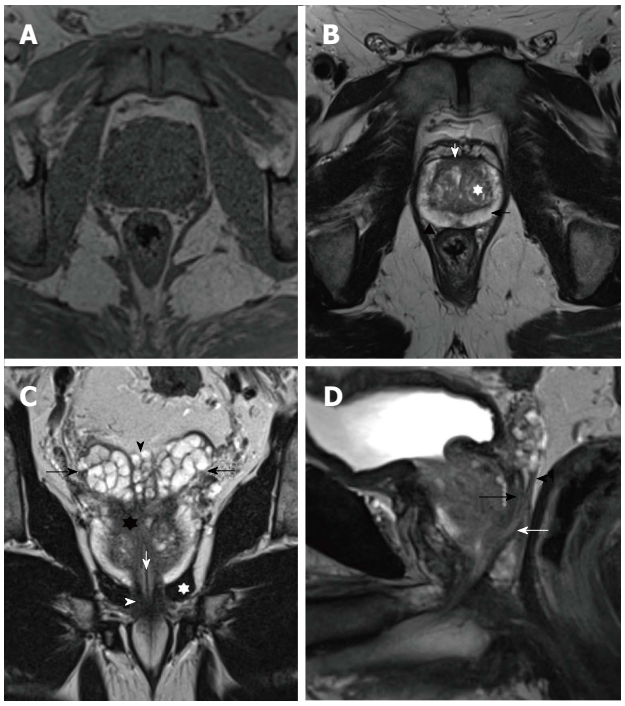

Figure 1 Normal prostate anatomy on magnetic resonance imaging - T1 and T2-weighted images. A: T1-weighted axial image of a normal prostate: Homogeneous gland, isointense to the adjacent pelvic muscles. It is not possible to differentiate any anatomical detail; B: Axial image of the prostate shows peripheral zone (PZ - black arrow) as a hyperintense area with a U-shape. The transitional zone (TZ - white asterisk) has a hypointense multinodular pattern ("organized chaos"). Anterior fibromuscular stroma (white arrow) is seen as a hypointense area anterior to the TZ and medial to both anterior PZ horns. The "capsule" (arrowhead) is seen as a hypointense rim surrounding the gland; C: Coronal image shows the hyperintense seminal vesicles located cephalad to the base of the prostate (black arrows). Ampulla of vas deferens (VD - black arrowhead) can be seen as paired structures medial to both seminal vesicles (SV). Prostate central zone (asterisk) is seen as a hypointense area located in the base of the prostate gland. Urethra (white arrow), levator ani muscle (white asterisk) and external sphincter (white arrowhead) are also shown; D: Sagittal image shows the vas deferens (black arrow), seminal vesicles (arrowhead) and the ejaculatory duct (white arrow).

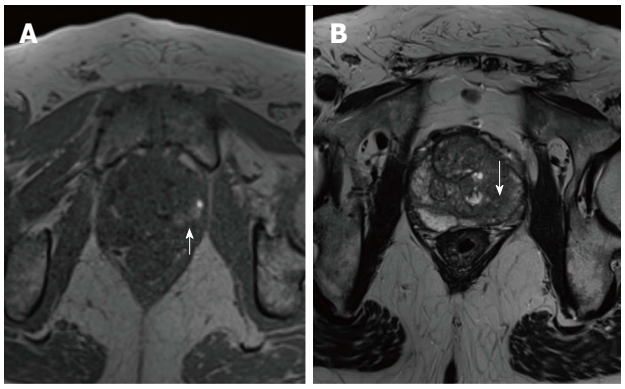

Figure 2 T1 (A) and T2-weighted images (B) of the midgland of the prostate. The left peripheral zone has a hyperintense area (white arrow, A) in T1-weighted image that correlates with a hypointensity (white arrow in B) in T2-weighted image, suggestive of bleeding in a patient with a recent transrectal ultrasound prostate biopsy.

limitation of the T2W sequence is that benign and malignant alterations often overlap. According to the Prostate Imaging Reporting and Data System (PIRADS)

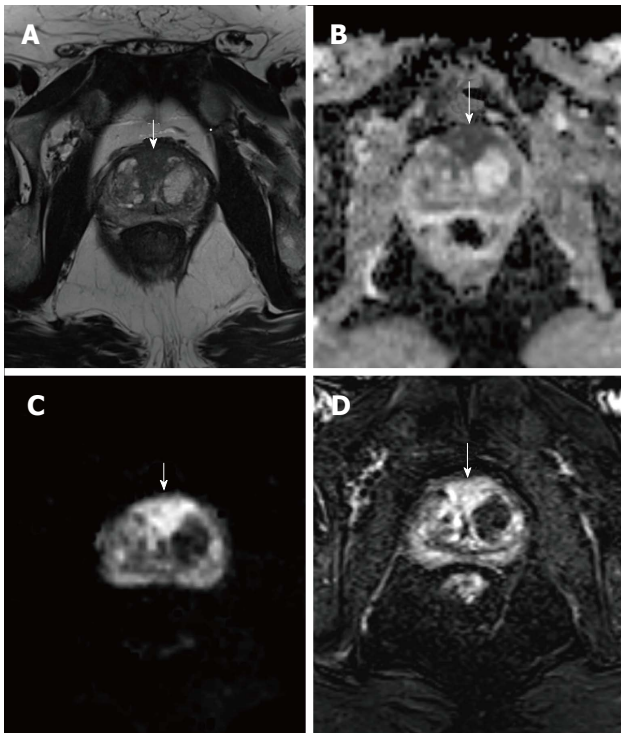

Figure 3 A 71-year-old patient. A: T2-weighted axial image at the level of the midgland of the prostate shows a hypointense nodular lesion at the transitional zone/anterior fibromuscular stroma, with a diameter of 26 mm (arrow); B and C: ADC map (B) and DWI image (C) show a marked hypo- and hyperintensity, respectively, in relation to restriction of diffusion (white arrows); D: DCE image with significant enhancement of the lesion (arrow). The characteristics of the nodule are compatible with a PIRADS 5 lesion and the marked restriction of the diffusion suggests a high-grade clinically significant prostate carcinoma, confirmed by the results of a MRI-guided transrectal ultrasound prostate biopsy (Gleason 4 + 4).

v2 model (see PIRADS reporting and interpretation model, version 2), the T2W sequence is key to the diagnosis of PCa in the transition zone[11] (Figure 3A). It is also useful in the diagnosis of local dissemination[14].

FUNCTIONAL SEQUENCES

Diffusion sequences

Diffusion sequences [diffusion-weighted MRI (DW-MRI) and apparent diffusion coefficient (ADC)] are performed in the axial plane and include the prostate and seminal vesicles (Figure 3B and C). These sequences are used primarily to evaluate the movement of the free water molecules in the interstitial space and through the cellular membrane. The behavior of lesions on DW-MRI and ADC is conditioned by cell density, the extracellular space, the integrity of cell membranes, and the extent of glandular organization. A correct assessment is based on a qualitative (high *b*-value DWI) and quantitative (ADC map) evaluation of the images. In PCa, the presence of impeded diffusion appears as a high signal intensity on the DWI and low intensity on the ADC map[13].

PCa presents architectural changes that restrict water diffusion. The more aggressive the tumour, the more pronounced these changes tend to be. For this

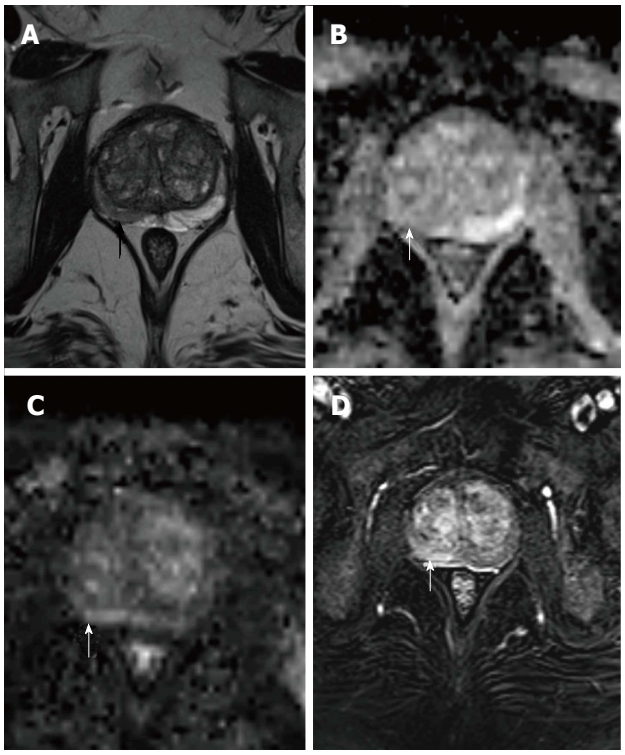

Figure 4 Prostate magnetic resonance imaging of a 64-year-old patient. A: T2-weighted image shows an area of hypointensity in the right peripheral zone of the midgland of the prostate (black arrow); B: A discrete restriction of diffusion in ADC map (white arrow); C: DWI image (white arrow); D: Enhancement on DCE image (white arrow), suggestive of prostatitis, confirmed by transrectal ultrasound biopsy.

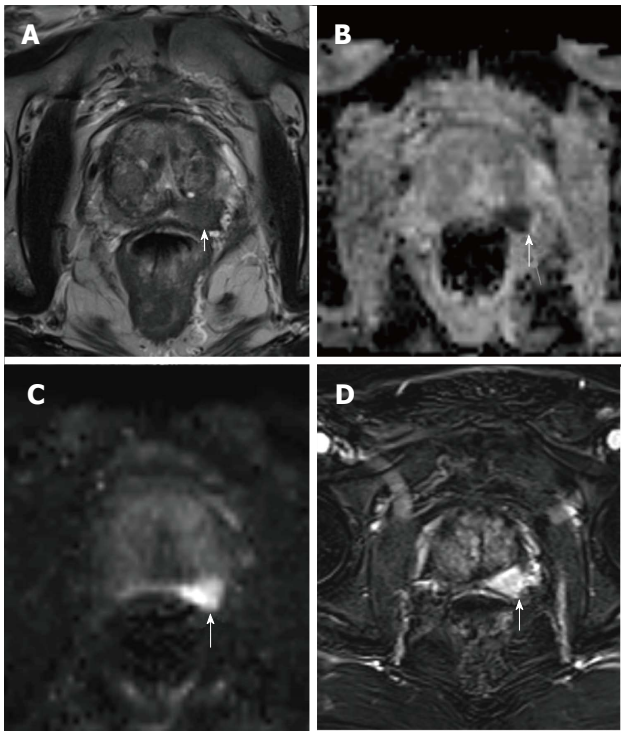

Figure 5 An 82-year-old patient with PIRADS 5 lesion in the basal left peripheral zone showing hypointensity (white arrows) in T2-weighted image (A), restriction of diffusion in apparent diffusion coefficient map (B) and diffusion-weighted magnetic resonance image (C), DCE shows significant early enhancement of the suspicious area (D). MRI-guided transrectal ultrasound biopsy confirmed a clinically significant prostatic carcinoma (Gleason 5 + 4). DCE: Dynamic contrast enhancement; MRI: Magnetic resonance imaging.

reason, diffusion sequences are valuable not only to characterise lesions likely to be malignant, but also to help to predict the Gleason score of the lesions[15,16] (Figure 3). Benign lesions, such as those occurring secondary to prostatitis, usually present less diffusion restriction (Figure 4)[17].

According to the PIRADS v2 model, diffusion sequences are crucial to the diagnosis of PCa in the peripheral zone and in characterising indeterminate lesions in the transition zone[12] (Figure 5B and C).

Dynamic contrast-enhanced sequences

Dynamic contrast-enhanced (DCE)-MRI is performed in the axial plane and includes the prostate and seminal vesicles. This sequence is performed prior to endovenous gadolinium administration and up to 4 min afterwards (Figure 5D).

In malignant lesions, the most common phenomenon observed on DCE-MRI is early uptake of the contrast material and early washout (Figure 5D). However, this behaviour is relatively variable and sometimes overlaps with that of benign lesions. According to PIRADS v2, although DCE sequences have a secondary value, their main value is in their contribution to the characterization of indeterminate lesions in the peripheral zone[12].

Pharmacokinetic models of DCE-MRI perfusion allow us to quantify various parameters to evaluate contrast perfusion, including k^{trans} (the volume transfer constant, which reflects the efflux rate of gadolinium contrast from the vascular compartment through the endothelium to the interstitial space), k_{ep} (rate of return to the vascular space), and Ve (the fractional volume of the extracellular tumour space). Using these data, it is possible to build parametric maps that represent the intratumoral heterogeneity of the spatial distribution of these parameters. However, at present, no conclusive results are available to support the use of these parameters for diagnostic purposes.

PIRADS ACQUISITION, INTERPRETATION, AND REPORTING MODEL, VERSION 2.0

A consensus-based model - PIRADS v2 - has been developed for interpreting and scoring mpMRI results. Several organizations, including the European Society of Urogenital Radiology (ESUR), American College of Radiology (ACR), and the AdMeTech Foundation, participated in the development of this model. The main objective of the model - aside from standardizing acquisition, interpretation and reporting protocols - is to predict the probability of clinically significant PCa by hierarchically organizing the information obtained in each MRI sequencing modality according to whether the lesion is located in the peripheral or transitional zone[11,12]. Although this model has some limitations, its implementation has served to reduce intra- and inter-

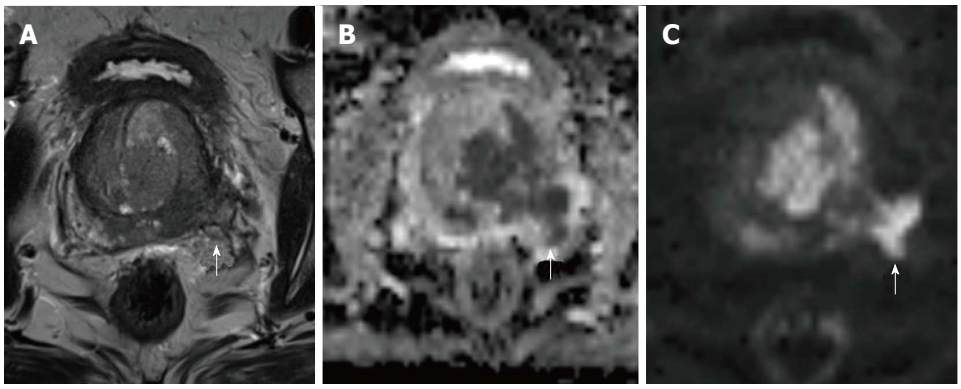

Figure 6 Prostate carcinoma with extracapsular extension in a 72-year-old patient. A: T2-weighted axial image of the pelvis at the level of midgland of the prostate shows a marked hypointensity in the left peripheral zone and disruption of the "capsule", distorting the normal anatomy of the left neurovascular bundle with measurable extracapsular extension (ESUR Score 5); B and C: ADC map (B) and DWI-images (C) demonstrate a significant restriction of diffusion, with hypointensity in the ADC and hyperintensity in the DWI-images that extend beyond the prostate "capsule" (arrows). Surgical specimen confirmed a pT3a prostate carcinoma. ADC: Apparent diffusion coefficient; DWI: Diffusion-weighted magnetic resonance imaging.

Table 1 European Society of Urogenital Radiology scoring of extracapsular extension in prostate cancer

Findings	Score
Abutment	1
Irregularity	3
Neurovascular bundle thickening	4
Bulge, loss of capsule	4
Measurable extra-capsular disease	5

observer variability as well as to increase the diagnostic yield of mpMRI.

EQUIPMENT

The minimum technological requirements necessary to guarantee that mpMRI assessment is performed to an acceptable quality standard have been defined by consensus agreement[11]. This consensus establishes, as a minimum requirement, that MRI equipment should be at least 1.5T magnetic field strength. The use of endorectal coil is only essential in older 1.5T equipment because newer MRI equipment at 1.5T and 3T are both capable of obtaining reliable image quality without the need for coils. The elimination of the need for endorectal coils is beneficial because it reduces imaging time, thus increasing patient comfort[11].

STAGING

Local staging

MRI is the imaging technique of choice to determine whether the tumor is organ-confined or extra-glandular. In the year 2012, the ESUR proposed a 5-point scale to establish the probability of extracapsular extension (ECE) based on direct and indirect signs[14] (Table 1 and Figure 6).

Schieda *et al*[18] evaluated the ability of this 5-point scoring system to predict ECE compared to a "non-standardized" reporting modality. Those authors concluded that the optimal sensitivity/specificity was achieved with a score of "≥ 3". In addition, the scale was more sensitive than the non-standardized modality (59.5% *vs* 24.5%, P = 0.01) without significant differences in specificity (68.0% *vs* 75.0%, P = 0.06).

Several clinical nomograms are available to predict the likelihood of ECE. The two most common nomograms are the Partin tables (which include several variables: PSA, biopsy-based Gleason score, and clinical stage)[19] and the nomogram developed at the Memorial Sloan-Kettering (MSK) Cancer Center (which adds prostate biopsy results - specifically, the percentage of positive cylinders)[20]. Recently, Feng *et al*[21] conducted a retrospective study of 112 patients who underwent mpMRI prior to RP to determine if mpMRI could improve the predictive capacity of the Partin tables and the MSK nomogram for ECE. The authors found that the area under the curve (AUC) for the Partin and MSK nomograms for predicting ECE was 0.85 and 0.86, respectively. When mpMRI was added, the AUC increased, respectively, to 0.92 and 0.94.

In the most recent guidelines published by the European Society of Urology, mpMRI has been included as a local staging technique in the following patient risk groups: High-risk disease; intermediate risk disease with predominantly Gleason pattern 4; and low-risk disease if mpMRI is considered necessary for treatment planning[11].

Staging of distant disease

The diagnosis of metastatic disease is essential to ensure proper therapeutic management and MRI has proven its value as a diagnostic tool for metastasis (Figure 7). In a recently published meta-analysis, Shen *et al*[22] compared the relative utility of choline PET/CT, MRI, and bone scintigraphy in the diagnosis of bone metastasis in patients with PCa. The authors reported sensitivity values, respectively of 91%, 97%, and 79% and specificity values of 99%, 95%, and 82%. These differences between these imaging modalities

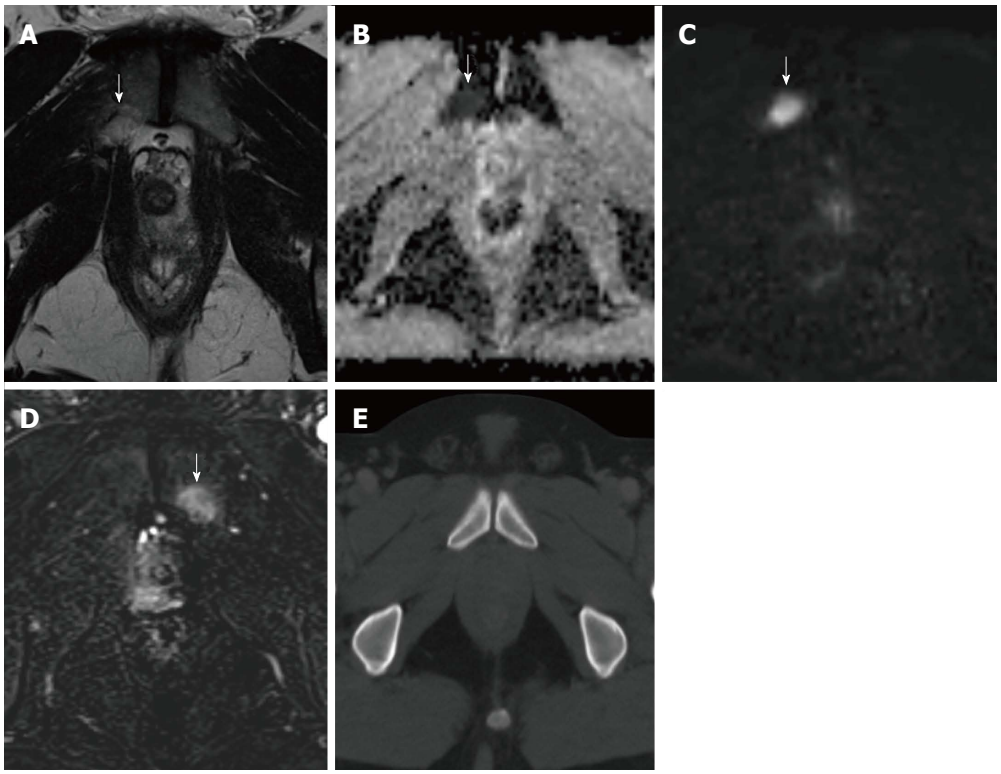

Figure 7 A 54-year-old patient with recently diagnosed prostate carcinoma. A: T2-weighted axial image of the pelvis shows an ill-defined hyperintense area in the right pubis (arrow); B and C: ADC map (B) and DWI images (C) show restriction of diffusion in the same area (arrow); D: DCE image presents enhancement of the lesion after IV gadolinium (arrow). MRI findings were suspicious for pelvic bone metastasis, with no evidence of such lesion on staging abdomino-pelvic CT scan performed a week earlier (E) and in a previous bone scintigraphy (not shown). Bone metastasis was confirmed on clinical evolution. MRI: Magnetic resonance imaging; DCE: Dynamic contrast-enhanced; CT: Computed tomography.

were statistically significant, with bone scintigraphy significantly less sensitive and less specific than the other two techniques. By contrast, MRI presented the highest diagnostic sensitivity for detecting metastasis.

Lecouvet *et al*[23] evaluated 100 patients at a high risk of metastasis to compare the diagnostic yield of whole-body DW-MRI *vs* CT and bone scintigraphy with Technetium Tc 99m (supported by simple X-ray when necessary) in the diagnosis of bone and nodal metastasis. Bone scintigraphy (± X-ray) plus CT had a sensitivity of 84% *vs* 91%-94% for whole-body DW-MRI ($P < 0.05$); specificity values were, respectively, 94%-97% *vs* 91%-96% ($P > 0.05$). The authors conclude that one-step, whole-body MRI can effectively assess nodal and bone metastasis in patients with high-risk PCa, thus eliminating the need for multimodal diagnosis (Figure 8).

Conde-Moreno *et al*[24] compared whole-body DW-MRI to choline PET/CT in the diagnosis of metastatic disease, finding that choline PET/CT had a greater sensitivity whereas whole-body DW-MRI had a greater specificity. Given these findings, the authors conclude that these techniques are complementary.

The value of MRI in the diagnosis of bone and nodal metastasis has been recognized by the ESUR[14]. The European Society of Urology also contemplates the use of MRI as an alternative technique to detect possible metastasis in intermediate and high-risk patients[11].

Re-staging following recurrence after radiotherapy

Reported 5-year biochemical relapse rates after radiotherapy range from 15% in low-risk patients to 67% in high-risk cases[9]. Both DW-MRI and DCE-MRI allow us to detect recurrences after radiotherapy. In a group of 24 patients who developed biochemical relapse following radiotherapy, Kim *et al*[25] performed prostate mpMRI at 3T (phased array coil), followed by TRUS-guided biopsy. They assessed the diagnostic performance of both DW-MRI and DCE-MRI to detect recurrent disease. The sensitivity, specificity, and diagnostic accuracy of DW-MRI were 49%, 93%, and 82%, respectively, *vs* 49%, 92% and 81% for DCE-MRI. Combined DW-MRI and DCE-MRI resulted in a sensitivity, specificity, and diagnostic accuracy of 59%, 91% and 83%, respectively.

Tamada *et al*[26] demonstrated the diagnostic value of mpMRI to assess recurrence after brachytherapy, reporting a sensitivity of 77%, specificity of 92%, and diagnostic accuracy of 90%.

IMPACT OF MPMRI ON TREATMENT DECISIONS FOR RADIOTHERAPY

Impact on the therapeutic strategy (EBRT)

Several studies have evaluated the impact of mpMRI staging on PCa risk group classification and on treatment decisions for EBRT (Table 2). Couñago *et al*[6] assessed

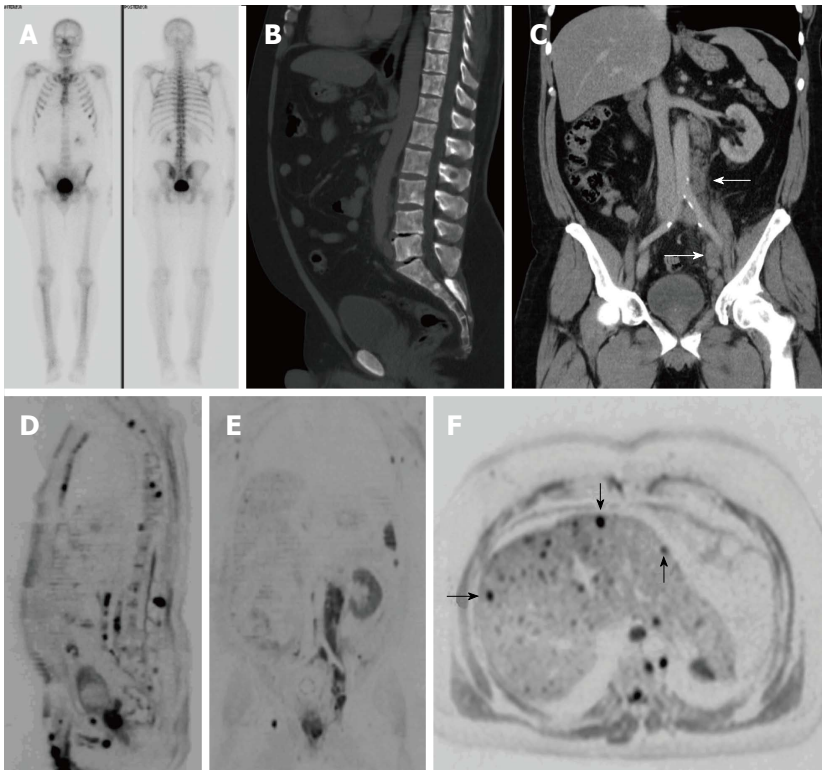

Figure 8 A 51-year-old patient with a history of Gleason 4 + 5 prostate carcinoma treated with hormone therapy. A: Re-staging bone scintigraphy was negative for bone metastases; B and C: Sagittal (B) and coronal (C) images of abdominal CT scan show a diffuse axial bone altered density that could not rule out bone metastases, along with multiple retroperitoneal and pelvic enlarged nodes suggesting malignant adenopathies (white arrows in C); D and E: Sagittal (D) and coronal (E) whole-body MRI images clearly show multiple bone metastases and adenopathies, along with hepatic nodules (black arrows in F, axial abdominal MRI) suspicious for metastatic disease. MRI: Magnetic resonance imaging.

274 patients staged initially by DRE and TRUS and subsequently by 3T mpMRI prior to the final EBRT treatment decision. The risk group classification shifted after mpMRI in 32.8% of cases after all factors (PSA, Gleason score and T-stage) were considered. In addition, in 43.8% of cases (52.5% depending on criteria used to indicate or not ADT in intermediate-risk patients), this led to a change in some aspect(s) of the radiotherapy treatment (treatment volume, dose, and ADT). Finally, the mpMRI results were validated in the subgroup of surgical patients, showing a 70.0% sensitivity and 93.8% specificity for ECE.

Panje *et al*[8] evaluated 122 patients staged using 1.5T or 3T (38% of sample) phased-array-body coil MRI. Most (53.3%) patients had received ADT prior to the mpMRI. The authors found that the use of mpMRI resulted in risk group modification in 28.7% of cases. Because the influence of 1.5T MRI and the use of ADT prior to mpMRI on the results is not known, it is difficult to perform a direct comparison with other studies.

Liauw *et al*[27] evaluated the role of endorectal coil mpMRI at 3T prior to EBRT in a group of 122 PCa patients, finding that mpMRI resulted in a change in therapeutic approach (indication for active surveillance, brachytherapy in monotherapy and dose modification, treatment volume, and use of ADT in EBRT) in 18% of patients. Recently, Pullini *et al*[28] prospectively evaluated 44 patients with PCa to determine the impact of mpMRI at 3T on staging and treatment decisions for EBRT, finding that staging by mpMRI resulted in a change in risk group classification in 41% of patients, thus potentially impacting the EBRT treatment decision.

Based on the studies described above, it is clear that mpMRI staging has a significant impact on radiotherapy treatment decisions, with risk group modifications ranging from 18% to 41% of patients, depending on the study. This wide variability may be attributable to numerous different factors, including the following: The MRI (magnet and coil, protocol used, experience of the radiologist); the initial clinical staging (experience of the clinician with DRE/TRUS, the use of CT to evaluate pelvic lymph nodes, *etc.*); the clinical characteristics of the patient cohort; the treatment protocol at each centre (dose, fractionation, target volume, indication for ADT, use of brachytherapy, *etc.*); the use of ADT prior to MRI; and finally the inclusion (or not) of metastatic patients in the final results.

Despite this heterogeneity, one finding common to all these studies is that a large percentage of patients staged by mpMRI are upstaged compared to conventional clinical staging. As a consequence, mpMRI staging implies that more patients will be classified as intermediate risk, high-to-very high risk, or metastatic patients. Nevertheless, it is worth noting that risk group downgrading has been reported in a small percentage (4%-12%) of patients[8,28].

Despite the clear influence of mpMRI staging on EBRT treatment decisions, numerous questions remain unresolved. For instance, we do not know which patient groups would benefit most, in terms of cost-effectiveness, from mpMRI staging. Nor is it clear if changes in therapeutic management based on MRI findings will increase survival and/or quality of life in these patients. The clearest example of this can be seen in low-risk patients in which upstaging after mpMRI is common (20%-50%) even though long-term biochemical control

Table 2 Studies evaluating the impact of the staging using magnetic resonance imaging in prostate cancer patients treated with radiotherapy

Ref.	Year	Type of MRI	No. of patients	Field strength	Coil	Tumor stage shift (%)	Risk group changes (%)	Change in RT (CTV, doses, HT) (%)	Technique validation
Jackson *et al*[75]	2005	Morphological	199	1.5T	PAB	55	NR	32.6[2]	No
Couñago *et al*[76]	2014	Multiparametric	103	3T	PAB	94.1	33.9	33.9	Yes
Chang *et al*[77]	2014	Morphological	115	1.5T	PAB	68.6	7	20[1]	No
Panje *et al*[8]	2015	multiparametric	122	1.5T and 3T	PAB	55.7	28.7	30	No
Horsley *et al*[78]	2015	Morphological	509	1.5T	PAB	20	9	18	No
Yamaguchi *et al*[79]	2015	Morphological	157	1.5T	PAB	25	9	8[1]	No
Couñago *et al*[6]	2015	Multiparametric	274	3T	PAB	90.4	32.8	43.8 or 52.5[3]	Yes
Pullini *et al*[28]	2016	Multiparametric	44	3T	PAB	65.9	40.9	NR	No
Liauw *et al*[27]	2016	Multiparametric	122	3T	PAB	NR	18	22	No

[1]Exclusive assessment of the CTV change; [2]Data from T1-T2 to T3-T4 upstaging; [3]Values according to the HT criteria in intermediate-risk patients. PAB: Phased-array-bodycoil; NR: Not reported; CTV: Clinical target volume; HT: Hormonal therapy.

in this risk cohort (staged exclusively by conventional DRE) and treated with EBRT is excellent (93% at 10 years' follow up)[29]. Therefore, we must exercise caution with regard to the changes in tumour stage that can result from the use of these newer, more precise imaging tests. In this sense, more prospective, multicentric studies are needed to better clarify the role of mpMRI prior to EBRT.

Contouring and treatment planning in EBRT

MRI has proven to be highly useful in radiation oncology in improving the accuracy of treatment volume delineation. The use of MRI allows for more precise identification of the prostate gland location and thus more accurate contouring, especially of the prostate apex, which can help to avoid over- or under-estimation of the microscopic volume that commonly occurs with CT-based contouring[30].

mpMRI can also help to rule out the presence of high-grade disease in the transition zone at the anterior base, thus allowing for lower doses to the bladder neck. In addition, MRI is highly useful in identifying the anatomic structures involved in erections: The internal pudenda artery, the periprostatic nerve fibers, and the penile bulb. The improved anatomic definition of these structures with mpMRI could help to limit the radiation dose to these areas, which could potentially lead to higher rates of erectile function preservation and, consequently, better quality of life[30]. Therefore, compared to CT-based treatment volumes, MRI allows for the delineation of a smaller clinical target volume (CTV), distinguishing the CTV from normal tissue, suggesting that MRI-based contouring can reduce treatment-related toxicity in PCa[8].

The reliability of mpMRI in tumour staging plays an increasingly important role in advanced radiotherapy techniques such as intensity modulated radiotherapy (IMRT) and volumetric modulated arc therapy (VMAT). In these highly-conformal techniques with narrow treatment margins, it is vitally important to accurately detect the presence of extracapsular disease or seminal vesicle invasion and to include these areas within the treatment volume to avoid geographic loss and, consequently, underdosing[8].

DETECTION OF RELAPSE AFTER RADICAL PROSTATECTOMY

PSA levels become undetectable after RP; however, depending on the pathological stage and other risk factors, up to 60% of patients with PCa will eventually develop biochemical relapse (defined as PSA > 0.2 ng/mL with two consecutive rises)[31].

Current cancer treatment guidelines recommend SRT after RP if the PSA remains elevated or if biochemical relapse is detected during follow-up[32]. In both of these clinical scenarios, the elevated PSA may be secondary to local disease (associated or not with a significant risk of metastasis) and/or distant disease. Although SRT is one of the potentially curative treatments in these cases, with cause-specific survival rates up to 3 times greater than observation alone[33], up to 50% of patients will develop a recurrence in the 10 years following SRT. These poor outcomes may be due to a variety of factors, including: (1) tumour-related factors associated with a greater risk of biochemical progression and worse disease-free survival, including the following: Initial PSA; Gleason 8; involvement of the seminal vesicles; infiltrated resection margins; early biochemical relapse; and PSADT; (2) delayed initiation of SRT when the PSA is > 1 ng/mL and palpable disease is evident on DRE; and (3) SRT-related factors (which merit more research to define the optimal therapeutic strategy for disease control): If the exact location of the relapse site is unknown, this can result in an inadequate CTV, underdosing, and the need for systemic treatment.

SRT is most effective when administered before the PSA reaches 0.5 ng/mL[34]. However, with such low PSA levels, conventional imaging tests - TRUS, bone scintigraphy, and CT - are of little use in detecting the recurrence. In recent years, mpMRI has become more widely used in the detection of local recurrences. Indeed, it is the only imaging technique recommended by the ESUR to evaluate pelvic recurrences in patients

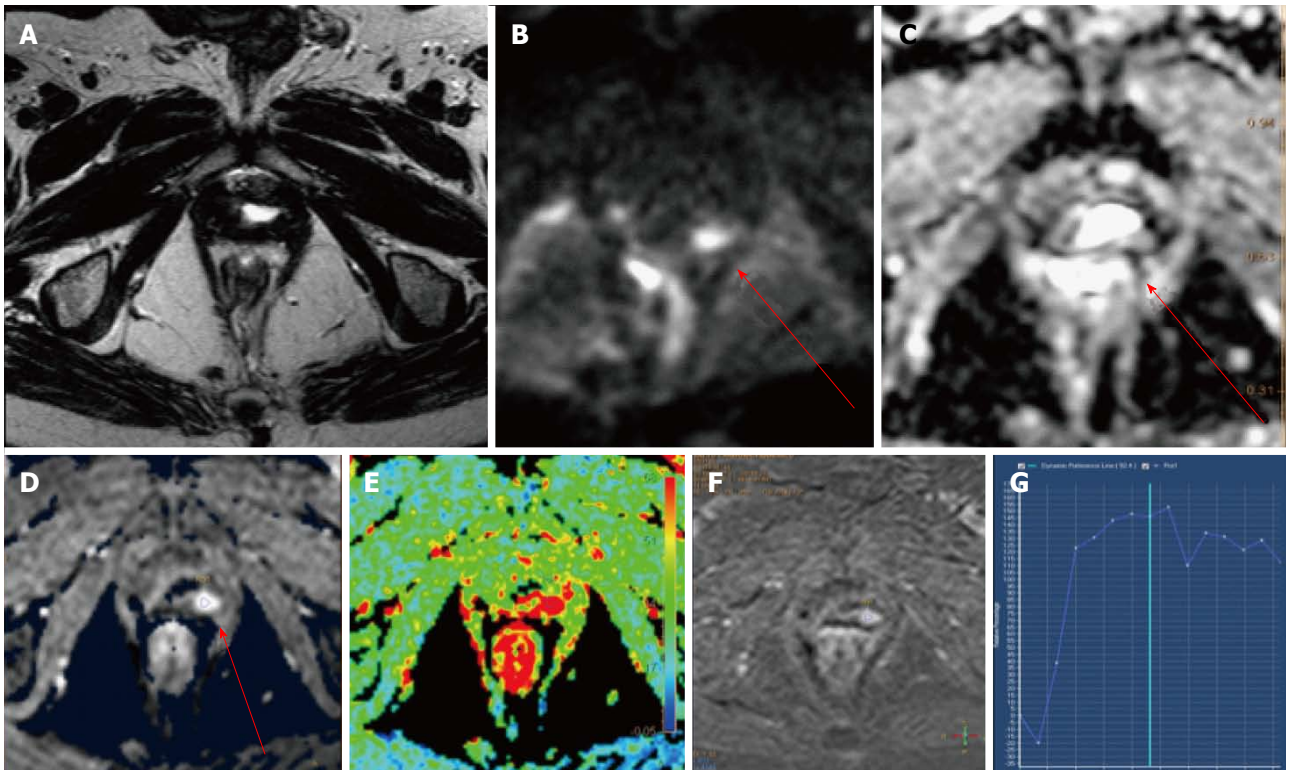

Figure 9 Multiparametric magnetic resonance imaging of local recurrence. A: Morphological study: Axial T2-weighted fast-spin echo image. No recurrence is detected; B-F: Functional study; B and C: Axial diffusion sequences showing restricted diffusion in ADC map (arrow); D-G: Axial gradient-echo T1-weighted images showing focal enhancement in dynamic study (D) (arrow), color map (E) and curve (G).

with low (0.2-2 ng/mL) PSA levels[14].

Although no protocol has yet been established for the use of MRI in SRT, in most published studies the approach does not differ from that used to assess the prostate in cases with a suspected tumour or local dissemination.

In local relapse, the most common patterns observed on MRI are slightly hyperintense lesions on T2W sequences and hypervascular lesions on the DCE-MRI. Cirillo *et al*[35] showed that adding DCE to T2W sequences improved detection rates for local relapse after RP: Unenhanced and contrast-enhanced MRI yielded, respectively, the following outcomes: Sensitivity (61.4% *vs* 84.1%), specificity (82.1% *vs* 89.3%), positive predictive value (84.4% *vs* 92.5%), negative predictive value (57.5% *vs* 78.1%), and accuracy (69.4% *vs* 86.1%). In diffusion imaging, the radiological appearance of local recurrence may be similar to the tumour, with high signals in the diffusion sequences and low signals in the ADC map (Figure 9). PIRADS v2 is not applicable in local relapses, although it may be in the future.

Incidence of recurrences detected by mpMRI

Multiple authors have investigated the capacity of mpMRI to detect post-prostatectomy relapses, with detection rates ranging from 84%-95% in series that use endorectal coil MRI in patients with median PSA > 1 ng/mL, many of whom also had palpable disease on DRE[9,35,36]. Other studies carried out in patients with lower PSA levels (median PSA, 0.3-0.59 ng/mL) by endorectal coil MRI[37,38] and/or pelvic MRI have reported recurrence rates ranging from 24%-91%[39-42] (Table 3). This variability among studies is likely due to several factors, including: Retrospective study design; sample size and sample heterogeneity; differences in the radiologists' experience level; the use of different MRI sequencing modalities; and difference in relapse criteria.

Results from a retrospective study[43] and from a meta-analysis[44] suggest that the ^{11}C or ^{18}F-choline PET/CT has a higher detection rate for local, nodal, or metastatic recurrences post RP when the PSADT is < 6 mo and PSA values are > 1 ng/mL. By contrast, in patients with lower PSA values, mpMRI has proven to be more sensitive in detecting local recurrences[22,45-48] and in small-sized (< 10 mm) local relapses[47]. Other authors have found that diagnostic rates for visible pelvic relapses are higher when mpMRI and PET/CT are combined *vs* MRI or choline PET/CT alone[49-51]. Recently, studies that investigated the use of PET/CT with PSMA (prostate-specific membrane antigen) ligands have reported higher detection rates for local relapse compared to choline PET/CT, even in cases with low PSA levels[52]. However, other authors, including Freitag *et al*[53], have found that adding MRI to PET provides additional value even when 68Ga-PSMA-11 is used as a tracer in PET/CT.

Table 3 Studies on multiparametric magnetic resonance imaging with low prostate-specific antigen levels for the diagnosis of local recurrence after radical prostatectomy

Ref.	Year	Desing	Coil and Magnet	No. of patients	PSA (mean or median)	Mean or median lesion size	Reference standard	% rRL	MRI sequences, Se, Spe, PPV, NPV, Acc
Linder *et al*[38]	2007	Retrospective	PAC + ERC 1.5T and 3T	187	0.59 ng/mL	10 mm	TRUS biopsy, PSA reduction after SRT or increased lesion size on serial imaging	91	T2 + DCE Se: 91% Spe: 45% PPV: 85% NPV: 60%
Rischke *et al*[39]	2012	Retrospective	PAC 1.5T	33	0.30 ng/mL	11 mm	PSA reduction after SRT and changes in MRI after SRT	67	T2 + DCE Se: 67% Spe: 100% PPV: 100% NPV: 83% Acc: 83%
Liauw *et al*[37]	2013	Retrospective	ERC 1.5T and 3T	88	0.30 ng/mL	0.26 cc	None	24	T2 + DCE + DWI
Park *et al*[56]	2014	Retrospective	PAC 1.5T and 3T	113	0.43 ng/mL	12 mm	TRUS biopsy	NR	T2 + DCE
Verma *et al*[42]	2014	Retrospective	PAC 3T	90	0.20 ng/mL negative MRI 0.40 ng/mL positive MRI	NR	None	22.2	T2 + DCE + DWI
Couñago *et al*[40]	2015	Retrospective	PAC 3T	57	0.40 ng/mL	15.2 mm	None	24.6	T2 + DCE + DWI
Hernandez *et al*[41]	2015	Retrospective	PAC 3T	70	0.38 ng/mL	8.5 mm	None	47 (local + lymph nodes) 38.6 (local)	T2 + DCE + DWI

mpMRI: Multiparametric MRI; RP: Radical prostatectomy; rRL: Radiographic local recurrence; T2: T2-weighted imaging; DCE: Dynamic contrast-enhanced imaging; DWI: Diffusion-weighted imaging; Se: Sensitivity; Spe: Specificity; PPV: Positive predictive value; NPV: Negative predictive value; Acc: Accuracy; PAC: Phased-array coil; ERC: Endorectal coil; NR: Not reported, SRT: Salvage radiation therapy.

Clinical factors associated with mpMRI findings

Several factors, including PSA levels at recurrence, the PSADT, and the presence of compromised resection margins, have all been significantly associated with mpMRI findings. Several authors have defined pre-radiotherapy PSA cut-off values, ranging from > 0.3[37] to > 0.5[40] or ⩾ 0.54[39] ng/mL, as clinical predictors of MRI positivity. Eifler *et al*[19] reported a higher probability of visible local relapse in patients with PSADT > 14 mo. In addition, a PSADT < 6 mo has been associated with higher incidence of nodal relapse, even with PSA levels < 1 ng/mL. Hernández *et al*[41] reported a median PSADT of 5.12 mo in patients with nodal recurrence *vs* 12.7 mo in patients without evidence of nodal disease on MRI (P = 0.17). Finally, a study that used MRI lymphography (ferumoxtran-10) to assess nodal involvement found that patients with positive lymphography presented a median PSADT of 3.8 mo[54].

Topography of recurrences

Most local recurrences occur in the peri-anastomotic and retrovesical regions[41,55,56], although up to 22% of recurrences have been diagnosed at the resection site of the vas deferens[57].

Efficacy of mpMRI to detect nodal relapse

Data on the efficacy of MRI to detect nodal relapses after RP are scant, particularly in patients with low PSA values. However, an incidence between 5%-10% has been estimated[37,41], most commonly involving the external iliac lymph node chains. It has been suggested that DW-MRI could increase the detection of nodal relapses, with a reported 90% efficacy rate in nodes < 1 cm[58] (Figure 10).

The two studies that used MRI with ferromagnetic contrast (ferumoxtran-10) to evaluate patients with biochemical relapse after RP reported positive nodes (< 1 cm) in 72% and 20% of patients, respectively, even with low PSA levels[54,59]. The main limitation of these studies is that ferumoxtran-10 was authorized only for research purposes.

Detection of bone metastasis

Recent research suggests that the use of whole-body MRI (WB-MRI), together with WB-DW-MRI, may allow assessment of nodal recurrence and bone metastasis with a single imaging modality. These approaches show greater sensitivity and specificity than conventional imaging and will facilitate the evaluation and monitoring of response to systemic treatments[60].

IMPACT OF MPMRI ON SRT TREATMENT DECISIONS

The impact of mpMRI on the efficacy of SRT is not known, but use of mpMRI is becoming increasingly

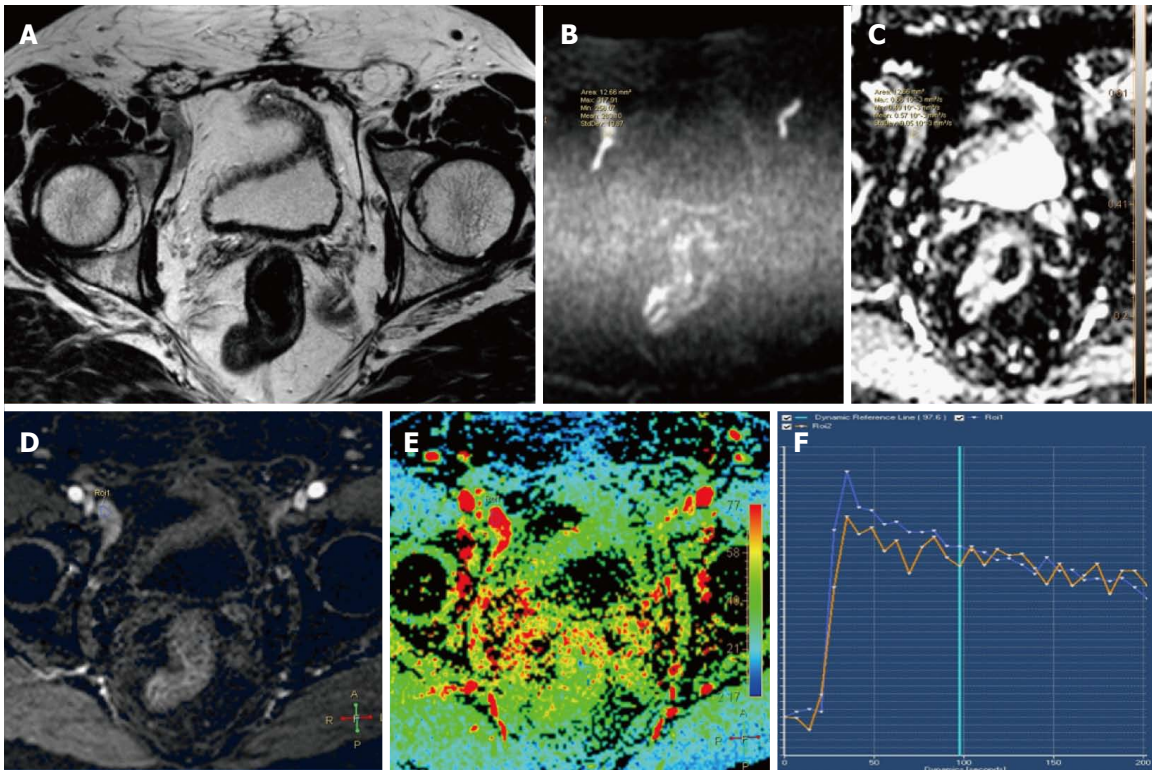

Figure 10 Lymph node study. A: Axial T2-weighted fast-spin echo image shows right external iliac lymph nodes; B and C: Axial diffusion sequences; C: Restricted diffusion in ADC map; D: Axial gradient-echo T1-weighted perfusion image showing a high peak enhancement; E: Color map; F: Curve; ADC: Apparent diffusion coefficient.

common in the evaluation of disease dissemination in patients with biochemical relapse after RP. The information provided by mpMRI is integrated into the decision-making process for SRT planning, as follows.

Definition of the CTV

Individualized treatment planning should assure that the relapse site is included within the CTV in accordance with published guidelines.

Irradiation (or not) of the lymph node stations: The benefits of elective pelvic irradiation in SRT is controversial since currently available data include only retrospective studies; however, findings from those studies suggest that pelvic irradiation increases both biochemical control[61] and biochemical relapse-free survival[62]. It has been suggested that RP may lead to changes in the lymphatic drainage pattern and that these are not adequately included in the CTV when contoured according to current recommendations[63,64].

Irradiation of oligometastatic bone disease: Currently, the decision to irradiate oligometastatic bone lesions is considered on an individual basis by consensus at multidisciplinary urological tumour boards, or in the context of a clinical trial.

It would be interesting to investigate the impact of irradiating only the nodal stations in patients with a short PSADT whose mpMRI images indicate the presence of nodal disease without prostate bed involvement. Similarly, it would also be interesting to conduct a study in which patients with a long PSADT (> 10 mo) and local disease alone received irradiation only to the local disease site. In both of these scenarios, the impact of ablative RT techniques such as stereotactic body radiotherapy (SBRT) should be investigated.

Dose escalation

We hypothesize that treating the prostatectomy bed at conventional doses while simultaneously increasing the dose to the relapse site detected on MRI could improve outcomes without increasing toxicity[65]. In fact, high dose irradiation delivered exclusively to the relapse site could be curative and this approach would also minimize irradiation of healthy tissue, thus reducing the risk of side effects.

Adjuvant systemic treatment

The addition of hormonotherapy to SRT remains controversial. In clinical practice, the trend is to administer combined treatment in patients with high-risk disease and/or poor prognostic factors. The RTOG 9601 study showed a significant increase in overall survival at 10 years in patients with post-RP biochemical relapse who were treated with SRT (64.8 Gy) plus bicalutamide 150 mg for 2 years *vs* patients who received RT alone [82% *vs* 78%, hazard ratio: 0.75 (95%CI: 0.58-0.98), P = 0.036][66]. The results of studies currently underway, such as RADICALS and RTOG 0534, should definitively determine the benefit of hormonotherapy in patients

who undergo postoperative radiotherapy. However, it should be pointed out that none of the aforementioned trials have included mpMRI or PET/CT for the purpose of diagnosing and locating tumour recurrences. As we have suggested above, more intensive treatment at the relapse site could improve SRT outcomes, with or without hormonotherapy.

FUTURE DIRECTIONS

The future of MRI in radiotherapy includes the following:

Monitoring response to radiotherapy

Various studies have shown the potential utility of ADC and K^{trans} to monitor response to radiotherapy in patients with PCa[67,68]. This application of MRI could be used to investigate the impact of new focal therapies administered early in patients with persistent disease or local relapse.

Technological advancements that increase detection rates [PET/MRI and new radiotracers (PSMA)]

PET/MRI is a new multimodal imaging technique that improves diagnostic imaging, with a promising future in the evaluation of PCa. In addition to diagnosis and staging, PET/MRI plays an important role in detecting recurrences in patients with biochemical relapse. In bone metastasis, the use of PET/MRI improves the detection and characterization of bone lesions, especially with the use of new radiotracers (^{18}F-FNa, PSMA, choline), providing functional information as well as greater anatomic information due to the incorporation of MRI[50,69,70]. These data are essential for ablative radiotherapy.

The future of imaging in PCa will be marked by improvements in equipment and in the sequences and antennas used, especially 3T equipment, diffusion sequences, and multichannel surface coils ($\geqslant$ 128 channels).

Implementation of MRI in the workflow of radiation oncology departments

It is worth highlighting the incipient but growing use of MRI in radiation oncology departments. MRI is used not only for simulations, workflow, and treatment planning, but it is also being incorporated into linear accelerators to guide radiotherapy treatment[71].

Genetic testing and MRI

At least one study has been conducted showing that MRI and genetic testing can improve the reliability for risk stratification in patients with PCa[72].

Guidelines for focal treatments

Several studies have assessed the role of MRI-guided dose escalation (EBRT or brachytherapy) to the dominant intraprostatic lesion[73,74].

CONCLUSION

The data obtained from mpMRI imaging are increasingly being integrated into PCa staging, recurrence detection, and therapeutic management. mpMRI is also being incorporated into the workflow of radiation oncology departments due to its capacity to help define target volumes for radical radiotherapy and SRT after prostatectomy. Technological advances, such as PET/MRI combined with new radiotracers such as PSMA, can improve staging and recurrence detection, and assist in planning more accurate treatments.

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MINIREVIEWS

Use of programmed cell death protein ligand 1 assay to predict the outcomes of non-small cell lung cancer patients treated with immune checkpoint inhibitors

Carmelo Tibaldi, Alice Lunghi, Editta Baldini

Carmelo Tibaldi, Alice Lunghi, Editta Baldini, Division of Oncology, Department of Oncology, S. Luca Hospital, 55100 Lucca, Italy

Author contributions: Tibaldi C, Lunghi A and Baldini E contributed equally to this work.

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Correspondence to: Dr. Carmelo Tibaldi, Division of Oncology, Department of Oncology, S. Luca Hospital, Via Guglielmo Lippi Francesconi 1, 55100 Lucca,
Italy. carmelo.tibaldi@uslnordovest.toscana.it
Telephone: +39-0583-055748
Fax: +39-0583-970161

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Abstract

The recent discovery of immune checkpoints inhibitors, especially anti-programmed cell death protein 1 (PD-1) and anti-programmed cell death protein ligand 1 (PD-L1) monoclonal antibodies, has opened new scenarios in the management of non-small cell lung cancer (NSCLC) and this new class of drugs has achieved a rapid development in the treatment of this disease. However, considering the costs of these drugs and the fact that only a subset of patients experience long-term disease control, the identification of predictive biomarkers for the selection of candidates suitable for treatment has become a priority. The research focused mainly on the expression of the PD-L1 receptor on both tumor cells and/or immune infiltrates determined by immunohistochemistry (IHC). However, different checkpoint inhibitors were tested, different IHC assays were used, different targets were considered (tumor cells, immune infiltrates or both) and different expression thresholds were employed in clinical trials. In some trials the assay was used prospectively to select the patients, while in other trials it was evaluated retrospectively. Some confusion emerges, which makes it difficult to easily compare the literature data and to translate them in practice management. This mini-review shows the possibilities and pitfalls of the PD-L1 expression to predict the activity and efficacy of anti PD1/PD-L1 monoclonal antibodies in the treatment of NSCLC.

Key words: Predictive biomarkers; Immunotherapy; Checkpoint inhibitors; Programmed cell death protein ligand 1; Non-small cell lung cancer

Core tip: Use of programmed cell death protein ligand 1 (PD-L1) assay to predict the outcomes of non-small cell lung cancer (NSCLC) patients treated with immune checkpoint inhibitors. This minireview underlines promises and pitfalls of the PD-L1 expression to predict the activity and efficacy of programmed cell death protein 1/PD-L1 inhibitors in NSCLC.

Tibaldi C, Lunghi A, Baldini E. Use of programmed cell death protein ligand 1 assay to predict the outcomes of non-small cell lung cancer patients treated with immune checkpoint inhibitors. *World J Clin Oncol* 2017; 8(4): 320-328 Available from: URL: http://www.wjgnet.com/2218-4333/full/v8/i4/320.htm DOI: http://dx.doi.org/10.5306/wjco.v8.i4.320

INTRODUCTION

Lung cancer is the leading cause of cancer-related deaths worldwide. Non-small cell lung cancer (NSCLC) accounts for more than 85% of primary lung cancers. Approximately two-thirds of NSCLC patients are diagnosed at an advanced stage and their prognosis remains poor[1].

The discovery of driver oncogene alterations such as epidermal growth factor receptor (EGFR) mutations, anaplastic lymphoma kinase (ALK) rearrangements, and identification of their targeted inhibitors, have dramatically improved the outcomes in highly selected patients[2,3]. Conversely, the last generation chemotherapy regimens date back more than 15 years and, unfortunately, the clinical results obtained with this strategy have reached a plateau.

The recent improvements in the knowledge of cancer immunoediting and the discovery of immune checkpoint inhibitors have led to new opportunities in the treatment of NSCLC and have paved the way to improve the outcomes for a considerable number of patients[4-6]. The immunoresponse, driven by T-lymphocytes, is regulated by a complicated balance between inhibitory checkpoints and activating signals. Some key immune checkpoint proteins have been identified: Cytotoxic T-lymphocytes antigen 4 (CTLA-4) and programmed death-1 (PD-1). In the priming phase, which occurs in lymph-nodes, the CTLA-4 receptor, located on the surface of the lymphocyte T cells binds the B7-receptor on the cellular membrane of the dendritic cell. In the effector phase, which occurs peripherally, the PD-1 located on the cellular membrane of lymphocyte T cells, binds programmed cell death protein ligand 1 (PD-L1) and PD-L2, which are expressed by tumor cells, stromal cells, or both. These observations have led to the development of a monoclonal antibody-directed against CTLA4 and PD1/PD-L1 proteins such as ipilimumab (anti-CTLA-4), nivolumab and pembrolizumab (anti PD1), atezolizumab, durvalumab, avelumab (anti-PD-L1). These new classes of drugs have gained a rising development in the treatment of NSCLC: So far, nivolumab, pembrolizumab and atezolizumab have been approved by the Food and Drug Administration for second-line treatment of advanced NSCLC. In this setting, all the above-mentioned drugs have shown a clear superiority in terms of activity and efficacy compared to standard chemotherapy. However, although well tolerated, these new drugs are highly effective only in a limited subset of patients; this fact, together with the high economic impact, has evidenced the need to identify of biomarkers able to select patients with the highest likelihood of benefit[7]. The attention of researchers and clinicians has focused mainly on the expression of PD-L1 on tumor cells and/or immune infiltrates determined by immunohistochemistry (IHC), since this protein seems to be critical in the PD-1/PDL-1 pathway. Unfortunately, the heterogeneity of tests, targets and scores has produced conflicting results in the literature.

ANTI-PD-1 ANTIBODIES

Nivolumab beyond first-line treatment

In a pivotal phase Ⅰ study published by Gettinger *et al*[8], 296 patients with advanced solid tumors, including 122 NSCLCs, were treated with an escalating dose of anti PD-1 antibody (BMS-936558). PD-L1 expression was evaluated by using a non-commercial anti PD-L1 monoclonal antibody (5H1) in formalin-fixed tumor specimens and fresh tumor tissues. Positivity was defined as $\geqslant$ 5% tumor cell membrane staining in a minimum number of 100 evaluable cells. PD-L1 expression was retrospectively evaluated in 10 patients: None of the 5 patients with negative tumors had an objective response whereas 1 out of 5 patients bearing positive tumors responded to treatment. This phase Ⅰ trial has been recently updated by recruiting an additional 129 patients who reported an overall response rate of 17%. A total of 68 samples were retrospectively tested for PD-L1 expression: Patients with positive tumors achieved an overall response rate of 15%, a median progression free survival (mPFS) of 3.3 mo (95%CI: 1.8-7.5), and a median overall survival (mOS) of 7.8 mo (95%CI: 5.6-21.7). Patients with negative tumors achieved an objective response rate of 14%, an mPFS of 1.8 mo (95%CI: 1.7-2.3), and a mOS of 10.5 mo (95%CI: 5.2-14.8). Responses were obtained regardless of histology (squamous or non-squamous), EGFR and KRAS status, PD-L1 positivity or negativity. Conversely, a smoking history seemed to be an interesting parameter: patients smoking more than 5 pack-years did much better (overall response rate of 30% *vs* 0% for < 5 pack-years). One intriguing observation, subsequently confirmed, was that some patients, who discontinued therapy for toxicity, maintained clinical remission in the absence of more than 9 months' treatment (Table 1).

In the CheckMate 063 multicenter phase Ⅱ study the nivolumab 3 mg/kg q 14 activity was evaluated in heavily pre-treated advanced squamous cell carcinoma of the lung[9]. The patient population was highly refractory to chemotherapy, with almost two-thirds having previously received three or more systemic treatments. A total of 117 patients were enrolled: The overall response rate, evaluated by an independent radiology review Committee, was 14.5% (95%CI: 8.7-22.2). Seventy-six tumors were retrospectively assessed for PD-L1 expression on formalin-fixed, paraffin-embedded (FFPE)

Table 1 Correlation between nivolumab activity and outcome and programmed cell death protein ligand 1 immunohistochemestry score

Author/study	Marker antibody	Tumor type	Treatment line	PD-L1 cutoff	N pts	Response (%)	mPFS mo (95%CI)	mOS mo (95%CI)
Nivolumab								
Gettinger *et al*[8]	Dako 28-8	NSCLC	> 2	⩾ 5 %	33	15	3.3 (1.8-7.5)	7.8 (5.6-21.7)
Phase Ⅰ				< 5%	35	14	1.8 (1.7-2.3)	10.5 (5.2-14.8)
Rizvi *et al*[9] CM 063	Dako 28-8	Squamous	⩾ 2	⩾ 5%	25	24	NR	NR
Phase Ⅱ		NSCLC		< 5%	51	14	NR	NR
Brahmer *et al*[10]	Dako 28-8	Squamous	> 1	⩾ 10%	36	19	3.7 (NR)	11 (NR)
CM 017		NSCLC		< 10%	81	16	2.3 (NR)	8.2 (NR)
Phase Ⅲ				⩾ 5%	42	21	4.8 (NR)	10 (NR)
				< 5%	75	15	2.2 (NR)	8.5 (NR)
				⩾ 1%	63	17	3.3 (NR)	9.3 (NR)
				< 1%	54	17	3.1 (NR)	8.7 (NR)
Borgheai *et al*[11]	Dako 28-8	Non squamous	> 1	⩾ 10%	86	37	5.0 (NR)	19.9 (NR)
CM 057		NSCLC		< 10%	145	11	2.1 (NR)	9.9 (NR)
Phase Ⅲ				⩾ 5%	95	34	5.0 (NR)	19.4 (NR)
				< 5%	136	14	2.1 (NR)	9.8 (NR)
				⩾ 1%	123	31	4.2 (NR)	17.7 (NR)
				< 1%	108	9	2.1 (NR)	10.5 (NR)
Gettinger *et al*[12]	Dako 28-8	NSCLC	1	⩾ 50%	12	50	NR	NR
CM 012				< 50%	34	15	NR	NR
Phase Ⅰ				⩾ 25%	18	44	NR	NR
				< 25%	28	11	NR	NR
				⩾ 10%	20	40	NR	NR
				< 10%	26	12	NR	NR
				⩾ 5%	26	31	NR	NR
				< 5%	20	15	NR	NR
				⩾ 1%	32	28	NR	NR
				< 1%	14	14	NR	NR
Rizvi *et al*[13] CM012	Dako 28-8	NSCLC	1	⩾ 1%	23	48	6.0 (< 0.1 ± 21.8)	20.2 (6.2-28.8+)
phase Ⅰ				< 1%	21	43	5.2 (0.9 ± 28.7+)	19.2 (4.5-29.7+)
Socinski *et al*[14]	Dako 28-8	NSCLC	1	⩾ 5%	NR	76.80	NR	NR
CM 026				< 5%	NR	NR	NR	NR
phase Ⅲ				⩾ 25%	NR	48.70	NR	NR
				< 25%	NR	NR	NR	NR
				⩾ 50%	NR	32.50	NR	NR
				< 50%	NR	NR	NR	NR
				⩾ 75%	NR	20.70	NR	NR
				< 75%	NR	NR	NR	NR

CM: CheckMate; NR: Not reported; pts: Patients; NSCLC: Non-small cell lung cancer; PD-L1: Programmed cell death protein ligand 1; mPFS: Median progression free survival; mOS: Median overall survival.

specimens with a commercially validated, automated immunohistochemical assay (Dako, Carpinteria, CA, United States) by using a 28-8 clone (rabbit anti-human PD-L1) with a 5% expression threshold to define PD-L1 positivity. Response rates were 24% and 14% in patients with positive *vs* negative tumors respectively (Table 1).

In the CheckMate 017 phase Ⅲ trial a total of 272 pre-treated patients with advanced squamous lung tumors were randomized to receive 3 mg/kg of nivolumab every 2 wk or 75 mg/m^2 of docetaxel every 3 wk. The primary end-point was overall survival OS[10]. This pivotal trial demonstrated a statistically and clinically significant survival advantage in favor of immunotherapy with a reduction in risk death of 41% [hazard ratio (HR) = 0.59, 95%CI: 0.44 to 0.79, P < 0.001]. The mOS was 9.2 mo (95%CI: 7.3 to 13.3) for nivolumab *vs* 6.0 mo (95%CI: 5.1 to 7.3) for docetaxel and the response rates were 20% and 9% respectively (P = 0.0008). PD-L1 protein expression was retrospectively evaluated in pretreatment tumor-biopsies with the Dako assay and the response rate was compared at pre-specified expression levels of 1%, 5% or 10%. The response rate was 17% in tumours with PD-L1 positivity ⩾ 1%; this rate of response was indistinguishable from that observed in PD-L1 negative specimens (< 1%). The response rate was 21% in tumors with PDL-1 positivity ⩾ 5% and 15% in tumors with PD-L1 < 5%. Ultimately, the response rates were 19% and 16% in PD-L1 positive tumors ⩾ 10% or < 10%, respectively (Table 1). It is noteworthy that the benefit of OS in this study was independent of the PD-L1 scores.

In the CheckMate 057 randomized phase Ⅲ trial, 582 pretreated advanced non squamous NSCLC patients received 3 mg/kg of nivolumab every 2 wk or 75 mg/m^2 of docetaxel every 3 wk[11]. Also in this study, the primary end-point was OS; mOS in the nivolumab arm was significantly longer than in the docetaxel arm, 12.2 mo *vs* 9.4 mo, respectively; the overall

response rates were 19% with nivolumab and 12% with docetaxel. The PD-L1 protein was retrospectively assessed with the Dako assay in pre-treatment archival or recent tumor-biopsy specimens. The response rate was compared at pre-specified expression levels of 1%, 5% and 10%. The response rate was 31% and 9% in tumors with PD-L1 positivity ⩾ 1% or < 1% respectively; the response rate was 36% and 10% in PD-L1 positive tumors ⩾ 5% or < 5%, and the response rate was 37% or 11% in PD-L1 positive tumors ⩾ 10% or < 10% respectively (Table 1).

Nivolumab for first-line treatment

In the CheckMate 012 study 52 treatment-naive advanced NSCLC patients received nivolumab at the dose of 3 mg/kg every 2 wk[12]. The response rate was 23% and the efficacy data were very encouraging: mPFS was 3.6 mo and mOS was 19.4 mo. On the whole, tumor shrinkage was obtained independently of the PD-L1 expression; however, the greater the PD-L1 positivity increase, the higher the probability of response. Conversely, there was no clear association between mPFS and mOS and PDL-1 expression (Table 1).

In the Rizvi *et al*[13]'s trial, patients with advanced NSCLC received 10 mg/kg of nivolumab every 2 wk in combination with cisplatin plus gemcitabine or pemetrexed or carboplatin plus paclitaxel; or, they received 5 mg/kg of nivolumab 5 mg/kg every 2 wk with carboplatin plus paclitaxel. The response rates were 33% in the nivolumab plus cisplatin/gemcitabine group, 47% in the cisplatin plus pemetrexed group, 47% in the carboplatin and paclitaxel group and 43% in the nivolumab 5 mg/kg plus carboplatin/paclitaxel group. In patients with PDL-1 expression ⩾ 1%, the response rate was 48%, whereas in patients with PD-1 < 1% the response rate was 43%. No relationship was observed between PDL-1 expression and mPFS and mOS (Table 1).

In the CheckMate 026 phase Ⅲ trial, patients with untreated advanced NSCLC and PD-L1 tumor positivity > 1% were randomized to receive 3 mg/kg IV of nivolumab 3 mg/kg IV every 2 wk or platinum-based chemotherapy every 3 wk for 6 cycles[14]. The primary end-point of the study was to demonstrate an improved PFS for patients with PD-L1 tumor-expression ⩾ 5%. Median PFS was 4.2 and 5.9 mo with nivolumab and platinum-based chemotherapy, respectively (HR = 1.15, 95%CI: 0.91-1.45, P = 0.25). Median OS was 14.4 mo for immunotherapy and 13.2 mo for chemotherapy. The preliminary results of this study presented at the ESMO meeting showed that the PD-L1 score did not predict the response rate (Table 1).

Pembrolizumab beyond first-line treatment

KEYNOTE-001 was a large phase Ⅰ study with an NSCLC expansion cohort including a total of 495 advanced NSCLC patients who received 2 mg or 10 mg/kg of pembrolizumab every 3 wk or 10 mg/kg every 2 wk[15]. One hundred and eighty-two patients were assigned to the "training group" recruited to define the PD-L1 positivity threshold on pre-treatment tumor biopsy (using the antibody clone 22C3-Dako-IHC assay). The remaining 313 patients were treated in the "validation group". According to the data obtained from the training group, a PD-L1 tumor expression of 50% was identified as threshold of positivity. The validation group patients with a tumor PD-L1 score ⩾ 50% had a response rate of 45.2% (95%CI: 33.5-57.3): This figure was 17% (95%CI: 9.9-25.1) in patients with a score 1%-49% and 3% (95%CI: 2.3-28.2) in patients with PD-L1 < 1% (Table 2). Noteworthy, a deterioration of the PD-L1 antigen was observed in tumor samples sectioned more than 6 mo before staining. The response rates were higher in former or current smokers compared to non-smokers (22.5% *vs* 10.3%). Treatment was effective at all tested doses and schedules, therefore an every-3-wk schedule was chosen for the phase Ⅲ study.

These data were confirmed in a large prospective randomized phase Ⅱ/Ⅲ trial (KEYNOTE-010). This study enrolled 1034 previously treated PD-L1-positive NSCLC patients (PD-L1 expression ⩾ 1% of tumour cells) and compared 2 mg or 10 mg/kg pembrolizumab every 3 wk *vs* 75 mg/m^2 docetaxel every 3 wk in terms of OS and PFS[16]. PD-L1 expression was evaluated in the archival tumor samples of 456 patients, while new biopsy material was collected before a study entry for the remaining patients. No differences in mPFS emerged between immunotherapy and chemotherapy. Overall survival was significantly longer in both pembrolizumab arms compared to the docetaxel arm: The HRs were 0.71 (95%CI: 0.58-0.88, P = 0.0008) and 0.61 (95%CI: 0.49-0.75, P < 0.0001) respectively for the two dose-levels of pembrolizumab. However, in patients with PD-L1 positivity ⩾ 50% the HRS for OS were 0.54 (P = 0.0002) in the pembrolizumab 2 mg/kg arm and 0.50 (P ⩽ 0.0001) in the Pembrolizumab 10 mg/kg treatment arm respectively; in addition, in this PD-L1 selected subgroup of patients also PFS was significantly longer than with chemotherapy (Table 2). In the total population, the response rates were 18% with pembrolizumab and 9% with docetaxel; in patients with PD-L1 positivity ⩾ 50% the response rate was about 30%, while it was 8% in patients with tumors showing a PD-L1 expression level < 50% (Table 2). Consistent with the results from the nivolumab trials, pembrolizumab was more tolerable than docetaxel and did significantly better in both squamous and non-squamous histology. Similarly, patients with EGFR mutated tumors seemed to have no survival advantage with immunotherapy over chemotherapy despite the small number of patients.

Pembrolizumab in first-line treatment

The KEYNOTE-024 was a phase Ⅲ trial in which 350 untreated NSCLC patients with a PDL-1 tumor

Table 2 Correlation between pembrolizumab activity and outcome and programmed cell death protein ligand 1 immunohistochemestry score

Author/study	Marker antibody	Tumor type	Treatment line	PD-L1 cutoff	N pts	Response	mPFS mo (95%CI)	mOS mo (95%CI)
Pembrolizumab								
Garon *et al*[15] KN001	Dako 22C3	NSCLC	⩾ 1	⩾ 50%	73	45.20%	6.4 (4.2-NR)	NR (NR-NR)
				1%-49%	103	17%	4.1 (2.3-4.4)	10.6 (7.3-NR)
				< 1%	28	3%	4 (2.1-6.2)	10.4 (5.8-NR)
Herbst *et al*[16] KN010	Dako 22C3	NSCLC	⩾ 2	⩾ 50%	290	30%	14.9 (10.4-NR)	5.0 (4.0-6.9)
				1%-49%	400	10%	17.3 (11.8-NR)	5.2 (4.1-8.1)
Reck *et al*[17] KN024 phase Ⅲ	Dako 22C3	NSCLC	1	⩾ 50%	305	44.80%	10.3 (6.7-NR)	NA
Langer *et al*[18] KN021 phase Ⅲ	Dako 22C3	Non squamous NSCLC	1	⩾ 50%	20	80%	13 (8.3-NR)	NA
				1%-49%	19	29%		
				< 1%	21	57%		

KN: KeyNote; NR: not reported; pts: Patients; NA: Not available; NSCLC: Non-small cell lung cancer; PD-L1: Programmed cell death protein ligand 1; mPFS: Median progression free survival; mOS: Median overall survival.

score of 50% or greater were randomized to receive pembrolizumab at a flat dose of 200 mg every 3 wk, or platinum-based chemotherapy for 4-6 cycles[17]. PD-L1 expression was assessed in formalin-fixed tumor specimens obtained at the time of diagnosis of the metastatic disease. Fine-needle aspirates were not considered appropriate. The primary endpoint of the study was PFS. A total of 1653 out of 1934 screened patients had evaluable PD-L1 material, and 500 (30.2%) patients had a PD-L1 positivity of 50% or greater. Median PFS was significantly longer in the pembrolizumab group [10.3 mo (95%CI: 6.7 to "not reached")] than in the chemotherapy group [6.0 mo (95%CI: 4.2-6.2)] with HR for disease progression or death of 0.50 (95%CI: 0.37-0.68, P < 0.001). The overall response rate was 44.8% (95%CI: 36.8%-53.0%) in the pembrolizumab group and 27.8% (95%CI: 20.8%-35.7%) in the chemotherapy group. At the time of the second interim analysis, OS was significantly longer with immunotherapy (HR for death: 0.60, 95%CI: 0.41-0.89, P = 0.005).

In the KEYNOTE-021 phase Ⅱ trial, a total of 123 treatment-naive advanced non-squamous NSCLC patients were randomized to receive 4 cycles of pembrolizumab (200 mg flat dose) plus carboplatin (AUC 5) and pemetrexed (500 mg/m^2) every 3 wk, followed by pemetrexed and pembrolizumab for 2 years, or to undergo the same strategy without pembrolizumab[18]. Randomisation was stratified by PDL-1 tumor proportion score (< 1% *vs* ⩾ 1%) assessed by the IHC 22C3 clone (Dako North America) in formalin-fixed tumour samples obtained at the time of diagnosis of metastatic disease. The primary end-point was the proportion of patients achieving an objective response. The response rate was 55% (95%CI: 42%-68%) in the pembrolizumab plus chemotherapy arm and 29% (95%CI: 18%-41%) in the standard arm with a 26% of difference in the response rate thus reaching statistical significance (95%CI: 9%-42%, P = 0.0016). In the experimental arm the response rate was 57% (95%CI: 34%-79%) in patients with a PDL-1 tumor score < 1% and 54% (95%CI: 37%-70%) in patients with a PDL-1 score of 1% or greater. Nevertheless, the probability of response increased according to the PD-L1 positivity level: 29% response rate in patients with PDL-1 positive tumors ranging from 1% to 49% and 80% response rate in those patients whose tumors scored 50% or greater (Table 2). Median PFS was longer with Pembrolizumab plus chemotherapy [13 mo (95%CI: 8.3 to "not reached")] with respect to chemotherapy alone [8.9 mo (95%CI: 4.4-10.3 mo)] with an HR of 0.53 (95%CI: 0.31-0.91, P = 0.01). However, no difference was observed in OS (HR = 0.90, 95%CI: 0.41-1.91, P = 0.39).

Anti- PD-L1 monoclonal antibodies

Atezolizumab

In the paper by Herbst and colleagues a total of 277 patients with advanced cancer were treated with escalating doses of MPDL3280A intravenously every 3 wk[19]. In advanced NSCLC patients (53/277 in total) the overall response rate was 21%. In this case PD-L1 was determined by using a novel IHC assay (Ventana SP142 North America) and positivity was categorized according to the expressing cell type [tumor cell (TC) or immune cell (IC)] and then scored along a gradient [< 1% (TC0 or IC0), 1%-4% (TC1 or IC1), 5%-49% (TC2) or 5%-10% (IC2), and ⩾ 50% (TC3) or <10% (IC3)]. A relationship was observed between PD-L1 scores and response rate: 83% of patients with score 3 responded to treatment, while only 20% of those with scores 0-2 obtained a remission (Table 3). However, not surprisingly, also 20% of patients with score 0 achieved a clinical response. In the subsequent randomized phase Ⅱ study (POPLAR) atezolizumab was compared to docetaxel, in terms of OS, in 285 pretreated advanced NSCLCs[20]. Patients were stratified according to the PD-L1 expression that was determined on TC as well as on IC by using the SP142 PD-L1 IHC assay (Ventana Medical Systems, Tucson, AZ, United States). The IHC scores were defined as follows: Score 0 =

Table 3 Correlation between atezolizumab activity and outcome and programmed cell death protein ligand 1 immunohistochemestry score

Author/study	Marker antibody	Tumor type	Treatment line	PD-L1 cutoff	N pts	Response (%)	mPFS mo (95%CI)	mOS mo (95%CI)
Atezolizumab							NR	NR
							NR	NR
							NR	NR
							NR	NR
Herbst *et al*[19]	Ventana SP142	NSCLC	≥ 2	Score 3	6	83	7.8 (2.7-12.3)	15.5 (9.8-NA)
phase I				Score 2	7	14	3.4 (1.4-6.9)	15.1 (8.4-NA)
				Score 1	13	15	3.0 (2.8-4.1)	15.5 (11.1-NA)
				Score 0	20	20	4.1 (2.7-5.6)	9.7 (6.7-12.0)
Fehrenbacher *et al*[20] POPLAR	Ventana SP142	NSCLC	≥ 2	Score 3	24	37.50	4.2 (2.9-7.0)	20.5 (17.5-NA)
PHASE II				Score 2	50	22.00	4.1 (2.8-5.3)	16.3 (13.3-20.1)
				Score 1	93	18.30	4.1 (2.9-4.3)	15.7 (12.6-18.0)
				Score 0	51	14.60	4.0 (3.1-4.2)	12.6 (9.6-15.2)
Rittmeyer *et al*[21]	Ventana	NSCLC	≥ 2	Score 3	72	30.60	7.3 (4.9-12.0)	26.9 (12.0-NA)
OAK	SP142			Score 2	129	22.50	7.3 (5.7-9.7)	23.5 (18.1-NA)
Phase III				Score 1	241	17.80		
				Score 0	80	7.80	7.6 (4.0-9.7)	23.5 (18.1-NA)
Wakelee *et al*[22] and Antonia *et al*[23]	Ventana	NSCLC	1	Score 3	65	34		
BIRCH	SP142			TC2/3 or IC2/3	138	25		
phase II				Score 2	73	18		

Score 3: PDL1 expression levels TC3 or IC3 (≥ 50% on TC or ≥ 10% on IC); Score 2: TC2 or IC2 (≥ 5%-< 50% on TC or ≥ 5% -< 10% IC); Score 1: TC1 or IC1 (≥ 1% - < 5% on TC or IC); Score 0: TC0 and IC0 (< 1% on TC and IC). IC: Tumor-infiltrating immune cell; TC: Tumor cell; NR: Not reported; pts: Patients; NA: Not available; NSCLC: Non-small cell lung cancer; PD-L1: Programmed cell death protein ligand 1; mPFS: Median progression free survival; mOS: Median overall survival.

PD-L1 expression on IC or TC < 1%; score = 1 TC or IC PD-L1 positivity between ≥ 1 and < 5%; score = 2 positivity between ≥ 5 and < 50% on TC or PD-L1 expression on IC between ≥ 5 and < 10%; score = 3 PD-L1 positive TC ≥ 50% or PD-L1 positive IC ≥ 10%. Median OS in the atezolizumab arm was 12.6 mo (95%CI: 9.7-16.4) compared to 9.7 mo (95%CI: 8.6-12.0) in the docetaxel arm (HR = 0.73, 95%CI: 0.53-0.99; *P* = 0.04). Overall survival improves according to the PD-L1 score level: TC3 or IC3 HR 0.49, TC2/3 or IC2/3 HR 0.54, TC 1/2/3 or IC1/2/3 HR 0.59; TC0 or IC0 HR 1.04. PFS also varied according to the different PD-L1 subgroups, but the differences did not reach any statistical significance (Table 3). In the immunotherapy arm the overall response rate was 37.5%, 22.0%, 18.3% and 16.7% in TC3 or IC3, TC2/3 or IC2/3, TC 1/2/3 or IC1/2/3. In the subgroup TC0 or IC0, the response rates were similar (14.6%) in both arms (Table 3).

In the phase III OAK trial, patients with squamous or non-squamous advanced NSCLC, pretreated with one or two chemotherapy regimens, were randomly assigned to 1200 mg of atezolizumab or 75 mg/m^2 of docetaxel every 3 wk[21]. The primary endpoint was OS. The mOS was 13.8 mo (95%CI: 11.8-15.7) in the atezolizumab arm and 9.6 mo (95%CI: 8.6-11.2) in the docetaxel arm (HR = 0.73, 95%CI: 0.62-0.87, *P* = 0.0003). Median OS was also analyzed according to the criteria of the previous study (20): In the TC1/2/3 or IC1/2/3 populations OS was 15.7 mo (95%CI: 12.6-18.0) with atezolizumab *vs* 10.3 (95%CI: 8.8-12.0) with docetaxel (HR = 0.74, 95%CI: 0.58-0.93, *P* = 0.0102) and in the TC0 or IC0 groups mOS was 12.6 mo *vs* 8.9 mo with atezolizumab and docetaxel respectively (HR = 0.75, 95%CI: 0.59-0.96). In the intention to treat population PFS did not differ between the two arms (HR = 0.95, 95%CI: 0.82-1.10, *P* = 0.4928) and in the different PD-L1 subgroups. Objective responses for atezolizumab were 30.6% in the TC3/IC3 subgroup, 22.5% in the TC 2/3 or IC 2/3 aubgroups, 17.8% and 7.8% in the TC 1/2/3 or IC1/2/3 and TC0 or IC0 subgroups, respectively (Table 3).

In phase II Birch trial patients with advanced NSCLC received atezolizumab in first or subsequent line of treatment at a flat dose of 1200 mg every three weeks[22]. The PDL-1 expression was evaluated by using the Ventana SP142 IHC assay and the study enrolled only patients with PDL1 expression > 5% in tumor cells or in immune cells (TC2/3 or IC 2/3). Efficacy data in the first line setting have been reported in a recent update[23]. Patients with PDL-1 TC3 or IC3 showed a 34% response rate and a mOS of 26.9 mo; PDL-1 TC2/3 or IC2/3 scores had an overall response rate of 25% and a mOS of 23.5 mo, and patients with PDL-1 TC2 or IC2 scores had an overall response rate of 18% and a mOS of 23.5 mo (Table 3).

Durvalumab

An ongoing phase 1/2 study is evaluating the safety and efficacy of durvalumab in patients with advanced NSCLC or with other solid tumor types[23]. Durvalumab was administered at 10 mg/kg every two weeks in previously untreated advanced NSCLC. Fifteen patients were initially enrolled regardless of the PD-L1 status. After a protocol amendment, enrolment was restricted to PD-L1 positive patients. PD-L1 status was assessed

Table 4 Correlation between durvalumab and avelumab activity and outcome and programmed cell death protein ligand 1 immunohistochemestry score

Author/study	Marker antibody	Tumor type	Treatment line	PD-L1 cutoff	N pts	Response (%)	mPFS mo (95%CI)	mOS mo (95%CI)
Durvalumab								
Gulley *et al*[24]	Ventana SP263	NSCLC	1	⩾ 25%	43	25		
Phase 1/2				< 5%	8	12		
Avelumab								
Verschraegen *et al*[25]	?	NSCLC	⩾ 2	⩾ 1%	118	14.40	11.7 wk	NR
Phase 1b				< 1%	20	10	5.9 wk	NR
Sheng *et al*[26]	?	NSCLC	1	⩾ 1%	35	20	NR	NR
Javelin phase Ib				< 1%	10	0	NR	NR

NR: Not reported; pts: Patients; NSCLC: Non-small cell lung cancer; PD-L1: Programmed cell death protein ligand 1; mPFS: Median progression free survival; mOS: Median overall survival.

with the companion Ventana SP263 assay. PD-L1 positivity was defined as a tumor cell membrane staining of ⩾ 25%. A total of 59 patients (48 PD-L1 positive; 9 PD-L1 negative) were included in the trial. The overall response rate was 25% in PDL-1 positive patients and 12% in PDL-1 negative patients (Table 4).

Avelumab

A phase-1b trial was designed to investigate the safety and activity of avelumab (MSB0010718C) in patients with advanced NSCLC progressing after platinum-based chemotherapy[24]. Patients were treated with avelumab at 10 mg/kg every two weeks. Tumor PD-L1 expression was assessed by immunohistochemistry. Objective responses were observed in 22 patients [12% (95%CI: 7.6%-17.5%)], while 70 patients (38%) achieved a stable disease. Median PFS was 11.6 wk (95%CI: 8.4-12.1). One hundred and eighteen (86%) evaluable patients were PDL1 positive (1% threshold of positivity). The overall response rate was 14.4% and 10.0% in PD-L1 positive and negative tumors, respectively. Median PFS in PD-L1 positive patients was 11.7 wk and 5.9 in PD-L1-negative patients.

The safety and activity of avelumab in chemotherapy-naive advanced NSCLC patients were investigated in a phase 1b trial[25]. Patients received 10 mg/kg of avelumab IV every 2 wk; PD-L1 expression was assessed by IHC with ⩾ 1% positivity threshold on tumor cell staining. The overall response rate was 18.7% (95%CI: 10.6, 29.3) and a disease stabilization was reported in 34 patients (45.3%). In 35 PD-L1 positive tumors the overall response rate was 20.0%; no patients with PD-L1 negative tumors achieved a response. Median PFS was 11.6 wk (95%CI: 6.7-17.9) for all treated patients (Table 4).

CONCLUSION

The literature data have clearly shown that immune checkpoint inhibitors might represent an important therapeutic option for NSCLC patients. However, in spite of exciting overall treatment outcomes, a considerable number of patients failed to achieve long-term clinical benefit.

Since the cost of these molecules impacts significantly on health care systems, the identification of predictive biomarkers to select patients who are more likely to benefit is a challenging area of ongoing research. The PD-L1 expression was early identified as potential indicator of benefit and the literature on this topic is plentiful. Several critical aspects might explain the conflicting results shown in clinical trials by using retrospective or prospective PD-L1 assays. Some of these results are strictly related to the PD-L1 nature, while others derive from the methodologies and material that have been used for testing. PD-L1 is a constitutively but also a functionally inducible receptor/ligand potentially expressed by tumor cells, stromal cells, inflammatory cells at tumor sites; it is heterogeneous and subject to pre-analytical variables. Furthermore, its expression is continuously distributed, it has varied significantly over time and may be affected by concurrent or prior treatments (radiation or chemotherapy)[26-28]. Classical predictive biomarkers such as hormone receptors, HER2 protein over-expression or gene amplification, EGFR activating mutations and ALK rearrangements are always present: These indicators define more clearly distinct tumor subgroups with different biology and clinical behavior. The PD-L1 expression is very dynamic, according to a constantly evolving immune response. Therefore, questions regarding reliability, consistency, feasibility and selection of an expression as a threshold remain artificial and controversial. This might explain why a significant proportion of PD-L1 negative patients benefited from treatment with immunotherapy in all studies. Conversely, even in highly PD-L1 selected cohorts, 25% to 50% of patients achieved no benefit. Moreover, it is not clear whether PD-L1 positivity has a different effect on outcome/response to treatment, compared to PD-L1 positivity on immune cells. PDL-1 expression was evaluated in tumor cells in the majority of studies. The immunoresponse is a delicate balance between inhibitory checkpoints and activating signals

such as LAG-3, OX40, *etc*. The discovery of these proteins has paved the way to new therapy strategies, whereas their potential predictive role as biomarkers of immunoresponse is actually unknown.

Technical aspects may also result in inconsistent data; tissue fixation, storage, and antigen recovery are not standardized. The quality of commercially available antibodies is also a reason for concern: The PD-L1 diagnostic test for nivolumab (Dako 28-8 pharmDx), pembrolizumab (Dako 22C3 pharmDx), atezolizumab (Ventana SP142) and durvalumab (Ventana SP263) showed variability in staining intensity and patterns creating uncertainties and doubts for their use in everyday practice. To address these concerns, som-eyears ago a task force was set up, formed by phar-maceutical companies, by representatives from Dako and Ventana, and by the scientific companies FDA, AACR, ASCO and IASCLC (International Association for the Study of Lung Cancer). The aim was to compare the performance of the four major PD-L1 companion assays. The recently published results of the pilot phase of the "Blueprint PDL1 IHC assay comparison project"[29] indicates that interchanging assays and cut-offs will lead to the misclassification of PD-L1 status for some patients, and therefore more data are required.

Summing up, the PD-L1 expression is likely to be related to the curative efficacy of immune checkpoint inhibitors. However, its role seems to be more informative in terms of probability and magnitude of the treatment effect rather than prediction of the effect itself, given that none of the available assays can conclusively identify non-benefitting patients.

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MINIREVIEWS

Platinum-induced neurotoxicity: A review of possible mechanisms

Ozkan Kanat, Hulya Ertas, Burcu Caner

Ozkan Kanat, Hulya Ertas, Burcu Caner, Department of Medical Oncology, Uludag University Faculty of Medicine, 16059 Bursa, Turkey

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Correspondence to: Ozkan Kanat, MD, PhD, Professor, Department of Medical Oncology, Uludag University Faculty of Medicine, Gorukle, 16059 Bursa, Turkey. ozkanat@uludag.edu.tr
Telephone: +90-224-2951321

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Abstract

Patients treated with platinum-based chemotherapy frequently experience neurotoxic symptoms, which may lead to premature discontinuation of therapy. Despite discontinuation of platinum drugs, these symptoms can persist over a long period of time. Cisplatin and oxaliplatin, among all platinum drugs, have significant neurotoxic potential. A distal dose-dependent symmetrical sensory neuropathy is the most common presentation of platinum neurotoxicity. DNA damage-induced apoptosis of dorsal root ganglion (DRG) neurons seems to be the principal cause of neurological symptoms. However, DRG injury alone cannot explain some unique symptoms such as cold-aggravated burning pain affecting distal extremities that is observed with oxaliplatin administration. In this article, we briefly reviewed potential mechanisms for the development of platinum drugs-associated neurological manifestations.

Key words: Cisplatin; Dorsal root ganglion; Mechanism; Oxaliplatin; Neurotoxicity; Neuropathic pain; Sodium channel

Core tip: Platinum drug-based chemotherapies may lead to intolerable neuropathic symptoms, preventing their administration at the optimal effective doses and duration. A better understanding of potential mechanisms underlying these symptoms can help clinicians better manage patients experiencing acute and/or cumulative neurotoxicity during treatment with platinum-containing chemotherapy.

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INTRODUCTION

Platinum drugs, including cisplatin (cis-diamminedi-

chloroplatinum Ⅱ), carboplatin (cis-diammine-1, 1-cyclobutane dicarboxylate platinum Ⅱ), and oxaliplatin (trans-R,R-cyclohexane-1,2-diamineoxalatoplatinum Ⅱ) have become an important part of the combination chemotherapy regimens used to treat different types of solid tumors. Despite their favorable anti-tumor properties, platinum drugs can cause serious side effects such as neurotoxicity[1-3].

Carboplatin neurotoxicity is negligible compared with that of cisplatin and oxaliplatin, however, it can develop, particularly high doses are administered[3,4]. Exposure of rat sensory neurons in culture to cisplatin, oxaliplatin or carboplatin *in vitro* caused a concentration-dependent increase in cell death and apoptotic cells[5]. However, carboplatin required a 10-fold higher drug concentration than cisplatin to induce a similar degree of cytotoxic effect. In addition, both cisplatin and oxaliplatin led to increased reactive oxygen species production and 8-oxoguanine DNA damage, but carboplatin did not[5]. These preclinical observations may partly explain why carboplatin has less neurotoxic effects.

Conversely, conventional-dose cisplatin- or oxaliplatin-based therapies can sometimes lead to intolerable neuropathic symptoms, preventing their administration at the optimal effective doses and duration. Large-diameter sensory nerve fibers appear to be the most affected by platinum drugs, leading to symmetrical glove and stocking type of sensory loss, numbness, tingling, pain, and burning sensation[4]. Some of these symptoms may persist for months or even years. Furthermore, in some cases, they may continue to worsen even after treatment cessation, a phenomenon known as "coasting"[6].

Platinum-induced neurologic symptoms become evident when certain cumulative drug doses have been administered. Cumulative doses of cisplatin and oxaliplatin of 350 mg/m^2 and 550 mg/m^2, respectively, have been considered as the threshold values for neurotoxicity development[6]. Some clinical and genetic features of patients may make them more susceptible to developing severe neurotoxicity during treatment with platinum drugs. A recent study by Velasco *et al*[7] found that among patients treated with oxaliplatin-based chemotherapy, male patients, patients experiencing more severe acute neuropathic symptoms, patients with abnormal findings on mid-treatment nerve conduction velocity studies, and patients receiving higher cumulative oxaliplatin doses have an increased risk of developing significant neuropathic symptoms. Several recent pharmacogenomics studies have suggested that patients with polymorphisms in the Glutathione S-transferases genes (GSTM1, GSTT1, and GSTP1) are more likely to develop grade 3-4 cumulative neuropathy during oxaliplatin treatment due to decreased drug detoxification[8].

Oxaliplatin may also cause acute dose-independent neurotoxicity, which can occur in approximately 90% of patients during or shortly after infusion, and is characterized by transient cold-induced paresthesias and dysesthesias affecting the distal extremities, and perioral and pharyngolaryngeal regions[9,10].

A better understanding of the potential mechanisms underlying cisplatin or oxaliplatin neurotoxicity will certainly help clinicians identify the optimal clinical management of this side effect. The aim of this review was, therefore, to summarize the current knowledge on the neuronal events induced during platinum-based therapy.

Nuclear DNA damage in dorsal root ganglion neurons

The accumulation of platinum compounds and their metabolites in the dorsal root ganglion (DRG) after their systemic administration and formation of platinum-DNA adducts are considered key steps in neurotoxicity development (Figure 1)[2,11]. The presence of an abundant fenestrated capillary network and the absence of blood-brain barrier in DRG allow platinum drugs to preferentially accumulate in DRG with easy access to sensory neurons[2,11,12].

Recently, it was demonstrated that the uptake of platinum drugs into DRG neurons may be facilitated by two different types of neuronal membrane transporters: Copper transporter-1 (CTR1) and organic cation transporter-2 (OCT2)[13-15]. The overexpression of these transporters in neurons, therefore, can contribute to the development or aggravation of neurotoxicity. For example, a 16- to 35-fold increase in the cellular oxaliplatin uptake was observed in neurons overexpressing mouse OCT2 or human OCT2, and this process resulted in significantly increased DNA platination and neurotoxicity[15].

Once the platinum drugs reach the neuronal cell nucleus, they attack the nuclear DNA to form adducts. They usually form same types of adducts on the same DNA sites, including 1,2-intrastrand d (GpG) (between adjacent guanine bases on the same DNA strand) and 1,2-intrastrand d (ApG) (between adenine and adjacent guanine bases on the same DNA strand) crosslinks. A correlation between adduct levels and the degree of neurotoxicity has been reported[16]. The platinum-DNA adduct levels produced by cisplatin were found to be approximately three times higher than those generated by equimolar oxaliplatin doses. Concordantly, *in vitro* cisplatin caused significantly more neuronal cell death than oxaliplatin[16]. DNA repair ability of DRG neurons for adducts (primarily performed by the nucleoid excision repair) is an important factor determining neurotoxicity severity[17]. Chronic cisplatin administration resulted in an accelerated accumulation of unrepaired platinum-DNA adducts in DRG neurons of DNA repair-deficient mice, which induced early neurophysiological alterations and led to an increase in neuronal cell death[17].

Inhibition of the global transcriptional activity of DRG neurons is one of the major consequences of DNA adduct formation[18]. DRG neurons need a high level of active transcription to sustain their large size, high metabolism, and long axons. Therefore, platinum-induced DNA damage leads to neuronal atrophy and disruption of their distant axonal connections[18].

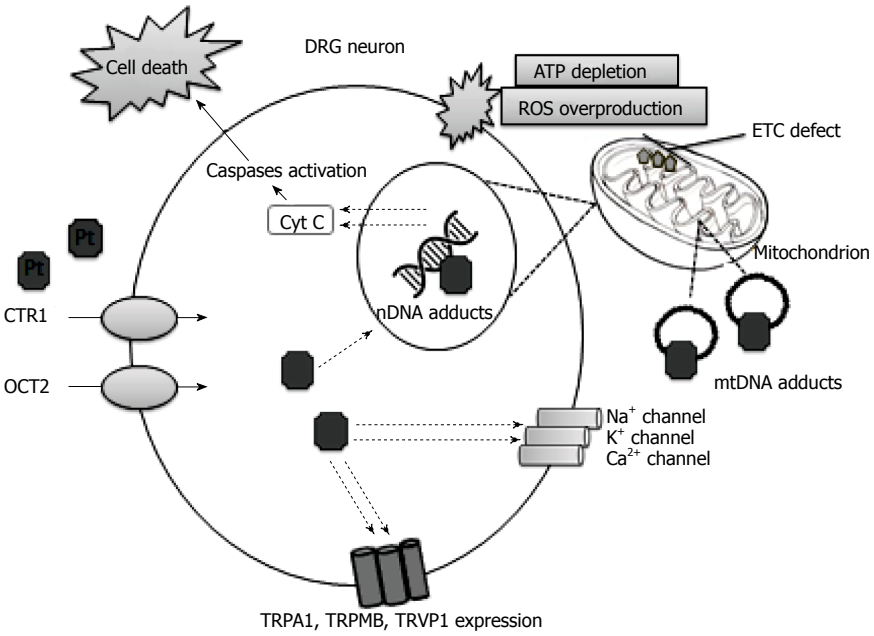

Figure 1 Proposed mechanisms of platinum-induced neurotoxicity. Dorsal root ganglion (DRG) is the main target of platinum drugs that preferentially accumulate in DRG neurons. Membrane transporters, copper transporter-1 (CTR1) and organic cation transporter-2 (OCT2), can facilitate the cellular uptake of platinum drugs. Platinum-DNA adducts inhibit replication and transcription, which results in caspase activation and subsequent cell death. Neuronal mitochondrial damage leads to cellular ATP depletion and increased reactive oxygen species (ROS) production. The voltage-gated sodium (Na^+), potassium (K^+) and calcium (Ca^{2+}) channels dysfunction, and the enhanced expression and responsiveness of transient receptor potential channels (TRPA1, transient receptor potential ankyrin-1; TRPM8, transient receptor potential melastatin 8; TRPV1, transient receptor potential vanilloid 1) play an important role in the development of platinum-induced neurotoxicity.

Several preclinical studies have reported that platinum-induced DNA damage also induces apoptosis and neuron loss in DRG both *in vivo* and *in vitro*[19-22]. Cisplatin has been shown to initiate several apoptotic events in neuronal cells, including p53 activation, Bax translocation, mitochondrial cytochrome c release, and activation of caspase-3 and caspase-9. Gill and Windebank demonstrated that following exposure to cisplatin, DRG neurons attempt to re-enter the cell cycle from G0 phase, and this event can be a prelude to triggering neuronal cell death[22].

Mitochondrial DNA damage

Mitochondrial dysfunction in DRG neurons was first described as a potential mechanism for platinum drugs neurotoxicity by Podratz *et al*[23]. They demonstrated that cisplatin also directly binds to mitochondrial DNA with similar binding affinity as nuclear DNA. Cisplatin-mitochondrial DNA adducts inhibit mitochondrial DNA transcription and replication, and cause morphological changes in the mitochondria. This can lead to disruption of the electron transport chain, loss of adenosine triphosphate (ATP) generation, energy failure, and overproduction of reactive oxygen species. All these events cause the opening of mitochondrial permeability transition pores, mitochondrial membrane depolarization, intracellular calcium accumulation, and expression of apoptotic proteins.

Cisplatin may also impair mitochondrial transport dynamics in neurons[24]. Proper mitochondrial transport in neurons is critical to cellular homeostasis. A new study in Drosophila has shown that cisplatin can significantly reduce mitochondrial movement frequency in axons[24]. This is probably caused by both ATP depletion and cellular calcium accumulation.

Some studies have demonstrated that cisplatin can alter the expression of mitochondrial fusion and fission proteins in peripheral nerves[25]. These proteins regulate mitochondrial shape, size, and number. Bobylev *et al*[25] detected a significant decrease in the mitochondrial fusion protein mitofusin 2 expression levels in DRG and tibial nerves of cisplatin-treated mice, resulting in mitochondrial swelling and vacuolization.

Voltage-gated ion channels dysfunction (channelopathies)

Oxaliplatin exhibits a tetrodotoxin-like inhibitory effect on the neuron voltage-gated sodium (Na^+) channels[26-30]. It remarkably slows their inactivation and reduces the peak Na^+ current, leading to an increase in the duration of the relative refractory period of sensory neurons. Oxaliplatin may also affect the Na^+ channels indirectly *via* the chelation of extracellular calcium ions by its metabolite oxalate (diaminocyclohexane-platinum-C2O4)[26]. Because of Na^+ channel dysfunction, sensory neurons become hyperexcitable and eventually generate spontaneous ectopic discharges.

Oxaliplatin can display isoform-specific effects on voltage-gated Na^+ channels leading to the development of unique neuropathy symptoms such as cold-aggravated peripheral pain[31,32]. It has been suggested that oxaliplatin-induced Nav1.6 dysfunction may play a

role in cold allodynia development[33,34]. Cooling in the presence of oxaliplatin increased Nav1.6-mediated persistent and resurgent Na^+ currents in large-diameter DRG neurons and resulted in the generation of action potential burst firing[31].

Peripheral nerve axonal excitability studies performed before and immediately after oxaliplatin administration have confirmed the above mentioned *in vitro* findings and revealed acute abnormalities in sensory nerve function related to Na^+ channel dysfunction, including decreased refractoriness and increased superexcitability[35]. Interestingly, it was shown that these excitability abnormalities can be detected in the initial oxaliplatin treatment cycles and may serve as a predictive tool to identify patients who are more likely to develop moderate or severe neurotoxicity.

Kagiava *et al*[33] suggested that altered voltage-gated potassium channel activity may be involved in oxaliplatin-induced neurotoxicity development. In their study, the effects of oxaliplatin on the compound action potential of rat sciatic nerve were observed to be similar to those with the potassium channel blockers 4-aminopyridine and tetraethylammonium. Oxaliplatin was found to cause broadening of action potentials and repetitive firing, suggesting its antagonistic effect on neuronal fast and slow potassium channels. This finding is indirectly supported by Sittl *et al*[34]. They showed that enhancement of axonal potassium conductance by flupirtine may reduce oxaliplatin-induced peripheral nerve hyperexcitability.

Conversely, voltage-gated potassium channels are unlikely to be the primary target for oxaliplatin because patch-clamp studies failed to show any effect of oxaliplatin on Shaker-type potassium channels[36]. Kagiava *et al*[37] found some evidence indicating that potassium channel dysfunction during oxaliplatin treatment can occur due to malfunction of the gap junction (GJ) channels and hemichannels in myelinated fibers. According to their findings, oxaliplatin causes prolonged opening of GJ channels and hemichannels, leading to excessive potassium accumulation in the periaxonal space and its osmotic swelling. This event is likely to have a disturbing effect on the voltage-gated potassium channel function.

Cisplatin does not appear to have a prominent effect on the neuronal sodium or potassium channel function. Initial studies using whole cell patch-clamp electrophysiological technique reported that cisplatin decreases the calcium channel currents, particularly in small-diameter neurons of rat DRG[38]. However, a new study revealed an increase in calcium influx through N-type calcium channels in rat DRG neurons after exposure to cisplatin[39]. This was mainly caused by the upregulation of the N-type calcium channels. Increased intracellular calcium levels led to caspase-3 activation and apoptosis induction.

Enhanced responsiveness of thermosensitive transient receptor potential ion channels

Sensory neurons express various types of transient receptor potential (TRP) channels, including TRPA1, TRPM8, and TRVP1, which all play an important role in the generation and sensation of inflammatory and neuropathic pain[40-45].

Nassini *et al*[40] showed that oxaliplatin- and cisplatin-induced mechanical and cold hyperalgesia in rats are mediated by transient receptor potential ankyrin-1 (TRPA1), and TRPA1 activation is most likely caused by glutathione-sensitive molecules. Subsequently, Zhao *et al*[44] reported that oxaliplatin-induced cold hyperalgesia could be related to increased responsiveness of TRPA1. Pretreatment of the cultured DRG neurons with oxaliplatin resulted in an increase in the number of allyl-isothiocyanate (a TRPA1 agonist)-sensitive neurons.

The results of a recent study suggested that aluminum accumulation in DRG may augment oxaliplatin-induced neuropathic pain through activation of TRPA1 and stimulation of apoptotic cell death[46]. In this study, aluminum concentration of in DRG was greater in mice treated with aluminum chloride and oxaliplatin than in those treated with aluminum chloride alone.

Gauchan *et al*[43] revealed that oxaliplatin treatment increased the cold receptor transient receptor potential melastatin 8 (TRPM8) expression in rat DRG neurons, which resulted in enhanced sensitivity to cooling stimulation. Capsazepine, a blocker of both TRMP8 and TRPV1 channels, but not the selective TRV1 blocker 5′-Iodoresiniferatoxin, was able to inhibit oxaliplatin-induced cold allodynia. These findings suggested that TRPM8 plays a role in cold allodynia caused by oxaliplatin.

Ta *et al*[41] showed that mice DRG neurons treated with cisplatin or oxaliplatin displayed an increase in transient receptor potential vanilloid 1 (TRPV1), TRPA1, and TRMP8 mRNA expression. Trigeminal ganglion neurons from the cisplatin-treated animals showed increased TRPV1 and TRPA1 mRNA expression, and this was associated with enhanced heat and mechanical hypersensitivity. Conversely, oxaliplatin affected only TRPA1 expression, which induced cold and mechanic hypersensitivity.

Glial activation

Di Cesare Mannelli *et al*[47,48] first suggested a link between oxaliplatin-induced neuropathic pain and glial activation. In a rat model with oxaliplatin-induced peripheral neuropathy, they showed a transient activation of microglia and astrocytes in the spinal cord and supraspinal areas involved with pain modulation accompanied by a decrease in mechanical and thermal pain thresholds following intraperitoneal oxaliplatin administration[48]. Intrathecal co-administration of microglial inhibitor minocycline was able to prevent microglial activation, but had no effect on the response of astrocytes. The astrocytic activation could be inhibited by intrathecal injection of fluorocitrate, an astrocyte specific metabolic inhibitor. Fluorocitrate did not influence oxaliplatin-induced microglial activation. Both drugs increased pain tolerance, but fluorocitrate produced greater pain relief than minocycline. However, neither minocycline nor fluorocitrate prevented oxaliplatin-

dependent morphological alterations in DRG neurons[48]. These findings provide some evidence for the participation of glial cells in oxaliplatin-induced neuropathy.

Involvement of nicotinic receptors

Oxaliplatin treatment was found to induce down regulation of alpha7 nicotinic acetylcholine receptor (nAChR) in the rat sciatic nerve, DRG, and spinal cord[49]. The administration of the selective alpha7 nAChR agonists (R)-ICH3 and PNU-282987 could prevent receptor down regulation and increase the pain threshold by oxaliplatin. These two agonists also could inhibit oxaliplatin-induced morphological changes in DRG and peripheral nerves, and upregulate glial cell density in the spinal cord, thalamus, and somatosensory area 1. CDP-choline, the other selective alpha7 nAChR agonist, was also found to be effective in reducing oxaliplatin-induced mechanical hyperalgesia when administered into the cerebral ventricles[50]. These findings suggested a neuroprotective role of alpha7 nAChR during oxaliplatin treatment.

DETECTION AND ASSESSMENT OF PLATINUM-INDUCED NEUROTOXICITY

Currently, no standard clinical method for the early detection and comprehensive assessment of platinum-induced neurotoxicity is known. The use of self-reporting questionnaires developed by the United States National Cancer Institute and European Organization for Research and Treatment of Cancer throughout the treatment course has been recommended as a simple clinical tool for determining and grading a pre-existing or new neuropathy[51,52]. These questionnaires contain items that evaluate the occurrence, severity, degree of distress, and frequency of neuropathic symptoms and their negative impacts on the patient daily activities.

Among neurophysiological techniques, nerve conduction velocity studies and electromyography remain the gold standard technique for detecting the location and extent of neuronal damage due to treatment with platinum drugs[1,6]. Nerve excitability studies performed before and immediately after oxaliplatin infusion have emerged as novel non-invasive tests for early identification of patients at high risk for severe neurotoxicity[35,53].

PREVENTION AND TREATMENT STRATEGIES

A recent Cochrane review examined the effects of the potential chemo-protective agents against neurotoxicity of platinum analogs[54]. This review included 29 randomized controlled trials (RCTs) and analyzed data from 2906 participants who received platinum-containing chemotherapy (cisplatin, carboplatin, or oxaliplatin) alone or in combination with a potential chemo-protectant, including amifostine, calcium/magnesium infusion, glutathione, Org 2766, acetylcysteine, oxcarbazepine, or vitamin E[54]. The data obtained in this study were found to be insufficient to recommend any particular agent to prevent or limit platinum drug neurotoxicity.

In 2014, the American Society of Clinical Oncology convened an expert panel to develop a clinical practice guideline for the prevention and treatment of chemotherapy-induced neuropathies in adult cancer survivors[55]. The experts reviewed 48 RCTs that investigated the efficacy of pharmacological agents, including antiepileptic drugs (carbamazepine and oxcarbazepine), antidepressants (amitriptyline, nortriptyline, venlafaxine and duloxetine), vitamins/minerals (calcium/magnesium infusions, vitamin E, and glutamine), and antioxidants (glutathione, N-acetylcysteine, and amifostine) against neuropathic pain caused by platinum compounds, paclitaxel or vinca alkaloids. They concluded that enough evidence to support routine clinical implementation of these agents for the prevention of platinum-induced peripheral neurotoxicity was not found. Conversely, duloxetine was found potentially useful for treating oxaliplatin-induced neuropathic pain.

CONCLUSION

The apoptotic loss of DRG neurons plays a central role in the initiation and progression of platinum-induced neurotoxicity. Recent evidence suggests that secondary mitochondrial dysfunction can mediate and aggravate cisplatin-mediated neuronal damage. Impaired activity of voltage-gated ion channels and/or increased sensitivity of TRP channels in sensory neurons seem to be the major events leading to the development of oxaliplatin-induced acute neurological side effects, including cold-induced paresthesias and painful dysesthesias. The potential roles of glial cells and nAChRs in platinum-induced neurotoxicity deserve further investigation to explore new strategies to prevent and to treat this side effect.

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ORIGINAL ARTICLE

Retrospective Study

Physician approaches to drug shortages: Results of a national survey of pediatric hematologist/oncologists

Jill C Beck, Baojiang Chen, Bruce G Gordon

Jill C Beck, Baojiang Chen, Bruce G Gordon, Department of Pediatric Hematology/Oncology, University of Nebraska Medical Center, Omaha, NE 68198-2168, United States

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Correspondence to: Jill C Beck, MD, Department of Pediatric Hematology/Oncology, University of Nebraska Medical Center, 982168 Nebraska Medical Center, Omaha, NE 68198-2168, United States. jill.beck@unmc.edu
Telephone: +1-402-5597257
Fax: +1-402-5596782

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Abstract

AIM

To evaluate personnel involved in scarce drug prioritization and distribution and the criteria used to inform drug distribution during times of shortage among pediatric hematologists/oncologists.

METHODS

Using the American Society of Pediatric Hematology/Oncology (ASPHO) membership list, a 20 question survey of pediatric hematologists/oncologists was conducted *via* email to evaluate personnel involved in scarce drug prioritization and distribution and criteria used to inform scarce drug distribution.

RESULTS

Nearly 65% of the 191 study respondents had patients directly affected by drug shortages. Most physicians find out about shortages from the pharmacist (n = 179, 98%) or other doctors (n = 75, 41%). One third of respondents do not know if there is a program or policy for handling drug shortages at their institution. The pharmacist was the most commonly cited decision maker for shortage drug distribution (n = 128, 70%), followed by physicians (n = 109, 60%). One fourth of respondents did not know who makes decisions about shortage drug distribution at their institution. The highest priority criterion among respondents was use of the shortage drug for curative, rather than palliative intent and lowest priority criterion was order of arrival or first-come first-served.

CONCLUSION

Despite pediatric hematology/oncology physicians and patients being heavily impacted by drug shortages, institutional processes for handling shortages are lacking. There is significant disparity between how decisions for distribution of shortage drugs are currently made and how study respondents felt those decisions should be made. An institution-based, and more importantly, a societal

approach to drug shortages is necessary to reconcile these disparities.

Key words: Pediatric hematology/oncology; Chemotherapy; Ethics

Core tip: The frequency of drug shortages are increasing and heavily impact physicians and patients. However, processes for handling drug shortages are lacking. An institution-based, and more importantly, a societal approach to drug shortages are necessary to reconcile these disparities.

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INTRODUCTION

Drug shortages occur whenever demand for a medication is greater than the available supply. The frequency of drug shortages has increased considerably over the last decade due to decreased availability of raw materials, disruptions along the supply chain, economic decisions by drug companies and health care systems, and increased demand[1-5]. Although drug shortages are a global issue, they have significantly impacted the United States due to low pharmaceutical company reimbursement rates for generic drugs with narrow cost margins[4,5].

Important ethical issues arise whenever the supply of an effective drug is insufficient to meet demand. The principles of beneficence, non-maleficence, and justice can guide prioritization of scarce drug distribution, but an individual's application of those principles may vary widely. The ethical principle of autonomy, which so commonly drives ethical decision making, is not relevant to drug shortages, perhaps making these decisions more difficult.

Several reports have evaluated the impact of drug shortages and ethical frameworks have been proposed for handling shortages, however, no studies have evaluated the key factors physicians use to determine drug distribution during times of shortage[3,6-13]. A more thorough understanding of the decision-making processes physicians are using will assist in developing frameworks and policies to more effectively manage drug shortages.

MATERIALS AND METHODS

Study review

The Institutional Review Board at University of Nebraska Medical Center (UNMC) in Omaha, Nebraska reviewed and approved the study.

Study population

The online membership directory from the American Society of Pediatric Hematology/Oncology (ASPHO) was used to identify pediatric hematology/oncology physicians in the United States.

Survey

A twenty question survey of pediatric hematologists/oncologists was conducted *via* email to evaluate demographics of study respondents, personnel involved in scarce drug prioritization and distribution, and criteria used to inform scarce drug distribution (Appendix Ⅰ). The survey was developed and piloted by the authors. The questions evaluating demographics and personnel involved in prioritization were multiple-choice closed-ended questions. Demographic information included current position, type of patients seen, practice type, and years since completing fellowship. Survey questions regarding experiences with drug shortages included whether drug shortages had directly affected the provider's patients and whether the provider knew of drug shortages at the institution but the shortage had not resulted in the provider's patient not receiving a needed medication. In addition, respondents were asked to identify whether the shortage drugs were chemotherapy or non-chemotherapy, however further specifics were not requested. Questions also addressed whether the respondent's institution has a program or policy to handle drug shortages, whether the provider felt a program or policy was necessary, who makes decisions about shortage drug distribution at their institution, and who should make decisions about shortage drug distribution. Respondents were asked to choose all decision makers with no limit on the number of answers and were asked to specify with an open-ended response if they chose other. The criteria for scarce drug distribution provided eight statements which were evaluated using a Likert scale ranging from 0 (strongly disagree) to 100 (strongly agree).

Survey questions did not specify definitions for relative terms such as length of survival, age, type or length of therapy, or dose. Study data was collected and managed using REDCap electronic data capture tools hosted at UNMC. REDCap is a secure, web-based application designed to support data capture for research studies. The invitation email provided an explanation of and electronic link to the voluntary survey. Participants entered responses directly into the online REDCap survey and responses were de-identified.

Survey population

At the time of this study, 1259 physicians in the United States had available email addresses in the ASPHO online membership roster and were emailed survey invitations. Twenty-nine addresses were undeliverable.

Table 1 Demographics

Characteristic	*n* (%)
Current position	
Attending physician	161 (84.7)
Fellow	23 (12.1)
Non-practicing physician	2 (1.1)
Type of patients	
Oncology	162 (85.7)
Hematology	145 (76.7)
Stem cell transplant	54 (28.6)
Do not see patients	2 (1.1)
Type of practice	
Academic medical center	160 (83.8)
Community/private institution	39 (15.7)
Years since completing fellowship	
Less than 5	64 (34.4)
5-10	44 (23.7)
11-20	33 (17.7)
More than 20	45 (24.2)
Gender	
Female	104 (55)
Male	85 (45)

Of the 1230 remaining physicians, 191 (15.5%) responded and were included in the study analysis.

The majority of respondents were attending pediatric hematologist/oncologists currently practicing (*n* = 161, 84.7%) and 12.1% (*n* = 23) were fellows in pediatric hematology/oncology (Table 1). The types of patients seen by the surveyed physicians were mostly oncology (*n* = 162) and hematology (*n* = 145), with 54 physicians seeing stem cell transplant patients. Sixty-seven percent of physicians saw multiple patient types. Most respondents practice in an academic medical center (*n* = 160, 83.8%) with the remainder practicing in a community or private institution (*n* = 30, 15.7%). The number of years since completing pediatric hematology/oncology fellowship was nearly evenly divided among respondents.

Statistical analysis

χ^2 or Fisher's Exact test was used to obtain *P*-values to evaluate association among categorical responses. A *P*-value less than 0.05 indicates statistical significance. Logistic regression was used to determine the odds ratios to study the association of the outcomes with all variables simultaneously. Responses to scaled questions were reported as means and compared using analysis of variance.

RESULTS

Nearly 65% of study respondents had patients directly affected by drug shortages where the provider was not able to prescribe a needed medication for his/her patient due to a shortage and 79% of study respondents knew of drug shortages at their institution, but the shortage did not result in a patient under the provider's care not receiving a needed drug. Physicians practicing in an academic medical center were more likely to have patients directly affected than physicians practicing in a community or private institution (OR = 2.61, 95%CI: 1.10-6.21). The physician's type of patients (hematology, oncology and/or stem cell transplant) did not impact the rate of patients directly affected by shortages.

Table 2 Decision makers for shortage drug distribution

	Who makes the decision about distribution of shortage drugs at your institution?	%	Who should make the decision about distribution of shortage drugs at your institution?	%
Pharmacist	128	70.3	147	80.3
Physician	109	59.9	152	83.1
Hospital administration	41	22.5	35	19.1
Panel/group	32	17.6	77	42.1
Ethics committee	8	4.4	35	19.1
Nurse	1	0.5	8	4.4
Parent	1	0.5	6	3.3
Do not know	46	25.3	13	7.1
Total responses	366		473	

Most physicians find out about drug shortages from the pharmacist (*n* = 179, 98%) or other doctors (*n* = 75, 41%). Other sources of information about drug shortages include a list or website (*n* = 69, 38%) and nurses (*n* = 13, 7%). Three respondents receive information from the Pharmacy and Therapeutics Committee or a drug shortage task force. Sixty-six percent found out from more than one of the above sources. One respondent stated they do not find out about drug shortages.

Sixty-two percent of respondents work at institutions that have a program or policy to handle drug shortages and 4% of institutions do not have a program or policy. One third of respondents do not know if there is a program or policy for handling drug shortages at their institution. However, 95% of respondents felt that a program or policy is necessary.

The pharmacist was the most commonly cited decision maker for shortage drug distribution (*n* = 128, 70%), followed by physicians (*n* = 109, 60%), hospital administration (*n* = 41, 23%), a panel or group (*n* = 32, 18%), ethics committee (*n* = 8, 4%), parent (*n* = 1, 0.5%), and nurse (*n* = 1, 0.5%) (Table 2). Sixty-six percent reported multiple decision makers. One fourth of respondents did not know who makes decisions about shortage drug distribution at their institution. In contrast, respondents felt that the physician (*n* = 152, 83%) and pharmacist (*n* = 147, 80%) should be the decision maker for shortage drug distribution. Other responses included a panel or group (*n* = 77, 42%), ethics committee (*n* = 35, 19%), hospital administration (*n* = 35, 19%), nurse (*n* = 8, 4%), and parent (*n* = 6, 3%). Seven percent of respondents did not know who should make the decision about distribution of shortage

Table 3 Prioritization of distribution criteria

	Mean	Criteria	Ethical Framework
Strongly disagree	0.0		
	37.1	Order of arrival should impact the priority given to a patient	First-come first-served
	41.8	A patient with fewer co-morbidities should be given priority over a patient who has more co-morbidities	Sickest first
	44.3	Younger patients should receive priority over older patients	Fair innings
	47.3	A patient with longer anticipated survival should be given priority over a patient with shorter anticipated survival	Saving the most
	51.8	A patient needing a small dose of a shortage drug should be prioritized over a patient needing a larger dose	Saving the most
	57.2	A patient using a drug for an approved indication should have priority over a patient using the drug for off-label use	Saving the most
	61.0	A patient who is starting therapy should be prioritized over a patient who has nearly completed therapy	Saving the most
	74.3	A patient using the shortage drug for curative intent should be prioritized over a patient using the drug for palliation	Saving the most
Strongly agree	100.0		

drugs.

Respondents ranked criteria for prioritizing scarce drug distribution on a scale of 0 (strongly disagree, low priority) to 100 (strongly agree, high priority) (Table 3). Respondents prioritized use of the shortage drug for curative, rather than palliative intent (a patient using the shortage drug for curative intent should be prioritized over a patient using the shortage drug for palliation, mean 74) as the most important criteria in determining which patient should receive a shortage drug.

Prioritization of patients starting *vs* completing therapy (a patient who is starting therapy should be prioritized over a patient who has nearly completed therapy, mean 61), using a drug for an approved indication (a patient using a drug for an approved indication should have priority over a patient using the drug for off-label use, mean 57), dose (a patient needing a small dose of a shortage drug should be prioritized over a patient needing a larger dose, mean 52), anticipated survival (a patient with longer anticipated survival should be given priority over a patient with shorter anticipated survival, mean 47), patient age (younger patients should receive priority over older patients, mean 44), and number of comorbidities (a patient with fewer co-morbidities should be given priority over a patient who has more comorbidities, mean 42) had closely ranked means. The priority ranking for anticipated survival (mean 44 *vs* 58, $P = 0.005$) and patient age (mean 40 *vs* 58, $P = 0.008$) was lower for physicians practicing at academic medical centers compared to community or private institutions. The priority ranking for using a drug for an approved indication was higher for fellows than attendings (mean 72 *vs* 54, $P = 0.02$). As the years after fellowship increased, the priority of mean rank of prioritizing patients starting *vs* completing therapy also increased (< 5 years mean 53, 5-10 years mean 63, 11-20 years mean 64, > 20 years mean 66, $P = 0.04$).

The lowest priority criterion was order of arrival [order of arrival (first come-first served) should impact the priority given to a patient, mean 37]. The priority ranking for order of arrival for physicians practicing at academic medical centers was significantly lower than physicians in community or private institutions (mean 34 *vs* 45, $P = 0.03$). Physicians whose patients were directly affected by drug shortages gave lower priority to order of arrival than physicians who did not have patients directly affected by shortages (mean 33 *vs* 42, $P = 0.01$).

DISCUSSION

While numerous reports have detailed the impact of drug shortages and several ethical frameworks have been proposed for handling shortages, this is the first survey of physicians evaluating individual approaches to prioritization[3,6-13]. The frequency of drug shortages have increased over the last decade and have disproportionately affected oncology due to quality issues, limited manufacturers, and complex production processes with specialized equipment[3,4]. In addition, chemotherapy medications often have no equivalent for substitution, whereas other drugs may have several alternatives within a medication class[5]. Unique issues arise when dealing with drug shortages in pediatric oncology due to the increased use of off-label drugs in pediatrics compared to adult medicine and dosing based on weight and size[2,3,7,14]. In addition, pediatric patients have surrogate decision makers, usually parents, determining the child's best interest in a situation in which no alternative may be ideal.

In this study, nearly 65% of respondents had patients unable to receive a needed drug due to shortages and 79% knew of drug shortages at their institution that had not directly affected their patients. Overall, physicians report they are informed about drug shortages from a variety of sources, most commonly pharmacists, other doctors, and/or a list or website. Despite being heavily impacted by and well informed about drug shortages,

institutional processes for handling shortages are lacking, or at least are not well known to the physicians caring for the patients: One third of respondents did not know if there is a program or policy for handling drug shortages at their institution.

There is significant disparity between how decisions for distribution of shortage drugs are made and how study respondents felt those decisions should be made. Currently, the pharmacist was the most commonly cited decision maker for shortage drug distribution, followed by physicians. Although respondents felt that physicians and pharmacists should be included in decisions regarding shortage drugs, many believed that a panel, group, or ethics committee should also be involved. This is not currently the practice at most institutions surveyed and may reflect the need to systematically involve more members of the health care team.

Physicians in academic medical centers were more likely to have patients directly affected by shortages than those in community or private institutions. It is not clear why this is the case, however, a report of adult oncologists found private practice providers were more likely to use brand name rather than generic drugs which more frequently have shortages[5]. The criteria of using a drug for an approved indication was given higher priority by fellows than attendings, likely reflecting increased comfort with using off-label medications as experience increased.

Important ethical issues arise whenever the supply of an effective drug is insufficient to meet demand. The principles of beneficence, non-maleficence, and justice can guide prioritization of scarce drug distribution, but as is demonstrated in this study, an individual's application of those principles may vary widely. The ethical principle of autonomy, which so commonly drives ethical decision making, is not relevant to drug shortages perhaps making these decisions even more difficult. Beneficence encourages safe and effective care such as utilizing evidence-based medicine and optimizing resource utilization. Non-maleficence promotes minimizing pain and suffering, a major potential effect on a patient unable to obtain a needed medication. Justice demands reasonable access to resources and is threatened by the many causes of shortages as well as institutional stockpiling of shortage drugs.

Prioritization frameworks have been proposed, some of which include "sickest first", "fair innings", "first-come first-served", and "saving the most", among others (Table 3)[3,13,15]. Sickest first" prioritizes patients based on degree of illness. Therefore, using the sickest first criteria to distribute shortage drugs, patients with the worst disease and the most co-morbidities would have highest priority. The fair innings approach argues that each person should have an equal opportunity to live a normal lifespan. The prioritization criterion of patient age relies on the fair innings approach. "First-come first-served" distributes shortage drug based on order of arrival. Finally, the goal of saving the most is to provide the most good to the most patients. In the case of drug shortages, this method focuses on the indication of the drug and goals of care while balancing risks and benefits[3].

In this survey, the highest priority criterion was use of the shortage drug for curative, rather than palliative, intent, which evokes the ethical framework of saving the most. The criteria of starting *vs* completing therapy, using a drug for an approved indication, dose, and anticipated survival completed the top five highest ranked criteria and all rely on the concept of saving the most. Therefore, the ethical framework of saving the most is used most often by pediatric hematologist/oncologists in the United States when considering shortage drug distribution.

Drug shortages require the physician to consider both the good of an individual patient and society at large. Using the framework of saving the most necessitates physicians to prioritize their patient within the larger context of the population. An institution-based, and more importantly, a societal approach to drug shortages is necessary to reconcile the physician-patient relationship with that of the larger population. Cooperative groups and medical societies can play an important role in this process.

Several groups have begun to address drug shortages in pediatric oncology. The Working Group on Drug Shortages in Pediatric Oncology has created recommendations for responding to drug shortages, COG gives guidelines for management of shortages within treatment protocols, the American Society of Health-System Pharmacists has provided guidelines and recommendations, and the American Board of Pediatrics, American Society of Clinical Oncology, and American Society of Pediatric Hematology/Oncology have provided commentary and position statements[6,7,11,16]. However, given the continued prevalence of drug shortages and their widespread impact, a continued coordinated effort is needed to ensure consistency and provide guidance for implementation. The recently published follow-up recommendations to the Working Group on Drug Shortages in Pediatric Oncology by Unguru *et al*[13] provides much needed concrete methods for shortage drug distribution in pediatric oncology that can ideally be adopted within and across institutions. Pediatric oncology is a prime subspecialty to formulate this coordinated endeavor given its established history of collaboration and could potentially set the stage as a model for other subspecialties impacted by drug shortages.

The lowest priority criterion in this survey was first-come first-served. Interestingly, this criterion may actually be used frequently in practice. One third of respondents did not know if there is a program or

policy for handling drug shortages at their institution. In addition, respondents who had patients directly affected by drug shortages gave first-come first-served lower priority than physicians who did not have patients directly affected by shortages. At those institutions, shortage drugs are rationed at the bedside and priority for shortage drug distribution is likely first-come first-served by default. If a program or policy for handling drug shortages is not in place, advance decision-making is unlikely to occur resulting in a process that may not be reasonable or transparent. This discrepancy between aims and reality highlights the need for clear institutional and ideally national guidelines developed prior to a drug shortage.

Limitations to this study include the limited sample size and voluntary nature of the survey resulting in possible selection bias. The small sample size offers trends in approaches to prioritization of shortage drug distribution. Non-practicing physicians were included because the decision processes are not limited to practicing providers. The survey statements were left purposefully broad to allow respondent interpretation, as is the case in clinical practice, however this approach may impact validity. However, this is the first national survey of physicians on the topic of physician prioritization criteria used for shortage drugs. The crucial finding of this study is the disparity between how decisions are made and how respondents feel they should occur, reinforcing the need for continued attention to organizational frameworks and policy development. Perspectives of other physician subspecialty groups, health professionals, patients, or family members are an important area for follow-up and such studies are underway by the authors.

COMMENTS

Background

The frequency of drug shortages is increasing. Important ethical issues arise whenever the supply of an effective drug is insufficient to meet demand. The principles of beneficence, non-maleficence, and justice can guide prioritization of scarce drug distribution, but an individual's application of those principles may vary widely. The ethical principle of autonomy, which so commonly drives ethical decision making, is not relevant to drug shortages, perhaps making these decisions more difficult.

Research frontiers

Little data is available evaluating the key factors physicians use to determine drug distribution in times of shortage. A more thorough understanding of the decision-making processes physicians are using will assist in developing frameworks and policies to more effectively manage drug shortages.

Innovations and breakthroughs

The frequency of drug shortages are increasing and heavily impact physicians and patients. However, processes for handling drug shortages are lacking. An institution-based, and more importantly, a societal approach to drug shortages is necessary to reconcile these disparities.

Applications

While numerous reports have detailed the impact of drug shortages and several ethical frameworks have been proposed for handling shortages, this is the first survey of physicians evaluating individual approaches to prioritization. Unique issues arise when dealing with drug shortages in pediatric oncology due to the increased use of off-label drugs in pediatrics compared to adult medicine and dosing based on weight and size. Given the continued prevalence of drug shortages and their widespread impact, a continued coordinated effort is needed to ensure consistency and provide guidance for distribution of shortage drugs. Pediatric oncology is a prime subspecialty to formulate this coordinated endeavor given its established history of collaboration and could potentially set the stage as a model for other subspecialties impacted by drug shortages.

Peer-review

The manuscript is well written and the topic is quite relevant.

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ORIGINAL ARTICLE

Retrospective Study

Trans-arterial chemoperfusion for the treatment of liver metastases of breast cancer and colorectal cancer: Clinical results in palliative care patients

Tatjana Gruber-Rouh, Marcel Langenbach, Nagy NN Naguib, Nour-Eldin M Nour-Eldin, Thomas J Vogl, Stephan Zangos, Martin Beeres

Tatjana Gruber-Rouh, Marcel Langenbach, Nagy NN Naguib, Nour-Eldin M Nour-Eldin, Thomas J Vogl, Stephan Zangos, Martin Beeres, Institute for Diagnostic and Interventional Radiology, University Hospital Frankfurt, 60590 Frankfurt am Main, Germany

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Correspondence to: Tatjana Gruber-Rouh, MD, Institute for Diagnostic and Interventional Radiology, University Hospital Frankfurt, Theodor-Stern-Kai 7, 60590 Frankfurt am Main, Germany. tatjana.gruber-rouh@kgu.de
Telephone: +49-69-63018720
Fax: +49-69-63017258

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Abstract

AIM

To evaluate the clinical value and efficiency of trans-arterial chemoperfusion (TACP) in patients with liver metastases from breast cancer (BC) and colorectal cancer (CRC).

METHODS

We treated 36 patients with liver metastases of BC (n = 19, 19 females) and CRC (n = 17; 8 females, 9 males) with repeated TACP. The treatment interval was 4 wk. TACP was performed with gemcitabine (1000 mg/m^2) and mitomycin (10 mg/m^2), administered within 1 h after positioning the catheter tip in the hepatic artery. Before treatment, the size, location, tumour volume, vascularization and number of liver tumours were evaluated using magnetic resonance imaging (MRI). Tumour response was evaluated according to the Response Evaluation Criteria in Solid Tumors guidelines.

RESULTS

TACP using gemcitabine and mitomycin for metastases from CRC and BC was performed without any serious side effects. The follow-up MRI showed a therapeutic response in 84.2% of the BC patients - stable disease 47.4% and partial response 36.8%. A progression was seen in 15.8%.

CRC patients showed a therapeutic response in 52.9% of cases. A progression of the disease was documented in 47.1% of the patients with CRC. These data show that TACP in patients with liver metastases of BC leads to a significantly better therapeutic response compared with CRC patients (P = 0.042). The median survival time was 13.2 mo for the BC patients, which is significantly longer than for CRC patients at 9.3 mo (P = 0.001).

CONCLUSION

TACP for liver metastases of BC appears to be a safe and effective palliative treatment with improved outcomes in comparison to patients with CRC.

Key words: Colorectal neoplasms; Breast neoplasms; Neoplasm metastasis; Neoplasms; Drug therapy

Core tip: Trans-arterial chemoperfusion could be an alternative treatment option for advanced stage palliative patients suffering from liver-dominant metastatic disease.

Gruber-Rouh T, Langenbach M, Naguib NNN, Nour-Eldin NEM, Vogl TJ, Zangos S, Beeres M. Trans-arterial chemoperfusion for the treatment of liver metastases of breast cancer and colorectal cancer: Clinical results in palliative care patients. *World J Clin Oncol* 2017; 8(4): 343-350 Available from: URL: http://www.wjgnet.com/2218-4333/full/v8/i4/343.htm DOI: http://dx.doi.org/10.5306/wjco.v8.i4.343

INTRODUCTION

Liver metastases are often found in malignant disease. In most cases, the appearance of liver metastases is associated with a poor prognosis of the disease. One third of all patients have metastases even at the time of the primary diagnosis of their cancer. Half of patients resected in an early tumour stage will develop metastases, especially in the liver. Currently, the surgical approach is seen as the only curative treatment for liver metastases. However, only in 20% of patients can curative surgery of their liver metastases be performed[1,2].

Here, other treatment options have to be considered, such as systemic chemotherapy, loco-regional chemotherapy or selective internal radiation therapy (SIRT). In all, the treatment of liver metastases is an interdisciplinary decision that should be discussed in an interdisciplinary tumour board.

Reasonable results have been achieved in the past using intra-arterial chemotherapy, especially in metastases of colorectal cancer (CRC), breast cancer (BC) and neuroendocrine tumours[3]. The main idea underlying the intra-arterial delivery of cytotoxic medication is that the liver predominantly derives its blood from the portal venous system, while the metastases predominantly use the arterial system for their blood supply[4]. As higher concentrations of the chemotherapeutic agent can be used, using the "first pass mechanism" of the liver, less cytotoxic medication arrives in the systemic circulation resulting in only minimal side effects.

In our study, 36 patients with unresectable, therapy-resistant advanced hepatic metastases of colorectal and breast cancer were treated with hepatic intra-arterial chemotherapy (HIC). Our palliative patient cohort consisted in most cases of patients with symptomatic disease. Gemcitabine as an antimetabolite was chosen because of its tolerable hematologic toxicity and its effect in tumour biology similar to fluorouracil (5-FU). Mitomycin C was added to the chemotherapy protocol because in previous studies at our department it has demonstrated good response rates, especially in HIC pre-treated patients[5]. Primary endpoints of our retrospective analysis were tumour response, patient survival and the time at which the maximum therapeutic effect could be observed.

MATERIALS AND METHODS

Pre-treatment evaluation

The patients' medical histories were evaluated and documented in detail. Patients were included if the liver was the only organ with metastases, except for BC patients who were included if they also had bone metastases. Patients were only included if the metastases could not be resected and other ablative treatment, *e.g.,* radiofrequency ablation (RFA), microwave ablation (MWA) or laser-induced thermotherapy (LITT), could not be performed. All patients had undergone surgery for their primary tumour and some of the patients (n = 13) also for their liver metastases. Each patient had undergone several therapies before, which were stopped because of progression of the disease or side effects with following progressive disease. Only adult patients with an Eastern Cooperative Oncology Group (ECOG) performance score of 0 or 1 and an estimated remaining survival time of $\geqslant$ 12 wk were treated. Female patients who were pregnant or breastfeeding were excluded. A minimum of three sessions in 4-wk intervals were performed in an outpatient setting. Sufficient coagulation parameters, bone marrow, renal and hepatic function were required. These parameters in general were evaluated before each treatment session. In the case of acute infection, dysfunction of the liver, kidney or bone marrow, as well as worsening of the general condition, therapeutic intervals were extended or the therapy was discontinued (Table 1). Before treatment, the size, location, vascularization and number of the liver tumours were evaluated using contrast-enhanced magnetic resonance imaging (MRI; 1.5 T; Magnetom Symphony, Siemens, Erlangen, Germany) as a baseline evaluation. Unenhanced T1- and T2-weighted spin-echo (SE) and gradient-echo (GE) sequences, as well as

Table 1 Indications and contraindications of trans-arterial chemoperfusion

Indications
Unresectable liver metastases
Liver-dominant metastatic disease
Minimum of three different chemotherapies before
No systemic chemotherapy available
Symptomatic liver metastases
Contraindications
ECOG >1
Tumour burden of the liver > 75%
Poor liver function (quick < 40%, PTT < 45 s, albumin < 2 g/dL)
Extensive amounts of ascites
Obstructive icterus (bilirubin > 3 mg/dL)
Acute infection
Myelodepression (leucocytes < 2000/mL, platelets < 100000/μL)
Limited kidney function (creatinine > 2 mg/dL)
Extensive heart insufficiency (> NYHA Ⅱ)

ECOG: Eastern Cooperative Oncology Group; PTT: Prothrombin time test; NYHA: New York Heart Association.

contrast-enhanced T1 sequences (True Fisp, HASTE, TSE, FLASH-2D in-phase and opposed phase and dynamic sequences), were used. Tumour response was evaluated according to the Response Evaluation Criteria in Solid Tumors (RECIST) guidelines.

Intervention

All patients were informed of the risks, side effects and other therapeutic options at least 24 h before the start of therapy. Informed consent was obtained. As pre- and concomitant medication for the most common side effects pethidine (Dolantin, Sanofi-Aventis, Frankfurt, Germany), granisetron (Kevatril, Roche, Mannheim, Germany) and dexamethasone were administered. After applying local anaesthesia, a commercially available angiographic catheter was introduced through the femoral artery using the Seldinger technique. In our cases, 4 or 5F gate (Introducer Ⅱ, Terumo, Eschborn, Germany) and Pigtail, Renegade (Boston Scientific, Munich, Germany), Sidewinder and Headhunter (Terumo, Eschborn, Germany) catheters were used. After an angiography of the aorta to rule out an abnormal anatomy of the vessels or atypical tumour vessels, an angiography of the upper abdomen was performed to evaluate the vascularization of the liver and the metastases. The catheter was then selectively placed in the right, the left or the common hepatic artery, depending on the tumour localization. In cases of anatomic variants or accessory hepatic arteries supplying the tumour, these arteries were selectively catheterized. Following our procedure, the two chemotherapeutic drugs were administered over 60 min using a perfusor (Perfusor, B. Braun; Melsungen, Germany). Our therapy consisted of 1000 mg/m^2 gemcitabine (Gemzar, Lilly, Bad-Homburg, Germany) and 10 mg/m^2 body surface mitomycin C (Mitomycin, Medac, Hamburg, Germany).

Response evaluation

Therapy response was evaluated after the third therapy cycle according to the RECIST criteria. "Complete response" (CR) was defined as the disappearance of all tumour lesions, "partial response" (PR) as a reduction of > 30%, "stable disease" (SD) as a reduction of < 30% or a growth of < 20% and "progressive disease" (PD) as a growth of > 20% or the occurrence of new lesions; all changes were relative to the baseline imaging (Table 2). Therapeutic response was defined as "complete response", "partial response" or "stable disease". The trans-arterial chemoperfusion (TACP) therapy was discontinued if tumour progression had occurred. In that case, alternative therapy options were discussed in an interdisciplinary tumour board and subsequently discussed with the patient. In the case of tumour response, therapy was continued as long as tumour growth could be controlled, or until it was possible to follow up with surgery or an interventional approach to remove the remaining tumour lesions.

Table 2 Response Evaluation Criteria in Solid Tumors

Category	RECIST
CR	Disappearance of all tumour lesions
PR	Reduction of > 30% in total tumour size
SD	Reduction of < 30% or a growth of < 20%
PD	Growth of > 20% or occurrence of new lesions

RECIST: Response Evaluation Criteria in Solid Tumors; CR: Complete response; PR: Partial response; SD: Stable disease; PD: Progressive disease.

Statistical analysis

Institutional Review Board approval for this retrospective study was obtained. All statistical analyses were performed in SPSS 15.0.1 (SPSS Inc.; United States, 2006). Survival data were assessed according to the Kaplan-Meier method. Groups were compared using the χ^2 test and the Cochran-Armitage trend test, as appropriate. The Mann-Whitney *U* test was used to evaluate tumour volumes because these data were not normally distributed. Survival times were compared with the log rank test. For each test, a *P*-value < 0.05 was considered to indicate a statistically significant difference.

Patients

In total, 36 patients with liver metastases of CRC (n = 17; 8 females, 9 males) and BC (n = 19, 19 females) were treated with repeated hepatic trans-arterial chemoperfusion (TACP). The median age of our patients at the start of the therapy was 60.5 years. In the patients with CRC (n = 17; 8 females, 9 males) the median age at the beginning was 64 years (range 43-84 years); in the patients with BC (n = 19, 19 females) the median age was 55 years (range 37-77). The median survival from the start of the TACP therapy in BC patients was 13.2 mo and survival from diagnosis was 75.2 mo. The median survival from the beginning of the TACP therapy in CRC patients was 9.3 mo and the median survival from diagnosis was 36.9 mo.

Defining complete response, partial response and

Table 3 Responders *vs* non-responders[1] *n* (%)

Carcinoma	Therapy response (CR + PR + SD)	Non responders (PD)
CRC	9 (52.9)	8 (47.1)
Breast-Ca	16 (84.2)	3 (15.8)

[1]The difference between responders and non-responders reached statistical significance ($P = 0.042$, χ^2 test). CR: Complete response; PR: Partial response; SD: Stable disease; PD: Progressive disease; CRC: Colorectal cancer.

Table 4 Number of treatments[1]

Carcinoma	Mean	Median	Min	Max
CRC	5.2	5	3	8
Breast-Ca	7.7	7	3	17

[1]The difference reached significance ($P = 0.0458$, Mann-Whitney *U* test). CRC: Colorectal cancer.

stable disease as the overall response and progressive disease as non-response, the results are shown in Table 3.

Colorectal carcinoma: In all, seventeen patients in our study suffered from CRC. All patients had undergone surgery for their primary tumour and a minimum of three courses of chemotherapy. Three patients had metastases of 2-4 cm, four patients had metastases of 4-7 cm and in ten patients the metastases were larger than 7 cm. Concerning the number of liver metastases, no patient had one metastasis, two patients had two metastases, one patient had 3-4 metastases, four patients had 5-9 metastases and ten patients had multiple liver metastases.

Breast cancer: This group consisted of 19 patients, all female. All patients had undergone surgery for their primary tumour and a minimum of three courses of chemotherapy. Four patients had metastases of 2-4 cm in size, nine patients had metastases of 4-7 cm and in six patients the metastases were larger than 7 cm. Concerning the number of liver metastases in BC patients, one patient had only one metastasis (> 7 cm), one patient had two metastases, two patients had 3-4 metastases, two patients had 5-9 metastases and thirteen patients had multiple metastases. Table 4 shows the number of treatments.

RESULTS

Trans-arterial chemoperfusion as a palliative treatment was tolerated well by all patients. During our therapy sessions no major technical problems occurred. Concerning therapy side effects, we did not observe severe common toxicity criteria (CTC) grade Ⅲ, Ⅳ or Ⅴ adverse events. CTC grade Ⅰ and Ⅱ side effects were common in our therapy cohort. These emerged in most cases as fatigue, nausea, vomiting and reduced appetite. The typical duration of these found to be 2-6 d after TACP. Haematological adverse events such as mild thrombocytopenia (grade Ⅰ and Ⅱ), dropped white blood cell count and reduced Hb values were observed as well. No serious side effects occurred that require hospitalisation or any other major medical intervention or treatment. Albeit we did not record a specific survey, all patients of our cohort rated the therapy side-effects as less severe compared to previous systemic chemotherapy treatment they all had undergone before.

Table 5 Partial response, stable disease, progressive disease

Carcinoma	Partial response	Stable disease	Progressive disease	Total
Colon	2	7	8	17
Breast	7	9	3	19

Overall, we had 0 CR, 9 PR, 16 SD and 11 PD (Table 5). Comparing the success rates and the therapeutic responses, the difference between the two tumour groups reached statistical significance ($P = 0.042$, χ^2 test and $P = 0.0232$, Cochran-Armitage trend test). Patients with liver metastases of BC survived significantly longer compared to patients with CRC (median 13.2 mo *vs* 9.3 mo, $P = 0.001$ - log rank test).

Survival data

Comparing the overall response (OR = CR, PR and SD) *vs* PD for each tumour group, there was a significant difference between BC (OR = 16 patients, 84.2%) and CRC (OR = 9 patients, 52.9%). The difference reached statistical significance ($P = 0.042$, χ^2 test), which means that our treatment is more effective in BC patients than in CRC patients.

Colorectal carcinoma

Median survival time after the first HIC session was 9.3 mo, and after initial diagnosis of the primary tumour 36.9 mo (Figure 1). We found no CR, two PR, seven SD and eight PD after the third cycle of TACP. However, none of the CRC patients are now alive, which might be due to the palliative setting of our study.

Breast carcinoma

The median survival after the first HIC session was 13.2 mo (Figure 1), after initial diagnosis of the primary tumour 75.2 mo. We found no CR, seven PR, nine SD and three PD after the third cycle of TACP. Two patients with PR were treated by LITT and microwave ablation to treat their last remaining metastasis and they are both alive today - 55 and 61 mo after the first therapy with TACP.

DISCUSSION

The main idea for this study was that in many cases of CRC or BC, liver metastases are a main factor influencing survival. In recent years, many advances

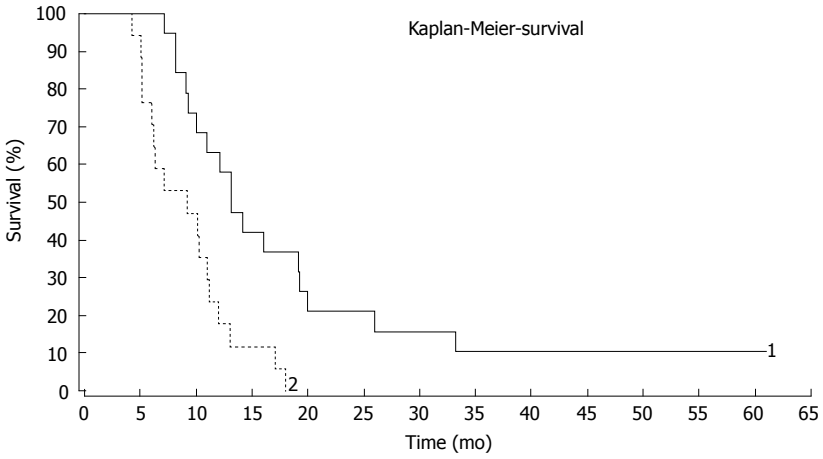

Figure 1 Kaplan-Meier survival curves. Survival time for BC *vs* CRC (P = 0.042, χ^2 test). 1: Survival data of all patients with liver metastases of BC after TACP (n = 19). Median survival time 13.2 mo; 2: Survival data of patients with liver metastases of CRC after TACP (n = 17). Median survival time 9.3 mo. BC: Breast cancer; CRC: Colorectal cancer; TACP: Trans-arterial chemoperfusion.

in therapy have been achieved. Without therapy, the median survival time with liver metastases of CRC is about 7.5 mo[6]. In BC, the time is about 6 mo[7,8]. The treatment of liver metastases is nowadays normally an interdisciplinary approach involving various departments, such as surgery, gynaecology, oncology and radiology. The standard therapy for liver metastases is still surgery, with the most promising outcome and the best long-term survival considering isolated liver metastases as a curable disease. Interventional radiological techniques, such as RFA, LITT and MWA have also been used as curative treatments of liver metastases. The limitations of such resection or ablative therapies are local spreading of tumours and unfavourable anatomical tumour localization[9]. Systemic chemotherapy can be viewed as standard in advanced disease. Nowadays, the systemic therapy regime consists of combinations of 5-FU, folinic acid, oxaliplatin, irinotecan, capecitabine and monoclonal antibodies bevacizumab or cetuximab. However, in general, such treatments are not suitable for all patients because of co-morbidities, major problems with the heart, liver or the kidneys (together with the tumour) or other disease. Loco-regional treatments can be an alternative to such general treatment. The loco-regional intra-arterial application of anti-tumour medication has now been an object of research for decades. In several studies, high tumour response rates have been achieved using this technique, but this does not necessarily lead to improved survival. In our study, this observation can be confirmed (overall response rate 69.5%; median survival 11 mo). The patients enrolled in this study were all in palliative care, they had all undergone surgery for their primary tumour and three courses of intravenous chemotherapy. Most patients had a high number of lesions concerning the liver (29 of 36 patients had more than 5 lesions and therefore disseminated liver disease), they were all multiply pretreated and therapy-resistant patients. Gemcitabine has not yet demonstrated high activity in CRC, but it has a more favourable toxicity profile compared to other cytostatic drugs and is well known in our institute as a treatment for palliative therapy[5].

Without therapy, the median survival time of patients with CRC liver metastases is between 3.8 to 21 mo[6,10,11]. The five-year survival rate is 3%-6.1%[6,8]. In liver metastases of BC, the median survival time is often less than 10 mo[12,13].

Currently, there are many different therapeutic strategies used for the treatment of liver metastases. Therapy for liver metastases now tends to be an interdisciplinary approach involving different clinical partners. For modern oncological therapy, concepts such as quality of life and the side effects of a therapy are increasingly important. These aspects are even more important in a palliative situation or when the malignant disease progresses. The gold standard therapy for liver metastases is the surgical approach, but this is only possible for 25% of patients with liver metastases with a curative intention[1,6,8,14]. If a complete R0 resection of the liver metastases is possible, this leads to 5-year survival rates in 10%-49% of patients and a median survival of up to 84 mo[6,15-17]. With adjuvant systemic chemotherapy, the 5-year survival time can be improved from 47.8% to 51.2%[18]. In a cohort study, the median survival time of the group treated with adjuvant chemotherapy (5-FU/FS) was 62 mo *vs* 46 mo in the control group[19]. The FOLFOX regimen is often used as an adjuvant chemotherapy protocol[20].

The indication for a surgical approach for metastases of BC is only given in patients with isolated liver metastases. However, only around 3%-5% of all BC patients show isolated metastases of the liver. With R0 resection of liver metastases in BC patients, 5-year survival rates of 33%-40% can be attained compared with R1 resection, depending on patient selection criteria[21]. In metastasized BC, chemotherapy is often used. Current therapy regimes are normally based on anthracycline or taxan chemotherapy protocols[22]. In some combination therapy studies, a median survival time of 10.3-24 mo and a 5-year survival rate of 18% maximum have been attained[23,24].

Many patients, even if their tumour is progressive, suffer from a liver-dominant metastatic disease. The side effects of loco-regional chemotherapy are often better tolerated by patients, which might be due

to the first pass effect of the chemotherapy in the liver[25,26]. Side effects are very rare during intra-arterial chemotherapy. For this reason, the therapy is performed on an outpatient basis[27]. We had no severe side effects (no CTC > 3). Intra-arterial chemotherapy remains a palliative treatment. In our study, we have shown a good response rate of 69.4%. In those patients, we achieved a partial response or stable disease after three courses of HIC. Especially in patients treated for liver metastases from BC, good tumour control was attained after the third session of TACP. In contrast to the stable disease or partial response in 84.2% of BC patients, the rate was only 52.9% among CRC patients, a statistically significant difference ($P = 0.042$, χ^2 test).

Thus, our results are similar to those of other studies. In 1999 a meta-analysis was published that showed a better response to HIC than systemic chemotherapy (41% *vs* 14%, *P*-value < 0.001) and a better median survival time (15 mo *vs* 11 mo, *P*-value < 0.009)[28]. Another study showed median survival in a group of patients receiving systemic chemotherapy of 20 mo compared to a median survival of 24.4 mo among patients treated with intra-arterial regional chemotherapy. Tumour response after systemic chemotherapy was 24% compared to 47% treated by intra-arterial chemotherapy[29]. The only limitation of this study was extrahepatic tumour progression, which the regional approach stopped for only 7.7 mo. The systemic approach in contrast stopped such progression for around 14.8 mo median. Intrahepatic tumour progression was better in the intra-arterial group (9.8 mo *vs* 7.3 mo). In recent years, more chemotherapeutic drugs have become available for intra-arterial chemotherapy. It has been documented that oxaliplatin, folinic acid and 5-FU intra-arterially administered (*via* a port system) attained a median survival time of 36.1 mo. The 2- and 3-year survival rates were 62% and 52% respectively[30].

The chemotherapy used in our study is normally used for the treatment of pancreatic cancer or BC[31]. For BC, gemcitabine is often administered, especially in second or third-line therapy[22]. In our institute, we have had good results using this combination[5].

In the treatment of liver metastases of CRC, many studies have shown that loco-regional treatment using intra-arterial chemotherapy is very promising[32-34]. Currently, for liver metastases of BC, intra-arterial chemotherapy is only rarely used[35].

If response to intra-arterial chemotherapy is documented, a repetition of the treatment is reasonable and generally the therapy can be repeated an unlimited number of time. This can lead to longer median survival and fewer side effects[29,31,36]. However, it is still a palliative therapy: Metastases can only be reduced and normally no general necrosis can be achieved. Nonetheless, intra-arterial chemotherapy in combination with local ablative procedures or with other therapeutic procedures is increasingly being used, for example in SIRT[37,38]. One study showed good response rates in primary and secondary liver tumours using combined TACE and LITT in a neoadjuvant setting[39]. Other promising studies have shown good response rates in combination with SIRT. This therapy might show good response rates especially in palliative care, without serious side effects[37,38,40].

Our results show that in the palliative care setting, a rather good response rate can be achieved using intra-arterial chemotherapy for liver metastases. However, perhaps the dosage of gemcitabine (1000 mg/m^2) was too low, or we should have used some embolization material in combination with our therapy protocol. In comparison to another study from our institute, we increased the mitomycin dosage to 10 mg/m^2 without serious side effects, but the effect was not as high as we expected[5]. Our palliative therapy should at least make the patient feel better, improve quality of life and suppress the symptoms of the disease. To achieve this, we used chemotherapy based on gemcitabine as, among the cytostatic drugs available, it has a good toxicity profile and provides clinical benefits. Based on the promising observations at our institute concerning the use of embolization material and SIRT, we aim to see what these therapy options will bring in further studies and we intend to use embolization material, other cytotoxic drugs and SIRT in earlier tumour stages.

In conclusion, our data indicate that repeated hepatic intra-arterial chemotherapy for liver metastases, especially of breast cancer appears to be a safe and effective palliative treatment with significantly improved outcomes in comparison to patients with colorectal cancer [χ^2 test *P*-value < 0.05 (= 0.042); statistically significant]. We observed good tumour response rates; indeed, although our treatment intention was palliative, two patients are still alive. In those two patients, suffering from breast cancer, an interventional ablative approach was performed following the intra-arterial chemotherapy to destroy their remaining lesions.

Our survival data lie within what we expected from the literature and our experience at the institute in the past. Selective and super-selective intra-arterial chemotherapy using gemcitabine and mitomycin for metastases from colorectal and breast cancer was performed without any serious side effects (CTC < 3). This is most likely due to the relatively low toxicity profile of gemcitabine and the loco-regional drug application, which resulted in lower systemic drug levels and lower side effects. More studies in the field of palliative care need to be undertaken to evaluate clearly the role of intra-arterial chemotherapy in the oncological therapy regime. Based on our findings to date, we think that this technique is an important therapy option that should be considered when a patient becomes palliative.

COMMENTS

Background

In malignant disease liver metastases can often be found. Currently, the surgical approach is seen as the only curative treatment for liver metastases.

However, only in 20% of patients can curative surgery of their liver metastases be performed. Here other treatment options have to be considered, such as systemic chemotherapy, locoregional chemotherapy, selective internal radiation therapy. Intraarterial chemotherapy showed reasonable results in the past, especially in metastases of colorectal cancer (CRC), breast cancer (BC) and neuroendocrine tumors. As higher concentrations of the chemotherapeutic agent can be used and, using the "first pass mechanism" of the liver, less cytotoxic medication arrives in the systemic circulation with only minimal side effects. Thuas, leading to the research question: To evaluate loco-regional chemoperfusion of liver metastases for tumor response, survival rate and therapy effect.

Research frontier

Loco-regional chemoperfusion is not in daily practise for tumor patients so far. However, the authors wanted to add this therapy as an additional tool for palliative cancer treatment, to open up this treatment as additional option to think of.

Innovations and breakthrough

In this study, the median survival time of CRC patients after the first hepatic intra-arterial chemotherapy (HIC) session was 9.3 mo, and after initial diagnosis of the primary tumor 36.9 mo. In breast cancer patients, the median survival time after first HIC session was 13.2 mo, and after initial diagnosis of the primary tumor 75.2 mo. This strengthened the authors' idea to keep this therapy as another option in mind.

Applications

This study suggests that loco-regional chemotherapy is useful in a palliative setting to treat liver dominant metastases without serious side effects.

Peer-review

The scientific question proposed in the manuscript were the results achieved with intra-arterial hepatic chemotherapy in 36 patients suffering from unresectable and therapy-resistant advanced and hepatically metastasized CRC and BC tumor response. It is a promising study to add another tool to the basket of palliative patient treatment; however, large population trials would be valuable in the future.

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ORIGINAL ARTICLE

Observational Study

Rescue associating liver partition and portal vein ligation for staged hepatectomy after portal embolization: Our experience and literature review

Charlotte Maulat, Antoine Philis, Bérénice Charriere, Fatima-Zohra Mokrane, Rosine Guimbaud, Philippe Otal, Bertrand Suc, Fabrice Muscari

Charlotte Maulat, Antoine Philis, Bérénice Charriere, Bertrand Suc, Fabrice Muscari, Department of Visceral Surgery, Toulouse-Rangueil University Hospital, 31059 Toulouse Cedex 9, France

Fatima-Zohra Mokrane, Philippe Otal, Department of Radiology, Toulouse-Rangueil University Hospital, 31059 Toulouse Cedex 9, France

Rosine Guimbaud, Department of Digestive Oncology, Toulouse-Rangueil University Hospital, 31059 Toulouse Cedex 9, France

Author contributions: Maulat C, Philis A, Charriere B and Muscari F performed research and wrote the paper; Mokrane FZ, Guimbaud R, Otal P and Suc B provided critical revision of the manuscript for important intellectual content.

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Correspondence to: Dr. Fabrice Muscari, Professor, Department of Visceral Surgery, Toulouse-Rangueil University Hospital, 1 Avenue du Pr Jean Poulhès, 31059 Toulouse Cedex 9, France. muscari.f@chu-toulouse.fr
Telephone: +33-56-1322088
Fax: +33-56-1322936

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Abstract

AIM

To report a single-center experience in rescue associating liver partition and portal vein ligation for staged hepatectomy (ALPPS), after failure of previous portal embolization. We also performed a literature review.

METHODS

Between January 2014 and December 2015, every patient who underwent a rescue ALPPS procedure in Toulouse Rangueil University Hospital, France, was included. Every patient included had a project of major hepatectomy and a previous portal vein embolization (PVE) with insufficient future liver remnant to body weight ratio after the procedure. The ALPPS procedure was performed in two steps (ALPPS-1 and ALPPS-2), separated by an interval phase. ALPPS-2 was done within 7 to 9 d after ALPPS-1. To estimate the FLR, a computed tomography scan examination was performed 3 to 6 wk after the PVE procedure and 6 to 8 d after ALPPS-1. A transcystic stent was placed during ALPPS-1 and remained opened during

the interval phase, in order to avoid biliary complications. Postoperative liver failure was defined using the 50-50 criteria. Postoperative complications were assessed according to the Dindo-Clavien Classification.

RESULTS

From January 2014 to December 2015, 7 patients underwent a rescue ALPPS procedure. Median FLR before PVE, ALPPS-1 and ALPPS-2 were respectively 263 cc (221-380), 450 cc (372-506), and 660 cc (575-776). Median FLR/BWR before PVE, ALPPS-1 and ALPPS-2 were respectively 0.4% (0.3-0.5), 0.6% (0.5-0.8), and 1% (0.8-1.2). Median volume growth of FLR was 69% (18-92) after PVE, and 45% (36-82) after ALPPS-1. The combination of PVE and ALPPS induced a growth of median initial FLR of +408 cc (254-513), leading to an increase of +149% (68-199). After ALPPS-2, 4 patients had stage Ⅰ-Ⅱ complications. Three patients had more severe complications (one stage Ⅲ, one stage Ⅳ and one death due to bowel perforation). Two patients suffered from postoperative liver failure according to the 50/50 criteria. None of our patients developed any biliary complication during the ALPPS procedure.

CONCLUSION

Rescue ALPPS may be an alternative after unsuccessful PVE and could allow previously unresectable patients to reach surgery. Biliary drainage seems to reduce biliary complications.

Key words: Rescue associating liver partition and portal vein ligation for staged hepatectomy; Associating liver partition and portal vein ligation for staged hepatectomy; Portal vein embolization; Liver resection; Future liver remnant

Core tip: Hepatic surgery appears as the best curative option for patients with primary or secondary malignant hepatic tumors. Several strategies have been developed to avoid postoperative liver failure, such as portal vein embolization (PVE). In 2012, associating liver partition and portal vein ligation for staged hepatectomy (ALPPS) was developed. It induces rapid and extensive hypertrophy of the future liver remnant, but with high morbidity and mortality. Therefore, some authors have suggested that ALPPS should be performed only as a "rescue", after failed PVE. We describe our results of rescue ALPPS after failure of previous PVE and we perform a literature review.

Maulat C, Philis A, Charriere B, Mokrane FZ, Guimbaud R, Otal P, Suc B, Muscari F. Rescue associating liver partition and portal vein ligation for staged hepatectomy after portal embolization: Our experience and literature review. *World J Clin Oncol* 2017; 8(4): 351-359 Available from: URL: http://www.wjgnet.com/2218-4333/full/v8/i4/351.htm DOI: http://dx.doi.org/10.5306/wjco.v8.i4.351

INTRODUCTION

Hepatic surgery appears as the best curative option for patients with primary or secondary malignant tumors of the liver[1,2]. As complete resection of the tumor load is directly linked to overall survival, it is sometimes necessary to perform major hepatectomies in order to achieve such a goal. The main complication after major hepatectomy is liver failure. Several studies have shown that the size of the future liver remnant (FLR) is a key element[3-5], as it is directly correlated to the postoperative liver function[6,7]. In 2013, a consensus statement established a FLR cut-off above which the risk of postoperative liver failure was considered too high for safe surgery: 20% in normal liver, 30% in liver pretreated with chemotherapy, and 40% in cirrhotic liver[8]. The FLR to body weight ratio (FLR-BWR) (%) is also used as a predictive factor for hepatic dysfunction: Patients with FLR-BWR < 0.5% have a major risk of liver failure and postoperative mortality[9,10].

Several strategies have been developed to lower the risk of postoperative liver failure, such as portal vein occlusion (PVO), either by ligature (PVL) or embolization (PVE). The aim of these techniques is to decrease the portal blood flow to the ipsilateral liver, inducing atrophy of the ipsilateral liver and hypertrophy of the contralateral liver[11]. It enables previously unresectable patients to have access to a surgical treatment by achieving an appropriate FLR volume[12]. Indeed, the PVE leads to an average hypertrophy of the contralateral liver (usually the left lobe) of 40% in 4-8 wk[13]. In 2012, a new surgical technique has been developed, "associating liver partition and portal vein ligation for staged hepatectomy" (ALPPS)[14].

This procedure induces rapid and extensive hypertrophy of the FLR in two steps. During the first surgical step of the original ALPPS procedure, called the "*in situ* splitting", the right portal vein is ligated (if there were no previous PVE), and the surgeon performs a transection of the hepatic parenchyma for extended right hepatectomy. The right hepatic artery, the right bile duct and the drainage veins are not ligated at this point. After the "*in situ* splitting", the right extended lobe is covered by a membrane or a bag to prevent adhesions. Several variations of ALLPS were later developed such as "left ALPPS", allowing left lobectomy, or "right ALPPS", allowing right posterior sectoriectomy[15].

The second surgery is usually performed within 7 to 15 d after the first step. During this step, the right liver is removed, after having dissected and ligated the remaining artery, bile duct and hepatic veins[14,16].

Although promising results were published by Schnitzbauer *et al*[14], several studies have described high perioperative morbidity and mortality, suggesting the necessity of a better selection of patients[17-19]. Some authors have suggested that the ALPPS procedure should be performed only as a "rescue", that is in case of insufficient liver hypertrophy after PVE[15,20-22]. Yet,

very few data relative specifically to the rescue ALPPS have been published[21-24] as most of the existing articles do not focus on this particular indication.

The aim of our study was to report the outcomes of patients with primary and secondary liver tumors undergoing a rescue ALPPS procedure in our center, after failure of previous portal embolization. We also performed a literature review.

MATERIALS AND METHODS

Patients and data collection

Between January 2014 and December 2015, every patient who underwent a rescue ALPPS procedure in Toulouse Rangueil University Hospital, France, was included. We evaluated the patients who could benefit from this strategy at our regional multidisciplinary team meeting, attended by senior hepatobiliary surgeons, hepatologists, oncologists, and radiologists.

The criteria for a rescue ALPPS procedure in our center were: A project of major hepatectomy, a previous PVE with insufficient liver hypertrophy after the procedure, age above 18, an absence of contraindication to surgery, and the approval of the patient after thorough information regarding the risks of the procedure.

In our center, a FLR-BWR < 0.5% was considered as a contraindication to perform major liver surgery. In case of fibrotic liver, previous chemotherapy, previous hepatectomy or multiple comorbidities, our center had higher ratio objectives. FLR-BWR around 1 was considered as optimal for major hepatic surgery. Patients who were eligible for a rescue ALPPS but did not complete the procedure were excluded from our study. All data were retrospectively collected in our local database, including patient characteristics, volumetric measurements, surgical characteristics and complications.

PVE procedure

PVE was performed in an interventional X-ray room or in an operating theater. For all patients, we used a floor-mounted image-guided system (Innova™ IGS 520, General Electric Healthcare, United Kingdom) to perform ultrasound-guided puncture of a portal branch of the left liver lobe, usually segment Ⅲ.

After a complete portography, we performed a contralateral embolization, using a mixture of 50% Lipiodol® (Guerbet, Vilepinte, France) and 50% Glu-bran2® (GEM SRL, Viareggio, Italy). We injected it selectively in each branch of the right portal tree, in order to occlude it. If occlusion of segment IV portal veins was necessary, it was performed after selective catheterization of the portal branches. Then, we administered 100, 250 and 400 μm microspheres (Embozene TM, Boston scientifics, Marlborough, MA, United States). This step was generally completed by 0.35 inch coils (Tornado®, Cook Medical, Bloomington, IN, United States).

After each PVE, we confirmed the complete occlusion of the right portal veins and the integrity of the left remaining ones by a final portography.

CT scans examinations

Before inclusion, each patient had a classical multi-slice computed tomography (MSCT) examination, including a portal phase.

A control CT scan examination was done 3 to 6 wk after the PVE procedure and 6 to 8 d after ALPPS-1. These examinations were performed using a 16-detector row CT scanner (Innova 411, General Electric Healthcare, United Kingdom). The acquisition parameters were: Voltage 120 KVp, intensity 650 mAs, and slice thickness 2 mm, collimation 1mm. Hepatic volumetry was evaluated on the portal phase of the MSCT examination using a semi-automatic method (Terarecon® software, Frankfort, Germany).

Surgical procedure

The ALPPS procedure was performed in two steps (ALPPS-1 and ALPPS-2), separated by an interval phase.

The surgical technique during ALPPS-1 was the following: Exploration of the abdominal cavity to look for signs of extra-hepatic metastases, which would be a contraindication to surgical resection. In case of cholangiocarcinoma, intra-hepatic metastases were also considered a contraindication; Ultrasonographic examination of the liver; Cholecystectomy; Introduction of a transcystic catheter, left in place after the first step (except for one patient who had a radiological biliary drainage); "Hanging maneuver"[25]; Splitting of the hepatic parenchyma for extended right hepatectomy, under intermittent clamping. We performed a complete parenchymal split; in case of metastases located in the FLR, wedge resections or thermoablations were performed during ALPPS-1.

Parenchymal transection was performed using Erbejet (Erbejet, RBE Elektromedizin GmbH, Waldhornlestrasse, Tubingen, Germany, ESM2 model, ref 10340-000) or ultrasonic dissector (Dissectron, Satelec Medical, ref DP 000108). The right hepatic artery, the bile duct and the hepatic veins were identified and surrounded with vessel loops to allow better identification during ALPPS-2. After *in situ* splitting, the two slices of the liver were covered using sheets of Tachosil with hemostatic aim. Instead of a bag, we placed COVA™ membranes (COVA+™, Biom' Up, France) around the liver, the hepatic pedicle and between the two hepatic slices. Silicone drainage was placed between the resection surfaces.

ALPPS-2 was performed within 7 to 9 d after the first step. After identifying the vascular and biliary structures, we performed the dissection and ligation of the remaining artery, the bile duct and the hepatic veins. Then the right liver was removed. For hilar cholangiocarcinoma, a Roux-en-Y hepaticojejunostomy was performed. Silicone drainage was placed near the resection surface.

Interval phase

Patients were hospitalized into intensive care unit during

Table 1 Preoperative characteristics of patients

Variable	Rescue ALPPS (n = 7)
Male/female gender	4/3
Age, yr (range)	61 (53-70)
Body mass index (range)	23 (21-27)
ASA 1-2	6
ASA 3	1
Colorectal liver metastases	4
Number of liver metastases (range)	5 (2-7)
Size of the largest metastases, mm (range)	45 (20-65)
Tumor location	
Right lobe ± segment Ⅳ	3
Right lobe + segment Ⅳ + left lateral segment	1
Previous colorectal resection	3
Previous hepatic resection or thermoablation	3
Preoperative chemotherapy	4
Oxaliplatin based	4
Irinotecan based	3
Angiogenesis inhibitor	1
Intra-arterial chemotherapy	1
Number of preoperative chemotherapy cycles (range)	16 (8-25)
Cholangiocarcinoma	3
Perihilar/intrahepatic	2/1
Preoperative chemotherapy	1
Gemcitabine and oxaliplatin	1
No. of preoperative chemotherapy cycles	3
Portal vein embolization	7
Right lobe	5
Right lobe + segment Ⅳ	2
Comorbidity	
Cardiovascular	2
Pulmonary	0
Diabetes	0
Prior history of cancer	2

ALPPS: Associating liver partition and portal vein ligation for staged hepatectomy.

the first few days, and the transcystic stent (or the radiological biliary drain) remained opened during the interval phase, in order to avoid biliary complications. We encouraged enhanced recovery by early removal of catheter, mobilization and transfer into standard care unit.

Variables

Postoperative liver failure was defined using the 50-50 criteria[26], which associates prothrombin time (PT) < 50% and serum bilirubin (SB) > 50 μmol/L at day 5. Postoperative complications were assessed according to the Dindo-Clavien Classification[27].

Literature review

Literature review was performed using PubMed, Google Scholar and the Cochrane Library Central. Articles reported were written in English and ALPPS procedures were limited to humans. The mesh terms were: "ALPPS", "Associating liver partition and portal vein ligation for staged hepatectomy", "Portal vein embolization", "rescue ALPPS", "salvage ALPPS".

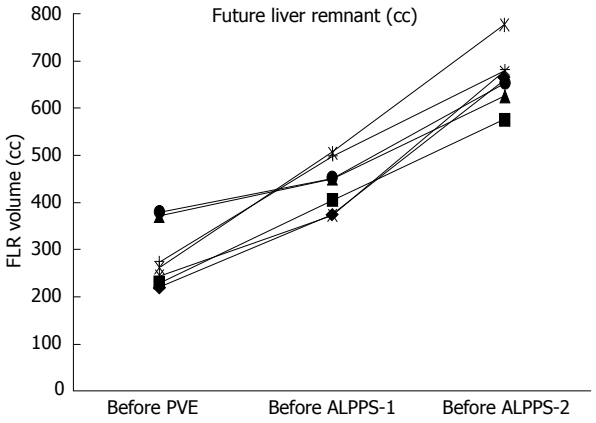

Figure 1 Future liver remnant volume increase among different steps of rescue associating liver partition and portal vein ligation for staged hepatectomy. FLR: Future liver remnant; PVE: Portal vein embolization; ALPPS: Associating liver partition and portal vein ligation for staged hepatectomy.

RESULTS

From January 2014 to December 2015, 10 patients were initially selected to undergo a rescue ALPPS procedure. Two patients had only an explorative laparotomy because their tumor was found unresectable during ALPPS-1. The third patient had more metastases in the left lobe than expected; therefore, the surgeon changed strategy during ALPPS-1 and performed a classical two-stage hepatectomy. These 3 patients were excluded from our analysis.

The characteristics of the 7 patients who underwent the rescue ALPPS procedure are detailed in Table 1. In our cohort, 4 patients had colorectal liver metastases (CRLM), and the others had cholangiocarcinoma. The 2 patients with a Bismuth-Corlette type Ⅲa perihilar cholangiocarcinoma (pCCA) had had a radiological biliary drainage prior to surgery. Among our 7 patients, one had a previous history of left lobectomy.

FLR and FLR/BWR volume increase among the different steps of rescue ALPPS are reported in Figures 1 and 2. Median FLR before PVE, ALPPS-1 and ALPPS-2 were respectively 263 cc (221-380), 450 cc (372-506), and 660 cc (575-776) (Figure 1). Median FLR/BWR before PVE, ALPPS-1 and ALPPS-2 were respectively 0.4% (0.3%-0.5%), 0.6% (0.5%-0.8%), and 1% (0.8%-1.2%) (Figure 2).

Median volume growth of FLR was 69% (18%-92%) after PVE, and 45% (36%-82%) after ALPPS-1. The combination of PVE and ALPPS induced a median growth of initial FLR of +408 cc (254-513), leading to a median increase of +149% (68%-199%).

Intermittent hilar or portal clamping was performed in all patients during ALPPS-1, with a median total duration of 20 min (15-35). ALPPS-1 had a median surgical duration of 240 min (180-300), and median blood losses were 750 mL (300-1000). ALPPS-2 median surgical duration was 90 min (60-120) and median blood losses were 300 mL (0-800). Six patients required

Table 2 Clinical outcomes and complications

Variable	Rescue ALPPS (*n* = 7)
Surgery	
Right trisegmentectomy extended to segment I	4/7
Right lobectomy	1/7
Right lobectomy combined with thermoablation	2/7
Days between ALPPS-1 and ALPPS-2 (range)	7 (7-9)
ALPPS-1	
Surgery duration ALPPS-1, min (range)	240 (180-300)
Blood loss during ALPPS-1, mL (range)	750 (300-1000)
Prothrombin ratio day 5, % (range)	76 (70-85)
Bilirubin day 5, μmol/L (range)	24 (15-70)
MELD score day 5 (range)	10 (8-15)
ALPPS-2	
Surgery duration ALPPS-2, min (range)	90 (60-120)
Blood loss during ALPPS-2, mL (range)	300 (0-800)
Prothrombin ratio day 5, % (range)	60 (41-73)
Bilirubin day 5, μmol/L (range)	43 (10-182)
MELD score day 5 (range)	14 (9-21)
Complications	
Liver failure after ALPPS-1	0/7
Liver failure after ALPPS-2	2/7
Complications after ALPPS-1 and before ALPPS-2	0/7
Complications after ALPPS-2	7/7
Clavien Ⅰ-Ⅱ	4/7
Clavien Ⅲ	1/7
Clavien Ⅳ	1/7
Clavien Ⅴ	1/7
30 d mortality	1/7
90 d mortality	1/7
R0 resection	6/7

ALPPS: Associating liver partition and portal vein ligation for staged hepatectomy; ALPPS-1: First stage ALPPS; ALPPS-2: Second stage ALPPS.

blood transfusions during ALPPS-1 and 4 patients during ALPPS-2. One patient required platelet transfusion during ALPPS-2. R0 resection was completed in 6 patients. One patient with CLRM had a R1 resection (surgical margin in contact with one metastasis) (Table 2).

Complications

The postoperative outcomes are detailed in Tables 2 and 3. There was no per-operative incident reported during ALPPS-1 or ALPPS-2 surgical steps, and we did not experience any complication, including biliary complications, during the interval phase between ALPPS-1 and ALPPS-2.

After ALPPS-2, postoperative complications occurred among all of our patients. Four patients had stage Ⅰ-Ⅱ complications: Ascites (*n* = 3), urinary infection (*n* = 1) or intraoperative blood transfusion (*n* = 6). Three patients had more serious complications. One had intra-abdominal abscess requiring radiological drainage (patient 4). Patient 6 developed a hemorrhage two hours after ALPPS-2, requiring an emergency revision surgery. A surgical clip on an arterial branch had slipped, causing massive internal bleeding. Six days later, she had septic shock, leading to another emergency revision surgery, but we could not find the cause of the septic shock. A radiological drainage was performed a few days later to drain an abdominal abscess. Afterwards, she progressively enhanced total recovery. We report one postoperative death 10 d after ALPPS-2, due to a peritonitis caused by bowel perforation (patient 1). Two patients suffered from postoperative liver failure according to the 50/50 criteria. None of our patients developed any biliary complication.

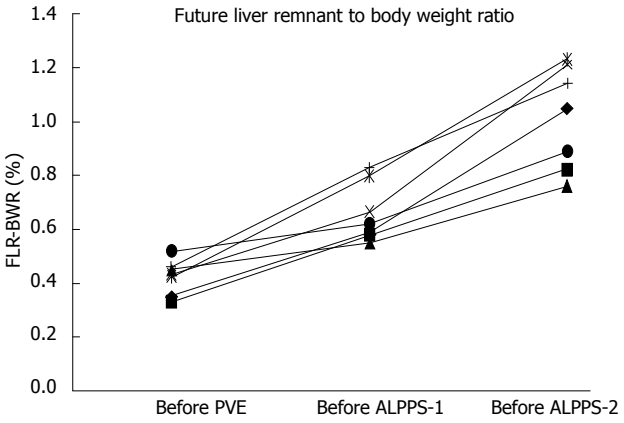

Figure 2 Future liver remnant to body weight ratio increase among different steps of rescue associating liver partition and portal vein ligation for staged hepatectomy. PVE: Portal vein embolization; FLR-BWR: Future liver remnant to body weight ratio; ALPPS: Associating liver partition and portal vein ligation for staged hepatectomy.

DISCUSSION

In this study, we report 7 cases of rescue ALPPS that illustrate how such a procedure can be used successfully after failure of PVE.

In our cohort, FLR volume increased despite a previously insufficient hypertrophy after PVE. The causes of insufficient volume growth of FLR after PVE are known: Technical failure during the procedure (impossibility of cannulating the portal system due to altered portal anatomy), portal vein recanalization, portal collateral development and poor quality of hepatic parenchyma[7,28,29]. In our study, 5 patients had chemotherapy before PVE, which induced histopathological damages (steatosis, sinusoidal obstruction syndrome, cholestasis, *etc*.), and affected the regenerative capacities of the liver after PVE.

It allowed 7 patients to reach surgery, while they were considered unresectable after PVE. Among them, 6 had R0 resection. Without the rescue ALPPS technique, they would have been considered unresectable despite PVE, and offered only palliative measures. Nonresection following PVE has been described by Abulkhir *et al*[7] in 2008. Their study focused on a cohort of 1088 patients undergoing PVE and showed a 15% failure rate, including inadequate hypertrophy of remnant liver in 2% of cases. These results show that while the inadequate hypertrophy of FLR after PVE must be feared, it remains infrequent. It explains the low number of patients in our study, and it also explains

Table 3 Patient characteristics

Patient number	Gender	Age	Tumor	Underlying liver function	FLR/BWR before PVE, %	FLR/BWR before ALPPS-1, %	FLR/BWR before ALPPS-2, %	ALPPS-2 day 5 Bilirubin, μmol/L	ALPPS-2 day 5 PT, %	Dindo-Clavien classification	Complications(by order of appearance)
1	M	68	pCCA	Cholestasis	0.35	0.59	1.05	182	45	V	Intraoperative blood transfusion, intra-abdominal abscess, pleural effusion, death due to peritonitis caused by bowel perforation
2	M	70	iCCA	-	0.33	0.58	0.82	43	60	Ⅱ	Intraoperative blood transfusion, transitory ascites
3	M	55	CRLM	FNH	0.45	0.55	0.76	43	41	Ⅱ	Intraoperative blood transfusion
4	F	66	pCCA	Cholestasis	0.43	0.66	1.21	44	73	Ⅲ	Transitory ascites and intra-abdominal abscess
5	F	59	CRLM	SOS and steatosis	0.42	0.80	1.23	10	69	Ⅱ	Intraoperative blood transfusion, urinary infection
6	F	53	CRLM	Dystrophy	0.52	0.62	0.89	87	50	Ⅳ	Intraoperative blood transfusion, internal hemorrhage, transitory hepatic insufficiency, infected ascites, septic choc, and intra-abdominal abscess
7	M	61	CRLM	-	0.46	0.83	1.14	13	67	Ⅱ	Intraoperative blood transfusion, transitory chylous ascites

pCCA: Perihilar cholangiocarcinoma; iCCA: Intrahepatic cholangiocarcinoma; CRLM: Colorectal liver metastases; FNH: Focal nodular hyperplasia; SOS: Sinusoidal obstruction syndrome; M: Male; F: Female; ALPPS: Associating liver partition and portal vein ligation for staged hepatectomy.

the scarcity of literature about rescue ALPPS performed after PVO, as is shown in our literature review (Table 4). However, the rate of insufficient FLR after PVE will probably increase in the years to come, due to more and more intensive chemotherapies, which greatly alter hepatic parenchyma. Most papers describe series of 1 to 3 cases and only 4 studies report small cohorts (9 to 11 patients) (Table 4). Therefore, the size of our cohort (7 patients) is consistent with the number of cases developed in literature.

We decided to perform ALPPS procedure for 2 patients who had a median FLR/BWR before PVE of 0.8% due to a high risk of liver failure after major hepatectomy: One patient had previous left lobectomy, wedge resections, thermoablation and neoadjuvant chemotherapy, inducing steatosis and sinusoidal obstruction syndrome. The second patient had multiple neoadjuvant chemotherapy cycles, suggesting that it was necessary to optimize its FLR volume to avoid postoperative liver failure.

In our cohort, median FLR growth between ALPPS-1 and ALPPS-2 was 45% (36%-82%), which might appear less than in the literature (70%-80%[14,18]). The impact of PVE before ALPPS might be an explanation to this result. Compared to the original ALPPS procedure, liver hypertrophy is developed in two steps: With PVE first, and then with the rescue ALPPS procedure. Therefore, it is more adequate to compare the FLR growth of the original ALPPS with the overall FLR growth of the complete rescue ALPPS procedure (from PVE to ALPPS). In our study, the median overall FLR growth of the complete rescue ALPPS procedure is 149%, which is far greater than the FLR growth induced by the "original ALPPS" described in the literature. Another factor which might explain our results regarding FLR growth between ALPPS-1 and ALPPS-2 is that the interval phase was shorter (7 d) than reported in literature: An average of 14 d was reported from 320 cases in the International ALPPS Registry by Schadde *et al*[30] in 2015.

Among our 7 patients, we report 43% of major complications (Clavien-Dindo > Ⅲ), including one death after ALPPS-2, due to bowel perforation, which is consistent with the literature of original ALPPS[31,32]. It is important to note that rescue ALPPS after PVE does not induce more major complication than the original ALPPS. It suggests that PVE does not have any impact on the rate of complications. Surgical complications after ALPPS procedure are partly linked to inflammatory adhesions around the liver and the hepatic pedicle, inducing many dissection difficulties during ALPPS-2.

Table 4 Literature review of rescue associating liver partition and portal vein ligation for staged hepatectomy

	Rescue ALPPS after PVO (PVE/PVL/PVE + PVL)	Tumor	Days between ALPPS-1 and ALPPS-2	FLR/BWR before PVO, %	FLR/BWR before ALPPS-1, %	FLR/BWR before ALPPS-2, %	Growth of FLR between PVO and ALPPS-1, % (range)	Growth of FLR between ALPPS-1 and ALPPS-2, % (range)	Clavien Dindo > III	30-d mortality
Conrad *et al*[37], 2012	1 (1/0/0)	CRLM	9	NC	NC	NC	-1	47	0/1	0/1
Gauzolino *et al*[15], 2013	1 (1/0/0)	CRLM	7	NC	NC	0.4	NC	26	0/1	0/1
Knoefel *et al*[21], 2013	3 (3/0/0)	NC	6	NC	NC	NC	46[2]	65[2]	1/2	1/2
Björnsson *et al*[20], 2013	2 (2/0/0)	CRLM (n = 1) HCC (n = 1)	9	NC	NC	NC	NC	NC	0/2	NC
Tschuor *et al*[22], 2013	3 (1/1/1)	CRLM	8	NC	NC	NC	61[2]	79[2]	2/3	0/3
Vyas *et al*[38], 2014	1 (1/0/0)	Neuroendocrine metastases	8	0.4	0.5	0.9	24	70	0/1	0/1
Nadalin *et al*[39], 2014	2 (2/0/0)	CRLM (n = 1) Pancreatic metastases (n = 1)	13	NC	0.5[2]	NC	NC	NC	1/2	1/2
Fard-Aghaie *et al*[40], 2015	1 (1/0/0)	CRLM	26	NC	NC	NC	69	50	1/1	1/1
Alavrez *et al*[41], 2015	1 (0/0/1)	CRLM	7	NC	NC	NC	38	65	1/1	0/1
Croome *et al*[42], 2015	2 (2/0/0)	CRLM	8	NC	NC	NC	NC	NC	NC	NC
Truant *et al*[23], 2015	9 (9/0/0)	NC	8	NC	NC	NC	NC	NC	NC	NC
Björnsson *et al*[43], 2016	10 (NC)	CRLM	8	NC	NC	NC	NC	NC	NC	0/10
Sparrelid *et al*[24], 2016	11 (7/4/2)	CRLM	7	0.3[1]	0.4[1]	0.7[1]	27[1] (7-67)	62[1] (19-120)	4/11	0/11
Ulmer *et al*[44], 2017	9 (9/0/0)	CRLM (n = 6), CCA (n = 2), others liver metastases (n = 1)	9	NC	NC	NC	30[2]	78[2]	6/9	1/9
Maulat, 2017	7 (7/0/0)	CRLM (n = 4), CCA (n = 3)	7[1]	0.4[1]	0.6[1]	1[1]	69[1]	45[1]	3/7	1/7

[1]Median; [2]Mean. PVE: Portal vein embolization; PVL: Portal vein ligation; PVE + PVL: Portal vein embolization associated with portal vein ligation; CRLM: Colorectal liver metastases; HCC: Hepatocellular carcinoma; CCA: Cholangiocarcinoma; NC: Not communicated; ALPPS: Associating liver partition and portal vein ligation for staged hepatectomy.

Using absorbable collagen membranes (COVA™ membranes) instead of bags at the end of ALPPS-1 helped prevent these inflammatory adhesions. We also performed ALPPS-2 within 7 to 9 d after ALPPS-1, which is shorter than the interval phase duration described in literature[30]. These two factors explain why we did not experience major inflammatory adhesions during ALPPS-2. It is interesting to note that we did not have any biliary complication in our cohort. In 2015, Truant *et al*[23] reported that among a series of 62 patients who underwent ALPPS procedure, 25 patients (40%) had biliary fistula: 19 (31%) after ALPPS-1 and 16 (27%) after ALPPS-2. Other studies are reporting bile leakage in up to 20% of patients after ALPPS procedure[14,18,33-35]. Biliary complications are the main cause of morbidity after ALPPS, and they are much more frequent than with ordinary hepatectomies (5%). It is even one of the main criticisms of this technique, as biliary fistula is known to alter the liver regeneration capacities, increase the risk for sepsis, extend the time of hospital stay, and increase postoperative mortality[36].

Therefore, it is of great importance to prevent biliary complication. Our results suggest that the use of biliary drainage during the interval phase (with transcystic catheter or radiological biliary drainage) is a promising technique to prevent biliary complications. To our knowledge, this is the first publication describing the use of a systematic biliary drainage between ALPPS-1 and ALPPS-2.

In conclusion, our study suggests that rescue ALPPS may be an alternative after unsuccessful PVE and could allow previously unresectable patients to reach surgery. It provides an opportunity for complete resection in cases otherwise eligible only to palliative treatments. Although the rate of complications is high, the use of PVE prior to the ALPPS procedure does not seem to increase morbidity. The use of a biliary drainage during the interval phase seems a promising technique to reduce biliary complications, although further studies should be performed to confirm these results.

COMMENTS

Background

Hepatic surgery appears as the best curative option for patients with primary or secondary malignant tumors of the liver. The main complication after major hepatectomy is liver failure. Several studies have shown that the size of the future liver remnant (FLR) is a key element as it is directly correlated to the postoperative liver function. Several strategies have been developed to lower the risk of postoperative liver failure, such as portal vein embolization (PVE). The aim of this technique is to decrease the portal blood flow to the ipsilateral liver, inducing atrophy of the ipsilateral liver and hypertrophy of the contralateral liver. In 2012, a new surgical technique has been developed, "Associating liver partition and portal vein ligation for staged hepatectomy" (ALPPS). This procedure induces rapid and extensive hypertrophy of the FLR in two steps. Several studies have described high perioperative morbidity and mortality. Therefore, some authors have suggested that ALPPS should be performed only as a "rescue", after failed PVE.

Research frontiers

Considering the high perioperative morbidity and mortality of ALPPS procedure, the current hotspots in this research field is the necessity of a better selection of patients and the necessity to minimize complications, and more specifically biliary complications.

Innovations and breakthroughs

Yet, very few data relative specifically to the rescue ALPPS have been published as most of the existing articles do not focus on this particular indication. Most papers describe series of 1 to 3 cases and only 4 studies report small cohorts (9 to 11 patients). Therefore, the size of the cohort (7 patients) is consistent with the number of cases developed in literature. Moreover, biliary complications are the main cause of morbidity after ALPPS, and they are much more frequent than with ordinary hepatectomies (5%). This study suggests that the use of a biliary drainage during the interval phase seems a promising technique to reduce biliary complications. To our knowledge, this is the first publication describing the use of a systematic biliary drainage between ALPPS-1 and ALPPS-2.

Applications

The results of the study suggest that in the future, ALPPS procedure should be performed only as a "rescue", in case of insufficient liver hypertrophy after PVE. Rescue ALPPS could allow previously unresectable patients to reach surgery. It provides an opportunity for complete resection in cases otherwise eligible only to palliative treatments.

Terminology

ALPPS: (Associating liver partition and portal vein ligation for staged hepatectomy) procedure was performed in two steps (ALPPS-1 and ALPPS-2), separated by an interval phase. During ALPPS-1, the surgeon performs a transection of the hepatic parenchyma. In this study, ALPPS-2 was performed within 7 to 9 d after ALPPS-1. During ALPPS-2, the right liver is removed, after having ligated the remaining artery, bile duct and hepatic veins.

Peer-review

The authors present a study on the interesting subject of rescue ALPPS.

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CASE REPORT

BRAF V600Q-mutated lung adenocarcinoma with duodenal metastasis and extreme leukocytosis

Ayman Qasrawi, Addison Tolentino, Mouhanna Abu Ghanimeh, Omar Abughanimeh, Sakher Albadarin

Ayman Qasrawi, Mouhanna Abu Ghanimeh, Omar Abughanimeh, Department of Internal Medicine, University of Missouri - Kansas City School of Medicine, Kansas City, MO 64108, United States

Addison Tolentino, Saint Luke's Cancer Specialists, Saint Luke's Hospital of Kansas City, Kansas City, MO 64111, United States

Sakher Albadarin, Division of Gastroenterology, University of Missouri - Kansas City School of Medicine, Kansas City, MO 64108, United States

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Correspondence to: Ayman Qasrawi, MD, Department of Internal Medicine, University of Missouri - Kansas City School of Medicine, 2411 Holmes Street, Kansas City, MO 64108, United States. qasrawia@umkc.edu
Telephone: +1-816-5727576
Fax: +1-816-9325793

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Abstract

Driver mutations in patients with non-small cell lung cancer (NSCLC) can lead to distinct behaviors and patterns of metastasis. Mutations in the proto-oncogene B-raf (*BRAF*) occur in approximately 3% of NSCLC cases. In the literature, reports of patients with lung adenocarcinomas metastasizing to the duodenum are rare, and most of the only 21 cases reported were from before the advent of next-generation sequencing. We present here a case involving a 57-year-old female who had a lytic lesion in her lesser trochanter. Biopsy showed metastatic adenocarcinoma of lung origin. Chest X-ray showed a large left upper lobe mass. Next-generation sequencing analysis confirmed the presence of *BRAF* V600Q mutation. The patient presented with persistent anemia and melena. Esophagogastroduodenoscopy confirmed the presence of duodenal metastasis. She also had suspected paraneoplastic leukemoid reaction. To our knowledge, this is only the second well-documented case of gastrointestinal metastasis from *BRAF*-mutated lung cancer.

Key words: *BRAF*; Lung adenocarcinoma; Duodenum; Metastasis; Gastrointestinal bleeding; Endoscopy; Leukocytosis

Core tip: We report a rare and interesting case of *BRAF*-mutated lung adenocarcinoma with metastases to the bone and duodenum, and extreme leukocytosis. We found next-generation sequencing to be helpful in prognostication and determination of some of the unique clinical behaviors of lung adenocarcinoma. This is only the second case of *BRAF*-mutated lung adenocarcinoma with well documented metastases to the gastrointestinal tract. The addition of this case to the literature should prompt interest in studying the propensity of *BRAF*-mutated malignancies to metastasize to the gastrointestinal tract.

Qasrawi A, Tolentino A, Abu Ghanimeh M, Abughanimeh O, Albadarin S. *BRAF* V600Q-mutated lung adenocarcinoma with duodenal metastasis and extreme leukocytosis. *World J Clin Oncol* 2017; 8(4): 360-365 Available from: URL: http://www.wjgnet.com/2218-4333/full/v8/i4/360.htm DOI: http://dx.doi.org/10.5306/wjco.v8.i4.360

INTRODUCTION

Non-small cell lung cancer (NSCLC) has been traditionally classified and treated as a single disease. However, recent research has helped us to better understand the molecular pathogenesis of lung cancers in general. For example, mutations in the epidermal growth factor receptor (*EGFR*) and rearrangements of the anaplastic lymphoma kinase (*ALK*) gene were discovered in 2004 and 2007, respectively[1]. These mutations, often called "driver mutations", have been shown to drive NSCLC tumorigenesis and are now being exploited as a targeted strategy for treatment-the application of which consisting mostly of tyrosine kinase inhibitors. Tumors with different driver mutations have been shown to have different clinical backgrounds, pathological features and prognoses[2]. In addition, different driver mutations can lead to distinct patterns of metastatic spread[3]. Generally, however, small bowel metastases from lung cancer is very uncommon, and duodenal metastases are particularly rare[4,5]. It is unknown if certain driver mutations can lead to an increased predisposition to gastrointestinal spread in patients with lung cancer.

In this report, we present a case of a metastatic lung adenocarcinoma with a V600Q mutation in the proto-oncogene B-raf (*BRAF*) and which had an atypical course of duodenal metastasis and extreme leukocytosis. Because this represents such a rare case, we also provide a review of the literature regarding *BRAF*-mutated lung cancers and of previous reports of duodenal metastasis originating from lung cancer. Finally, we also provide reasoned hypotheses as to the causes of the accompanying leukocytosis.

CASE REPORT

Our patient was a 57-year-old female with a known history of metastatic lung adenocarcinoma. Her history dated back to December 2015, when she developed left hip pain. It was initially treated conservatively and imaging examination was not performed. However, over the ensuing 4 mo, the pain worsened and became gnawing and constant. She was afebrile. Results from laboratory work-up revealed leukocytosis (19.3 × 10^9/L; reference range: 4.3-11 × 10^9/L) with 84% neutrophils, microcytic anemia (7.1 g/dL; reference range: 12-15.5 g/dL) with mean corpuscular volume (MCV) of 71 fL, and thrombocytosis (595 × 10^9/L; reference range: 150-450 × 10^9/L). At 4 mo prior, her hemoglobin had been 13.0 g/dL (reference range: 12-15.5 g/dL).

A computed tomography (CT) scan of the left hip was obtained, and showed marked irregularity of the lesser trochanter with cortical bone destruction. A soft tissue mass was also seen in the region of the cortex. A plain chest film revealed a large left lung mass. Iron studies revealed a ferritin level of 86 ng/mL, iron of < 10 μg/dL (reference range: 50-160 μg/dL) and total-iron binding capacity of 353 μg/dL (reference range: 270-380 μg/dL). Other causes of anemia were ruled out. A peripheral smear showed microcytic hypochromic anemia and granulocytosis without left-shift.

The patient was transfused with a unit of packed red blood cells (PRBCs) and taken to the operating room. She underwent intralesional curettage, partial excision of the lesser trochanter, and open arthotomy of the left hip with extraction of the mass. Pathological examination of the extracted bone and soft-tissue mass revealed poorly differentiated metastatic adenocarcinoma cells. Immunohistochemical staining revealed strong reactivity for cytokeratin 7 (CK7) and thyroid transcription factor-1 (TTF-1) and negative reactivity for cytokeratin 20 (CK20) and GATA binding protein 3 (GATA3); these findings are most consistent with lung origin. CT scans revealed a large left upper lobe mass, measuring 8.5 cm × 6.7 cm × 10.7 cm, with extensive local invasion. In addition, there was a left adrenal mass indicative of metastatic disease.

Genetic testing of the tumor was carried out using Caris Molecular Intelligence® (Caris Life Sciences, Irving, TX, United States). The next-generation sequencing (NGS) analysis revealed exon 15 *BRAF* V600Q and exon 7 TP53 G215V mutations. No mutations or rearrangements were found in the genes for Kirsten ras viral oncogene (*KRAS*), neuroblastoma ras viral oncogene (*NRAS*), anaplastic lymphoma receptor tyrosine kinase (*ALK*), tyrosine-protein kinase Met (*cMET*), *EGFR*, *ROS1*, retinoblastoma-1 (*RB1*), phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit alpha (*PIK3CA*), or ret proto-oncogene (*RET*).

The patient received palliative radiotherapy to the left femur. Her anemia was considered likely multifactorial, given the active malignancy with possible iron deficiency. Ferrous sulfate supplementation was initiated (oral; 325 mg regular-release twice daily), but only minimal improvement in her anemia was observed. Of note, her leucocyte count remained elevated after the surgery (18.9-56.4 × 10^9/L). She had no signs of

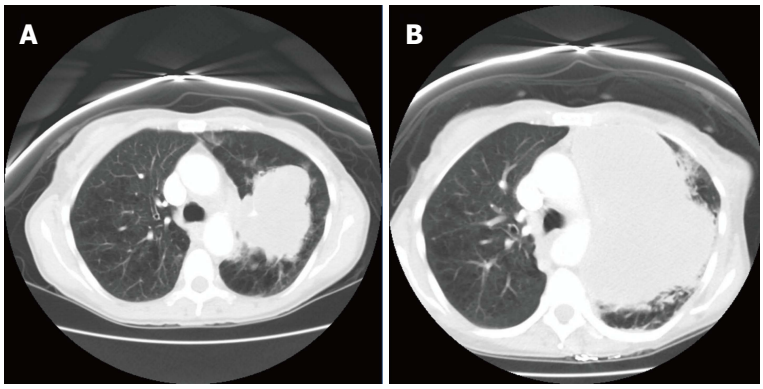

Figure 1 Computed tomography scan of the chest showing the left lung mass. A: At the time of diagnosis; B: Explosive growth of the tumor after two cycles of chemotherapy.

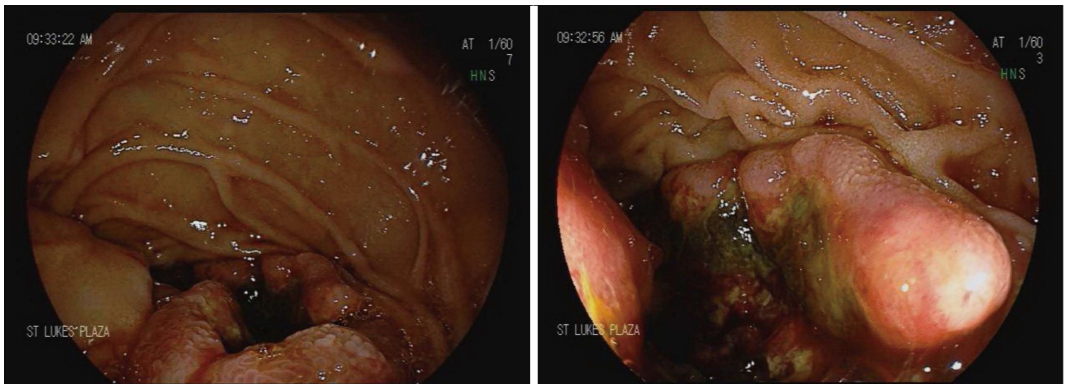

Figure 2 Esophagogastroduodenoscopy showing the malignant-appearing 1-cm mass in the second part of the duodenum. The scope could not traverse the lesion and the exam could not be finished. Cold forceps biopsies were taken for histology.

infection or inflammation.

After the radiotherapy, two cycles of carboplatin and pemetrexed were administered. However, shortly after the second cycle, the patient presented to the emergency room with increasing shortness of breath and weakness. She also reported intermittent melanotic stool for the past few days. Physical exam revealed pallor and almost no air entry into the left part of the chest on auscultation. She was afebrile. Laboratory investigations showed a leucocyte count of 80.2 × 10^9/L with 92% neutrophils, 6% monocytes and 2% lymphocytes, hemoglobin of 6.0 g/dL, and platelet count of 519 × 10^9/L. Guaiac fecal occult blood test was positive. CT scan showed extensive growth of the upper lobe mass (to 14.5 cm × 10.0 cm × 17.4 cm) with progressive mediastinal invasion (Figure 1).

The patient was admitted to the hospital and transfused with 1 U of PRBCs. Esophagogastroduodenoscopy was performed, and an ulcerated bleeding 1-cm mass with malignant appearance was found in the second part of the duodenum (Figure 2). The scope could not traverse the lesion, and the exam could not be finished. Cold forceps biopsies were taken. On pathological exam, poorly differentiated adenocarcinoma was determined. The morphological and immunohistochemical characteristics of the tumor were similar to the findings on the original bone biopsy, being consistent with lung origin.

The patient's leukocytosis worsened (up to 102 × 10^9/L, with 92% neutrophils, 3% monocytes, 2% myelocytes, 1% metamyelocytes, 1% promyelocytes and 1% lymphocytes). She did not have fever or other signs of infection. She did not receive any granulocyte-stimulating agent with her chemotherapy and did not receive steroids. A peripheral smear showed absolute neutrophilia with coarse toxic granulation and Döhle bodies in numerous neutrophils and with occasional metamyelocytes and myelocytes. In addition, rare nucleated red blood cells were observed. Peripheral flow cytometry did not show any increase in blast count. Mutational analysis showed no mutation in the genes for Janus kinase-2 (*JAK-2*), calreticulin (*CALR*) and colony-stimulating factor 3 receptor (*CSF3R*). In addition, reverse-transcriptase polymerase chain reaction assay of peripheral blood gave negative results for *BCR-ABL b2a2*, *b3a2*, and *e1a2* fusion gene transcripts. This finding lessened the likelihood of chronic myeloid leukemia as well as of chronic myeloproliferative disorders. Given the patient's very poor prognosis, bone marrow examination was not performed.

Considering the patient's rapid course of progression and development of resistance to front-line chemotherapy, she was started on the off-label combination of dabrafenib with trametinib, which has Federal Drug Administration approval for use in *BRAF*-mutated melanoma. Unfortunately, her clinical condition deteriorated quickly and she died around 2 wk after her presentation.

DISCUSSION

The *BRAF* gene on chromosome 7 (7q34) is a proto-oncogene that encodes the serine/threonine specific protein kinase family member *BRAF*[6]. The BRAF protein participates in the mitogen-activated protein kinase (MAPK)/extracellular signal-regulated kinase (ERK) pathway, which is also known as the Ras-Raf-mitogen-activated protein kinase kinase (MEK)-ERK pathway[1]. It is a chain of proteins that functions in the signaling from cell surface to the nucleus[1]. Activation of this pathway leads to synthesis of transcription factors that are important in cell cycle regulation[7]. Mutations in *MAPK/ERK* can lead to uncontrolled growth and neoplastic transformation. BRAF mutations were first described in 2002 and occur in varying frequencies in melanoma, colorectal carcinomas, and lung, thyroid and other types of malignancies[8].

BRAF is mutated in approximately 3% of patients with NSCLC (mainly of adenocarcinoma type)[9]. The most commonly observed mutation in BRAF is the valine (V) to glutamic acid (E) substitution at codon 600 (*BRAF* V600E) on exon 15[10]. *BRAF* V600E accounts for about 50% of the *BRAF* mutations in NSCLC cases[11]. A large meta-analysis found that the *BRAF* V600E mutation was more frequent in women and was closely related a history of never-smoking[9]. In addition, one study showed that V600E-mutated tumors had an aggressive histotype and were significantly associated with shorter disease-free and overall survival rates[12]. Two other studies showed that V600E-mutated tumors responded less favorably to platinum-based chemotherapy, although the finding did not reach statistical significance[13,14]. In contrast, other studies have shown that overall survival was not statistically different between patients with wild-type *BRAF* and those with V600E or non-V600E BRAF mutations[10,11,15].

Our patient had metastasis to the duodenum. The immunohistochemical pattern of her bone and duodenal biopsies was suggestive of adenocarcinoma originating in the lung. In general, positive staining for TTF-1 and CK7, in addition to negative CK20 staining (*i.e.,* $TTF1^+$/$CK7^+$/$CK20^-$ pattern) strongly supports a lung origin, as opposed to a gastrointestinal origin[16]. Metastases to the small bowel from lung cancer are very rare and usually asymptomatic[4]. According to a literature review by Hillenbrand *et al*[4] published in 2005, clinically-manifested small bowel metastasis was documented in 58 reports between 1961 and 2003. The most common symptoms were perforation and/or obstruction, or less commonly, bleeding.

Duodenal metastasis from lung cancer is exceedingly rare. AlSaeed *et al*[5] reported a case of duodenal metastasis from lung adenocarcinoma and cited another 11 previous reports. In addition, we found 9 more cases of lung cancer with duodenal metastasis[17-23]. Out of the 21 total cases reported, 9 showed adenocarcinoma histology, 7 showed squamous cell histology, 2 showed large cell histology, 2 showed small cell histology, and 1 was deemed unspecified NSCLC. Symptoms of gastrointestinal bleeding and/or iron deficiency occurred in 13 cases. Other reported clinical features include obstructive jaundice, abdominal pain, obstruction, and perforation. None of the reported cases had accompanying molecular analyses data for driver mutations.

We have previously reported a case of *BRAF* V600E-mutated lung adenocarcinoma, which had an aggressive clinical course and gastric metastases[24]. Herein, we present another case with a similar aggressive course and duodenal metastasis. Previous reports and studies have not indicated the association between certain genetic mutations in lung cancer and the predilection to gastrointestinal metastasis. In addition, gastric or intestinal metastases are rare and difficult to diagnose on imaging, and most of the reported cases of gastrointestinal metastases from lung cancer occurred before the discovery of driver mutations. Therefore, it cannot be proven that the *BRAF* mutation contributed to the gastric or duodenal metastasis in the previously reported cases.

We suggest that in cases of lung cancer with *BRAF* V600E mutations, an aggressive behavior must be expected. In addition, gastrointestinal tract involvement must be kept in mind. Clinical research on a larger scale (rather than relying on the rare case reports) will be imperative to understand the incidence of gastrointestinal involvement. In addition to mutated *BRAF*, our patient had a missense mutation in codon 245 of exon 7 of the *TP53* gene (G245V). This mutation occurred in the DNA-binding site of the protein and was previously reported in lung cancer[25]. A previous, large meta-analysis found that *TP53* mutations conferred worse clinical outcomes in patients with NSCLC, especially in those with adenocarcinoma histology[26]. It is possible that the TP53 mutation in our patient contributed to the aggressive clinical course and resistance to chemotherapy.

Another interesting feature of our case was the extreme neutrophilia. Infections and myeloproliferative disorders were unlikely in the absence of fever or signs of infection, *JAK-2* mutations, or *BCR-ABL* rearrangements. Bone marrow infiltration from adenocarcinoma cells was a possibility, given the presence of rare nucleated red blood cells in the peripheral smear. Unfortunately, the patient's bone marrow was not examined. It is also possible that her poor outcome was related to a paraneoplastic leukemoid reaction. Interestingly, neutrophilic leukemoid reaction was reported in a patient with *BRAF* V600E-mutated metastatic melanoma[27]. In another report, a case of squamous cell carcinoma with peritoneal carcinomatosis and an eosinophilic leukemoid reaction showed coexistence of the *BRAF* V600E and oncogenic *KRAS* G12A mutations[28]. Up-regulation of granulocyte colony-stimulating factor (G-CSF) through RAS/RAF/MEK pathway activation can lead to a paraneoplastic leukemoid reaction[29].

Finally, dabrafenib and trametinib are small molecule

inhibitors of BRAF and MEK1/MEK2, respectively. Their oral administration combination is approved for treatment of *BRAF* V600E-mutated melanoma and is currently being investigated for lung cancer harboring the mutation[30]. In that trial, the overall response rate was 63%, with the median duration of response being 9 mo. We started our patient on dabrafenib and trametinib. Unfortunately, she died of her disease before any response was observed.

In conclusion, certain molecular mutations in NSCLC might lead to unique clinical behaviors. We have described a case of lung adenocarcinoma which had an atypical and aggressive clinical course, with duodenal metastasis and extreme leukocytosis. We have performed molecular analysis using NGS, which showed the mutations of exon 15 *BRAF* V600Q and exon 7 *TP53* G245V. To the best of our knowledge, this is only the second reported case of well-documented *BRAF*-mutated lung adenocarcinoma with metastases to the gastrointestinal tract. Indeed, the continued use of modern molecular methods, such as NGS, will allow us to explore possible correlations between certain mutations and clinical behaviors.

COMMENTS

Case characteristics

A 57-year-old female with metastatic lung adenocarcinoma mutation in the proto-oncogene B-raf (*BRAF*) gene with presentation of fatigue, increasing shortness of breath and melena.

Clinical diagnosis

Pallor and almost no air entry into the left part of the chest on auscultation.

Differential diagnosis

Peptic ulcer disease, esophagitis, gastritis, duodenitis, vascular lesions or tumors.

Laboratory diagnosis

Anemia, extreme leukocytosis, and positive hemoccult stool test.

Imaging diagnosis

Computed tomography chest scan showing rapid progression of the cancer. Esophagogastroduodenoscopy with duodenal mass demonstrating a metastatic deposit of lung origin.

Pathological diagnosis

The morphological and immunohistochemical characteristics of the tumor were similar to the findings on the original biopsy, being consistent with lung origin.

Treatment

Dabrafenib and trametinib were started, but the patient died before any response could be measured.

Related reports

This is only the second well-documented case of gastrointestinal metastasis from *BRAF*-mutated lung cancer.

Term explanation

The *BRAF* gene is a proto-oncogene that encodes the serine/threonine specific protein kinase family member *BRAF*. The BRAF protein participates in the mitogen-activated protein kinase/extracellular signal-regulated kinase pathway.

Experiences and lessons

BRAF-mutated lung adenocarcinoma can be aggressive. Further studies are needed to explore possible correlations between *BRAF* mutations and clinical behaviors. Furthermore, treatment with dabrafenib and trametinib has promising results.

Peer-review

The object is interesting and the manuscript clearly reported. This is the second well-documented case of gastrointestinal metastasis from *BRAF*-mutated lung cancer.

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CASE REPORT

Intimal sarcoma of the pulmonary artery with multiple lung metastases: Long-term survival case

Sonia García-Cabezas, Macarena Centeno-Haro, Simona Espejo-Pérez, Elvira Carmona-Asenjo, Alberto L Moreno-Vega, Rosa Ortega-Salas, Amalia Palacios-Eito

Sonia García-Cabezas, Amalia Palacios-Eito, Department of Radiation Oncology, Reina Sofia University Hospital, 14004 Cordoba, Spain

Macarena Centeno-Haro, Rosa Ortega-Salas, Department of Pathological Anatomy, Reina Sofia University Hospital, 14004 Cordoba, Spain

Simona Espejo-Pérez, Department of Radiology, Reina Sofia University Hospital, 14004 Cordoba, Spain

Elvira Carmona-Asenjo, Department of Nuclear Medicine, Reina Sofia University Hospital, 14004 Cordoba, Spain

Alberto L Moreno-Vega, Department of Medical Oncology, Reina Sofia University Hospital, 14004 Cordoba, Spain

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Correspondence to: Amalia Palacios-Eito, PhD, Department of Radiation Oncology, Reina Sofia University Hospital, Avda. Menéndez Pidal, s/n, 14004 Cordoba, Spain. amalia.palacios.sspa@juntadeandalucia.es
Telephone: +34-95-7011415
Fax: +34-95-7011414

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Abstract

Pulmonary artery intimal sarcoma (PAIS) is a rare tumor with a very poor prognosis. Clinical and radiological findings usually mimic thromboembolic disease, leading to diagnostic delays. The treatment of choice is surgery, and adjuvant chemotherapy and radiotherapy have limited results. We report the case of a 48-year-old male patient, initially suspected with pulmonary thromboembolism. The angio-CT revealed a filling defect in the pulmonary artery trunk. The patient underwent surgery, resulting in with complete resection of the mass with a diagnosis of PAIS. The tumor progressed rapidly in the lung, requiring surgery of multiple lung metastases. The patient was treated with stereotactic body radiation therapy (SBRT) on two occasions for new pulmonary lesions. In the last follow-up (4 years after initial diagnosis), the patient was disease-free. In conclusion, SBRT proved to be an alternative treatment to metastasectomy, allowing palliative chemotherapy to be delayed or omitted, which may result in improved quality of life.

Key words: Intimal sarcoma of the pulmonary artery; Lung metastases; Metastasectomy; Stereotactic body radiation therapy; Treatment

Core tip: Intimal sarcoma of the pulmonary artery is a rare tumor with a very poor prognosis. It has been described in a limited number of reports. This case is a uncommon patient with long-term survival despite having rapid metastatic progression, who maintains a complete remission after initial surgical treatment, completed after occurrence of progression with stereotactic body radiotherapy.

García-Cabezas S, Centeno-Haro M, Espejo-Pérez S, Carmona-Asenjo E, Moreno-Vega AL, Ortega-Salas R, Palacios-Eito A. Intimal sarcoma of the pulmonary artery with multiple lung metastases: Long-term survival case. *World J Clin Oncol* 2017; 8(4): 366-370 Available from: URL: http://www.wjgnet.com/2218-4333/full/v8/i4/366.htm DOI: http://dx.doi.org/10.5306/wjco.v8.i4.366

INTRODUCTION

Pulmonary artery intimal sarcoma (PAIS) is a rare tumor first described by Mandelstamm[1] in 1923. Since then, about 300 cases have been reported in the literature[2,3]. The prognosis is generally poor, with a median overall survival of approximately 17 mo[2-4]. Clinical and radiological findings usually mimic thromboembolic disease, leading to diagnostic delays[5]. Surgical resection of the primary tumor is the best therapeutic option to prolong survival and adjuvant chemotherapy and radiotherapy have limited results[2,3]. The treatment of choice is surgery, as adjuvant chemotherapy and radiotherapy have limited results. When metastases occur, they may be resected in specific patients[6,7]. Otherwise, treatment is generally systemic and palliative in nature. In recent years, stereotactic body radiation therapy (SBRT) for lung metastases, a high-precision external radiotherapy technique, alternative to metastasectomy, has undergone significant development. Prospective phase Ⅰ/Ⅱ studies have shown that SBRT is safe and effective as treatment of lung metastases in oligometastatic patients who are not candidates for surgery[8,9]. SBRT of inoperable lung metastases is today considered routine in many centers. We report the case of a rapidly metastatic PAIS, with sustained complete remission following surgical resection and SBRT.

CASE REPORT

A 48-year-old male patient presenting with sudden-onset symptoms of sweating, dizziness and falling to the ground, with loss of consciousness, and spontaneous recovery. After observing electrocardiographic changes, he was hospitalized with suspected acute coronary syndrome. An angio-computed tomography (CT) was performed, resulting in a diagnosis of pulmonary thromboembolism, with no improvement after anti-coagulant therapy. The patient was transferred to our hospital, where a repeat angio-CT was performed, revealing a filling defect in the pulmonary artery trunk, extending from the subvalvular area to the origin of the right pulmonary artery, with no change in size with respect to the previous angio-CT (Figure 1). The patient was operated on for a suspected primary tumor of the pulmonary artery, resulting in with complete resection of the mass, whose pathological result was an intermediate-grade malignant tumor suggestive of PAIS (Figure 2). Following an extension study, CT showed only a nonspecific pulmonary nodule of 4 mm in diameter in the right upper lobe. No adjuvant treatment was given. Three months later, a positron emission tomography-computed tomography (PET-CT) found that the previously mentioned nodule measured 6 mm, suggestive of metastasis. Another 4 mm *de novo* nodule was found and two more of 2-3 mm in size, possibly granulomas, all without an increase in metabolic activity. The patient underwent surgery where 4 bilateral pulmonary lesions, compatible with metastasis, were resected. Fifteen months later, a new PET-CT reveals a subpleural nodule in the left upper lobe, again suspicious of metastasis (Figure 3A), as well as several millimeter-size nodules reported in the previous CT, not metabolically characterizable. After discussion in a multidisciplinary committee, the patient was given treatment with SBRT (12 Gy × 5 fractions), with excellent control. Six months later, growth of the two new pulmonary lesions noted in the previous CT was observed (Figure 3B and C). Chemotherapy was prescribed, which was rejected by the patient, and a second course of SBRT was given on both pulmonary lesions. Nine months after SBRT, the patient is disease-free by PET-CT.

DISCUSSION

PAIS is characterized by insidious growth, causing extensive local invasion and hematogeneous metastases. Because it is a rare tumor, only case reports and small case series have been published, most of them focused on the histopathological findings and surgical aspects of its management[3,4,10-12]. Few patients achieve long-term survival and they are those without disease dissemination. The largest analysis of outcomes of this tumor reported better median survival of patients who received multimodality treatment with respect those who had single treatment (median survival of 24.7 and 8.0 mo, respectively). However, single treatment was defined as either surgery, chemotherapy or radiotherapy alone, instead of surgery without postoperative treatment[2]. Mussot *et al*[3] described a surgical series of 31 patients. They concluded that there appeared to be no statistical survival benefit in those who received adjuvant treatment compared to those who did not. A recent study[4] analyzed 20 patients diagnosed with PAIS obtaining a median overall survival of 17 mo: Patients who received postoperative chemo and radiotherapy showed a trend towards better survival compared to those who had surgery alone (24 mo *vs* 8 mo, $P = 0.3417$). Successful

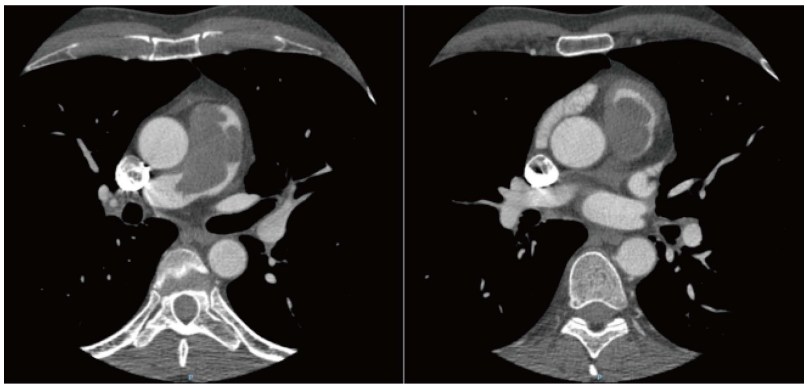

Figure 1 Angio-computed tomography: Filling defect in pulmonary artery trunk measuring 58 mm × 32 mm × 44 mm, extending from the subvalvular area to the origin of the right pulmonary artery.

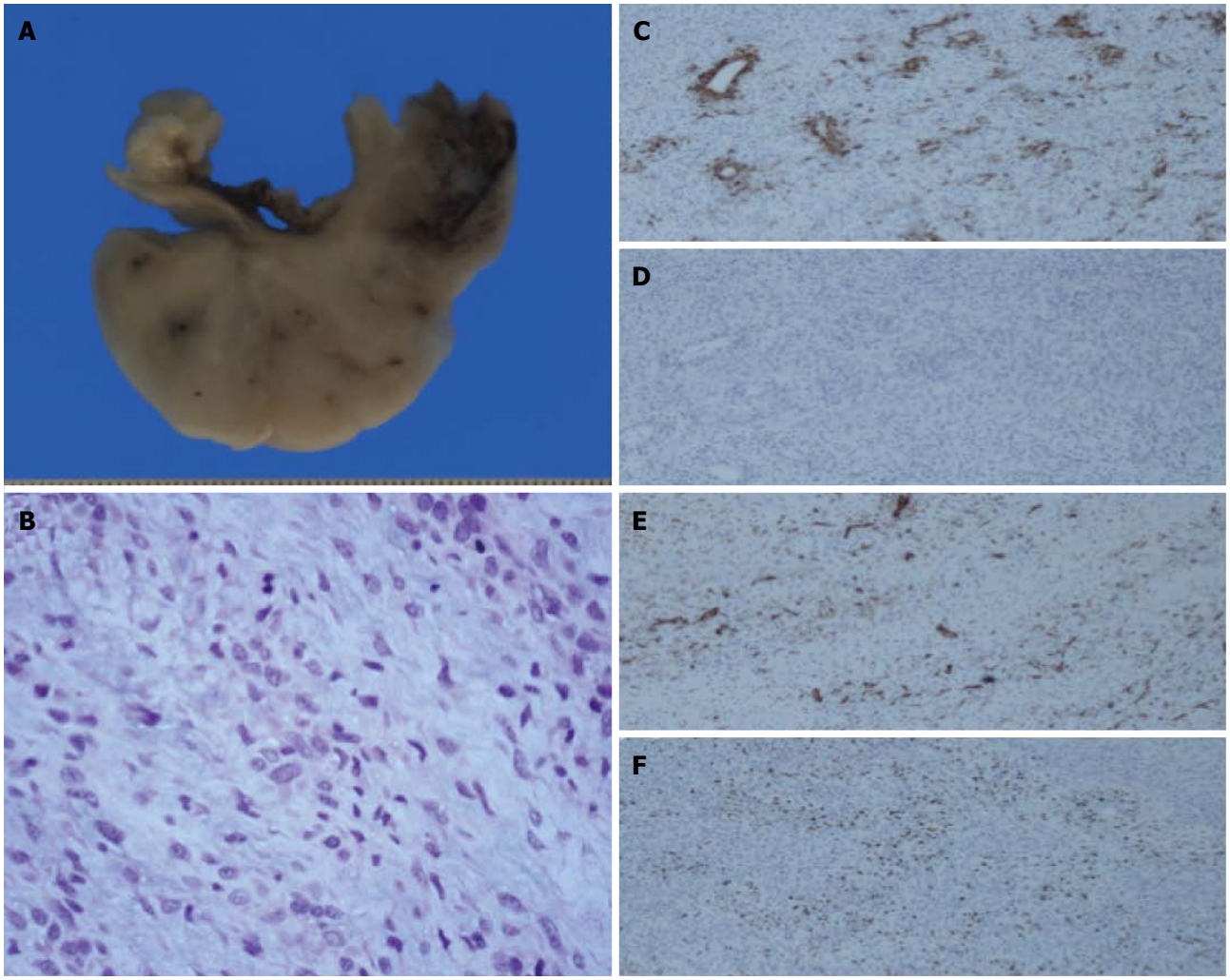

Figure 2 The patient was operated on for a suspected primary tumor of the pulmonary artery, resulting in with complete resection of the mass, whose pathological result was an intermediate-grade malignant tumor suggestive of Pulmonary artery intimal sarcoma. A: Macro: View of the pulmonary artery transversal section with infiltrating sarcoma on the lumen; B: High power of the tumor. Note the variable atypia 200 ×. Immunohistochemical stainings 100 ×; C: Smooth muscle actin: Focal tumor cell reaction 100 ×; D: Desmine: Negative 100 ×; E: CD31: Reaction of the endothelium surrounded by negative tumor cells 100 ×; F: Ki-67: Variable proliferation index in tumor cells 100 ×.

cases reported in metastatic patients are anecdotal. Thus, Said *et al*[13] reported a case of pulmonary artery angiosarcoma, with a follow-up of 5 years and multiple repeat lung metastasectomies, which has a disease-free interval of 1 year. Choi *et al*[14] published a case of PAIS with metastases in the thyroid and adrenal glands, 4.7 and 6.3 years, respectively, after initial surgery. Both metastases were surgically resected, with an unusual

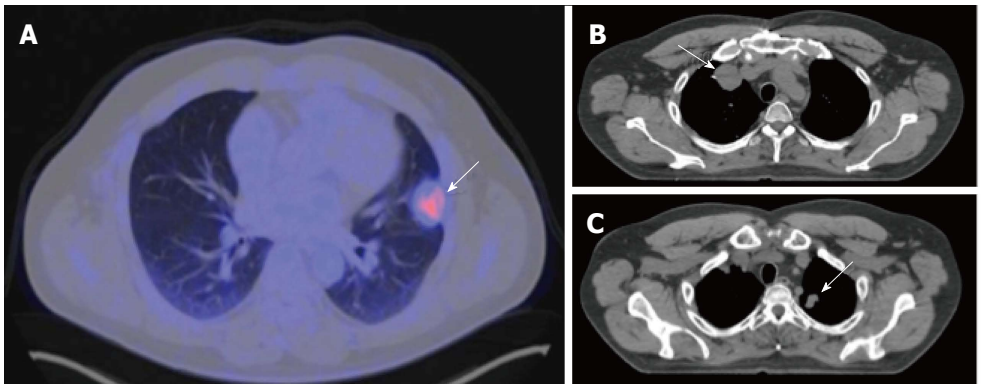

Figure 3 Positron emission tomography–computed tomography, three months later. A: Subpleural hypermetabolic nodular lesion in left upper lobe (SUV max 3.8) measuring 27 mm × 30 mm, suggestive of tumor activity; B and C: Computed tomography, a 38-mm nodule in right upper lobe and another 20-mm nodule in left upper lobe, compatible with metastasis.

survival of 12.5 years up to the last follow-up. In our case, dissemination occurred much earlier, as the patient was operated on for lung metastases 5 mo after the initial surgery, though it is likely that patient was already metastatic at diagnosis. He is currently disease-free, 4 years after diagnosis. To our knowledge, this is the first published case of metastatic PAIS with long-term survival treated with surgery and SBRT.

In conclusion, SBRT proved to be an alternative treatment to metastasectomy, allowing palliative chemotherapy to be delayed or omitted, which may result in improved quality of life.

COMMENTS

Case characteristics

A 48-year-old male presenting sudden-onset symptoms of sweating, dizziness and fall, with momentary loss of consciousness.

Clinical diagnosis

Acute coronary syndrome.

Differential diagnosis

Thromboembolic disease.

Imaging diagnosis

Angio-computed tomography: Filling defect in the pulmonary artery trunk, extending from the subvalvular area to the origin of the right pulmonary artery.

Pathological diagnosis

Intermediate-grade malignant tumor suggestive of pulmonary artery intimal sarcoma.

Treatment

The patient underwent surgery. The tumor progressed rapidly in the lung, requiring surgery of multiple lung metastases. Finally, he was treated with stereotactic body radiation therapy on two occasions for new pulmonary lesions.

Experiences and lessons

This case report describes a uncommon patient with exceptional long-term survival despite having rapid metastatic progression. This case teaches us that SBRT is an alternative treatment to metastasectomy, allowing palliative chemotherapy to be delayed or omitted.

Peer-review

The article presents an unusual case of intimal sarcoma of the pulmonary artery. There are a small number of cases reported. This is a rare sarcoma with very good response to treatment with radiotherapy.

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CASE REPORT

Long-term stabilization of metastatic melanoma with sodium dichloroacetate

Akbar Khan, Doug Andrews, Jill Shainhouse, Anneke C Blackburn

Akbar Khan, Doug Andrews, Medicor Cancer Centres Inc, Toronto, ON M2N 6N4, Canada

Jill Shainhouse, Insight Naturopathic Clinic, Toronto, ON M4P 1N9, Canada

Anneke C Blackburn, the John Curtin School of Medical Research, the Australian National University, Canberra, ACT 2601, Australia

Author contributions: Khan A treated the patient and wrote most of the case report; Andrews D assisted in development of the natural medication protocol for reduction of DCA side effects, and wrote a portion of the case report; Shainhouse J treated the patient with natural therapy; Blackburn AC interpreted the case report in the context of the literature on *in vitro* and *in vivo* DCA research, wrote parts of the introduction and discussion, and reviewed the manuscript overall.

Informed consent statement: The patient described in this manuscript has given consent to publish his case anonymously.

Conflict-of-interest statement: One of the authors (Khan) administers dichloroacetate therapy for cancer patients through Medicor Cancer Centres at a cost, and without profit. The clinic is owned by a family member of this author. The other authors have nothing to disclose.

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Correspondence to: Akbar Khan, MD, Medical Director, Medicor Cancer Centres Inc, 4576 Yonge St., Suite 301, Toronto, ON M2N 6N4, Canada. akhan@medicorcancer.com
Telephone: +1-416-2270037
Fax: +1-416-2271915

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Abstract

Sodium dichloroacetate (DCA) has been studied as a metabolic cancer therapy since 2007, based on a publication from Bonnet et al demonstrating that DCA can induce apoptosis (programmed cell death) in human breast, lung and brain cancer cells. Classically, the response of cancer to a medical therapy in human research is measured by Response Evaluation Criterial for Solid Tumours definitions, which define "response" by the degree of tumour reduction, or tumour disappearance on imaging, however disease stabilization is also a beneficial clinical outcome. It has been shown that DCA can function as a cytostatic agent *in vitro* and *in vivo*, without causing apoptosis. A case of a 32-year-old male is presented in which DCA therapy, with no concurrent conventional therapy, resulted in regression and stabilization of recurrent metastatic melanoma for over 4 years' duration, with trivial side effects. This case demonstrates that DCA can be used to reduce disease volume and maintain long-term stability in patients with advanced melanoma.

Key words: Dichloroacetate; Cancer; *BRAF*; Melanoma; Cytostatic

Core tip: Sodium dichloroacetate (DCA) has been studied as a metabolic cancer therapy since 2007. It has been shown that DCA therapy can result in a classic response which is measured by reduction or disappearance of

tumours on imaging. However, DCA can also halt cancer cell growth without causing apoptosis (cytostatic effect). This can result in long-term stabilization of metastatic cancer. We present a case of oral DCA therapy resulting in reduction and stabilization of metastatic melanoma in a 32-year-old male for over 4 years, with only minor side effects.

Khan A, Andrews D, Shainhouse J, Blackburn AC. Long-term stabilization of metastatic melanoma with sodium dichloroacetate. *World J Clin Oncol* 2017; 8(4): 371-377 Available from: URL: http://www.wjgnet.com/2218-4333/full/v8/i4/371.htm DOI: http://dx.doi.org/10.5306/wjco.v8.i4.371

INTRODUCTION

Sodium dichloroacetate (DCA) caught the attention of the medical community in 2007, when Bonnet *et al*[1] published the first *in vitro* and *in vivo* study illustrating the value of DCA as a metabolic cancer therapy, through its inhibitory action on the mitochondrial enzyme pyruvate dehydrogenase kinase. Previously, Stacpoole *et al*[2-4] had published several studies of DCA for the treatment of congenital lactic acidosis in mitochondrial diseases[2-5]. These studies demonstrated that oral DCA is a safe drug for human use. DCA was noted to have an absence of renal, pulmonary, bone marrow and cardiac toxicity[4]. Most DCA side effects were modest, with the most serious one being reversible peripheral neuropathy[6]. Reversible delirium has also been reported[7]. Elevation of liver enzymes (asymptomatic and reversible) has been noted in a small percentage of patients[3]. The prior human research in mitochondrial disorders has enabled the rapid translation of DCA into human use as an off-label cancer therapy. Several reports of clinical trials using DCA as cancer therapy have now been published, confirming its safety profile, and indicating an increasing recognition of the potential usefulness of DCA in the cancer clinic[8-11]. One limitation of these studies involving late stage patients is that they have only reported on treatment for short periods of time.

In Bonnet's 2007 publication[1], DCA treatment was shown to reduce mitochondrial membrane potential which promoted apoptosis selectively in human cancer cells. Aerobic glycolysis inhibition (the Warburg effect) and mitochondrial potassium ion channel activation were identified as the mechanisms of action of DCA. Further investigations of DCA *in vitro* have confirmed the anti-cancer activity against a wide range of cancer types, which have been reviewed recently by Kankotia and Stacpoole[12]. In addition, DCA is also able to enhance apoptosis when combined with other agents[13-15]. Other anticancer actions of DCA have also been suggested, including angiogenesis inhibition[16], alteration of HIF1-α expression[17], alteration of cell pH regulators V-ATPase and MCT1, and other cell survival regulators such as p53 and PUMA[18]. However, many *in vitro* studies use unreasonably high concentrations of DCA that are not clinically achievable, in an effort to show cytotoxic activity[12]. In other studies, more modest DCA concentrations were used, demonstrating that DCA could be cytostatic. The second report in 2010 of its *in vivo* anti-cancer activity found DCA alone to be cytostatic in a metastatic model of breast cancer[19], inhibiting proliferation without triggering apoptosis. This suggests a role for DCA as a cancer stabilizer, similar to angiogenesis inhibitors.

In response to the 2007 report of the anti-cancer actions of DCA, Khan began using DCA for the treatment of cancer patients with short prognosis or who had stopped responding to conventional cancer therapies. A natural medication protocol was developed in collaboration with a naturopathic physician (Andrews) to address the dose-limiting neurologic toxicity of DCA. This consisted of 3 medicines: Acetyl L-carnitine[20-22], R-alpha lipoic acid[23-25] and benfotiamine[26-28], for neuropathy and encephalopathy prevention. In over 300 advanced stage cancer patients, observational data revealed that DCA therapy benefitted 60%-70% of cases. The neuropathy risk when natural neuroprotective medicines were combined with DCA was approximately 20% using 20-25 mg/kg per day dosing on a 2 wk on/1 wk off cycle (clinic observational data published online at www.medicorcancer.com). Here, a patient case report illustrating both the apoptotic and anti-proliferative effects of chronic DCA treatment over a period of over four years is presented.

CASE REPORT

A 32 years old previously healthy fair-skinned male originally noted that a mole on his left calf began to change in 2006. He consulted a doctor and the mole was excised. A pathologic diagnosis of melanoma was made. A sentinel node dissection was carried out, and was negative for metastatic disease. In 2007, the patient noted enlargement of left inguinal lymph nodes, and small melanocytic lesions on the skin of his left leg. He was treated with interferon alpha under a clinical trial at a regional cancer hospital, with reduction of the nodes and resolution of the skin metastases. Interferon was stopped after 9 mo due to side effects.

The patient remained well until 2010, when a new left leg skin metastasis appeared. This was surgically excised. In late 2011, another new cutaneous metastasis was identified on the left leg, within the scar from the original melanoma surgery. This was biopsied and a diagnosis of recurrent melanoma was confirmed. He was then treated with wide excision and skin graft.

In March 2012, the patient was diagnosed with a recurrence within the left leg skin graft. This was excised and a new skin graft procedure was performed. Pathology revealed positive margins of the excised

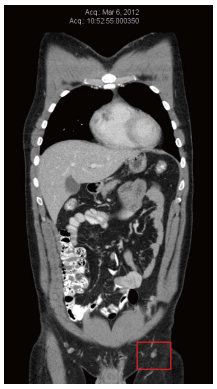

Figure 1 Computed tomography scan from March 2012 prior to natural therapies and prior to dichloroacetate therapy. Largest node measured 8 mm in diameter.

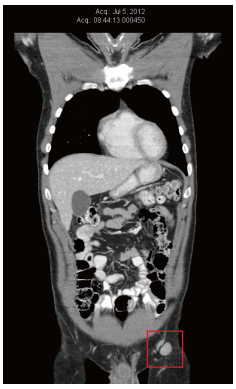

Figure 2 Computed tomography scan from July 2012 after 3 mo of natural therapy alone, just prior to the start of dichloroacetate therapy. Largest node measured 22 mm × 20 mm.

metastasis, so a re-excision was performed, again with positive margins. At the same time, needle biopsy of a left inguinal lymph node confirmed the presence of BRAF-positive metastatic melanoma. A Computed tomography (CT) scan performed in Mar 2012 revealed no evidence of distant metastases. The largest left inguinal node was 8mm in diameter, which was reported as "insignificant by size criteria" (Figure 1).

In April 2012, the patient consulted a naturopathic doctor (Shainhouse) and began therapy with the following oral natural anti-cancer agents: Active hexose correlated compound or AHCC (mushroom extract)[29], dandelion root[30], curcumin[31], and astragalus root[32]. Parenteral therapy was also started, which consisted of intravenous vitamin C twice weekly[33] and subcutaneous European mistletoe extract[34]. The patient also changed to a vegan diet.

In May 2012, the patient attended the author's clinic (Khan) looking to pursue additional non-traditional therapies. DCA therapy was discussed, but the patient decided to give the natural anti-cancer therapies (prescribed by Shainhouse) an adequate trial first. CT scan was performed again in May 2012 (after only 1 mo of natural therapy) and indicated mild growth of multiple inguinal and external iliac nodes, with sizes ranging from 10 mm × 11 mm to 14 mm × 15 mm.

In July 2012, CT scan was repeated to assess the patient's natural anti-cancer therapies. At that time, the left inguinal and external iliac nodes had enlarged again, and ranged in size from 13 mm × 16 mm to 22 mm × 20 mm (Figure 2). PET scan was also performed in preparation for entering a clinical trial in Boston, MA (United States), and confirmed increased glucose uptake in the left inguinal nodes. There was new low intensity (2/10) aching pain in the left inguinal region. Examination revealed a 20 mm non-tender left inguinal lymph node, and two small skin metastases within the left calf skin graft.

The patient was thus diagnosed with disease progression. At that point he decided to initiate DCA therapy. He began oral DCA 500 mg 3 times per day, which was equivalent to 17 mg/kg per day (manufacturer: Tokyo Chemical Industry, United States) in addition to maintaining the other natural therapies. The DCA treatment cycle was 2 wk on and 1 wk off. To minimize the occurrence of DCA side effects, 3 additional natural medications were prescribed: Oral acetyl L-carnitine 500 mg 3 times a day, oral benfotiamine 80 mg twice a day and oral R-alpha lipoic acid 150 mg 3 times a day. These supplements were taken daily (no cycle). Routine baseline blood tests were performed (Table 1). These were all normal, except for low creatinine which was felt to be insignificant.

In November 2012, 4 mo after the addition of DCA to his original natural anti-cancer therapies, the patient was re-assessed. He felt generally well. Two new symptoms were reported to have begun only after initiation of DCA therapy: Slightly reduced sensation of the finger tips and toes, and slightly reduced ability to concentrate during the 2 wk periods in which he was taking DCA. The mild sensory loss was not worsening and was felt to be mild DCA-related neuropathy. Both the numbness and reduced concentration were reported to resolve during the weeks when the patient was off DCA. Blood panel from October 2012 showed no significant changes (Table 1). August 2012 and November 2012 CT scans revealed significant regression of all previously enlarged lymph nodes. The largest node was 10 mm, and there was no evidence of intra-thoracic or intra-abdominal disease, and no bone metastases (Figure 3).

The patient continued to feel well on DCA therapy, and did not notice any new skin metastases or new enlargement of inguinal nodes. He continued to have frequent clinical monitoring with his naturopathic doctor (Shainhouse), and annual follow-up with his medical doctor (Khan). The listed natural anti-cancer therapies (prescribed by Shainhouse) and DCA therapy were maintained into 2016. Blood panel results in June 2016 continued to be normal (Table 1). CT scan was repeated in August 2016, showing no evidence of metastatic melanoma, after a full 4 years of ongoing DCA therapy, combined with natural anti-cancer therapy (Figure 4). By December 2016, the patient reported an increase in work-related stress and a reduction in compliance

Table 1 Blood panel prior to and during dichloroacetate therapy

Blood test	July 12 pre-DCA	October 12 3 mo DCA	June 16 4 yr DCA	Units	Normal range
Hemoglobin	154	150	157	g/L	135-175
White cell count	4.5	4.1	5	$\times 10^9$/L	4.0-11.0
Platelets	220	214	229	$\times 10^9$/L	150-400
Glucose	-	4.6	4.9	mmol/L	3.6-7.7
Urea	3.9	3.2	3.9	mmol/L	2.5-8.0
Creatinine	49[1]	50[1]	55[1]	μmol/L	62-115
Calcium	2.47	2.41	2.47	mmol/L	2.15-2.60
Albumin	48	45	47	g/L	35-50
Bilirubin	8	10	13	μmol/L	< 22
Sodium	139	141	140	mmol/L	135-147
Potassium	4	4.3	3.9	mmol/L	3.5-5.5
Chloride	106	107	105	mmol/L	100-110
Alkaline Phosphatase	77	69	71	U/L	45-129
LDH	139	135	144	U/L	120-246
GGT	18	19	20	U/L	15-73
AST	18	25	21	U/L	7-37
ALT	18	28	19	U/L	12-49

[1]Indicates abnormal value. DCA: Dichloroacetate; LDH: Lactate dehydrogenase; GGT: Gamma-glutamyltransferase; AST: Aspartate aminotransferase; ALT: Alanine aminotransferase.

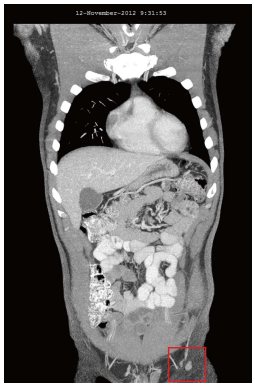

Figure 3 Computed tomography scan from November 2012 after 4 mo of dichloroacetate therapy. Largest node measured 10 mm.

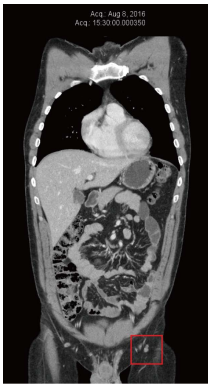

Figure 4 Computed tomography scan after 4 years of dichloroacetate therapy without any concurrent conventional cancer therapies. Scan demonstrates absence of cancer re-growth. All nodes measure less than 10 mm.

with his medications. At the time, he noted a new left inguinal mass. Ultrasound imaging was obtained, which revealed a new conglomerate of enlarged lymph nodes measuring 40 mm × 25 mm × 23 mm, with colour Doppler showing blood flow within the mass. This was interpreted as re-growth of melanoma, after approximately four and a half years of continuous DCA therapy. Further workup was performed including a PET/CT scan, which confirmed disease recurrence in 3 left inguinal nodes (SUV_{max} ranging from 13 to 17.8).

In summary, the patient received conventional therapy for recurrent stage 3 melanoma over a period of 6 years, consisting of primary surgical excision with lymph node dissection, interferon alpha and surgical excisions for recurrent cutaneous metastases on 5 occasions. The patient then received natural anti-cancer therapy alone (prescribed by Shainhouse) for 3 mo with no response, evidenced by steady disease progression on serial CT scans. Finally the patient added oral DCA therapy to the natural anti-cancer therapy, with 3 concurrent neuroprotective medicines (lipoic acid, acetyl L-carnitine and benfotiamine) and no concurrent conventional cancer therapies. The result was a complete radiological remission lasting for over 4 years, followed by recurrence. During the course of DCA therapy, the patient experienced trivial side effects consisting of slight neuropathy and slight reduction of concentration. The patient maintained ECOG level 0 function, and he was able to work full time.

DISCUSSION

The use of oral DCA in the metastatic melanoma patient described herein demonstrates tumour shrinkage and long-term disease stability according to clinical status and CT imaging. Disease stability was maintained for over 4 years while taking DCA in the absence of any concurrent conventional therapy, with a survival time since the initial diagnosis of 10 years. According to the National Cancer Institute's SEER cancer statistics, the survival of this patient who showed no evidence of distant metastases is

not remarkable (62.9% 5-year survival rate for melanoma with spread to regional lymph nodes, https://seer.cancer.gov/statfacts/html/melan.html). What is remarkable is that in a situation where involved lymph nodes were clearly enlarging, the addition of oral DCA therapy was efficacious in shrinking the enlarging nodes (Figures 2 and 3), and in achieving a remission lasting over 4 years. It is possible that the natural anti-cancer therapies the patient received synergized with DCA, but it is also clear that these natural therapies alone cannot account for the disease regression. DCA has been reported to have both apoptotic and cytostatic effects[14,17,19,35,36], which is consistent with this patient's clinical course of regression (apoptotic) and prolonged remission (cytostatic). The recurrence after 4 years coincided with reduced compliance, suggesting that this method of cancer management with DCA requires the metabolic pressure to be maintained continuously. Despite recurrence, the patient remained clinically well and planned to start new immunotherapy medications. It remains to be seen if a change in therapy can once again achieve disease regression or stability.

In addition to the maintenance of remission for over 4 years, this case illustrates that DCA can be well-tolerated in a cancer patient for a prolonged time period, as compared to all published DCA cancer clinical trials. Notably, this patient was able to tolerate 17 mg/kg per day in a regime of 2 wk on/1 wk off for 4 years with minimal side effects. This is similar to our previous case report of chronic DCA usage in colon cancer[37], where the patient was able to tolerate 16 mg/kg per day (but not 25 mg/kg per day) in the same regime, but contrasts with the clinical trials for DCA, which recommend a lower dose of 10-12.5 mg/kg per day given continuously[9,11]. The 1 wk break or the neuroprotective supplements may both contribute to the ability of the patients in the case reports to tolerate the higher dose. Genetic polymorphisms in *GSTZ1*, the liver enzyme that metabolises DCA, may also contribute to the dose of DCA that can be tolerated[9,38]. Variable drug levels have been reported in the trials, but not all of them have considered this pharmacogenetic aspect of DCA therapy[9,11], and further studies are needed to clarify if this is a significant contributor to DCA tolerance. As of this writing, a DCA multiple myeloma human trial is ongoing, which is examining both *GSTZ1* genotypes and drug levels to contribute to our understanding of these issues (Australia New Zealand Clinical Trials Register #ACTRN12615000226505, http://www.anzctr.org.au).

This case report shows that chronic DCA therapy can be used without reducing quality of life, as compared to conventional melanoma therapies such as interferon. To determine the optimal protocol for maximum tolerable acute or chronic treatment with DCA, human trials are needed. But more importantly, it still remains to be clarified what dose is required for on-target effects that will be efficacious against cancer. This information is necessary before investing in larger, long term studies on patient outcomes. DCA deserves further investigation in clinical trials as a non-toxic cancer therapy due to its modest cost and low toxicity, and deserves consideration as an off-label cancer therapy.

ACKNOWLEDGMENTS

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COMMENTS

Case characteristics

The 32-year-old male patient presented with a pigmented lesion on his leg.

Clinical diagnosis

The patient was diagnosed with a melanoma.

Laboratory diagnosis

Melanoma confirmed by excisional biopsy.

Imaging diagnosis

Enlarged inguinal node confirmed to be involved with melanoma (needle biopsy).

Pathological diagnosis

Melanoma, BRAF positive.

Treatment

Excision of primary lesion with skin graft, sentinel node dissection, multiple excisions of recurrent cutaneous metastases. Traditional therapy stopped and natural anti-cancer therapies started (AHCC, dandelion root, curcumin, astragalus root, *i.v.* vitamin C, *s.c.* European mistletoe). Progression after 3 mo, dichloroacetate (DCA) added. Regression and remission following addition of DCA lasting for over 4 years.

Related reports

Computed tomography scan reports demonstrate the course of the disease and response to therapies.

Term explanation

DCA: Dichloroacetate sodium; RECIST: Response Evaluation Criteria for Solid Tumours; ECOG: Eastern Cooperative Oncology Group.

Experiences and lessons

DCA can act as a pro-apoptotic and cytostatic drug, and can thus achieve regression as well as long-term stabilization of metastatic cancer without serious side effects, as illustrated by this melanoma case.

Peer-review

Dr. Khan described a 32-year-old man received DCA therapy, with other medications from natural therapists and maintained in a stabilization state (metastatic melanoma) for over 4 years. It is an interesting case.

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Contents

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Contents

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REVIEW

Further the liquid biopsy: Gathering pieces of the puzzle of genometastasis theory

Ana García-Casas, Dolores C García-Olmo, Damián García-Olmo

Ana García-Casas, Universidad Complutense de Madrid, 28050 Madrid, Spain

Dolores C García-Olmo, Centre de Recerca Experimental Biomèdica Aplicada(CREBA), IRBLLEIDA, 25138 Lleida, Spain

Damián García-Olmo, Department of Surgery, Universidad Autónoma de Madrid, Instituto de Investigación Sanitaria, Fundación Jiménez Díaz, 28050 Madrid, Spain

ORCID number: Ana García-Casas (0000-0002-1186-8054); Dolores C García-Olmo (0000-0001-9071-547X); Damián García-Olmo (0000-0002-9369-2338).

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Correspondence to: Dr. Damián García-Olmo, MD, PhD, Director, Professor, Surgeon, Department of Surgery, Universidad Autónoma de Madrid, Instituto de Investigación Sanitaria, Fundación Jiménez Díaz, Avda Reyes Católicos 2, 28050 Madrid, Spain. damian.garcia@uam.es
Telephone: +34-669-845297

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Abstract

Metastasis is the major cause of mortality in cancer disease and still constitutes one of the most controversial mechanism, not yet fully understood. What is almost beyond doubt is that circulatory system is crucial for cancer propagation. Regarding this system, much attention has been recently paid to liquid biopsy. This technique is aimed to detect circulating tumor cells (CTCs) and circulating nucleic acids so it can be used as a tool for diagnostic, prognostic and follow-up of patients. Whereas CTCs tend to be scarce in serum and plasma from cancer patient, abundant circulating nucleic acids can be detected in the same location. This fact, together with the genetic origin of cancer, stands out the relevance of circulating nucleic acids and shed light into the role of nucleic acids as drivers of metastasis, a recently discovered phenomenon called Genometastasis. This innovative theory supports the transfer of oncogenes from cancer cells to normal and susceptible cells located in distant target organs through circulatory system. What is more, many biological processes haven been described to deliver and secrete circulating nucleic acids into the circulation which can allow such horizontal transfer of oncogenes. In this review, we focus not only on these mechanisms but also we demonstrate its putative role in cancer propagation and give insights about possible therapeutic strategies based on this theory. Our objective is to demonstrate how findings about cell-to-cell communications and previous results can agree with this unprecedented theory.

Key words: Genometastasis; Cancer metastasis; Circulating Nucleic acids: Circulating tumor cells; Liquid biopsy; Exosomes; Virtosomes

Core tip: Liquid biopsy not only constitutes a promising tool for cancer diagnostic and patient follow-up but also it may help in the comprehension of metastasis. This technique has revealed how circulating tumor cells are limited in blood, while circulating nucleic acids are much more abundant. This property, together with the demonstrated capability of circulating nucleic acids to transform susceptible cells, strongly support the theory of genometastasis. This theory sustains that cancer propagation relies on gene transfer from malignant cells to normal cells. We pretend to gather all these concepts, also including cell-to-cell communication mechanisms to demonstrate this phenomenon.

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CONCEPT OF LIQUID BIOPSY

Liquid biopsy

Traditionally, tissue biopsy has been used to diagnose and manage diseases. In cancer, biopsies are used to determine histological properties of the tumor as well as its genetic profile for diagnostic, prognostic purposes and prediction of response to therapies. However, the characteristic heterogeneity of tumors makes it necessary to analyze different parts of the same tissue which results in repeated sampling. Obtaining several tissue biopsies involves a high risk for the patient as well as economic cost for the system. As an alternative to tissue biopsy, liquid biopsy constitutes a promising and less invasive technique.

Liquid biopsy consists on the detection of cancer-derived molecular biomarkers, such as tumor cells or cell-free nucleic acids (cfNA) in biological fluids, mainly in blood. Given the non-invasiveness properties of the technique, it is possible to take repeated samples and so, to follow the progression and evolution of the disease in contrast to the static image from tissue biopsy.

The effectiveness of this approach has been demonstrated in different malignancies including breast, pancreatic and colorectal cancer (CRC)[1]. In the case of pancreatic cancer, liquid biopsy provides an advantageous technology regarding the anatomical and clinical difficulties for pancreatic tissue[2]. It would also help in the early detection of this disease, which is usually diagnosed at an advanced stage because it develops with no symptoms. For its part, CRC is mainly characterized by its heterogeneous genetic profile, in which new mutations constantly appear during tumor development[3]. These new mutations may confer proliferative capacities to tumor cells and, thus, molecular and genetic analysis of the whole tumor might be crucial during CRC follow-up. Similarly, tumor genotyping is also required in the case of anti-EGFR therapies, to which only the patients with KRAS wild-type gene respond. Thus, liquid biopsy can be conceived not only for recording tumor progression but also for selecting the most suitable treatment.

As mentioned before, liquid biopsy can be intended to detect circulating tumor cells (CTCs) and/or circulating cfNA.

CTCs

CTCs can be secreted into circulation by primary and metastatic tumor deposits. In 1869, during autopsy of a breast cancer patient, CTCs were first identified as cells similar to those of the primary tumor, presented in the bloodstream[4]. These cells are mainly found in patients with malignant diseases like carcinomas, being extremely rare in healthy subjects and patients with nonmalignant diseases[5].

CTCs can be difficult to obtain given its heterogeneous morphology and its limited amount in the circulation: They constitute one cell per 1×10^9 normal bloodstream cells in patients with metastatic cancer[6]. In other terms, in 7.5 mL of blood from metastatic carcinoma patients, only 5 to 50 CTCs are presented on average[7]. This small cell number makes it difficult to detect CTCs, especially small subpopulations of tumor cells, which can harbor crucial mutation for tumorigenesis. However, many attempts and approaches have been designed to isolate CTCs. Most of them are based on antibody identification of cell surface markers, such as EpCAM, or size differences between CTCs and the rest of blood cells[8]. Once CTCs are obtained, they have to be further analyzed through genome sequencing. Nevertheless, these isolating techniques might not provide the whole spectrum of CTCs, uncovering the tumor heterogeneity. As an example, basal-like breast cancer CTCs with low levels of EpCAM may not be captured using this cell surface marker determinant[7]. On the other hand, false-positive CTC results can also be found in the case of patients with benign inflammatory disease such as Crohn disease. It has been shown that 11% to 19% of these patients present small numbers of circulating epithelial cells detectable that can be confused with CTCs[9]. In addition, although correlation between cell number and disease severity have been established in metastatic patients from breast, colon and prostate cancer[10-12], less is known about early-stage tumors and CTC number. Altogether, more studies are required to elucidate the relationship between tumor burden and the number of CTCs in order to verify the clinical utility of CTCs as prognostic markers[7].

It is also worth noting that CTCs are difficult to grow in culture, which questions the functionality of these cells. Thus, it can be hypothesized that these cells are more likely to constitute death cells, poured by tumor mass, than active cells responsible for metastasis emergence.

Circulating nucleic acids

Regarding to circulating nucleic acids we can make a distinction between circulating cell-free DNA (cfDNA) and cell-free RNA (cfRNA).

Circulating cfDNA: The first association between cancer and the presence of circulating cfDNA was established in 1977 by Leon *et al*[13] who detected a higher concentration of DNA in serum from cancer patients. Ten years later, Stroun *et al*[14] confirmed this relation by isolating and characterizing DNA obtained from the plasma of cancer patients. Moreover, it was further shown that patients with malignant tumors have higher circulating cfDNA levels than patients suffering benign disease[15]. The tumor origin of such cfDNA was also confirmed by the identification of tumor-specific abnormalities such as loss of heterozygosity (LOH) of microsatellites and methylation of CpG islands[16,17]. In addition from tumor cells, plasma cfDNA may come from blood cells and other tissue-specific cells. However, the proportion of DNA derived from different origins widely varies. In fact, circulating tumor DNA proportion range between 0.01% and 93% in cancer patients[7,18].

cfDNA is usually found in plasma as with a double-stranded structure, although single-stranded circulating DNA has also been identified[19-21]. It should be noted that DNA molecule need to be protected by different complexes or other molecules, described in detail below, in order to avoid its degradation by serum nucleases.

Circulating cfRNA: Circulating cfRNA was first isolated in 1987 from serum of patients with malignant disorders and culture media of different malignant cell lines. It was initially found in the form of RNA-proteolipid complex[22]. As it happens with DNA, it is no surprising to detect cfRNA associated with other molecules since it alone can be very labile due to the increased amounts of RNases present in the circulation.

Circulating RNA consist of messenger RNA (mRNA) and microRNA (miRNA). Regarding to mRNA, different transcripts have been identified to be overexpressed in plasma of tumor patients, especially human transcriptase reverse telomerase (hTERT) mRNA levels in malignancies such as breast cancer or colon cancer[23-25]. miRNA molecules are fragments of 19-25 bp non-coding RNA molecules which derive from 70-100 bp hairpin precursor molecules. By posttranscriptional regulation, they modulate the expression of target genes involved in many physiological and pathological process such as development, cell proliferation, differentiation or apoptosis[26,27].

Circulating nucleic acids as biomarkers: Although the term nucleic acids refers to both types of molecules, special attention has been paid to cfDNA in the field of liquid biopsy because it carries the tumor-associated mutations and thus, it represents an attractive biomarker.

As commented before, circulating DNA gives more detailed information about the heterogeneity of the tumour because it may come from different cells with presumably different genomic alterations, which can be detected by sequencing.

Likewise, circulating cfDNA is much easier to isolate than CTCs because it is abundantly present in blood, especially in patients with advanced disease[28]. Indeed, circulating DNA extraction can be performed following a simple protocol that does not exceed 5 h[18]. Once it is isolated, PCR, followed by DNA sequencing can be used to detect tumor-specific genetic aberrations which may also help in the comprehension of tumor dynamics. In this issue, droplet digital PCR, together with genome-wide high throughput sequencing, provide a high sensitivity and specificity for detecting mutations[29]. These new tools for DNA analysis are also contributing to give a more profound insight into the presence and role of circulating DNA, among its value as a biomarker.

CTCs and circulating cfDNA

It would be reasonable to suspect that tumor cfDNA found in the circulation can be released by CTCs. However, the discrepancy between the number of CTCs and the quantity of circulating DNA discards this theory. Considering the average amount of circulating DNA in a ml of plasma from advanced-stage cancer patients (17 ng) and the amount of DNA contained by a single human cells (6 pg), more than 2000 CTCs would be required if CTCs were the primary source of circulating DNA. Conversely, less than 10 CTCs per 7.5 mL of blood are found on average[18]. Therefore, tumor cfDNA might come from different regions within the tumor and thus, it may better represent tumor genetic heterogeneity. This fact, together with its high concentration in blood, suggests that circulating DNA might be a better liquid biopsy-derived biomarker. In the following section, we will focus on the reasons why DNA can be released into circulation.

WHERE DO CIRCULATING NUCLEIC ACIDS COMES FROM AND HOW DO THEY CIRCULATE IN THE BLOOD STREAM

Depending on how they are released, circulating nucleic acids can be found in different forms including molecular or macromolecular complex, linked to serum proteins or internalized in vesicles such as exosomes or microvesicles (Figure 1). In general terms, circulating nucleic acids can be either passively released, by apoptotic and necrotic cells, or actively released by living cells.

Passive release

During cell-death mechanisms, such as necrosis or apoptosis, both circulating DNA and circulating RNA can be liberated into bloodstream by dying or dead cells.

In necrosis, cellular DNA is incompletely and nonspecifically digested. In this condition, a smearing pattern

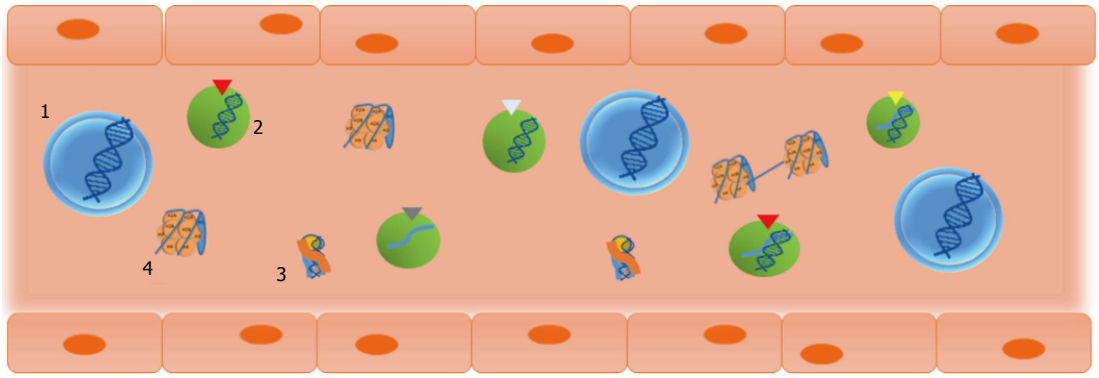

Figure 1 Circulating nucleic acids can be present in different forms. If actively released, circulating nucleic acids can be found inserted in exosomes (1) and microvesicles (2), or associated with RNA and lipoproteins forming a complex called the virosome (3). Circulating nucleic acids can be also passively released, mainly through apoptosis, in the form of oligo- or mono-nucleosome (4).

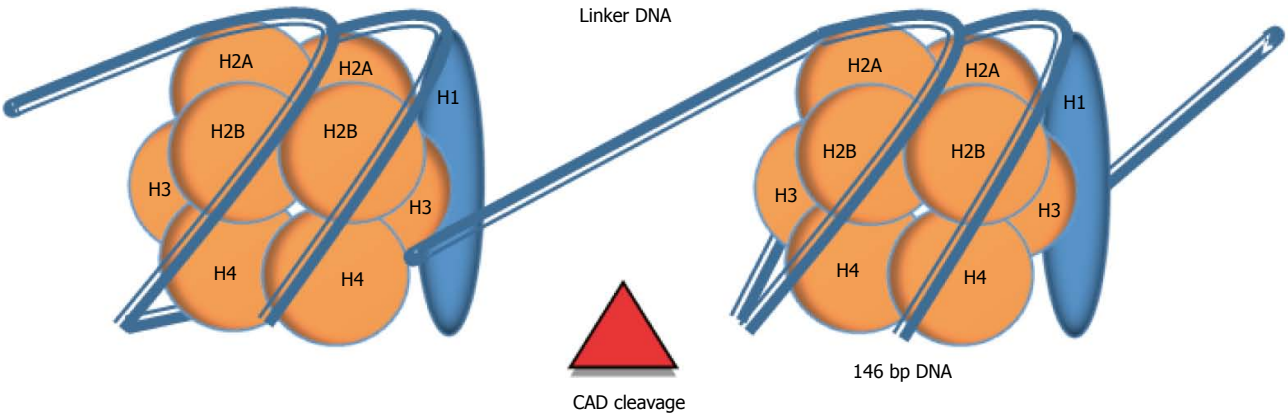

Figure 2 Chromatin cleavage during apoptosis can be a source of circulating DNA. Circulating DNA can be found in the form of nucleosomes. Each nucleosome is composed of 147 of DNA wrapped around an octamer of histones (H2A, H2B, H3 and H4). An extra histone (H1) stabilizes this complex. During apoptosis, CAD enzyme cleaves in the naked DNA that links each nucleosome (DNA linker), releasing oligo- or mono-nucleosomes. In cancer, where a higher cellular turnover is required, this process can be overloaded and nucleosomes can be secreted into circulation. CAD: Caspase-activated deoxyribonuclease.

would be observed when DNA is run electrophoretically in agarose gel. However, when circulating DNA is analyzed by agarose-gel electrophoresis, a ladder pattern is observed. This feature indicates that necrosis is not the major source of circulating DNA although it may be a possible contributor given the presence of DNA fragments ranging from 21 kb to 80 kb in length in blood plasma samples[14,30].

The mentioned ladder pattern is formed by fragments ranging from 180 bp to 1000 bp which matches with the fragments released from chromatin cleavage into nucleosomes, a process that occurs during apoptosis[30].

Nucleosome: Nucleosomes are molecular complexes that allow DNA stabilization and packing into the nucleus. In each nucleosome, 146 bp of double-stranded DNA are wound on an octamer of positively-charged proteins called histones (H2A, H2B, H3 and H4), through electrostatic interaction. Nucleosomes are linked by 10 to 100 bp of naked DNA, termed as linker DNA. An extra histone (H1), which is localized outside the octamer, stabilizes the tertiary structure of the chromatin chain[31] (Figure 2). Cell death by apoptosis implies the activation of a set of caspases that catalyze the hydrolysis of cellular components. Some of these caspases (*e.g.*, Caspase-3) trigger the activation of endonucleases, especially the caspase-activated deoxyribonuclease (CAD). Endonucleases cleavage chromatin through linker DNA, the most accessible region, generating oligo- and mono-nucleosomes that are packed into vesicles called apoptotic bodies. Apoptotic bodies are subsequently released from the cells and phagocytosed by macrophages and dendritic cells. Nevertheless, in conditions when higher cellular turnover is required, such as inflammation or tumor cell proliferation, this process collapses and nucleosomes are liberated into circulation[30,31]. Then, cell-free nucleosomes can be internalized into cells by crossing plasma membrane and penetrating into the nucleus from where it can alter gene expression[32].

It is worth noting that the octamer of histones of the nucleosome protects DNA molecule from its degradation by circulating endonucleases. It also should be noted that tumor-derived circulating DNA may be more fragmented than DNA derived from healthy cells as recent publications have shown[33].

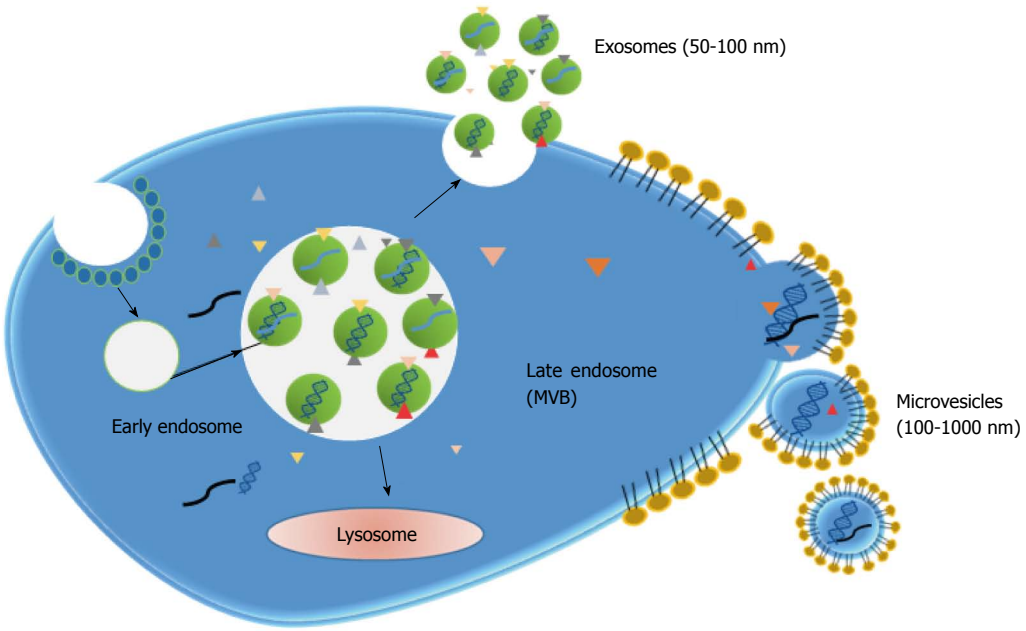

Figure 3 Exosomes and microvesicles can harbour nucleic acids. Exosomes and microvesicles are generated by different pathways. Exosomes derive from the recycling endosomal pathway, in which the late endosomes (MVB) merge with the plasma membrane instead of with the lysosome, releasing the exosomes. Exosomes encapsulate cytoplasm material such as proteins, RNA or DNA. Microvesicles result from plasma membrane budding, containing cellular components from both cell membrane and cell cytoplasm, including nucleic acids. MVB: Multivesicular bodies.

The apoptotic origin is confirmed by the existence of circulating mitochondrial DNA (mitDNA). In contrast to nuclear DNA, mitDNA is a circular and smaller (16.5 kb) molecule of DNA, not protected with histones[34]. It can be secreted to the circulation during cell death (*e.g.,* apoptosis) and mitophagy, which consist on the elimination of damaged mitochondria through autophagy[35]. Due to its elevated copy number, circulating mitDNA may account for a high proportion of the total circulating DNA found in blood. It can be present in circulation in both protein-associated and free form[34]. Circulating mitDNA measurement and mutation analysis has been proposed to diagnose different malignancies such as breast tumors or epithelial ovarian cancer and hepatocellular or colorectal cancer, respectively[1,36].

Active release

In addition to passive secretion, circulating nucleic acids can be actively released through cell-derived vesicles, such as exosomes and microvesicles, from living cells. The phenomenon of spontaneously released DNA was first described in lymphocytes, frog auricles and cultured cell lines[37-43]. Like nucleosomes, vesicles protect cell-free nucleic acids from the circulating nucleases and hinder the recognition by the immune system[32].

Exosomes: Exosomes are small lipid membrane vesicles (50-100 nm) secreted from various cell types including dendritic cells, B cells, T cells, tumor cells and epithelial cells[44]. Exosomes result from the recycling endosomal pathway. During endocytosis, vesicles are generated at the plasma membrane and enter into the cell forming early endosomes. These early endosomes are transformed into late endosomes which then develop multivesicular bodies (MVB). MVBs can fused with lysosomes for degradation of its content or with the plasma membrane. In this last case, internal vesicles are liberated into the extracellular space and termed exosomes[45] (Figure 3). Therefore, exosomes contain membrane and cytoplasmic components such as lipids, proteins and RNA (mainly mRNA and miRNA). Additionally, the presence of single-stranded and double-stranded DNA was further demonstrated[46].

Furthermore, exosomes are capable to enter in recipient cells by either binding to cell surface receptors through adhesion molecules or being internalized through mechanism similar to endocytosis and so can act as cellular communicators. What is more, these vesicles can travel to distant sites of the organism and release the packed biomolecules into local and remote cells. Exosomes can bear different proteins including transmembrane proteins, such as major histocompatibility complex (MHC), and other intraluminal proteins and oncoproteins such as mutant KRAS[47]. Proteins delivered by exosomes can activate or inhibit different signalling pathways, altering cell function. For its part, exosomes-derived miRNA can modulate gene expression by posttranscriptional regulation.

Particularly in cancer cells, exosomes secretion is usually increased. Tumor-derived (TD) exosomes may favour tumor growth by inhibiting apoptosis and increasing cellular proliferation. As an example, it was demonstrated that exosomes increased cellular proliferation in gastric cancer cell lines by activating Akt phosphorylation[48]. Moreover, it has been described that

TD exosomes can also facilitate cancer invasion and metastasis by regulating stromal cells, remodelling the extracellular matrix and stimulating angiogenesis[47,49].

Regarding to nucleic acids, the presence of mRNAs, miRNAs and DNA highlights the role of exosomes as carriers of genetic information too. Indeed, the role of exosome-derived miRNA has been widely demonstrated. Depending on its target gene, miRNA can act either as a tumor suppressor or as a tumor enhancer. For instance, miR-198 has been demonstrated to be released by T-lymphoblast exosomes performing a tumor suppressor role in lung, liver and colorectal cancer[50-55]. Conversely, other miRNAs favour tumor progression such as miR-21, which can also be secreted through exosomes[56,57]. It should be considered that exosomes generally carry more than one kind of miRNA, so its effects depend on the combination of miRNAs presented[58].

Microvesicles: Microvesicles emerge from plasma membrane budding and the following fission of the vesicles from the plasma membrane. They have a larger size (100-1000 nm) than exosomes and membrane composition is more similar to that of plasma membrane than exosome membrane composition (Figure 3). Thus, tumor-derived microvesicles constitute a representation of the tumor proteomic signature. Microvesicles can be secreted by different cell types including hematopoietic cells, endothelial cells, mesenchymal stem cells and cancer cells[59]. It has to be taken into consideration that, despite their differences, the terms exosomes and microvesicles are usually interchanged. Moreover, in most studies, vesicles are obtained by approaches that cannot discriminate both types of vesicles and so it may be difficult to classify published information according to each type.

As well as exosomes, microvesicles are key elements in cell-to cell communication, modulating the recipient cell phenotype. For instance, it has been shown that cultured hematopoietic progenitor cells can be reprogrammed by microvesicles derived from embryonic stem cells. In fact, these microvesicles contained mRNA for several pluripotent transcription factors demonstrating an additional mechanism of horizontal transfer of genetic material[60].

In cancer scenario, microvesicles from tumor and non-tumor cells can also be secreted to transfer miRNA and other oncogenic proteins to facilitate invasion and tumor growth. Likewise, it was reported that tumor-derived microvesicles carrying surface determinants of tumor cells, like chemokine receptors, and mRNA for growth factors, such as vascular endothelial growth factor (VEGF) or hepatocyte growth factor (HGF), were able to internalize in monocytes and so, change its phenotype and biology activity[61]. Furthermore, it was also published that tumor-associated macrophages can secrete microvesicles containing miRNA that can promote breast tumor cell invasiveness[62].

Virtosomes: The existence of virtosomes was first described by Stroun and Gahan[63]. The virtosome is a macromolecular complex formed by newly synthesized DNA and RNA associated with lipoproteins, which is spontaneously released from living cells. To form this structure, newly DNA is synthesised in the nucleus and then transferred to the cytosol. In cytosol, DNA associates with a lipoprotein, which serves as a protector from nuclease digestion, and before leaving the cell, an RNA molecule is attached to the complex. The complex can exit the cell in an energy-dependent way and entering other cells by mechanism not well understood[63].

Viral nucleic acids: Viral DNA as well as viral RNA can be found in plasma and serum from patients[30]. Given the relation between some viral infections and particular malignancies, detection of viral DNA might be used as a biomarker for certain neoplastic disease. As an example, cell-free DNA from Epstein-Barr virus (EBV) serves as diagnostic and prognostic marker for nasopharyngeal carcinoma[64].

Nucleic acids can be attached to cell surface

cfDNA and RNA can also be found attached to the exterior part of the plasma membrane from where they can be detached and released into circulation. DNA is usually found in the cell surface of leucocytes and erythrocytes and can be internalized by receptor recognition or remain associated with the surface[30].

GATHERING PIECES

Liquid biopsy has been commonly proposed as a tool for cancer diagnostic, characterisation and prognostic in patients as both CTCs and cfNA provide relevant information from the tumor. Nonetheless, very much attention has been paid for this practical application without taking full account of the possible biological roles of cfNA in blood. Although it is known how circulating nucleic acids can be presented in blood (as it has been described), its function in this location is still controversial. Considering the above commented discrepancy between CTCs number and cfDNA quantity as well as the active mechanisms of cfNA release, cfNA presence in blood does not appear to be a mere coincidence. What is more, many evidences point to cfNA as a key driver of metastasis, which is the essence of the theory of genometastasis.

GENOMETASTASIS: A PUTATIVE MECHANISM INVOLVED IN THE ORIGIN OF METASTASIS

Metastasis is an enormously complex process that remains to be a major problem in the management of

cancer. The metastatic properties of tumor cells were extensively investigated from 1970s, although so much earlier (as soon as 1889) it was proposed the "seed an soil" theory that today is still alive and even under constant reformulation (*e.g.,*[65])

During the seventies, some theories were proposed, such as that most primary tumor cells have a low metastatic potential, and that during later stages of tumorigenesis rare cells acquire metastatic capacity through additional somatic mutations (reviewed in[66]). This suggested mechanism had contrary evidence in other studies that concluded that metastases are a random representation of disseminated tumor cells, all of which have the ability to form a metastasis[66]. On the whole, it might be said that the discussion of "dynamic heterogeneity" models *vs* "clonal dominance" theories prevailed during two decades, always under the premise of a circulatory view of cancer progression. In fact, nowadays, many authors appears to not conceive any other way, as showed in the recent literature, *e.g.,* "Metastasis is the consequence of a cancer cell that disperses from the primary tumor, travels throughout the body, and invades and colonizes a distant site"[65].

This view does not explain some questions such as the lack of correlation between the sites of development of metastasis and the anatomic vascular filters[67]. Several million cells per gram of tumor can be shed daily into the lymphatic system or bloodstream. However, insufficient data exists to quantify the fraction of shed tumor cells that successfully seed secondary tissues. Moreover, the fate of blood borne tumor cells is controversial and many experimental evidence are contradictory: Whereas in some models most circulating cells die, in others most survive and extravasate. Nevertheless, all studies show that most cells entering the vasculature fail to form macroscopic foci at distant sites (reviewed in[68]). On the other hand, an unquestionable fact is that the identification and characterization of CTC require extremely sensitive and specific analytical methods, much more than detection of cell-free tumor DNA.

In connection with the circulatory theory, surgical maneuvers for tumor resection (particularly, gastrointestinal tumors) have classically been designed to avoid blood dissemination of cancer cells, which hypothetically results in a lower risk of recurrence and metastasis (reviewed in[69]). However, benefits of such procedures have not been fully demonstrated yet. At late 1990s, our group challenged that technical axiom-not sufficiently supported-, and performed a study in colorectal cancer patients that showed that the use of no-touch isolation techniques in colorectal cancer was not justified, based on lack of evidence indicating the detachment of cells from the tumor at surgery[70]. Apart from the clinical discussion (which has not been finished yet), the fact was that circulation of tumor cells appeared to have lesser value than attributed. In parallel, the evidence of high levels of cell-free nucleic acids in plasma of cancer patients and tumor-bearing animals led us to examine the biological role of such molecules[1,29,71]. Firstly in cancer models using immunocompetent animals and later in clinical studies with colon cancer patients, we demonstrated the biological feasibility of gene transfer and of the transformation of cells by cell-free tumor-derived nucleic acids in the plasma[41,72,73]. In the light of such results, we proposed that cell-free nucleic acids in the plasma participate in tumorigenesis and the development of metastases *via* transfection-like uptake of such nucleic acids by susceptible cells. This putative phenomenon was named as "genometastasis" (Figure 4).

Albeit, at first, some authors exhibited more criticism than enthusiasm for this hypothesis[74], later experimental evidence supported the existence of the genometastasis. Moreover, the assays that substantiated this theory were repeated and enlarged by other authors, who confirmed our results[75-78].

Consistently with our theory, recently Mittra *et al*[79] have asserted that circulating nucleic acids, far from being biologically inert particles, have significant deleterious functions in the host. According with their results, they concluded that circulating nucleic acids are ubiquitous and continuously arising, and freely can enter healthy cells integrate into their genomes, inflicting repeated damage to the somatic DNA. Moreover, the authors have suggested that the somatic genome may not be stable, but rather remains in a state of turmoil characterized by dsDNA breaks, genomic instability and apoptosis affected by integration of circulating DNA. These events may lead to deletions, duplications and rearrangements causing DNA mosaicism[79]. Once demonstrated the existence of this phenomena and connecting all previous results, it would be even naive to think that progression of cancer is not related to triggering genetic events and consequent genomic rearrangements.

Nonetheless, despite the soundness of results, some authors were still showing their reticence to accept the genometastasis as a feasible mechanism for metastasis, arguing mainly that such theory is not able to explain the tropism of metastasis[80]. In our opinion, this is an erroneous assessment perhaps motivated by a partial view of the phenomenon that we described, because, precisely, in both own and other authors' studies, it was shown that not all kind of cells were transformed by plasma[73,75,76]. Our model is, not only incompatible with the idea of specific tropism for metastasis, but it is really proper to search tropism mechanism.

Mittra *et al*[79] clearly demonstrated that cellular/nuclear uptake of DNA is energy dependent and requires an active metabolic machinery of the recipient cells, which might be a first selection. However, it is possible that the key is not only in the characteristics of susceptible cells ("soil"), but also in the particles circulating in the transforming plasma ("seed"). In fact, there is an increasing stream of studies about the potential of extracellular vesicles on induction of cellular transformation and most of those observations are fully

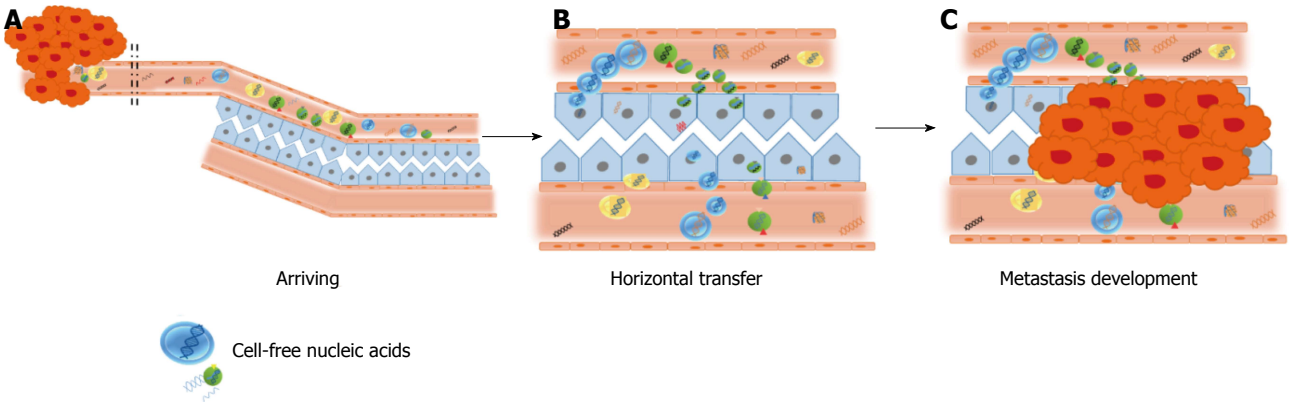

Figure 4 The theory of genometastasis. The putative mechanism of genometastasis: A: Releasing of nucleic acids to the blood stream; B: Transfection of susceptible cells; C: Malignization of transfected cells.

consistent with the theory of genometastasis[81-83].

LOOK TO THE FUTURE: TREATMENTS BASED ON THE GENOMETASTASIS PHENOMENON

Traditionally, treatments directed to prevent metastasis have been based on the use of cytotoxic substances that avoid circulation, homing and reproduction of malignant cells. If we assume that circulating nucleic acids in cancer patients have a role in the production of metastasis, a new scenario can be opened up. We can imagine a variety of strategies for interfering with these circulating nucleic acids either during their travel or during the horizontal transfer at the target organ.

Perhaps, the most immediate approach appears to be the use of enzymes to degrade circulating nucleic acids. The idea of enzymes-based therapies for cancer hovered since four decades ago[84], and in the last years, some convincing approaches have been reported. For example, Trejo-Becerril *et al*[85] have reported that systemic treatment with DNAse I and a protease mix in rats decreased DNA and proteins from serum and had antitumor effects. Interestingly, Patutina *et al*[86] have reported that tumor-bearing animals treated with RNase A and DNase I had a general systemic and immunomodulatory effect that led to a drastic suppression of metastasis development. Undoubtedly, those results support the role of the genometastasis phenomenon in the development of metastasis and encourage deepening.

Other potential therapeutic approach might be based on the use of potentially transfecting particles charged by "good sequences" of nucleic acids. It has not been enough tested but, theoretically, it is possible that such particles promote a "competitive" effect with cell-free tumor nucleic acids and, then, avoiding metastasis. In this line, virtosomes (*i.e.*, the mentioned DNA-RNA-lipoprotein complex) might constitute a useful tool. These particles are spontaneously released from healthy human, other mammalian, avian, amphibian and plant cells in a regulated and energy-dependent manner[63]. Likewise, these released virtosomes have been demonstrated to enter other cells[87-89]. More importantly, the biology of the recipient cells may be also modified if virtosomes come from a different cell type. Experiments with virtosomes in an immunocompetent animal model of colorectal cancer, showed a virtosomal effect in blocking cell multiplication in both *in vitro* and *in vivo* studies, resulting in a scape from inhibition at times after inhibition initiation. These results could indicate the existence of a response derived from the initiation of an immune reaction[90].

In other way, some previous studies have suggested the possibility of silencing these circulating oncogenic signals through RNA interference. As an example, the use of some micro-RNA can determine a novel regulatory pathway in KRAS-driven cancers, which offers a potential therapeutic target for their eradication[91], if this microRNAs are harboured by particles such as virtosomes or exosomes.

Indeed, a lot of strategies can be suggested in order to interfere with the horizontal transfer mechanism, responsible for the transformation of healthy and normal cell into malignant cell. Nonetheless, as happens with all new paradigms, lots of further lines of research are required in this field.

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MINIREVIEWS

Stereotactic radiotherapy for prostate cancer: A review and future directions

Yusef A Syed, Ami K Patel-Yadav, Charlotte Rivers, Anurag K Singh

Yusef A Syed, Department of Radiation Oncology, Winship Cancer Institute of Emory University, Atlanta, GA 30342, United States

Ami K Patel-Yadav, Charlotte Rivers, Department of Radiation Oncology, University at Buffalo, Buffalo, NY 14263, United States

Anurag K Singh, Department of Radiation Oncology, Roswell Park Cancer Institute, Buffalo, NY 14263, United States

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Correspondence to: Dr. Anurag K Singh, Professor, Department of Radiation Oncology, Roswell Park Cancer Institute, Elm and Carlton Streets, Buffalo, NY 14263, United States. anurag.singh@roswellpark.org
Telephone: +1-716-8451180
Fax: +1-716-8457616

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Abstract

Prostate cancer affects over 200000 men annually in the United States alone. The role of conventionally fractionated external beam radiation therapy (RT) is well established as a treatment option for eligible prostate cancer patients; however, the use of stereotactic body radiotherapy (SBRT) in this setting is less well defined. Within the past decade, there have been a number of studies investigating the feasibility of SBRT as a potential treatment option for prostate cancer patients. SBRT has been well studied in other disease sites, and the shortened treatment course would allow for greater convenience for patients. There may also be implications for toxicity as well as disease control. In this review we present a number of prospective and retrospective trials of SBRT in the treatment of prostate cancer. We focus on factors such as biochemical progression-free survival, prostate specific antigen (PSA) response, and toxicity in order to compare SBRT to established treatment modalities. We also discuss future steps that the clinical community can take to further explore this new treatment approach. We conclude that initial studies examining the use of SBRT in the treatment of prostate cancer have demonstrated impressive rates of biochemical recurrence-free survival and PSA response, while maintaining a relatively favorable acute toxicity profile, though long-term follow-up is needed.

Key words: Stereotactic body radiotherapy; Prostate cancer; Radiation therapy; Hypofractionation; Toxicity; Stereotactic ablative radiotherapy

Core tip: Initial studies examining the use of stereotactic body radiotherapy (SBRT) in the treatment of prostate cancer have demonstrated impressive rates of biochemical recurrence-free survival and prostate specific antigen response, while maintaining a relatively favorable acute toxicity profile. Here we review a number of recent

prospective and retrospective studies to evaluate the efficacy and toxicity of SBRT in the treatment of low, intermediate, and high-grade prostate cancer.

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INTRODUCTION

According to the National Cancer Institute's Surveillance, Epidemiology and End Results (SEER) database there were 220800 new cases of prostate cancer diagnosed in 2015 and an estimated 27540 deaths[1]. Since the advent of routine prostate specific antigen (PSA) screening, the majority of cases are confined to the prostate and radiation therapy (RT) is often employed as an alternative to surgical resection. Currently, the National Comprehensive Cancer Network guidelines recommend a combination of observation, radical prostatectomy, conventionally fractionated external beam RT, and androgen deprivation therapy (ADT), depending on stage and risk profile. Stereotactic body radiation therapy (SBRT), which entails five or fewer fractions of at least 5 Gray (Gy), is not currently included in the national guidelines.

A number of studies have evaluated the efficacy of conventionally fractionated external beam RT. With follow up ranging from 5 to 20 years and total doses ranging from 78 to 86 Gy, reported biochemical control was greater than 80% for the favorable risk group compared to approximately 60% for the high risk group. Total dose was also a factor as biochemical control was approximately 60% at lower doses and greater than 80% for higher doses with an estimated overall risk reduction of 40%-50% with respect to biochemical failure[2-5]. This review will examine the evidence for SBRT in comparison to conventional fractionation in the era of modern treatment, and the future direction of SBRT in the treatment of prostate cancer.

Stereotactic radiosurgery has already been applied with great success in other types of cancer, most notably malignancies of the lung and brain (*i.e.,* stereotactic radiosurgery). In the case of lung malignancies, SBRT offers an overall survival benefit as compared with conventionally fractionated RT and offers an alternative when patients are not surgical candidates. Recent work has sought to extend SBRT to prostate cancer with the goal of demonstrating improved outcomes. However, as described above, the threshold for proving non-inferiority is high given excellent results with conventionally fractionated radiation therapy, surgery, or even observation in low risk patients[6].

In this review we present trials of SBRT in the treatment of prostate cancer. Data from these studies are relatively immature with a maximum median follow-up time of 60 mo. Since overall survival at 60 mo or less is expected to be high even in the absence of intervention, we focus on factors such as biochemical recurrence-free survival (bRFS), PSA response, and toxicity. Here we attempt to provide a balanced perspective on the benefits and challenges associated with the use of SBRT in the treatment of prostate cancer.

RESEARCH

Studies included in this review were identified by performing a search of existing literature appearing in the PubMed database, using the keywords "prostate" and "SBRT", which returned a total of 270 results. To qualify for inclusion, treatments must have been delivered in five fractions or fewer, with the exception of one study that employed SBRT as a boost upon conclusion of a conventionally fractionated course. Both prospective and retrospective studies were included. In addition, only those studies that provide detailed results for both PSA response and toxicity were considered for inclusion. Computed tomography and/or magnetic resonance imaging were used for treatment planning in all studies, and treatment positioning was achieved with either daily or real-time imaging. A total of 14 studies met these criteria and are presented here. The remaining 256 published works were excluded for a variety of reasons, including: Insufficient follow-up, lack of toxicity data, or irrelevance to the topics addressed in this review.

SBRT AS A DEFINITIVE THERAPY IN PROSTATE CANCER

SBRT is currently an evolving treatment approach, with no established standard fractionation schedule. There have been a number of single-institution experiences reported with promising results that show local control rates comparable to conventional fractionation, albeit with a much shorter length of follow-up. While hypofractionated radiation therapy has been used in the treatment of prostate cancer since the 1960's, it has historically been undertaken with 2D planning, as described in Lloyd-Davies *et al*[7]. The emergence of advanced technologies, such as intensity modulated radiation therapy (IMRT) and image guided radiation therapy (IGRT), have greatly improved toxicity. However, this review of 209 patients treated with a six-fraction regimen over three weeks established the feasibility of hypofractionation with good local control and an absence of significant morbidity[7].

Among the earliest published studies, Madsen *et al*[8] reported initial findings from their SHARP trial in which forty enrolled patients were treated with five fractions of 6.7 Gy. The authors assumed an alpha/beta ratio of 1.5, similar to other prostate SBRT studies, resulting in a biologically equivalent dose of 78 Gy.

However, the advantage is that hypofractionated dose prescriptions produce an acute effect profile consistent with a significantly lower conventionally fractionated prescription. Enrolled patients were all categorized as low-risk with combined Gleason scores of six or less. All patients achieved a PSA nadir below 2.0 ng/mL and thirteen achieved a nadir below 0.5 ng/mL. There were three biochemical failures resulting in a bRFS rate of 90% at 48 mo. The group also reported an acute toxicity profile comparable to a conventionally fractionated trial conducted at the Cleveland Clinic[9]. The five-year follow-up shows an overall survival of 75% with no prostate cancer related deaths and a resolution of all GU and GI toxicities; however, 50% of the twenty-six patients who were potent at the time of treatment subsequently became impotent[10]. The median PSA nadir was 0.65 ng/mL at a median time of 24 mo.

Building upon past studies utilizing HDR brachytherapy as a monotherapy, King *et al*[11] enrolled 67 low- to favorable intermediate-risk patients in their phase Ⅱ trial. All participants were treated in 5 fractions of 7.25 Gy[12]. They report a four-year bRFS rate of 94% and a median PSA of 0.5 ng/mL at follow-up. There were, however, two biopsy proven failures, but neither of these patients were found to have metastatic disease. Furthermore, patients tolerated the treatment relatively well; there were no grade 3 or higher rectal toxicities, and the grade 3 urinary toxicity rate was 3.5% with no grade 4 urinary toxicities. The toxicity profile compared favorably to past conventionally fractionated dose-escalation and hypofractionated studies. The authors attribute this, in part, to the relatively narrow expansion margins that SBRT affords (in this study, 5 mm overall and 3 mm posteriorly). One unique feature of this trial is that the first twenty-two patients were treated QD (*i.e.,* five consecutive days) while the balance were treated every other day (QOD). Interestingly, the QOD cohort experienced fewer grade 1 to 2 urinary and rectal toxicities, with no change in the rate of grade 3 urinary toxicity.

Boike *et al*[13] conducted a multicenter dose escalation study, enrolling a total of forty-five stage T1-2 patients with Gleason scores of seven or less. Their dose prescriptions were based upon prior nude mouse xenograft studies and radiobiologic modeling of established high dose rate (HDR) brachytherapy[14]. Patients were divided into three cohorts, each of which was treated in 5 fractions of 9, 9.5, or 10 Gy. The study began with the 9 Gy cohort and a ninety day observation period was enforced to evaluate for acute toxicity before the subsequent higher dose cohort was treated. PSA response was favorable in all cohorts with an overall mean nadir of less than 0.4 ng/mL. The authors were particularly focused on evaluating the toxicity associated with this protocol, as comparable preceding studies limited the total dose to 36.25 Gy or less[15,16]. Acute toxicity was generally limited, with only grade 1 or 2 symptoms reported. A limited number of higher-grade late toxicities arose as follows: One case of a grade 4 rectal ulcer, and one case each of grade 3 cystitis and dysuria.

Hannan *et al*[17] report the five-year follow-up results of Boike *et al*[13] and add a phase Ⅱ portion consisting of 47 patients treated to 50 Gy in 5 fractions. This study achieved a remarkable collective five-year bRFS rate of 98.6%, which the authors acknowledge may be overestimated due to their follow-up protocol. This rate exceeded those previously reported by groups that employed other modalities, including: Intensity modulated radiation therapy, hypofractionated radiation therapy, and radical prostatectomy. The majority of acute grade 2 toxicities and all late grade 3 to 4 toxicities occurred in the 50 Gy arm. Three out of a total of four grade 4 toxicity events affected the rectum. Though the stoppage criteria for severe toxicity were not met, the authors ultimately concluded that doses less than 50 Gy are advisable.

Katz *et al*[18] recruited 304 low, intermediate, and high-risk patients. Expansion margins of 5 mm overall and 3 mm posteriorly were employed and patients were treated with five fractions of either 7 or 7.25 Gy. No acute grade 3 or 4 toxicity was reported, and of the 48 patients who reached the twelve-month follow-up at the time of publication, only one late grade 3 toxicity occurred. Quality of life (QOL) was measured using the Expanded Prostate Cancer Index Composite (EPIC) questionnaire. Patients reported an initial decrease in bowel and urinary QOL, but returned to baseline. However, sexual QOL decreased by approximately 10% and remained at that level. By twelve months, 28% of patients achieved a PSA nadir of less than 0.5 ng/mL. A total of four individuals failed biochemically. Long term follow-up revealed a seven-year biochemical disease free survival of 95.6%, 89.3%, and 68.5% for low, intermediate and high-risk cases, respectively[19]. Minimal late toxicity was reported.

Jabbari *et al*[20] treated 20 low- or favorable intermediate-risk patients with four fractions of 9.5 Gy while another 18 intermediate- and high-risk patients were treated with EBRT and ADT combined with an SBRT boost consisting of two fractions of 9.5 Gy. Four patients received an integrated 1 Gy/fraction boost to the dominant intraprostatic lesion. Treatment was planned so as to mimic HDR brachytherapy in terms of dose heterogeneity and outside-of-target sparing. No acute grade 3 or higher toxicity was observed and two patients experienced late grade 3 toxicities. With a median follow-up of 18.3 mo, the median PSA nadir for the monotherapy group was 0.47 ng/mL and 0.10 ng/mL for the combined therapy group. No patients experienced biochemical failure at the time of publication. Though the results are generally favorable, the authors caution that additional accrual and follow-up is needed to ensure durable relapse-free survival. Bolzicco *et al*[21] treated the spectrum of low- to high-risk patients and also stratified PSA response based upon ADT use. The authors note a trend towards lower nadirs with the addition of ADT (median nadir of 0.62

ng/mL *vs* 0.18 ng/mL at 3 years), though statistical significance was not reported. Oliai *et al*[22] undertook a dose escalation trial for low- to high-risk patients and also stratified PSA response by ADT use, reporting a mean PSA nadir that decreased from 0.4 ng/mL to less than 0.1 mg/mL with the addition of ADT.

The Naples and Stanford groups compiled a combined cohort of 41 patients with a median follow-up time of 5 years[23]. The Stanford patients were treated with 5 fractions of 7.25 Gy and the Naples patients were treated with 5 fractions of 7 Gy. The reported five-year biochemical progression free survival rate was 92.7% with a mean PSA nadir of 0.35 ng/mL, though the Stanford subset had a mean nadir of 0.18 ng/mL, significantly lower than entire the cohort average. There were three biopsy proven failures. Treatment was generally well tolerated, though acute toxicities were not explicitly reported. There was one reported case of late grade 3 toxicity and no late grade 4 toxicities.

McBride *et al*[24] reported on 45 patients who received 5 fractions of 7.25 to 7.5 Gy. Biochemical progression free survival at three years was reported as 97.7% and the median PSA nadir at twelve months was 0.91 ng/mL. One late grade 3 urinary obstruction and two late grade 3 proctitis events were noted. There was a statistically significant decrease in the Sexual Health in Men (SHIM) survey score, along with the EPIC bowel and sexual function scores. All three of the reported grade 3 toxicities resolved with corrective intervention.

In 2013, the American Society for Radiation Oncology (ASTRO) released a policy statement supporting the use of SBRT as an appropriate alternative to conventional RT for low- to intermediate-risk disease. This allowed researchers to begin focusing attention on addressing specific technical challenges associated with SBRT. Mantz *et al*[25] tried to control for prostate movement with the implementation of reliable organ tracking techniques to ensure adequate dose localization and to minimize toxicity to surrounding sensitive tissues. Towards this end, they enrolled 102 low-risk patients who were subsequently treated using a proprietary technology, the Calypso® System (Varian Medical Systems, Palo Alto, CA, United States) that uses implanted transponders to track the prostate in real-time during treatment. Patients are then treated on conventional linear accelerators. Other studies used a competing real-time tracking platform, CyberKnife® (Accuray, Inc., Sunnyvale CA), which is comprised of a 6 MV linear accelerator mounted to a robotic arm. Patients received five fractions of 8 Gy, and achieved a mean PSA of 0.27 ng/mL at 24 mo. The toxicity profile was among the best of the prostate SBRT studies with no grade 2 or higher rectal events and only two grade 3 urinary events, both acute. Twelve-month EPIC scores showed a return to near-baseline after an initial decline. These results suggest that real-time tracking may provide a means of reducing toxicity without compromising efficacy.

Recently, efforts have been made to expand the use of SBRT in the treatment of intermediate- and high-risk prostate cancer. Anwar *et al*[26] built upon the previously discussed Katz study, delivering a two-fraction boost of either 9.5 or 10.5 Gy total to 50 patients who had already received a course of conventionally fractionated EBRT to doses of 45-50 Gy. The reported five-year bRFS for all patients was 83%, with a median PSA nadir of 0.05 ng/mL achieved at a median time of 26.2 mo. No grade 3 or higher toxicity was noted. Four cases of disease progression were recorded, all of which occurred outside of the field of radiation. By comparison, a multi-institutional analysis found five-year bRFS rates of 84% and 81% for intermediate- and high-risk patients, respectively[27]. The results of this work compare favorably to HDR boost therapy, suggesting that SBRT boost may be a viable option for intermediate- and high-risk prostate cancer patients. Additionally, this work showed that SBRT resulted in an increased rate of PSA decline as compared to conventionally fractionated EBRT, which is a feature associated with improved clinical outcomes.

Davis *et al*[28] analyzed outcomes for a total of 437 localized prostate cancer patients treated with SBRT at one of seventeen centers in the United States and Australia. Patients were enrolled between 2006 and 2015 and all risk categories were represented. Two-year bRFS was found to be 99.0%, 94.5%, and 89.8% for low, intermediate and high-risk groups, respectively. Higher Gleason score was associated significantly with lower biochemical disease-free survival. Fifteen patients experienced biochemical failure. In general, the SBRT treatments were well tolerated; no patients experienced high-grade genitourinary or gastrointestinal toxicity. The authors corroborated an assertion others had made that SBRT does induce a rapid twelve-month decline in PSA, as observed across multiple studies. A similar pooled analysis by King *et al*[27] that included 1100 patients treated at eight institutions from 2003 to 2013 found collective bRFS rates of 95%, 84% and 81% for low-, intermediate- and high-risk patients, respectively. Patients were treated to a total dose of 35 to 40 Gy over five fractions. Biochemical failure at a median follow-up time of 36 mo was low, at 4.5%, and a subset of these patients were determined to have a PSA bounce that subsequently declined. Interestingly, neither total dose nor the use of ADT had a statistically significant effect on bRFS. The authors conclude that SBRT compares favorably to other definitive treatments and should be considered as an alternative therapy in low- and intermediate-risk prostate cancer. The relative paucity of high-risk patients prevented the authors from extending a similar recommendation to this subset.

DISCUSSION

Initial results and follow-up duration

The use of SBRT for prostate cancer has received considerable attention in recent years and multiple studies have demonstrated short-term outcomes comparable to established therapies. Currently, 8.8% of low-risk patients treated with RT at academic centers are receiving SBRT[29]. Advantages include the potential for

Table 1 Summary of stereotactic body radiotherapy prostate trials and retrospective analyses

Study	No. of patients	Dose	Median follow-up	Biochemical RFS	Overall survival	PSA response	BF[1]	PSA bounce
Madsen (*IJROBP*, 2007)	40	6.7 Gy × 5 Fx	41 mo	90% at 48 mo		18 mo time to nadir	3	"Few"
Pham (*IJROBP*, 2010)	40	6.7 Gy × 5 Fx	60 mo	93% at 60 mo	75% at 60 mo	Median nadir of 0.65 ng/mL at median time of 24 mo		22.50%
Boike (*JCO*, 2011)	15/15/15 (45 tot)	9/9.5/10 Gy × 5 Fx	30/18/12 mo	100% at median follow-up	100% at median follow-up	Mean < 0.4 ng/mL at 12 mo for all cohorts	0	"Multiple"
Katz (*BMC Urol*, 2010)	50/254 (304 tot)	7/7.25 Gy × 5 Fx	30/17 mo	35 Gy: 88% < 1 ng/mL PSA at 30 mo 36.25 Gy: 81% < 1 ng/mL PSA at 24 mo[2]	94%/99% at median follow-up. No deaths due to prostate cancer	28.1% < 0.5 ng/mL at 12 mo	4	37
Jabarri (*IJROBP*, 2012)	20/18 (38 tot)	9.5 Gy × 4/2 Fx	18.1/23.5 mo	100% at median follow-up		Median of 0.35 ng/mL at 18.3 mo	0	
King (*IJROBP*, 2012)	67	7.25 Gy × 5 Fx	2.7 yr	94% at 4 yr		Median of 0.50 ng/mL at follow-up	2	
McBride (*Cancer*, 2012)	34/10/1 (45 tot)	7.5/7.25 Gy × 5 Fx, 1 received "other regimen"	44.5 mo	95.5%/97.5% at 3 yr	97.7% at 3 yr	Median of 0.2 ng/mL at follow-up	0	9
Anwar (*Rad Oncol*, 2016)	24/26 (50 tot)	9.5/10.5 Gy boost in 2 Fx	42.7 mo	95%/95%/90% at 3/4/5 yr		Median nadir of 0.05 ng/mL at median time of 26.2 mo	4	2
Mantz (*Fontiers Rad Oncol*, 2014)	102	8 Gy × 5 Fx	Min. of 5 yr	99% at 6 yr		Mean of 0.27 ng/mL at 24 mo	1	15
Hannan (*Eur J Cancer*, 2016)	92	9/9.5/10 Gy × 5 Fx	54 mo (pooled phase Ⅰ/Ⅱ)	98.6% at 5 yr	94%/89.7% at 3/5 yr	Median of 0.125 ng/mL at 42 mo	1	19
Freeman (*Rad Oncol*, 2011)	41	7-7.25 Gy × 5 Fx	5 yr	93% at 5 yr		Median nadir of 0.3 ng/mL at follow-up	3	
Davis (*Cureus*, 2015)	437	7-7.4 Gy × 5 Fx, 9.5 Gy × 4 Fx, 19.5-29 Gy boost	20 mo	96.1% combined at 2 yr 99.0%/94.5%/89.8% for low/intermediate/high-risk at 2 yr		Median of 0.4 ng/mL at 24 mo	15	35

[1]Biochemical failure; [2]Values reflect only patients who did not receive hormone therapy.

improved therapeutic control and a reduced number of patient visits. However, the lack of long-term toxicity data combined with a relatively small number of patients enrolled in prospective trials prevents SBRT from superseding conventionally fractionated RT at the present time. Clinical results, discussed above and compiled in Tables 1-3, have demonstrated consistently favorable outcomes over the short-term using a variety of SBRT fractionation schedules for definitive and boost treatment. Overall, five fractions of 6.7 to 10.5 Gy per fraction were utilized. With range of follow-up varying from 18 to 60 mo, biochemical recurrence free survival was excellent, as later trials reported rates of greater than 93%.

Conventionally fractionated RT often requires long courses of treatment consisting of eight or more weeks of daily visits. The accelerated schedule that SBRT offers improves the logistic feasibility of treatment. While these initial SBRT reports are encouraging, longer follow up will be required to confirm that bRFS and an acceptably low rate of late toxicity can be maintained over the long term. Most current studies have yet to report data beyond five years and thus are not sufficient to allow for an unequivocal endorsement of SBRT in the treatment of prostate cancer.

Toxicity and dose per fraction

While continued follow-up and additional large-scale prospective studies are needed, certain conclusions can be inferred from the body of existing literature. Firstly, increasing per fraction dose beyond approximately 8 Gy appears to worsen toxicity without offering significantly improved progression-free survival. High-grade toxicity has not been reported in studies with doses between 7 and 8 Gy. Beyond 8 Gy per fraction, reports of both low- and high-grade toxicities increase measurably. Though rectal toxicity and early urinary toxicity are comparable to those seen with conventional fractionation, late urinary toxicity remains a concern[27,30]. The majority of studies evaluated here reported at least one instance of late grade 3 or higher urinary toxicity, often requiring instrumentation or transurethral resection of the prostate (TURP). This flare phenomenon has been found to peak between 12 and 18 mo post-treatment, though symptoms resolve by 24 mo in a majority of cases[31]. However, this trend remains a concern and should be further elucidated prior to large-scale adoption of SBRT

Table 2 Summary of genitourinary toxicities for included stereotactic body radiotherapy trials

Study	Acute			Late			Clinical notes
	Gr. 1	Gr. 2	Gr. ⩾ 3	Gr. 1	Gr. 2	Gr. ⩾ 3	
Madsen (*IJROBP*, 2007)	28%	21.50%	1 tot	25%	20%	0%	Gr. 3 event was urinary obstruction that resolved
Pham (*IJROBP*, 2010)				22.50%	12.50%	2.50%	All toxicities resolved
Boike (*JCO*, 2011)[1]	28.80%	22.20%	0%	13.30%	8.80%	2 tot	Gr. 3 events due to dysuria and cystitis
Katz (*BMC Urol*, 2010)[1]	74.60%	4.60%	0%	4.70%	5.10%	1 tot	
Jabarri (*IJROBP*, 2012)	29%	42%	0%	1 tot	8%	2 tot	One case each of urge incontinence and irritation requiring catheterization
King (*IJROBP*, 2012)[1]	Not reported	Not reported	Not reported	22.80%	5.30%	2 tot	Gr. 3 patients both underwent repeated urologic instrumentation for post-SBRT dysuria
McBride (*Cancer*, 2012)	59%	19%	0%	17%	17%	1 tot	Gr. 3 event was urinary obstruction requiring TURP
Anwar (*Rad Oncol*, 2016)	48%	37%	0%	21%	25%	1 tot	Gr. 3 event was urinary obstruction
Mantz (*Fontiers Rad Oncol*, 2014)	32.3% frequency, 16.6% dysuria, 7.8% retention		2 tot	19.6% frequency, 2.9% dysuria, 4.9% retention		0%	Gr. 3 events were urinary frequency
Hannan (*Eur J Cancer*, 2016)[1]	48.40%	22.00%	0%	24.20%	20.90%	5.50%	1 late Gr. 4 event (cystitis requiring ureteroileal diversion)
Freeman (*Rad Oncol*, 2011)	Not reported	Not reported	Not reported	25%	7%	1 tot	Gr. 3 event after repeated urologic instrumentation
Davis (*Cureus*, 2015)	19%/3%/3%[2]	2%/1%/1%[2]	0%	25%/4%/5%[2]	8%/2%/2%[2]	0%	

[1]Aggregate values for all cohorts; [2]Notation as follows: urinary frequency/urinary retention/cystitis. TURP: Transurethral resection of the prostate.

for low- and intermediate-risk prostate cancer.

Relatively few studies attempt to rigorously evaluate the impact of prostate volume on outcomes, and the overall conclusions are equivocal. A subset includes maximum volume cutoffs in the exclusion criteria, while others simply note the range of organ volumes among those enrolled. Chen *et al*[30] prospectively collected quality of life data for 204 prostate cancer patients treated with SBRT, with median follow up time of 3.9 years. Patients were treated to a dose of 35-36.25 Gy in 5 fractions. At 3 years post SBRT, EPIC-UI (Urinary Incontinence) score declined significantly; however, this was of borderline clinical significance. Notably, prostate volume was associated with UI score. Similarly, a second study evaluated 515 patients treated with SBRT to a dose of 35-36.25 Gy in 5 fractions. Of 336 patients with available prostate volumes, there was a higher incidence of grade 2 and 3 urinary toxicity with prostate volumes greater than 60 cc that trended towards statistical significance[19]. Conversely, a third study evaluated 216 patients treated with 35-36.25 Gy in 5 fractions, and found no correlation between urinary symptoms and prostate volume at the 2 year mark[31]. It is important to note that the mean prostate volumes for the first two studies were 39 cc and 65.3 cc, respectively; median prostate volume for the third study was 38 cc. For men with prostate volumes greater than 50 cc, Janowski *et al*[32] conducted a retrospective review of 57 patients with a median prostate volume of 62.9 cc (range 50-138.7cc). All patients were treated to 35-36.25 Gy in 5 fractions, and followed for a median of 2.9 years. The rate of grade 3 urinary toxicities was low, occurring in two patients. As there is limited data regarding toxicity with SBRT in the setting of larger volume prostates (> 100 cc), caution should be used when treating these patients.

Similar to large prostate volume, prior TURP may predict for worse toxicity, although large-scale data are unavailable. Bolzicco *et al*[21] prospectively accrued 100 patients for treatment with SBRT, to a dose of 35 Gy in 5 fractions. Of seven patients with prior TURP, three had late urinary toxicities (1% Grade 1, 1% Grade 2, 1% Grade 3). Also of note, there was only one patient with Grade 3 late urinary toxicity, and this patient had undergone urologic tests including cystoscopy and urethral dilatation. Similarly, Chen *et al*[33] report a single case of Grade 3 late urinary toxicity, in a patient with a large prostate and two prior TURP procedures.

Real-time tracking of the prostate

The role of improved technology cannot be overstated. Though rigorous evaluations of prostate movement are limited, it is commonly accepted that translation of 5 mm or more during a single treatment session is likely[34]. Real-time tracking of the prostate has the potential to markedly improve dose delivery to tumor tissue and minimize the exposure of surrounding non-involved structures. The majority of studies presented here made use of either CyberKnife or Calypso, and while there are no marked differences in toxicity, the prevailing sentiment among authors strongly favors real-time tracking. Furthermore, catheterization during treatment simulation improves urethral contour accuracy and may be advisable.

Table 3 Summary of gastrointestinal toxicities for included stereotactic body radiotherapy prostate trials

Study	Acute			Late			Clinical notes
	Gr. 1	Gr. 2	Gr. ⩾ 3	Gr. 1	Gr. 2	Gr. ⩾ 3	
Madsen (*IJROBP*, 2007)	26%	13%	0%	30%	7.50%	0%	Gr. 2 events were proctitis
Pham (*IJROBP*, 2010)				22.50%	7.50%	0.00%	All toxicities resolved
Boike (*JCO*, 2011)[1]	33%	22.50%	0%	22.20%	2 tot	1 tot	Gr. 4 event due to rectal ulcer
Katz (*BMC Urol*, 2010)[1]	74.90%	3.60%	0%	5.10%	2.30%	0%	
Jabarri (*IJROBP*, 2012)	21%	11%	0%	2 tot	1 tot	0%	
King (*IJROBP*, 2012)[1]	Not reported	Not reported	Not reported	14.00%	1 tot	0%	
McBride (*Cancer*, 2012)	31%	7%	0%	7%	7%	2 tot	Gr. 3 events were proctitis requiring ablation
Anwar (*Rad Oncol*, 2016)	42%	10%	0%	12.50%	0%	0%	
Mantz (*Fontiers Rad Oncol*, 2014)	0%	0%	0%	3 tot	0%	0%	Toxicity was rectal bleeding
Hannan (*Eur J Cancer*, 2016)[1]	37.40%	20.90%	2 tot	25.30%	13.20%	6.60%	1 acute and 2 late Gr. 4 events (one rectal bleed)
Freeman (*Rad Oncol*, 2011)	Not reported	Not reported	Not reported	13%	1 tot	0%	
Davis (*Cureus*, 2015)	4%/1%/1%[2]	1%/0%/0%[2]	0%	4%/3%/3%[2]	0%	0%	

[1]Aggregate values for all cohorts; [2]Notation as follows: Diarrhea/constipation/proctitis.

Future directions

Ongoing clinical trials seek to address some of the concerns discussed above. The SMART trial, initiated in 2009, is a phase Ⅱ study for stage T1-T2c prostate cancer using Calypso for real-time tracking and IMRT plan reoptimization. Patients are treated to 37 Gy in five fractions with a primary endpoint of urinary and gastrointestinal toxicity at 3 years, placing the focus on late complications. Enrollment has closed, though no results have been published to date. RTOG 0938 is a phase Ⅱ trial comparing 36.25 Gy delivered in 5 fractions to 51.6 Gy delivered in 12 fractions. This work builds upon RTOG 0415, an equivalence study comparing 70 Gy in 28 to a conventionally fractionated course of 73.8 Gy in 41 fractions. RTOG 0938 includes patients with T1-2a disease and mandates the use of intrafraction motion tracking. The primary endpoint is QOL at 1 year post-treatment, assessed by EPIC score. Again, the importance of toxicity is highlighted.

To date, the field has emphasized the role of SBRT in treating early stage, low- to intermediate-risk disease. A subset of studies presented here included high-risk patients and reported favorable results. Katz *et al*[18] noted a 7-year bRFS of 68.5% for high-risk cases while Anwar *et al*[35] reported 81% bRFS at 5 years. Additionally, Oliai *et al* report a 3-year freedom from biochemical failure of 77.1% for their high-risk cohort. These results, among others, suggest that SBRT may offer improved biochemical control as compared with conventionally fractionated RT and should be explored further in this context.

CONCLUSION

Initial studies examining the use of SBRT in the treatment of prostate cancer have demonstrated impressive rates of biochemical recurrence-free survival and PSA response, while maintaining a relatively favorable acute toxicity profile. Doses of 8 Gy or less per fraction have lower reported rates of toxicity with similar biochemical control rates compared to higher doses per fraction. Though we are cautiously optimistic that SBRT has the potential to serve as an alternative to conventionally fractionated RT in the treatment of prostate cancer, long-term follow-up is needed in order to evaluate whether biochemical control, overall survival, and late toxicity are maintained, or improved, as compared to the current standard of care.

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ORIGINAL ARTICLE

Basic Study

Characteristics of *Clostridium difficile* infection in patients hospitalized with myelodysplastic syndrome or acute myelogenous leukemia

Kamini Shah, Bryan F Curtin, Christopher Chu, Daniel Hwang, Mark H Flasar, Erik von Rosenvinge

Kamini Shah, Division of Infectious Diseases, Department of Medicine, University of Maryland School of Medicine, Baltimore, MD 21201, United States

Bryan F Curtin, Digestive Diseases Branch, National Institute of Diabetes and Digestive and Kidney Diseases, Bethesda, MD 21201, United States

Christopher Chu, Daniel Hwang, Department of Medicine, University of Maryland School of Medicine, Baltimore 21201, MD, United States

Mark H Flasar, Erik von Rosenvinge, Division of Gastroenterology and Hepatology, Department of Medicine, University of Maryland School of Medicine and VA Maryland Health Care System, Baltimore, MD 21201, United States

Author contributions: Shah K and Curtin BF collected data and wrote the manuscript; Chu C and Hwang D collected data; Flasar MH analyzed and interpreted the data; von Rosenvinge E supervised the study; all authors read and approved the final manuscript.

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Correspondence to: Erik von Rosenvinge, MD, Division of Gastroenterology and Hepatology, Department of Medicine, University of Maryland School of Medicine and VA Maryland Health Care System, Baltimore, MD 21201, United States. evonrose@medicine.umaryland.edu
Telephone: +1-410-6057000
Fax: +1-410-3288315

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Abstract

AIM

To evaluate factors associated with *Clostridium difficile* infection (CDI) and outcomes of CDI in the myelodysplastic syndrome (MDS) and acute myeloid leukemia (AML) population.

METHODS

After IRB approval, all MDS/AML patients hospitalized at the University of Maryland Greenebaum Comprehensive Cancer Center between August 2011 and December 2013 were identified. Medical charts were reviewed for demographics, clinical information, development of CDI, complications of CDI, and mortality. Patients with CDI, defined as having a positive stool PCR done for clinical suspicion of CDI, were compared to those without CDI in order to identify predictors of disease. A *t*-test was used for comparison of continuous variables and chi-square or Fisher's exact tests were used for categorical

variables, as appropriate.

RESULTS

Two hundred and twenty-three patients (60.1% male, mean age 61.3 years, 13% MDS, 87% AML) had 594 unique hospitalizations during the study period. Thirty-four patients (15.2%) were diagnosed with CDI. Factors significantly associated with CDI included lower albumin at time of hospitalization ($P < 0.0001$), prior diagnosis of CDI ($P < 0.0001$), receipt of cytarabine-based chemotherapy ($P = 0.015$), total days of neutropenia ($P = 0.014$), and total days of hospitalization ($P = 0.005$). Gender ($P = 0.10$), age ($P = 0.77$), proton-pump inhibitor use ($P = 0.73$), receipt of antibiotics ($P = 0.66$), and receipt of DNA hypomethylating agent-based chemotherapy ($P = 0.92$) were not significantly associated with CDI.

CONCLUSION

CDI is common in the MDS/AML population. Factors significantly associated with CDI in this population include low albumin, prior CDI, use of cytarabine-based chemotherapy, and prolonged neutropenia. In this study, we have identified a subset of patients in which prophylaxis studies could be targeted.

Key words: *Clostridium difficile*; Acute myeloid leukemia; Cytarabine-based chemotherapy; Myelodysplastic syndrome; Neutropenia

Core tip: This study evaluates factors associated with the development and outcomes of *Clostridium difficile* infection (CDI) in patients with Myelodysplastic syndrome (MDS) or acute myelogenous leukemia (AML). Our findings demonstrate a high incidence of CDI with 15.2% of patients diagnosed with CDI during the 28-mo study period. Risk factors associated with the development of CDI include low albumin, prior history of CDI, chemotherapy within 30 d of hospitalization, cytarabine-based chemotherapy within 30 d of hospitalization, and increased duration of neutropenia and hospitalization.

Shah K, Curtin BF, Chu C, Hwang D, Flasar MH, von Rosenvinge E. Characteristics of *Clostridium difficile* infection in patients hospitalized with myelodysplastic syndrome or acute myelogenous leukemia. *World J Clin Oncol* 2017; 8(5): 398-404 Available from: URL: http://www.wjgnet.com/2218-4333/full/v8/i5/398.htm DOI: http://dx.doi.org/10.5306/wjco.v8.i5.398

INTRODUCTION

Clostridium difficile is a gram-positive, spore-forming, anaerobic bacterium that is the major cause of nosocomial diarrhea in the developed world. Over the last two decades the rate, morbidity, mortality, and costs of *C. difficile* infection (CDI) have risen dramatically[1]. Data from the United States Centers for Disease Control and Prevention show that the discharge diagnosis rate of CDI doubled from the 1990's into the 2000's[2,3]. CDI rates have increased considerably since that time, with a current estimate of almost half a million cases and 29000 deaths per year occurring in the United States alone[1]. This increase has not only been observed in hospitalized, elderly, and immunocompromised patients, but also in younger adults without significant comorbidities[4]. Patients who develop CDI have significant increases to their length of hospitalization[5]. According to a recent systematic review, attributable mean CDI costs range from $8911 to $30049 for hospitalized patients[6]. The sheer burden of CDI necessitates a search for more effective means of preventing and combating this infection.

Current statistics indicate that approximately 53000 new cases of leukemia will be diagnosed in the United States this year, 20000 of which will be acute myeloid leukemia (AML)[7]. Patients receiving treatment for myelodysplastic syndrome (MDS) or AML are at increased risk for developing CDI given their frequent neutropenic episodes, as well as exposure to antibiotics and chemotherapy[8]. Antineoplastic agents have antimicrobial properties, and numerous chemotherapeutic drugs have been associated with the development of CDI, including cisplatin, etoposide, bleomycin, paclitaxel, vinblastine, 5-fluorouracil, cyclophosphamide, methotrexate, doxorubicin, and cytarabine-based regimens[8]. Several risk factors for CDI in leukemia patients have been recently identified, which include receipt of chemotherapy, age > 65 years, admission at a teaching hospital, increased length of stay, diagnosis of acute rather than chronic leukemia, sepsis, and neutropenia[9,10].

The aim of this study is to evaluate factors associated with CDI and outcomes of CDI in the MDS and AML population. Outcomes of interest include mortality and severe morbidity such as Intensive Care Unit (ICU) admission, need for surgical intervention, or recurrence of CDI.

MATERIALS AND METHODS

The Institutional Review Board of the University of Maryland, Baltimore, approved this study and waived the requirement for informed consent (IRB# HP-00058296). All patients with a diagnosis of MDS or AML were identified through an electronic medical record database utilized by the University of Maryland Medical Center (UMMC). Inclusion criteria were: Age greater than or equal to 18 years, a diagnosis of MDS or AML, and hospitalization at the UMMC Greenebaum Comprehensive Cancer Center between August 2011 and December 2013. Charts were reviewed for demographics, clinical information, development of CDI, complications of CDI, and mortality. The starting point of data collection was identified as August 2011, when UMMC began to utilize the illumigene® *C. difficile* DNA amplification assay (Meridian Bioscience, Inc.). The assay uses loop-

Table 1 Characteristics of Patients with *C. difficile* infection *n* (%)

Variable (per patient)	CDI (*n* = 34)
Diagnosis	
AML	31 (91)
MDS	3 (9)
Gender	
Male	15 (44.1)
Female	19 (55.9)
PPI therapy[1]	22 (64.7)
Prior history of CDI	5 (14.8)
Receipt of chemotherapy[2]	31 (91)
Type of chemotherapy	
Cytarabine-based chemotherapy	21 (61.7)
DNA hypomethylating agent-based chemotherapy	11 (32.3)
Death/referral to hospice	8 (23.5)
Severity of CDI[3]	
Mild-moderate	29 (85.2)
Severe	4 (11.7)
Severe-complicated	1 (2.9)
Total number of CDI episodes during hospitalization	
1	31 (91)
2	3 (9)
Recurrence of CDI	4 (11.7)
ICU admission	8 (23.5)
Bowel perforation	0
Need for surgical intervention	1 (3)

[1]PPI therapy defined as use of PPI documented at the time of hospital admission; [2]Receipt of chemotherapy defined as being given within 30 d of hospital admission; [3]Severity as defined by the SHEA/IDSA Guidelines[12]. CDI: Clostridium difficile infection; AML: Acute myeloid leukemia; MDS: Myelodysplastic syndrome; PPI: Proton-pump inhibitor; ICU: Intensive care unit.

mediated isothermal DNA amplification to detect the tcdA 5′ region present in all toxigenic *C. difficile,* and has a sensitivity and specificity of 95.2% and 95.3%, respectively[11]. Our facility currently does not implement a two-step detection method for CDI.

Demographics and clinical data were recorded per patient encounter and included: Documented diagnosis of MDS or AML, age at diagnosis, gender, proton pump inhibitor (PPI) use during hospitalization or the within 30 d prior to hospitalization, any prior documented history of CDI, type of chemotherapy received during hospitalization or within 30 d prior to hospitalization, antibiotic use during hospitalization or within 30 d prior to hospitalization, total length of stay in days, albumin level at admission, duration of neutropenia during hospitalization, current episode of CDI as a recurrence, and documentation of death or referral to hospice. Data collected included factors previously associated with CDI and focused on investigating the primary aim of our study as described above. CDI was defined as a positive stool *C. difficile* test done in the setting of diarrhea, defined as the passage of 3 or more unformed stools in 24 or fewer consecutive hours[12]. Our laboratory policy does not permit *C. difficile* testing on formed stool, thus we are reasonably confident all patients had diarrhea. Recurrence of CDI was defined as CDI in the setting of a positive *C. difficile* stool assay as well as receipt of CDI treatment in the 8 wk prior to the current episode. Severity of CDI was determined based on the criteria set forth by the Society for Healthcare Epidemiology of America (SHEA) and Infectious Diseases Society of America (IDSA) guidelines[12]. Chemotherapeutic regimens were defined as cytarabine-based, DNA hypomethylating agent-based, or other regimens. Neutropenia was defined as an absolute neutrophil count of 500 cells/μL or less.

MDS and AML patients with CDI were compared to those patients that were not diagnosed with CDI in order to identify factors related to disease. A *t*-test was used for comparison of continuous variables and χ^2 or Fisher's exact tests were used for categorical variables, as appropriate (SAS, version 9.2). Statistical significance was defined as $P < 0.05$. As some patients were hospitalized multiple times, data analysis was performed on variables per hospital encounter. Total days of neutropenia as well as total days of hospitalization during the study period were analyzed per patient. A biomedical statistician performed the statistical review.

RESULTS

We identified 223 patients with MDS or AML that had 594 unique hospitalizations between August 2011 and December 2013. Sixty point one percent of the patients were male, the mean age was 61.3 years, 87% had AML, and 13% had MDS. Thirty-four of the patients (15.2%) were diagnosed with CDI during the study period. Of these, 44% were male, the mean age was 59.2 years, 91% had AML, 9% had MDS, and 35% were on a PPI at time of admission. Sixty point seven percent received cytarabine-based chemotherapy, and 32.3% received DNA hypomethylating agent-based chemotherapy. None of the patients with MDS who developed CDI received cytarabine-based chemotherapy. Eighty-five percent received antibiotics during hospitalization or within the 30 d prior to hospitalization. Twelve percent had recurrent CDI, eight required intensive care unit admission, and one underwent colectomy for CDI. According to the classification criteria set forth by the SHEA/IDSA guidelines, 85.2% had mild-moderate disease, 11.7% had severe disease, and 2.9% had severe-complicated disease[12]. Twenty-three point five percent of these patients died or were referred to hospice (Table 1).

Several factors were significantly associated with CDI when analyzed by hospital encounter (Table 2), including a lower albumin at the time of hospitalization (mean 2.8 g/dL in the CDI group *vs* 3.5 g/dL in the non-CDI group, $P < 0.0001$), prior history of CDI ($P < 0.0001$), receipt of any chemotherapy in within 30 d of hospitalization (92.1% in the CDI group *vs* 78.8% in the non-CDI group, $P = 0.048$), and receipt of cytarabine-based chemotherapy within 30 d of hospitalization (63.4% in the CDI group *vs* 45.5% in the non-CDI group, $P = 0.015$).

As some factors did not lend themselves to a per-hospital encounter analysis, we performed a per patient

Table 2 Comparison of myelodysplastic syndrome and acute myeloid leukemia patients with and without *Clostridium difficile* infection *n* (%)

Variable	No CDI (*n* = 556)	CDI (*n* = 38)	Significance (*P* value)	No CDI (*n* = 189)	CDI (*n* = 34)	Significance (*P* value)
Per encounter analysis						
Age on admission, mean (95%CI)	58.4 (57.0-59.8)	59.2 (54.6-63.9)	0.77			
Albumin level (g/dL) on admission, mean (95%CI)	3.5 (3.4-3.5)	2.8 (2.6-3.1)	< 0.0001			
AML (*vs* MDS) diagnosis	506 (91.0)	35 (92.1)	0.82			
Male gender	338 (60.8)	18 (47.4)	0.1			
Female gender	218 (39.2)	20 (52.63)	0.1			
Use of PPI therapy[1]	206 (37.0)	13 (34.21)	0.73			
Prior history of CDI	15 (2.7)	10 (23.32)	< 0.0001			
Antibiotic use	465 (84)	31 (83)	0.66			
Any chemotherapy[2]	438 (78.8)	35 (92.1)	0.048			
Cytarabine-based chemotherapy[2]	253 (45.5)	25 (65.79)	0.015			
DNA hypomethylating agent-based chemotherapy[2] (*n*)	165 (29.7)	11 (29.0)	0.92			
Other chemotherapy[2]	25 (4.5)	0 (0)	0.18			
Death or referral to hospice	82 (14.8)	8 (21.05)	0.29			
Per patient analysis						
Total days of neutropenia during study period				13.7 (11.3-16.0)	21.6 (14.5-28.7)	0.014
Total days of hospitalization during study period				22.7 (19.9-25.5)	40.8 (28.9-52.7)	< 0.0001

[1]PPI therapy defined as use of PPI documented at the time of hospital admission; [2]Receipt of chemotherapy defined as being given within 30 d of hospital admission. CDI: *Clostridium difficile* infection; MDS: Myelodysplastic syndrome.

analysis (Table 2) for total days of neutropenia during the study period (mean 21.6 d in the CDI group *vs* 13.7 d in the non-CDI group, $P = 0.014$), and total days of hospitalization during the study period (mean 40.8 d in the CDI group *vs* 22.7 d in the non-CDI group, $P = 0.005$).

DISCUSSION

Our findings demonstrate a high incidence of CDI in our MDS and AML population with 15.2% of patients diagnosed with CDI during the 28-mo study period. This is comparable to previous reports. In a retrospective study of AML patients receiving chemotherapy, the incidence of CDI was 18%[13]. In another similar study, the incidence was 12%[14]. Within this overall high-risk group, we identified several factors associated with CDI. Specifically, CDI is significantly associated with low albumin level at time of hospitalization, prior history of CDI, receipt of any chemotherapy within 30 d of hospitalization, receipt of cytarabine-based chemotherapy within 30 d of hospitalization, total length of neutropenia and total length of hospitalization.

Similar to previously published findings, we found a higher rate of CDI in women, though this result was not statistically significant ($P = 0.10$)[1]. While other studies have identified associations between age and PPI use and risk for CDI[13], age ($P = 0.77$) and PPI use ($P = 0.73$) were not associated with CDI in our population. In addition, use of DNA hypomethylating agent-based chemotherapy ($P = 0.92$) was not associated with CDI. No differences in mortality or referral to hospice rates during the study period were identified between CDI and non-CDI groups ($P = 0.29$). It is well established that the greater the antibiotic exposure, the greater the risk of CDI[12,13]. However, infections during a neutropenic state are associated with high mortality rates, and thus antibiotic prophylaxis is indicated in patients with high-risk neutropenia per American Society of Clinical Oncology guidelines[15]. Fluoroquinolones are generally the agents of choice in these situations. The emergence of the NAP1/BI/027 hypervirulent strain is associated with an increased incidence of CDI over the past 15 years[16]. Fluoroquinolone resistance characterizes the NAP1/BI/027 strain[16], which may be one reason for increased risk of CDI in this population. In our study, 85% of patients received antibiotic therapy. Interestingly, antibiotic usage was not a significantly associated with CDI ($P = 0.66$). This likely reflects insufficient power to detect a difference given the high rate of antibiotic use in this population. Previous studies examining strategies to improve antibiotic prescribing practices of providers have shown mixed results in the reduction of CDI incidence[17]. However, reducing the duration and potency of antibiotics used, particularly after initial presentation of CDI, would be an interesting area of study for the MDS and AML population.

Consistent with our findings, low albumin levels have previously been established as a risk factor for the development of CDI[18]. A low albumin level may indicate poor baseline health status, malnutrition, or the presence of other comorbidities such as cirrhosis or nephrotic syndrome, all which may increase susceptibility to CDI[19,20]. Also, low albumin may be found in cases of diarrhea and loss of protein due to mucositis/enterocolitis in patients after chemotherapy for AML. A prior history of CDI was associated with CDI, as demonstrated in previous studies[21]. Prior history of CDI may predispose a patient to future episodes of CDI due to patient colonization, environmental contamination, or the presence of persistent risk factors.

The majority of patients in our study had a confir-

med diagnosis of AML and received treatment with cytarabine-based chemotherapy. Based on our findings, any chemotherapy, and cytarabine-based therapy in particular, was associated with development of CDI. This may be related to neutropenia, as total days of neutropenia was also significantly increased in patients that developed CDI. Chemotherapeutics are also known to disrupt enteric bacterial populations and the resulting dysbiosis may predispose to CDI[8]. While there is a paucity of data on the effect of different chemotherapeutic regimens on the gastrointestinal microbiome, cytotoxic changes may create a favorable environment for the proliferation of *C. difficile*. Microbial data suggests that chemotherapeutics may select for colonization of *C. difficile* and *Enterococcus faecium*[22].

We evaluated several factors that did not prove to be significantly associated with CDI in our MDS and AML population, including age, gender, PPI use, use of DNA hypomethylating agent-based chemotherapy, and antibiotic use. In theory, PPI therapy may increase the risk of CDI by increasing the ability of *C. difficile* spores to survive in the lumen of the gastrointestinal tract. While there has been controversy regarding their significance, a meta-analysis demonstrated a significant association between PPI use and risk of developing CDI (OR = 1.74, 95%CI: 1.47-2.85)[23]. Within the same study there appears to be increased risk with concomitant use of antibiotics and PPIs, and increased risk of recurrence with PPI use[23]. Non-cytarabine based chemotherapy, which in the case of our study was primarily DNA-hypomethylating agents, was not associated with CDI. We hypothesize that cytarabine-based agents are generally more caustic and induce a greater period of neutropenia, thus providing a more favorable environment for CDI in comparison to less cytotoxic agents.

We believe that our findings will inform future CDI prophylaxis studies in the high-risk MDS and AML population. We have identified a subset of this population, namely those with low albumin, prior CDI, or receipt of cytarabine-based chemotherapy, who can be identified at time of hospital admission as being especially high-risk for CDI. Recently, metronidazole prophylaxis has been proposed as a possible strategy for CDI prevention, however data specifically looking at patients with malignancies has not been supportive of prophylactic antibiotic treatment to prevent CDI[24,25]. In addition, the anti-toxin monoclonal antibody bezlotoxumab was recently approved by the FDA, and a toxoid vaccine in phase Ⅲ clinical study is likely to be available soon[26,27]. Studies of these agents for CDI prophylaxis in our high-risk patient population are warranted.

Our study is not without limitations. Our study is retrospective and took place in a single tertiary medical center. We included primarily AML patients with a high degree of medical complexity, and our findings may not be generalizable to other populations. Additionally, many of the patients had prolonged hospital stays or numerous admissions throughout the testing period, and our study design was ill-equipped to evaluate temporal relationships between chemotherapy and CDI onset. Another limitation is our inability to analyze the degree in which antibiotics predict development of CDI in this population. While antibiotic usage was not found to be associated with CDI in our study, the widespread use of antibiotics makes this difficult to assess. While antibiotic exposure is not necessary for the development of CDI, it is likely to contribute to our population's overall CDI risk[10].

In conclusion, CDI is common in our MDS/AML population. Factors significantly associated with CDI include low albumin, prior history of CDI, use of cytarabine-based chemotherapy, and prolonged neutropenia. Length of hospitalization is also associated with CDI; however, this is likely both a cause and effect of CDI. Prophylactic strategies to lower the burden of CDI in MDS/AML patients are needed. In this study, we have identified a subset of this high-risk population in which prophylaxis studies could be targeted. These findings are novel and increase our understanding of CDI in this patient population as well as open new frontiers of research.

COMMENTS

Background

Acute myelogenous leukemia (AML) and Myelodysplastic syndrome (MDS) are blood borne malignancies that require strong treatments with heavy doses of chemotherapy, which leaves these patient's susceptible to opportunistic infections. *Clostridium difficile* infection (CDI) remains a major cause of nosocomial diarrhea and is of significant importance to the immunosuppressed population, such as those receiving chemotherapies for AML and MDS.

Research frontiers

CDI has been recognized as a major contributor of increased morbidity and mortality in hospitalized patients. New treatment regimens, such as vaccinations, immunotherapy, and fecal transplantation are currently undergoing evaluation. It is essential to identify certain susceptible populations in which targeted therapy for CDI can be investigated. Patients with AML and MDS are particularly susceptible to CDI and further characterization of CDI in this population is warranted.

Innovations and breakthroughs

The authors have found that CDI is common in this specific patient populations. Factors significantly associated with CDI in this population include low albumin, prior CDI, use of cytarabine-based chemotherapy, and prolonged neutropenia. The authors have identified a subset of patients in which prophylaxis studies could be targeted

Applications

By identifying and characterizing CDI within this specific patient population, the authors have identified a cohort of patients that would benefit from future novel CDI therapies and possible CDI prophylaxis. The authors have also identified risk factors that would enable providers to recognize patients that are particularly susceptible for identifying CDI and adjusting their management accordingly.

Terminology

CDI was defined as a positive stool *C. difficile* test done in the setting of diarrhea, defined as the passage of 3 or more unformed stools in 24 or fewer consecutive hours. Recurrence of CDI was defined as CDI in the setting of a positive *C. difficile* stool assay as well as receipt of CDI treatment in the 8 wk prior to the current episode. Severity of CDI was determined based on the

criteria set forth by the Society for Healthcare Epidemiology of America (SHEA) and Infectious Diseases Society of America guidelines. Chemotherapeutic regimens were defined as cytarabine-based, DNA hypomethylating agent-based, or other regimens. Neutropenia was defined as an absolute neutrophil count of 500 cells/μL or less.

Peer-review

The authors have shown that CDI is common in the MDS/AML population. Factors significantly associated with CDI in this population include low albumin, prior CDI, use of cytarabine-based chemotherapy, and prolonged neutropenia. The findings are worthy of sharing with the scientific community.

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ORIGINAL ARTICLE

Retrospective Cohort Study

Factors influencing response to ingenol mebutate therapy for actinic keratosis of face and scalp

Nevena Skroza, Ilaria Proietti, Nicoletta Bernardini, Veronica Balduzzi, Alessandra Mambrin, Anna Marchesiello, Ersilia Tolino, Sara Zuber, Giuseppe La Torre, Concetta Potenza

Nevena Skroza, Ilaria Proietti, Nicoletta Bernardini, Veronica Balduzzi, Alessandra Mambrin, Anna Marchesiello, Ersilia Tolino, Sara Zuber, Concetta Potenza, Dermatology Unit "Daniele Innocenzi", Department of Medical and Surgical Sciences and Biotechnologies, Sapienza University of Rome, 04019 Terracina, Italy

Giuseppe La Torre, Department of Public Health and Infectious Diseases, Sapienza University of Rome, 04019 Terracina, Italy

Author contributions: Skroza N, Proietti I, Bernardini N and Potenza C designed the research; Balduzzi V, Mambrin A, Marchesiello A, Tolino E and Zuber S performed the research; La Torre G analyzed the data.

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Correspondence to: Nevena Skroza, MD, Dermatology Unit "Daniele Innocenzi", Department of Medical and Surgical Sciences and Biotechnologies, Sapienza University of Rome, Via Firenze snc Polo Pontino, 04019 Terracina, Italy. nevena.skroza@uniroma1.it
Telephone: +39-773-708811
Fax: +39-773-708399

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Abstract

AIM

To determine factors independently influencing response to ingenol mebutate therapy and assess efficacy on clinical setting of non-hypertrophic non-hyperkeratotic actinic keratosis (AK).

METHODS

Consecutive patients affected by non-hypertrophic non-hyperkeratotic AKs of the face or scalp were enrolled to receive ingenol mebutate 0.015% gel on a selected skin area of 25 cm^2 for 3 consecutive days. Local skin reactions were calculated at each follow up visit using a validated composite score. Efficacy was evaluated by the comparison of clinical and dermoscopic pictures before the treatment and at day 57, and classified as complete, partial and poor response.

RESULTS

A number of 130 patients were enrolled, of which 101 (77.7%) were treated on the face, while 29 (22.3%) on the scalp. The great majority of our study population (n = 119, 91.5%) reached at least a 75% clearance of AKs and, in particular, 58 patients (44.6%) achieved a complete response while 61 (46.9%) a partial one.

Logistic backward multivariate analysis showed that facial localization, level of local skin reaction (LSR) at day 2, the highest LSR values and level of crusts at day 8 were factors independently associated with the achievement of a complete response.

CONCLUSION

Ingenol mebutate 0.015% gel, when properly applied, is more effective on the face than on the scalp and efficacy is directly associated to LSR score.

Key words: Ingenol mebutate; Actinic keratosis; Facial and scalp lesions; Skin reactions; Dermoscopic feature

Core tip: Ingenol mebutate 0.015% gel is an effective treatment for non-hypertrophic non-hyperkeratotic actinic keratosis of face and scalp. Facial lesions are more prone to achieve a complete response to this therapy than those located on the scalp. Facial localization and the highest levels of local skin reaction, in particular the amount of crusting, are predictive for complete response to ingenol mebutate 0.015% gel therapy in a real clinical setting.

Skroza N, Proietti I, Bernardini N, Balduzzi V, Mambrin A, Marchesiello A, Tolino E, Zuber S, La Torre G, Potenza C. Factors influencing response to ingenol mebutate therapy for actinic keratosis of face and scalp. *World J Clin Oncol* 2017; 8(5): 405-411 Available from: URL: http://www.wjgnet.com/2218-4333/full/v8/i5/405.htm DOI: http://dx.doi.org/10.5306/wjco.v8.i5.405

INTRODUCTION

For a long time dermatologists have questioned if actinic keratosis (AK) should be considered as a precancerous lesion or an early squamous cell carcinoma (SCC). Apart from academic debate, it is actually clear that AKs have a low but definite potential to become invasive and even metastatic and that this risk increases over time[1].

Since it is impossible to predict which AK will progress to SCC and given the high prevalence of AKs in people with fair photo-types, chronically exposed to ultraviolet (UV) rays, treatment is recommended[2].

Conventional treatments for AK include cryotherapy, laser-therapy, surgical excision, photodynamic therapy, diclofenac 3% gel, imiquimod 5% and 5-fluorouracil creams[3,4].

Ingenol mebutate 0.015% gel, obtained by the sap of the plant Euphorbia peplus, has been recently approved in Europe for the treatment of non-hypertrophic non-hyperkeratotic AKs of face and scalp, which mainly correspond to Ⅰ and Ⅱ histopathologic categories[5,6].

The mechanism of action of ingenol mebutate has been partially explained with a rapid cytotoxic activity at higher concentration and with the activation of immune system at lower concentration[7]. The long-lasting immune surveillance and the clearance of single tumour cell clones within cancerization field, could justify the low recurrence rates of AKs observed after treatment[8].

To the best of our knowledge, no studies have assessed factors independently influencing the response to ingenol mebutate therapy. Efficacy data of phase Ⅲ trials have not been widely confirmed on a large real clinical setting to date[9-11].

These studies reported a higher efficacy of ingenol mebutate 0.015% gel in patients experiencing more severe local skin reactions (LSRs); however they didn't investigate how the single components of the composite LSR score could influence the response to treatment.

We conducted a prospective study to determine which factors, among age, gender, head site and LSR score, could independently predict the response to 0.015% ingenol mebutate treatment and to assess the efficacy of this therapy in a real clinical setting.

MATERIALS AND METHODS

Study population

We (GLV and RP) enrolled consecutive patients, aged ⩾ 18 years, affected by non-hypertrophic non-hyperkeratotic AKs of face and scalp, who were attending our outpatient clinic from April 2014 to March 2015.

The diagnosis of AK was performed both clinically and dermoscopically, respectively based on the presence of erythematous macular lesions with or without a slightly scaly surface, and on the identification of the typical red pseudonetwork, corresponding to grade Ⅰ AK, or strawberry pattern, corresponding to grade Ⅱ AK[12].

The presence of a skin cancer other than AK in the selected skin area was considered as an exclusion criteria. Furthermore, if at least one AK of the selected area had been treated by non-ablative methods within the previous year, patient was excluded from the study.

Treatment procedure

Ingenol mebutate 0.015% gel was applied by the same physician (GLV) for 3 consecutive days on a selected skin area of 25 cm^2, which included 4 to 8 AKs.

Each enrolled patient gave written informed consent for clinical and dermoscopic digital documentation and the ethical committee approval was waived.

Outcome assessment

Clinical and dermoscopic pictures were collected at baseline and at each control visit (day 2, 3, 8, 15, 29 and 57).

Local skin reactions (LSR) score was calculated at each control visit, using a validated composite score (ranging from 0 to 24) given by the sum of 6 single scores for erythema, flaking/scaling, crusting, swelling, vesiculation/pustulation and erosion/ulceration; with grade 0 representing no reaction while grade 4 indicating a skin reaction extending beyond the treated are[13].

Table 1 Demographic and response data of the whole study population *n* (%)

Factors		Value
Age (yr), mean ± SD		72.2 ± 10.3
Gender	M	91 (70)
	F	39 (30)
	Total	130
Head site	Face	101 (77.7)
	Scalp	29 (22.3)
	Total	130
Response	Poor	11 (8.5)
	Partial	61 (46.9)
	Complete	58 (44.6)
	Total	130

Efficacy was evaluated comparing clinical and dermoscopic pictures at baseline and at day 57 and response was classified as complete, partial (⩾ 75% clearance) or poor (< 75% clearance).

Statistical analysis

Statistical analyses were performed using the IBM SPSS 21.0 package (Statistical Package for Social Sciences, SPSS Inc., Chicago, Ill.).

Data is expressed as mean standard deviation. To analyse factors influencing efficacy of 0.015% ingenol mebutate therapy, we used Spearman's rho coefficient to assess significant correlations, which were subsequently quantified *via* univariate logistic regression. Furthermore, a logistic multivariate regression backward model was constructed to identify major independent factors that showed a significant difference ($P < 0.10$) on univariate analysis, that have an influence on complete response. The statistical significance was set at $P < 0.05$.

RESULTS

Study population and efficacy data

Demographic and efficacy data are listed in Table 1. A number of 130 patients were enrolled, 91 (70.0%) were males and 39 (30.0%) were females, with a mean age (standard deviation) of 72.2 (10.3) years. All the patients completed the 3 applications of ingenol mebutate 0.015% gel, as scheduled; the majority, 101 (77.7%) were treated on the face, while 29 (22.3%) on the scalp.

Regarding efficacy, the great majority of our study population (119, 91.5%) reached at least a 75% clearance of AKs, in particular 58 patients (44.6%) achieved a complete response and 61 (46.9%) a partial one; while poor responders were only 11 (8.5%).

Figure 1 shows the clinical and dermoscopic pictures of a patient treated on the scalp, before and after the therapy.

Local skin reaction data

Figure 2 and Table 2 report data about the "number of patients with positive scores" and "mean values" of LSR composite and single scores at each follow up visit.

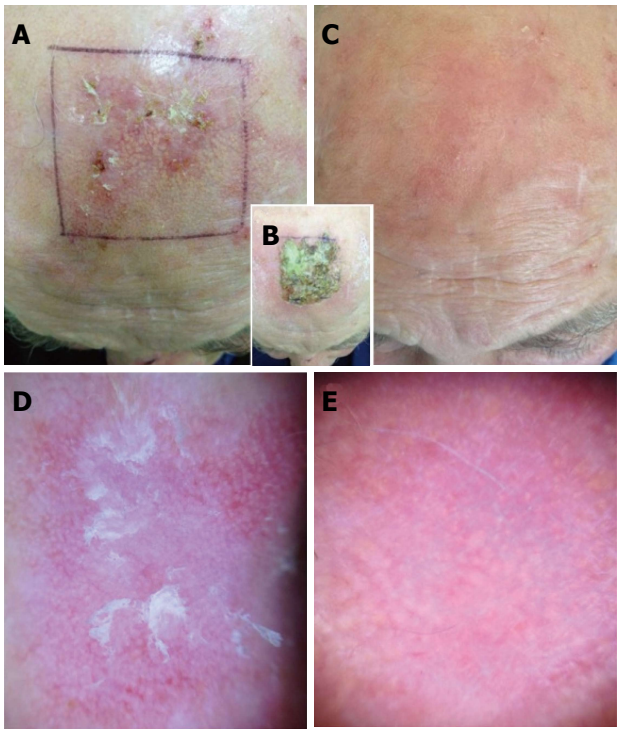

Figure 1 Patient treated with ingenol mebutate for actinic keratosis of the scalp. A and C: Clinical images of the treated area before and after (day 57) the therapy, respectively; B: Local skin reaction to ingenol mebutate at day 8 showing a grade 3 crusting reaction and erythema exceeding the treated area (grade 4); D: Dermoscopic image of an actinic keratosis of the treated area at baseline showing red pseudonetwork and scaling in the central area; E: Dermoscopic picture of the same skin area at day 57 showing the complete disappearance of the preexisting actinic keratosis.

Each patient enrolled experienced at least one LSR, but no one reported systemic symptoms.

The highest number of patients involved and the highest mean scores were reached at day 3 for both composite and all single scores, with the exception of crusting and flaking/scaling, reaching the highest level at day 8 and 15, respectively.

These 2 components were the less represented at day 2 [4 patients (3.1%) had flaking/scaling and only 2 (1.5%) had crusts, with mean values of 0.05 ± 0.28 and 0.02 ± 0.12, respectively] and totally disappeared in the whole population since day 57.

Erythema was the only LSR component involving the entire study population (at day 2 and 3) and the only, still present at day 57 in 49 patients (37.7%), with a mean score of 0.38 ± 0.50.

Swelling reached the highest levels at day 2 and 3 [114 (87.7%) and 125 (96.2%) patients, with 1.48 ± 0.82 and 2.38 ± 1.08 mean scores, respectively], but quickly reduced afterward, becoming totally absent since day 57.

Grade 4 swelling was observed in 23 (17.7%) patients and presented as periorbital edema following the application of ingenol mebutate gel on forehead and temporal areas; it resolved within day 15 in all cases.

Vesiculation/pustulation were the first signs to

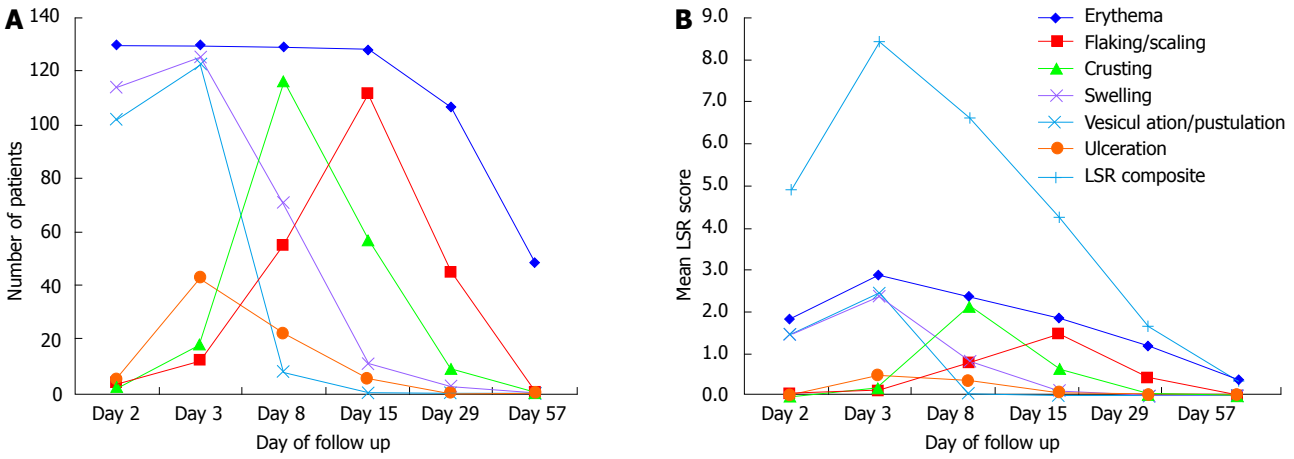

Figure 2 Number of skin reactions and scores at each follow up visit. A: The number and features of different skin reactions over the time; B: Mean values describing the severity of each skin reaction and the LSR composite score (light blue line). Skin reactions included: Erythema (blue), flaking/scaling (red), crusting (green), swelling (purple), vesiculation/pustulation (light blue), and ulceration (orange). LSR: Local skin reaction.

Table 2 Number of patients with positive scores and mean values of local skin reaction composite and single scores at each follow up visit

		Day 2	Day 3	Day 8	Day 15	Day 29	Day 57
Erythema	*n* (%)	130 (100)	130 (100)	129 (99.2)	128 (98.5)	107 (82.3)	49 (37.7)
	mean ± SD	1.82 ± 0.68	2.87 ± 0.58	2.38 ± 0.78	1.86 ± 0.81	1.19 ± 0.77	0.38 ± 0.50
Flaking/scaling	*n* (%)	4 (3.1)	12 (9.2)	55 (42.3)	112 (86.2)	45 (34.6)	0
	mean ± SD	0.05 ± 0.28	0.11 ± 0.39	0.78 ± 1.00	1.49 ± 0.87	0.43 ± 0.65	0
Crusting	*n* (%)	2 (1.5)	18 (13.8)	116 (89.2)	57 (43.8)	9 (6.9)	0
	mean ± SD	0.02 ± 0.12	0.17 ± 0.45	2.16 ± 0.98	0.65 ± 0.89	0.10 ± 0.39	0
Swelling	*n* (%)	114 (87.7)	125 (96.2)	71 (54.6)	11 (8.5)	2 (1.5)	0
	mean ± SD	1.48 ± 0.82	2.38 ± 1.08	0.85 ± 0.98	0.13 ± 0.55	0.02 ± 0.12	0
Vesiculation/pustulation	*n* (%)	102 (78.5)	123 (94.6)	8 (6.2)	0	0	0
	mean ± SD	1.48 ± 0.93	2.45 ± 0.86	0.06 ± 0.24	0	0	0
Ulceration	*n* (%)	5 (3.8)	43 (33.1)	22 (16.9)	5 (3.8)	0	0
	mean ± SD	0.04 ± 0.19	0.48 ± 0.74	0.36 ± 0.90	0.08 ± 0.45	0	0
LSR composite	mean ± SD	4.89 ± 2.14	8.43 ± 2.38	6.62 ± 2.44	4.25 ± 1.72	1.66 ± 1.28	0.35 ± 0.49

LSR: Local skin reaction.

disappear, being widely present at day 2 and 3 [102 (78.5%) and 123 (94.6%) patients, with 1.48 ± 0.93 and 2.45 ± 0.86 mean scores, respectively], but only observable in 8 patients (6.2%) at day 8 and completely absent since day 15.

Ulceration was the least observed LSR component, being present in a maximum of 43 patients (33.1%) at day 3 and early disappearing in the entire population since day 29.

Spearman's correlation

Spearman rho analysis highlighted significant correlations among response and gender, head site and the maximum level of the LSR composite score ($\rho = 0.189$, $P = 0.031$; $\rho = -0.258$, $P = 0.003$; $\rho = 0.449$, $P < 0.001$, respectively).

Furthermore, all the maximum levels of single scores, but flaking/scaling, resulted to be correlated to response (erythema: $\rho = 0.351$, $P < 0.001$; vesiculation: $\rho = 0.329$, $P < 0.001$; crusting: $\rho = 0.255$, $P = 0.003$; swelling: $\rho = 0.365$, $P < 0.001$; ulceration: $\rho = 0.194$, $P = 0.027$).

Regarding the single follow up visits, a significant correlation with response was reported for LSR composite score at day 2, 3, 8 and 15 ($\rho = 0.455$, $P < 0.001$; $\rho = 0.484$, $P < 0.001$; $\rho = 0.325$, $P < 0.001$; $\rho = 0.234$, $P = 0.007$, respectively), for erythema at every follow up visit (day 2: $\rho = 0.400$, $P < 0.001$; day 3: $\rho = 0.351$, $P < 0.001$; day 8: $\rho = 0.314$, $P < 0.001$; day 15: $\rho = 0.270$, $P = 0.002$; day 29: $\rho = 0.282$, $P = 0.001$; day 57: $\rho = 0.189$, $P = 0.032$), for crusting, swelling, vesiculation/pustulation and ulceration at days 3 ($\rho = 0.180$, $P = 0.041$; $\rho = 0.372$, $P < 0.001$; $\rho = 0.329$, $P < 0.001$; $\rho = 0.215$, $P = 0.014$, respectively) for swelling and vesiculation at day 2 ($\rho = 0.357$, $P < 0.001$; $\rho = 0.418$, $P < 0.001$, respectively) and for crusting and swelling at day 8 ($\rho = 0.288$, $P = 0.001$; $\rho = 0.237$, $P = 0.007$, respectively).

Univariate analysis

The univariate logistic regression analysis confirmed that the factors highlighted by Spearman's correlation were all good predictors of complete response to

Table 3 Univariate logistic regression analysis

			OR	95%CI for OR		*P* value
				Lower	Upper	
Gender			2.30	1.07	4.94	0.033[a]
Head site			4.07	1.53	10.83	0.005[a]
Max values	LSR composite		1.55	1.27	1.89	< 0.001[a]
		Erythema	3.90	1.86	8.19	< 0.001[a]
		Crusting	1.85	1.18	2.92	0.008[a]
		Swelling	2.24	1.50	3.35	< 0.001[a]
		Vesiculation/pustulation	2.76	1.55	4.94	0.001[a]
Day 2	LSR composite		1.70	1.36	2.12	< 0.001[a]
		Erythema	3.83	2.05	7.17	< 0.001[a]
		Vesiculation/pustulation	2.82	1.74	4.56	< 0.001[a]
		Swelling	2.73	1.64	4.55	< 0.001[a]
Day 3	LSR composite		1.65	1.34	2.05	< 0.001[a]
		Erythema	3.90	1.86	8.19	< 0.001[a]
		Vesiculation/pustulation	2.76	1.55	4.94	0.001[a]
		Swelling	2.22	1.51	3.28	< 0.001[a]
		Ulceration	1.72	1.06	2.79	0.028[a]
Day 8	LSR composite		1.25	1.07	1.47	0.006[a]
		Erythema	2.44	1.45	4.13	0.001[a]
		Swelling	1.55	1.06	2.25	0.022[a]
		Crusting	1.76	1.17	2.64	0.006[a]
Day 15	LSR composite		1.27	1.02	1.59	0.030[a]
		Erythema	1.93	1.22	3.05	0.005[a]
Day 29		Erythema	2.06	1.26	3.37	0.004[a]
Day 57		Erythema	2.03	1.01	4.09	0.047[a]

Factors predicting the response to ingenol mebutate 0.015% therapy. [a]$P < 0.05$. OR: Odds ratio; LSR: Local skin reaction.

ingenol mebutate 0.015% therapy, with the exclusion of crusting at day 3 and the highest values of ulceration (OR = 2.32, 95%CI: 0.99-5.46, $P = 0.053$ and OR = 1.35, 95%CI: 0.93-1.96, $P = 0.113$, respectively) (Table 3).

More specifically, females were 2 times more likely to risk facial lesions than males, and were almost 4 times more likely to achieve a complete response than scalp ones.

Concerning local skin reactions, both the maximum levels and the values at day 2, 3, 8 and 15 of the composite score were associated with increased odds to achieve a complete response, ranging from 1.27 to 1.70.

Similarly, for erythema, both the maximum values and the levels at each follow up visit were associated with a complete response.

The maximum levels of crusting, swelling and vesiculation/pustulation gave also an increased odd to achieve a complete response, as well as the scores of swelling and vesiculation/pustulation at day 2 and 3 and of swelling and crusting at day 8.

Finally, ulceration at day 3 was also predictive of complete response to therapy.

Multivariate analysis

Multivariate backward logistic regression analysis showed that patients with facial lesions were almost 5 times more likely to achieve a complete response than those treated on the scalp (OR = 5.19, 95%CI: 1.51-17.86, $P = 0.009$); LSR composite score at day 2 resulted as a predictive factor of complete response, with 14.6% higher odds for each point of score added (OR = 1.46, 95%CI: 1.08-1.97, $P = 0.014$). Furthermore, also the maximum level of LSR composite score was associated with complete response to ingenol mebutate therapy, but with a lower statistical significance (OR = 1.50, 95%CI: 1.02-2.21, $P = 0.038$). Finally, regarding single scores, we found that patients with higher crusting reactions at day 8 were more likely to achieve a complete response, with 19.4% higher odds for each point of score added (OR = 1.94, 95%CI: 1.18-3.20, $P = 0.009$) (Table 4).

DISCUSSION

Ingenol mebutate gel was recently introduced as a safe and effective therapeutic option for non-hypertrophic non-hyperkeratotic AK at the dosage of 0.015% for face and scalp[14,15].

Phase Ⅲ trials reported complete clearance rates of 42.2% and partial response rates of 63.9%, for the treatment of facial and scalp AKs with ingenol mebutate, 5 however less is known about the factors influencing the response to treatment[16].

In the present study, we achieved complete and partial responses in 44.6% and 46.9% of cases, respectively; furthermore, ingenol mebutate 0.015% gel therapy resulted to be independently related to both the head site and the level of LSR, with a higher efficacy on facial lesions, compared to scalp ones and in case of more severe LSRs. Level of crusting at day 8 was independently associated with the achievement of a complete response.

Table 4 Multivariate logistic regression backward analysis[1]

	OR	95%CI for OR		*P* value
		Lower	Upper	
Head site	5.19	1.51	17.86	0.009[a]
LSR composite day 2	1.46	1.08	1.97	0.014[a]
Crusting day 8	1.94	1.18	3.20	0.009[a]
LSR composite max	1.50	1.02	2.21	0.038[a]

[1]Factors predicting response to ingenol mebutate 0.015% therapy. Logistic backward multivariate regression model. Reported OR mutually adjusted for all variables in the model. Variables in the model: Gender: Male (M), female (F); head site: Scalp, face; erythema at day 2, 3, 8, 15, 29, 57 and max; crusting at day 8 and max; swelling at day 2, 3, 8 and max; vesiculation/pustulation at day 2, 3 and max; ulceration at day 3; LSR composite at day 2, 3, 8, 15 and max. [a]$P < 0.05$. OR: Odds ratio; LSR: Local skin reaction.

Previous studies showed a greater efficacy of ingenol mebutate on AKs located on the face compared to scalp lesions, but the reason has not been clarified so far. In our opinion a possible explanation could be related to the lower rate of self-application errors on face than on scalp; however, in the present study, we obtained the same results even performing a physician-assisted application[17]. Therefore, other factors should be investigated to explain these findings, such as local differences in skin architecture, microbiota and ph.

Regarding the LSR composite score, we observed that both the highest levels and the values at day 2 were independently associated to complete response. The vast majority of our study population reached the highest values of LSR composite score at day 2.

The weight of each component of the composite score at each follow up visit was further evaluated and related to drug efficacy.

Erythema was the only component present at each evaluation and it was closely associated with response in univariate logistic regression analysis. Intriguingly, in multivariate analysis, when the weight of each variable was mutually adjusted for all variables in the model, erythema no longer could be associated with the response to therapy.

The highest levels of swelling and vesiculation/pustulation and the levels of these components reported in the first week after treatment were significantly associated to response in univariate analysis, but not in the multivariate model.

Conversely, the level of crusting at day 8 was the only single component of LSR composite score independently associated with the achievement of a complete response to ingenol mebutate therapy. A possible explanation of this finding could be related to the fact that the other parameters, in particular swelling and vesiculation/pustulation, probably reached their peak between day 3 and 8 follow up visits, so we couldn't register the highest levels of these reactions. This is also supported by the fact that crusts are strictly related to the occurrence of vesicles and pustules, resulting from the drying of their fluid content.

Differently from phase Ⅲ trials in which the first follow up was set at day 4, we evaluated LSRs at day 2 and 3, during physician-assisted application of ingenol.

Physician assisted application seems to be very effective in limiting withdrawal due to LSRs therefore improving adherence, in particular in elderly patients; however, a direct comparison with self-application was not performed.

Other limitations of the present study were the absence of long term efficacy, safety and cosmetic data, the absence of a quantitative evaluation of symptoms, such as pruritus, burn and pain and the low number of patients treated on the scalp, compared to the face group. However, facial localization demonstrated to be independently associated to complete response in multivariate analysis; whereas, this was not the case for patients treated on the scalp, due to the low number of patients that were treated. To obtain a more reliable result a test should be made on a higher number of patients.

On the basis of our findings we suggest that physician-assisted application of ingenol mebutate, at least for the first 2 d, could be very effective in order to improve adherence and patient satisfaction, maximize the results and minimize the risk of application errors. The severity of LSRs at day 2 and the level of crusting at day 8 should be considered as the best predictors of response to treatment.

In conclusion, our experience demonstrates that ingenol mebutate 0.015% gel is safe and effective when applied correctly. This treatment seems to be more effective on the face than on the scalp and the efficacy seems to be directly related to the level of LSR.

COMMENTS

Background

Actinic keratosis (AK) is considered an *in situ* squamous cell carcinoma, therefore treatment is mandatory.

Research frontiers

Ingenol mebutate 0.015% gel was recently approved for the treatment of non-hypertrophic non-hyperkeratotic AK of face and scalp.

Innovations and breakthroughs

This study considers severe local skin reaction (LSR) the most important factor influencing the response to ingenol mebutate therapy for actinic keratosis.

Applications

This study demonstrates that ingenol mebutate 0.015% gel is safe and effective when applied correctly. This treatment seems to be more effective on the face than on the scalp and the efficacy seems to be directly related to the level of LSR.

Peer-review

This is an interesting study regarding the use of ingenol mebutate therapy for actinic keratosis of face and scalp, and the factors which may affect the treatment response. The study was well-performed, the results are novel and interesting, and the findings should be clinically relevant and useful.

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ORIGINAL ARTICLE

Observational Study

Prophylactic lateral pelvic lymph node dissection in stage Ⅳ low rectal cancer

Hiroshi Tamura, Yoshifumi Shimada, Hitoshi Kameyama, Ryoma Yagi, Yosuke Tajima, Takuma Okamura, Mae Nakano, Masato Nakano, Masayuki Nagahashi, Jun Sakata, Takashi Kobayashi, Shin-ichi Kosugi, Hitoshi Nogami, Satoshi Maruyama, Yasumasa Takii, Toshifumi Wakai

Hiroshi Tamura, Yoshifumi Shimada, Hitoshi Kameyama, Ryoma Yagi, Yosuke Tajima, Takuma Okamura, Mae Nakano, Masato Nakano, Masayuki Nagahashi, Jun Sakata, Takashi Kobayashi, Toshifumi Wakai, Division of Digestive and General Surgery, Niigata University Graduate School of Medical and Dental Sciences, Niigata 951-8510, Japan

Shin-ichi Kosugi, Department of Digestive and General Surgery, Uonuma Institute of Community Medicine, Niigata University Medical and Dental Hospital, Minamiuonuma 949-7302, Japan

Hitoshi Nogami, Satoshi Maruyama, Yasumasa Takii, Department of Surgery, Niigata Cancer Center Hospital, Niigata 951-8586, Japan

Author contributions: Tamura H and Shimada Y designed the report; Kameyama H, Tajima Y, Okamura T, Nakano M, Nakano M, Nagahashi M, Sakata J, Kobayashi T, Kosugi S, Nogami H, Maruyama S and Takii Y were attending doctors for the patients and performed surgical operation; Tamura H and Yagi R collected the patient's clinical data; Tamura H and Shimada Y analyzed the data and wrote the paper; Wakai T approved the final version of the manuscript.

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Correspondence to: Yoshifumi Shimada, MD, PhD, Division of Digestive and General Surgery, Niigata University Graduate School of Medical and Dental Sciences, 1-757, Asahimachi-dori, Chuo-ku, Niigata 951-8510, Japan. shimaday@med.niigata-u.ac.jp
Telephone: +81-25-2272228
Fax: +81-25-2270779

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Abstract

AIM

To assess the clinical significance of prophylactic lateral pelvic lymph node dissection (LPLND) in stage Ⅳ low rectal cancer.

METHODS

We selected 71 consecutive stage Ⅳ low rectal cancer patients who underwent primary tumor resection, and enrolled 50 of these 71 patients without clinical LPLN metastasis. The patients had distant metastasis such as liver, lung, peritoneum, and paraaortic LN. Clinical LPLN metastasis was defined as LN with a maximum diameter

of 10 mm or more on preoperative pelvic computed tomography scan. All patients underwent primary tumor resection, 27 patients underwent total mesorectal excision (TME) with LPLND (LPLND group), and 23 patients underwent only TME (TME group). Bilateral LPLND was performed simultaneously with primary tumor resection in LPLND group. R0 resection of both primary and metastatic sites was achieved in 20 of 50 patients. We evaluated possible prognostic factors for 5-year overall survival (OS), and compared 5-year cumulative local recurrence between the LPLND and TME groups.

RESULTS

For OS, univariate analyses revealed no significant benefit in the LPLND compared with the TME group (28.7% *vs* 17.0%, $P = 0.523$); multivariate analysis revealed that R0 resection was an independent prognostic factor. Regarding cumulative local recurrence, the LPLND group showed no significant benefit compared with TME group (21.4% *vs* 14.8%, $P = 0.833$).

CONCLUSION

Prophylactic LPLND shows no oncological benefits in patients with Stage Ⅳ low rectal cancer without clinical LPLN metastasis.

Key words: Prophylactic lateral pelvic lymph node dissection; Stage Ⅳ; Low rectal cancer

Core tip: The clinical significance of prophylactic lateral pelvic lymph node dissection (LPLND) in stage Ⅳ low rectal cancer has not been proven. In this study, we showed two main findings concerning treatment strategy in these patients. First, prophylactic LPLND was not a significant prognostic factor for overall survival and did not contribute local control. Second, R0 resection was an independent prognostic factor for overall survival. These results suggest that prophylactic LPLND is not an important component of surgical treatment in stage Ⅳ low rectal cancer patients.

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INTRODUCTION

In rectal cancer, lymphatic spread accords with the anatomical level of the tumor[1,2]. When the tumor is located above the peritoneal reflection, lymphatic cancer metastasis is predominantly associated with upward mesenteric spread along perirectal vessels originating from the inferior mesenteric artery. In contrast, when the tumor is located at or below the peritoneal reflection, lymphatic cancer metastasis can show upward mesenteric spread and lateral extramesenteric spread along the internal iliac vessels. Based on the rationale of lateral extramesenteric spread, lateral pelvic lymph node dissection (LPLND) is performed to eradicate LPLN metastasis in patients with rectal cancer located at or below the peritoneal reflection[3-9].

The management of LPLN associated with low rectal cancer differs considerably between Western countries and Japan. In Western countries, LPLN metastasis is generally considered as a metastatic disease, and preoperative chemoradiation and total mesorectal excision (TME) is the standard treatment[10]. In contrast, LPLN metastasis is regarded as a local disease in Japan, and TME with LPLND is performed for patients with locally advanced low rectal cancer[11]. Large-scale retrospective studies in Japan evaluated the survival outcome of patients with LPLN metastasis, and concluded that LPLN could be considered as regional lymph nodes in low rectal cancer[12].

LPLN metastasis was identified in approximately 20% of Japanese patients with T3 or T4 tumors who underwent LPLND[11,13]. Nevertheless, the clinical significance of LPLND has not been fully proven and a prospective study is needed to resolve whether LPLND has any survival benefit in patients with low rectal cancer. Accordingly, a randomized controlled trial was conducted to clarify the clinical significance of prophylactic LPLND for clinical stage Ⅱ and Ⅲ low rectal cancer (JCOG0212)[14]. However, to date, no studies have addressed the surgical outcome of TME with LPLND for stage Ⅳ low rectal cancer, and the clinical significance of LPLND for stage Ⅳ low rectal cancer is still unclear.

We retrospectively evaluated 50 consecutive stage Ⅳ low rectal cancer patients without clinical LPLN metastasis to assess the survival benefit of prophylactic LPLND in patients with stage Ⅳ low rectal cancer. We analyzed various prognostic factors including LPLND with respect to overall survival (OS), and evaluated cumulative local recurrence of patients with LPLND.

MATERIALS AND METHODS

Patients

We selected patients from our colorectal cancer databases with stage Ⅳ low rectal cancer according to the AJCC 7th edition[15], applied the following inclusion criteria: Adenocarcinoma confirmed on histological examination, preoperative pelvic computed tomography (CT) scan negative for clinical LPLN metastasis, and primary tumor resection undertaken at Niigata University Medical and Dental Hospital or Niigata Cancer Center Hospital between January 2000 and December 2015. We selected 71 consecutive stage Ⅳ low rectal cancer patients who underwent primary tumor resection, and enrolled 50 of these 71 patients without clinical LPLN

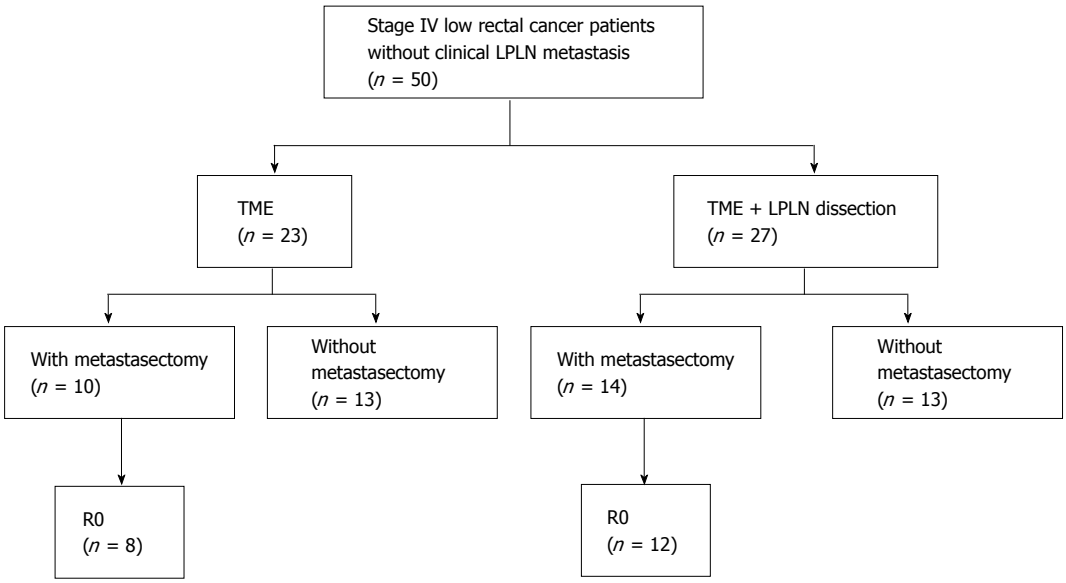

Figure 1 Flowchart of surgical treatment. TME: Total mesorectal excision; LPLN: Lateral pelvic lymph node.

metastasis (Figure 1, Table 1). All the patients had negative circumferential resection margin. Twenty of these 71 patients were excluded in the present study because they were diagnosed as positive for clinical LPLN metastasis by preoperative pelvic CT scan, and 1 patient was excluded because of loss of follow-up. Clinical LPLN metastasis was defined as LN with a maximum diameter of 10 mm or more on preoperative pelvic CT scan. In this study period, "therapeutic LPLND" was carried out for patients with clinical LPLN metastasis. For patients without clinical LPLN metastasis, whether "prophylactic LPLND" was performed or not was determined by preoperative conference. Neoadjuvant chemoradiotherapy (NACRT) was not administered at the participating institutions because it is uncertain whether this approach improves OS[16,17]. Distant metastasis was classified according to the JSCCR classification[18]. Liver metastases were classified into three categories (H1: 1-4 metastatic tumors all of maximum diameter 5 cm or less; H2: Those other than H1 or H3; H3: 5 or more metastatic tumors at least one of which has a maximum diameter of more than 5 cm). Lung metastases were classified into three categories (LM1: Metastasis limited to one lobe; LM2: Metastasis to more than one lobe in one side of lung; LM3: Metastasis to both sides of lungs). Peritoneal metastases were classified into three categories (P1: Metastasis localized to adjacent peritoneum; P2: Metastasis limited to distant peritoneum; P3: Diffuse metastasis to distant peritoneum). This retrospective study was performed in accordance with the Helsinki Declaration, and the Ethics Committee of the School of Medicine, Niigata University approved the study protocol (approval number: 2330), waiving patient consent.

Procedure of TME with LPLND and postoperative complications

Twenty-three patients underwent only TME ("TME group"), and 27 patients underwent TME with LPLND ("LPLND group"). Regarding LPLND, 26 procedures were performed as open surgery and 1 procedure was done as laparoscopic surgery. The LPLN were classified into five areas (distal internal iliac, proximal internal iliac, obturator, external iliac and common iliac) according to the JSCCR classification[18]. In the LPLND group, LPLND was carried out in accordance with previously reported methods[3,13,14]. Bilateral LPLND was performed simultaneously with primary tumor resection in LPLND group. Post-operative complications were monitored for 90 d after surgery and graded according to a standard classification[19]. Major complications were defined as grade $\geqslant$ 3.

Metastasectomy and residual tumor status

To achieve R0 resection of metastatic lesion, simultaneous or staged metastasectomy was planned according to the patients' condition. Essentially, simultaneous metastasectomy was performed when the patients had resectable intra-abdominal metastasis such as solitary liver metastasis which could be respected by partial hepatectomy, limited peritoneal dissemination, or paraaortic lymph nodes. Staged metastasectomy was planned when the patients had extra-abdominal metastasis such as lung metastasis, or liver metastasis which needed major hepatectomy such as right hepatic lobectomy. In this cohort, there were no patients who received conversion therapy such as hepatectomy for initially unresectable multiple liver metastasis. We classified the patients according to residual tumor status, *i.e.*, the patients who received R0 resection of both primary lesion and distant metastasis were classified as "R0", and the other patients in whom R0 resection could not be achieved were classified as "R2".

Prognostic factors

We evaluated possible prognostic factors including LPLND

for OS, and compared cumulative local recurrence rates between the TME and LPLND groups. To elucidate the factors influencing OS after surgery, 16 variables were tested in all 50 patients: Age (< 65 *vs* ⩾ 65 years), sex, preoperative Carcinoembryonic antigen (CEA) level (< 20 ng/mL *vs* ⩾ 20 ng/mL), tumor size (< 60 mm *vs* ⩾ 60 mm), T category (T2, 3 *vs* T4), histopathological grading (G1, 2 *vs* G3), lymphatic invasion (absence *vs* presence), venous invasion (absence *vs* presence), lymph node metastasis (absence *vs* presence), LPLND (absence *vs* presence), number of metastatic organs (1 *vs* 2), metastatic organ (liver only *vs* others), Grade 3 complication of primary tumor resection (absence *vs* presence), residual tumor status (R0 *vs* R2), Preoperative chemotherapy (absence *vs* presence), and Postoperative chemotherapy (absence *vs* presence).

Statistical analysis

After the operation, the patients were followed-up by physical examination, laboratory testing, and imaging. CEA and carbohydrate antigen 19-9 were monitored periodically. Disease recurrence and tumor progression were determined mainly by chest-abdominal-pelvic CT scans. Colonoscopy was performed to detect local recurrence at the anastomotic site. The median follow-up period of all 50 patients was 23.6 mo (range: 1-130). Statistical analyses were performed with IBM SPSS Statistics 22 (IBM Japan Inc., Tokyo, Japan). The relationships between each of the clinicopathological variables and residual tumor status were analyzed using Fisher's exact test. Five-year OS and cumulative local recurrence rates were estimated using the Kaplan-Meier method. The log-rank test was used to assess for significant difference between the subgroups by univariate analysis. To investigate independent prognostic factors for OS, factors with a *P* value of less than 0.10 in univariate analyses were entered into multivariate analysis. The Cox proportional hazards regression model was used to identify factors that were independently associated with OS after surgery. *P* values less than 0.05 were considered statistically significant.

RESULTS

Procedure and postoperative complications of primary tumor resection

All patients received R0 resection of primary site with the operative procedure as follows: 31 patients received low anterior resection, 18 patients received abdominoperineal resection, 1 patient received pelvic exenteration. Dysuria was observed in 20 patients, and all of them were grade 1 or 2. Major complications (grade ⩾ 3) were observed in 12 of 50 patients (24.0%); anastomotic leakage, surgical site infection, and anastomotic stenosis were observed in 4, 7, and 1 patients, respectively. Postoperative histopathological analysis revealed LPLN metastasis in 12 of 27 patients (44.4%) who received prophylactic LPLND, with a median number of 1 metastatic node per patient (range: 1-4). The sites of LPLN metastases were as follows: distal internal iliac nodes, proximal internal iliac nodes, obturator nodes, external iliac nodes, and common iliac nodes in 6, 5, 3, 1, and 1 patients, respectively.

Metastasectomy

Of the 50 patients, 24 received metastasectomy and 20 received R0 resection of both primary and metastatic sites (Figure 1). Sixteen patients simultaneously underwent primary tumor resection and metastasectomy. The details of the metastasectomy sites are as follows: Liver in 8 patients, limited peritoneal dissemination in 7 patients, and liver and paraaortic lymph node in 1 patient. Successful R0 resection was achieved in 14 of 16 patients; however, two patients who had liver and lung metastases underwent only hepatectomy because of progression of lung tumor after hepatectomy. In contrast, 8 patients underwent staged metastasectomy after primary tumor resection. The details of the metastasectomy sites are as follows: Liver in 4 patients, lung in 3 patient, liver and lung in 1 patient. R0 resection was achieved in 6 of these 8 patients; however, 1 patient who had liver and lung metastases underwent only hepatectomy because of progression of lung tumor after hepatectomy, and 1 patient who had lung metastasis underwent margin positive surgery. In contrast, 26 of 50 patients did not undergo metastasectomy because of tumor progression or development of new metastatic lesions after primary tumor resection.

Factors influencing OS after primary tumor resection

A comparison of clinicopathological characteristics between the LPLND and TME groups showed that there were no significant differences in 15 tested variables (Table 2). Five-year overall cumulative survival rates after primary tumor resection were 74.0% at 1 year, 43.7% at 3 years, and 23.4% at 5 years. Univariate analyses revealed that the LPLND group showed no significant benefit compared with TME group (28.7% *vs* 17.0%, *P* = 0.523) (Table 3 and Figure 2), and that age (⩾ 65 years) and R0 resection were factors whose *P* values were less than 0.10 for OS. Multivariate analysis identified R0 resection as significant independent prognostic factor for OS (*P* < 0.001) (Table 3).

Efficacy of prophylactic LPLND for local control

Five of the 50 patients showed local recurrence. The details of local recurrence sites are as follows: Anastomotic site in 2 patients, and the other intrapelvic space in 3 patients. One patient who had LPLND showed local recurrence of the right LPLN area. Twenty-seven patients with LPLND showed no significantly improved 5-year cumulative local recurrence rate compared with the 23 patients without LPLND (21.4% *vs* 14.8%, *P* = 0.833) (Figure 3).

DISCUSSION

In the present study, we showed that prophylactic

Table 1 Clinicopathological characteristics of the 50 patients

Variable	
Age (yr)[1]	58.5 (31-78)
Sex	
Male:female	43:7
Preoperative CEA level (ng/mL)[1]	24.5 (1.6–6856.5)
Tumor size (mm)[1]	63.0 (22–130)
T category	
T2:T3:T4	2:31:17
Histopathological grading	
G1:G2:G3	1:35:14
Lymphatic invasion	
Absence:Presence	6:44
Venous invasion	
Absence:Presence	10:40
Lymph node metastasis	
Absence:Presence	9:41
Pathological LPLN metastasis	
Absence:Presence	15:12
No. of metastatic organs	
1:2:3	44:5:1
Metastatic organ	
Liver:Lung:Peritoneum:Paraaortic LN:Bone	28:16:10:1:2
Grade of liver metastasis[2]	
H1:H2:H3	14:5:9
Grade of lung metastasis[2]	
LM1:LM2:LM3	8:7:1
Grade of peritoneal metastasis[2]	
P1:P2:P3	8:1:1
Grade ⩾ 3 Complication of primary tumor resection	
Absence:Presence	38:12
Residual tumor status	
R0:R2	20:30
Preoperative chemotherapy	
Absence:Presence	41:9
Postoperative chemotherapy	
Absence:Presence	7:43
Chemotherapy regimen	
5FU-LV and/or S-1 and/or capecitabine	25
FOLFOX and/or CapeOX and/or FOLFIRI	33
Bevacizumab	18
Cetuximab or panitumumab	4

[1]Data are expressed as median (range); [2]Distant metastasis was classified according to the Japanese Society for Cancer of the Colon and Rectum classification (See material and method). FOLFOX oxaliplatin, leucovorin, and 5FU, CapeOX oxaliplatin and capecitabine, FOLFIRI irinotecan, leucovorin, and 5FU. CEA: Carcinoembryonic antigen; LPLN: Lateral pelvic lymph node; LN: Lymph node; TME: Total mesorectal excision; 5FU: 5-Fluorouracil; LV: Leucovorin.

Table 2 Clinicopathological characteristics of patients in the lateral pelvic lymph node dissection and total mesorectal excision groups

Variable	TME group (*n* = 23)	LPLND group (*n* = 27)	*P* value
Age (yr)			
< 65	13	19	0.382
⩾ 65	10	8	
Sex			
Male	20	23	0.999
Female	3	4	
Preoperative CEA level (ng/mL)			
< 20	10	11	0.999
⩾ 20	13	16	
Tumor size (mm)			
< 60	7	8	0.999
⩾ 60	16	19	
T category			
T2, 3	11	14	0.999
T4	12	13	
Histopathological grading			
G1, 2	19	17	0.206
G3	4	10	
Lymphatic invasion			
Absence	3	3	0.999
Presence	20	24	
Venous invasion			
Absence	7	3	0.155
Presence	16	24	
Lymph node metastasis			
Absence	7	2	0.062
Presence	16	25	
No. of metastatic organs			
1	21	23	0.647
2, 3	2	4	
Metastatic organ			
Liver only	12	13	0.999
Others	11	14	
Grade ⩾ 3 complication of primary tumor resection			
Absence	17	21	0.999
Presence	6	6	
Residual tumor status			
R0	8	12	0.569
R2	15	15	
Preoperative chemotherapy			
Absence	18	23	0.715
Presence	5	4	
Postoperative chemotherapy			
Absence	4	3	0.689
Presence	19	24	

CEA: Carcinoembryonic antigen; LPLND: Lateral pelvic lymph node dissection; TME: Total mesorectal excision.

LPLND was not a significant prognostic factor for OS and did not contribute to local control. These results suggest that prophylactic LPLND is not an important component of surgical treatment in stage Ⅳ low rectal cancer.

It is possible that there are several acceptable treatment strategies in stage Ⅳ low rectal cancer patients without clinical LPLN metastasis. When the primary and metastatic sites are resectable, the patient can be treated with a staged or simultaneous resection to achieve R0 resection of both primary and metastatic sites. To achieve R0 resection of the primary site, the options are: (1) TME only; (2) TME with LPLND; (3) NAC followed by TME; and (4) NACRT followed by TME, *etc*. However, optimal treatment of patients with primary metastatic rectal cancer is controversial[20,21].

The NCCN guidelines state that NACRT is a standard treatment for stage Ⅱ/Ⅲ rectal cancer[10], however, it is also associated with increased toxicity (*e.g.*, radiation-induced injury, hematological toxicities). To date, the clinical significance of NACRT for stage Ⅳ low rectal cancer remains still unclear. van Dijk *et al*[20] reported that radical surgical treatment of all tumor sites carried out after short-course radiotherapy, and bevacizumab-

Table 3 Univariate and multivariate analyses of different prognostic factors for overall survival

Variable	Modality	*n*	Univariate		Multivariate	
			5-yr OS (%)	*P* value	HR (95%CI)	*P* value
Age (yr)	< 65	32	27.9	0.095	1	
	⩾ 65	18	14.8		1.5 (0.8–3.0)	0.197
Sex	Male	43	19.8	0.618		
	Female	7	42.9			
Preoperative CEA level (ng/mL)	< 20	21	23.7	0.671		
	⩾ 20	29	22.9			
Tumor size (mm)	< 60	15	29.6	0.634		
	⩾ 60	35	20.9			
T category	T2, 3	25	17.3	0.515		
	T4	25	32.5			
Histopathological grading	G1, 2	36	25	0.348		
	G3	14	21.4			
Lymphatic invasion	Absence	6	0	0.446		
	Presence	44	24.2			
Venous invasion	Absence	10	40	0.215		
	Presence	40	19.1			
Lymph node metastasis	Absence	9	0	0.904		
	Presence	41	27.5			
LPLND	Absence	23	17	0.523		
	Presence	27	28.7			
No. of metastatic organs	1	44	23.8	0.866		
	2	6	22.2			
Metastatic organ	Liver only	25	36	0.241		
	Others	25	10.6			
Grade ≥ 3 complication of primary tumor resection	Absence	38	28.8	0.398		
	Presence	12	9.5			
Residual tumor status	R0	20	59	< 0.001	1	
	R2	30	3.6		2.1 (1.4–3.0)	< 0.001
Preoperative chemotherapy	Absence	41	17.6	0.254		
	Presence	9	55.6			
Postoperative chemotherapy	Absence	7	38.1	0.397		
	Presence	43	24.3			

OS: Overall survival; CEA: Carcinoembryonic antigen; LPLND: Lateral pelvic lymph node dissection.

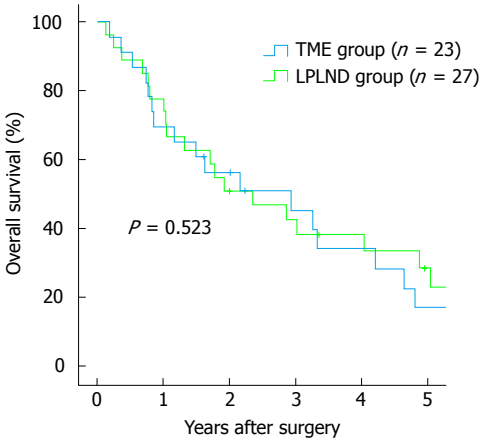

Figure 2 Comparative overall survival rates of patients with total mesorectal excision and lateral pelvic lymph node dissection groups. TME: Total mesorectal excision; LPLND: Lateral pelvic lymph node dissection.

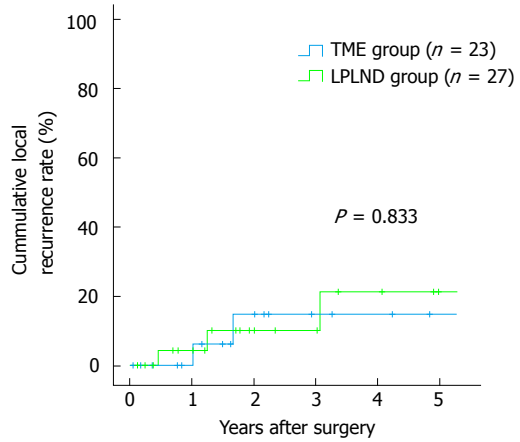

Figure 3 Comparative cumulative local recurrence rates of patients with total mesorectal excision and lateral pelvic lymph node dissection groups. TME: Total mesorectal excision; LPLND: Lateral pelvic lymph node dissection.

capecitabine-oxaliplatin combination therapy is a feasible and potentially curative approach in primary metastasized rectal cancer. Conversely, Butte *et al*[21] reported that selective exclusion of radiotherapy may be considered in rectal cancer patients who are diagnosed with simultaneous liver metastasis, because systemic

sites were overwhelmingly more common than pelvic recurrences after primary tumor resection. In stage Ⅳ patients, we surmised that subsequent metastasectomy and systemic chemotherapy are essential for cure; hence, a treatment strategy without NACRT could be a reasonable and acceptable approach to avoid the toxicity associated with NACRT.

To the best of our knowledge, this is the first report regarding the clinical significance of prophylactic LPLND in stage Ⅳ low rectal cancer patients without clinical LPLN metastasis. We demonstrated that prophylactic LPLND has no oncological benefits regarding OS and cumulative local recurrence in this setting. Previous studies reported that TME with LPLND is associated with significant morbidity, longer operative time, greater blood loss, and functional impairment, particularly impotence and bladder dysfunction[3,12-25]. To avoid the post-operative complications associated with LPLND and achieve early induction of postoperative chemotherapy, we think that prophylactic LPLND could be omitted for stage Ⅳ low rectal cancer patients without clinical LPLN metastasis.

We recognize several limitations in this study. First, this retrospective study included a small sample size. Second, we could not investigate how many patients, such as those who had multiple distant metastases, were excluded from the indications for primary tumor resection, because those patients were generally not referred to surgeons. Third, we could not investigate detailed parameters such as resectability criteria of distant metastases, comorbidity and response to chemotherapy. Fourth, it is possible that the LPLND group included patients with suspicious clinical LPLN metastasis of maximum diameter less than 10 mm, because histopathological LPLN metastases were observed in 12 of 27 patients (44.4%) patients in the LPLND group. Fifth, we included only patients without clinical LPLN metastasis. Hence, we could not assess the value of therapeutic LPLND for patients with clinical LPLN metastasis, and the clinical significance of LPLND for these patients is still unclear. In future, a multicenter prospective study is required to clarify the clinical significance of LPLND for stage Ⅳ low rectal cancer patients.

In conclusion, prophylactic LPLND shows no oncologic benefits in patients with stage Ⅳ low rectal cancer without clinical LPLN metastasis.

COMMENTS

Background

No studies have addressed the surgical outcome of total mesorectal excision with lateral pelvic lymph node dissection (LPLND) for stage Ⅳ low rectal cancer, and the clinical significance of LPLND for stage Ⅳ low rectal cancer is still unclear.

Research frontiers

There is little clinical information relating to LPLND for stage Ⅳ low rectal cancer.

Innovations and breakthroughs

This study is the first report regarding the clinical significance of prophylactic LPLND in stage Ⅳ low rectal cancer patients without clinical LPLN metastasis.

Applications

Prophylactic LPLND shows no oncologic benefits in patients with stage Ⅳ low rectal cancer without clinical LPLN metastasis.

Peer-review

To assess the clinical significance of prophylactic lateral pelvic lymph node dissection is the first research in stage Ⅳ low rectal cancer. The article is well-designed and important for clinical practice.

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CASE REPORT

First report of small cell lung cancer with PTHrP-induced hypercalcemic pancreatitis causing disconnected duct syndrome

Eric M Montminy, Stephen W Landreneau, Jordan J Karlitz

Eric M Montminy, Jordan J Karlitz, Division of Gastroenterology, Tulane University Medical Center, New Orleans, LA 70112, United States

Stephen W Landreneau, Division of Gastroenterology, LSU Health Sciences Center, New Orleans, LA 70112, United States

Author contributions: Montminy EM and Landreneau SW wrote manuscript and directly cared for the patient while hospitalized; Karlitz JJ was overseeing author and provided edits to manuscript.

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Correspondence to: Eric M Montminy, MD, Internal Medicine Resident, Tulane University Medical Center, 1430 Tulane Avenue, SL-50, New Orleans, LA 70112, United States. emontmin@tulane.edu
Telephone: +1-630-3060555
Fax: +1-504-9883971

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Abstract

Here we report a patient diagnosed with small cell lung cancer after first presenting with parathyroid hormone-related peptide-induced hypercalcemic pancreatitis and developed walled-off necrosis that resulted in disruption of the main pancreatic duct. Disconnected duct syndrome (DDS) is a rare syndrome that occurs when the main pancreatic duct exocrine flow is disrupted resulting in leakage of pancreatic enzymes and further inflammatory sequela. To date, no prior reports have described DDS occurring with paraneoplastic reactions. Diagnostic imaging techniques and therapeutic interventions are reviewed to provide insight into current approaches to DDS.

Key words: Disconnected duct syndrome; Parathyroid hormone-related peptide; Hypercalcemic pancreatitis

Core tip: Acute recurrent pancreatitis flares should raise concern for disconnected duct syndrome (DDS). This case is the first reported case of DDS caused by paraneoplastic hypercalcemia. Paraneoplastic syndromes may predispose patients to prolonged hypercalcemic pancreatitis and in turn, may predispose patients to DDS. Furthermore, this case report reviews the current approach and treatment difficulties of DDS as well as pancreatic walled-off necrosis.

Montminy EM, Landreneau SW, Karlitz JJ. First report of small cell

lung cancer with PTHrP-induced hypercalcemic pancreatitis causing disconnected duct syndrome. *World J Clin Oncol* 2017; 8(5): 420-424 Available from: URL: http://www.wjgnet.com/2218-4333/full/v8/i5/420.htm DOI: http://dx.doi.org/10.5306/wjco.v8.i5.420

INTRODUCTION

Disconnected duct syndrome (DDS) is a pancreatic syndrome where the main pancreatic duct is occluded and pancreatic exocrine flow leaks into the pancreatic parenchyma[1]. This syndrome frequently results in further inflammatory reactions such as sepsis, development of pseudocysts, and fistulizing disease. Etiologies of DDS are more commonly from mass-like lesions such as large pseudocysts, walled-off necrosis, or neoplasms obstructing the main pancreatic duct[1]. DDS often is difficult to treat due to narrow or complete occlusions requiring cannulation and increased surgical morbidity and mortality. Additionally, this case report discusses a unique cause of DDS and the current approaches used for diagnosis and treatment. To date, this is the first report of a DDS being related to a paraneoplastic syndrome.

CASE REPORT

A 38-year-old man with newly diagnosed small cell lung cancer (SCLC) presented in late July 2016 with acute onset epigastric pain, nausea, and vomiting. He was admitted one month prior for acute pancreatitis secondary to a calcium of 13.7 mg/dL (normal 8.4-10.3 mg/dL). He denied alcohol history or previous gall stones at that time, and imaging work up was only positive for pancreatic inflammation and a lung mass determined by biopsy to be SCLC. No evidence of bone metastasis was seen on imaging. During the July 2016 admission, vital signs at presentation were blood pressure 143/99 mmHg, heart rate 120 beat/min, respiratory rate 14 breaths/min, oxygen saturation 100%, temperature 36.6 ℃, and physical exam was only positive for epigastric tenderness. Labs demonstrated a serum lipase of 2030 U/L (normal < 90 U/L), serum calcium of 11 mg/dL (normal 8.4-10.3 mg/dL), parathyroid hormone less than 9 pG/mL (normal 12-65 pG/mL) and parathyroid-related peptide of 3.9 pmol/L (normal < 2 pmol/L). Triglycerides were normal. Abdominal ultrasound revealed no evidence of gallstones. MRI of the abdomen with magnetic resonance cholangiopancreatography (MRCP) showed multiple cystic areas with rim enhancement replacing large portions of the pancreatic body with the largest centered in the mid-body of the pancreas measuring 3.5 cm × 6.2 cm compressing the main pancreatic duct as well as a 2 cm × 4.3 cm collection extending into the pancreatic groove (Figure 1). MRCP displayed complete lack of enhancement of the main pancreatic duct (Figure 2). A diagnosis of DDS was made based off of these findings. Development of the walled-off necrosis and pancreatic inflammation was thought to be secondary to repeated paraneoplastic-induced pancreatitis episodes. ERCP-guided cannulation of main pancreatic

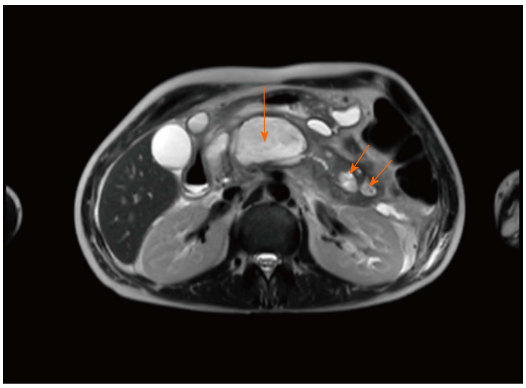

Figure 1 Magnetic resonance imaging of abdomen with and without contrast during July 2016 presentation. Image displays large walled-off necrosis within the body and tail of the pancreas (arrows).

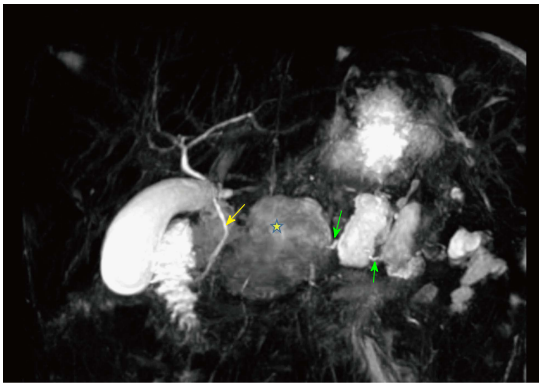

Figure 2 Magnetic resonance cholangiopancreatography performed during July 2016 admission. Image displays poorly defined main pancreatic duct (green arrows) throughout the pancreas. Common bile duct defined well (yellow arrow) with lack of contrast accentuating the main pancreatic duct. A large walled-off necrosis well imaged again (star).

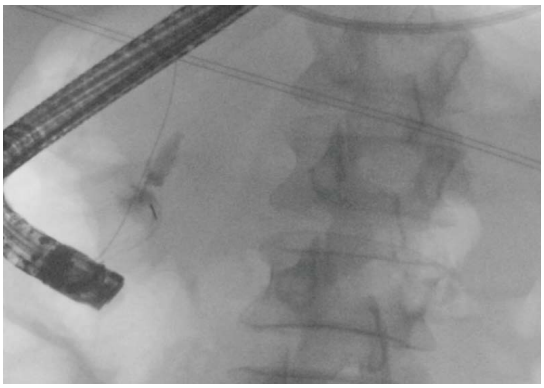

Figure 3 Endoscopic retrograde cholangiopancreatography performed during July 2016 admission. Image displays failure of contrast dye to define pancreatic duct and failure of guidewire to cannulate pancreatic duct. Guidewire continues to be diverted to common bile duct which provides evidence of pancreatic duct obstruction.

Table 1 Definitions and descriptions of structural complications of acute pancreatitis

Structural complications of acute pancreatitis[2]	
Acute peripancreatic fluid collection	Defined as peripancreatic fluid within the first 4 wk of interstitial edematous pancreatitis Homogeneous collection with fluid density No visible encapsulating wall around fluid collection Adjacent to pancreas
Pancreatic pseudocyst	Defined as an encapsulated fluid collection usually forming > 4 wk from initial pancreatitis event with visible inflammatory wall typically outside the pancreas with minimal or no necrotic features forming Homogeneous fluid density with no non-liquid components
Acute necrotic collection	Defined as a fluid collection with variable amounts of fluid and necrosis without a visible encapsulating wall Only can occur with necrotizing pancreatitis Can involve pancreatic parenchyma and/or peripancreatic tissue Heterogeneous and non-liquid density of varying degrees
Walled-off necrosis	Defined as a mature collection of pancreatic and/or peripancreatic necrosis with an encapsulating inflammatory wall typically requiring > 4 wk from initial pancreatitis to form Only can occur with necrotizing pancreatitis Heterogeneous with liquid and non-liquid density with varying degrees of loculation

duct past the pancreatic head was unsuccessful due to complete occlusion of the duct (Figure 3). Pancreatic duct stent placement was unsuccessful. Endoscopic ultrasound visualized the walled off necrosis, but transmural drainage was avoided due to symptomatic improvement with conservative management. The patient was managed conservatively with pain management and bisphosphonates over the following 24 wk until cholecystectomy and surgical necrosectomy were performed. The surgery was uncomplicated. Currently, the patient was transitioned to home hospice due to progression of his cancer.

DISCUSSION

Acute pancreatitis is defined by the Atlanta Classification as having: (1) Typical pain; (2) imaging showing pancreatic inflammation; and (3) elevation in amylase or lipase > 3 × the upper limit of normal. Two of the three criteria must be present to confirm the diagnosis[2]. Acute pancreatitis can be complicated by the formation of fluid collections which have been defined and characterized by the 2012 revised Atlanta Classification (Table 1)[2]. Major distinguishing features of fluid collections are the required time for formation, the presence of an encapsulating inflammatory wall, and heterogeneity[2]. An acute peripancreatic fluid collection (APFC) is a collection of adjacent fluid that develops within the first four weeks of the initial pancreatitis[2]. APFC is not contained by a visible encapsulating inflammatory wall and is a homogeneous collection with fluid density[2]. In contrast, a pancreatic pseudocyst is a fluid collection usually outside of the pancreas and typically requires four weeks or more to develop[2]. A pseudocyst has a visible encapsulating inflammatory wall and is homogeneous with only fluid components[2]. If acute pancreatitis progresses to necrotizing pancreatitis, an acute necrotic collection (ANC) can develop. An ANC develops usually less than four weeks from initial event and does not have visible encapsulating walls. ANC can be distinguished from an APFC by a heterogeneous appearance from localized liquid and necrotic pancreatic tissue. After approximately four weeks, an ANC will develop an encapsulated inflammatory wall which is termed a walled-off necrosis (WON). A WON will continue to have a heterogeneous appearance from accumulated fluid and necrotic pancreatic tissue[2].

Acute recurrent pancreatitis is a clinical condition that is defined as two or more attacks of pancreatitis without evidence of underlying chronic pancreatitis[3]. Acute recurrent pancreatitis is often attributed to gallstones, alcohol ingestion, or idiopathic causes[3]. Furthermore, acute recurrent pancreatitis can progress to necrotizing pancreatitis and develop inflammatory fluid collections that obstruct pancreatic duct drainage, termed DDS. DDS should be considered on a differential diagnosis particularly when a patient presents with repeated bouts of pancreatitis and enlarging pancreatic fluid collections. DDS is a syndrome that starts with an episode of acute pancreatitis that typically develops a large fluid collection or necrosis. This initial fluid collection results in compression of the main pancreatic duct. Disruption of the main pancreatic duct flow, most commonly in the neck or body of the pancreas[4], results in blockage and leakage of distal drainage of pancreatic enzymes. Leakage of these enzymes into the pancreatic parenchyma results in further inflammatory sequela such as more fluid collections, fistulas, or sepsis. Causes of DDS all result in a significant narrowing or complete occlusion of the main pancreatic duct. More frequently encountered causes of duct obstruction are large pseudocysts, necrotic lesions, trauma, and abdominal neoplasms[4,5]. Less commonly, causes such as intra-ductal pancreatic mucinous neoplasm or calculi can result in DDS. Of note, acute recurrent pancreatitis as a presenting feature of SCLC is rare and if present, pancreatitis is more commonly from metastatic lesions obstructing the pancreatic duct rather than PTHrP-induced hypercalcemia[6]. Hypercalcemia results typically from either elevated PTHrP production or osteolytic activity from bone metastasis. Paraneoplastic

hypercalcemia is most commonly associated with squamous cell carcinoma of the lung as opposed to small cell lung cancer[7]. The presence of paraneoplastic hypercalcemia in lung cancer has been associated with poorer survival outcomes[8]. No previous cases have reported DDS developing from PTHrP-induced hypercalcemic pancreatitis. There is no clear consensus on which cause of pancreatitis is most likely to result in DDS. This patient possessed persistent hypercalcemia and an aggressive malignancy, both risk factors for pancreatitis, and in turn, risks for development of DDS.

Patients presenting with acute pancreatitis will often have already received an abdominal ultrasound and/or CT abdomen with and without contrast in the emergency department to visualize causes of pancreatic inflammation. In patients with suspected DDS, MRCP is particularly useful by providing detailed mapping of the pancreaticobiliary ducts[4]. ERCP is no longer routinely used for diagnostic purposes as MRCP can provide the same information without the risks associated with ERCP, but is undertaken with therapeutic intentions such as relieving obstructions *via* stent placement and displaying resolution of obstruction on repeat fluoroscopy[9]. Additionally, endoscopic ultrasound (EUS) is utilized for more accurate visualization of the pancreatic duct and ultrasound-guided drainage of large fluid collections causing obstruction of the duct[10]. For cases of DDS involving WON, endoscopic necrosectomy can be coupled with EUS to relieve obstructions by debriding and opening necrotic septa through gastric or duodenum access[11].

Whenever possible, definitive intervention is delayed 4 wk or more to allow organization of necrotic collections and development of an encapsulating wall[12]. Initially, if the patient is clinically stable, a minimally invasive approach can be performed to relieve ductal compression with endoscopic/percutaneous approaches favored over open surgical necrosectomy[12,13]. If endoscopic interventions are unsuccessful, surgical intervention (*i.e.* necrosectomy, Roux-en-Y, or debridement) is required to relieve obstructions. While data suggests that minimally invasive approaches are superior to surgical intervention for necrosectomy, whether endoscopic or surgical intervention is superior for DDS is still a subject of debate[14,15]. For DDS, endoscopic intervention is typically first-line and less invasive than surgery, but success is dependent on cannulation of narrow strictures and stent placement in cases of ERCP and optimal positioning of lesions for drainage in cases of EUS[14]. Surgical interventions are often successful at relieving obstructions, but often are associated with higher morbidity and mortality compared to endoscopy[11,13,15]. This case demonstrates the approach to a unique case of DDS and highlights the difficulty associated with treatment of DDS. Additionally, this case is evidence of the importance of earlier detection of lesions prior to complete ductal obstructions. In complete pancreatic duct obstructions, ERCP efficacy may be limited and result in patients having to undertake greater morbidity and mortality risks to relieve obstructions.

COMMENTS

Case characteristics

A 38-year-old man with small cell lung cancer presented with acute onset epigastric pain, nausea and vomiting.

Clinical diagnosis

Tenderness in the epigastric region of the abdomen and tachycardia.

Differential diagnosis

Acute hypercalcemic pancreatitis, acute recurrent pancreatitis, gastric ulcer, erosive gastropathy, cholelithiasis, choledocholithiasis.

Laboratory diagnosis

Labs demonstrated lipase 2030 U/L (normal < 90 U/L), serum calcium of 11 mg/dL (normal 8.4-10.3 mg/dL), parathyroid hormone less than 9 pG/mL (normal 12-65 pG/mL), parathyroid-related peptide of 3.9 pmol/L (normal < 2 pmol/L), and normal triglycerides.

Imaging diagnosis

Magnetic resonance imaging with magnetic resonance cholangiopancreatography showed multiple cystic areas with rim enhancement replacing large portions of the pancreatic body with the largest centered in the mid-body of the pancreas measuring 3.5 cm × 6.2 cm compressing the main pancreatic duct as well as a 2 cm × 4.3 cm collection extending into the pancreatic groove.

Treatment

Unsuccessful endoscopic retrograde cholangiopancreatography-guided main pancreatic stent placement followed by successful surgical necrosectomy and cholecystectomy.

Related reports

Disconnected duct syndrome (DDS) is rare syndrome that often presents with recurrent pancreatitis flares. The syndrome is more commonly caused by mass lesions obstructing the main pancreatic duct. Paraneoplastic hypercalcemia is more often associated with squamous cell lung cancer as opposed to small cell lung cancer.

Term explanation

DDS is a pancreatic syndrome where the main pancreatic duct is occluded and pancreatic exocrine flow leaks into the pancreatic parenchyma. This syndrome frequently results in further inflammatory reactions such as sepsis, development of pseudocysts, and fistulizing disease.

Experiences and lessons

Acute recurrent pancreatitis should raise concerns for DDS due to exocrine leakage into pancreatic parenchyma causing repeated inflammatory reactions. Although less common than squamous cell lung cancer, small cell lung cancer can result in paraneoplastic hypercalcemia which can expose patients to prolonged risks of pancreatitis. This prolonged risk of pancreatitis may increase the risk for develop of DDS.

Peer-review

This is an interesting case for physician.

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CASE REPORT

Charcot-Marie-Tooth hereditary neuropathy revealed after administration of docetaxel in advanced breast cancer

Hampig Raphael Kourie, Nicolas Mavroudakis, Philippe Aftimos, Martine Piccart

Hampig Raphael Kourie, Oncology Department, Faculty of Medicine, Saint Joseph University, Beirut 880, Lebanon

Nicolas Mavroudakis, Philippe Aftimos, Martine Piccart, Oncology Department, Jules Bordet Institute, 1000 Brussels, Belgium

Author contributions: Kourie HR and Aftimos P initiated and wrote this case; Mavroudakis N and Piccart M reviewed and commented on this paper.

Institutional review board statement: The Bordet Institute's Ethics Committee provides a favorable opinion on the disclosure/publication of a patient clinical history to be reported as a "case report".

Informed consent statement: The authors undertake to respect the confidentiality, anonymity as well as the quality of the published information, The authors have recorded the consent of the patient in his medical record.

Conflict-of-interest statement: The authors confirm that they do not have any conflict of interest.

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Correspondence to: Hampig Raphael Kourie, MD, MSc, Oncology Department, Faculty of Medicine, Saint Joseph University, Damascus Street, Beirut 880, Lebanon. hampig.kourie@usj.edu.lb
Telephone: +961-3-321899
Fax: +961-1-877787

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Abstract

Charcot-Marie-Tooth (CMT) neuropathy is the most common hereditary cause of neuropathy. Diagnosis is usually not made during the childhood but in adolescence or late adulthood. It is reported in the literature that some neurotoxic chemotherapeutical agents can reveal an asymptomatic CMT IA hereditary neuropathy. To our knowledge, we report here the first case of CMT IA revealed in a 55-year-old woman after the administration of docetaxel/trastuzumab/pertuzumab for metastatic breast cancer. This case stresses again the necessity to obtain a complete personal and familial anamnesis and to perform a neurologic examination before the administration of neurotoxic chemotherapeutical agents to prevent the clinical expression of these hereditary neuropathies.

Key words: Charcot-Marie-Tooth IA; Docetaxel; Breast cancer; Neurotoxicity; Peripheral neuropathy

Core tip: This case report represents the first case of Charcot-Marie-Tooth IA revealed after the administration of docetaxel/trastuzumab/pertuzumab for metastatic breast cancer. This paper will help to focus on the revelation of rare hereditary neuropathies after the administration of chemotherapies.

Kourie HR, Mavroudakis N, Aftimos P, Piccart M. Charcot-Marie-Tooth hereditary neuropathy revealed after administration of

docetaxel in advanced breast cancer. *World J Clin Oncol* 2017; 8(5): 425-428 Available from: URL: http://www.wjgnet.com/2218-4333/full/v8/i5/425.htm DOI: http://dx.doi.org/10.5306/wjco.v8.i5.425

INTRODUCTION

Charcot-Marie-Tooth (CMT) type Ⅰ neuropathy is the most common hereditary cause of neuropathy. Seventy percent to 80% of these patients present the subtype IA. This disease involves the motor and sensory peripheral nerves. Age of onset is variable; diagnosis is usually made between the age of 5 and 25 years. The diagnosis of CMT IA is confirmed with genetic testing and electro-diagnostic studies. There is no approved medical therapy to prevent the progression of CMT[1].

To our knowledge, we report here the first case of CMT IA revealed in a patient after receiving docetaxel/trastuzumab/pertuzumab for metastatic breast cancer. We first discuss the originality of our case and the potential role of chemotherapies in revealing or accentuating these syndromes. Then, we review the reported cases in the literature describing the relationship between these diseases and different chemotherapeutical agents.

CASE REPROT

We present the case of a 55-year-old patient, with unremarkable past medical history, who was diagnosed with HER2-positive and hormonal receptor positive stage Ⅳ metastatic breast cancer in 2014 with a primary tumor of 2.7 cm and one liver metastasis. Her first-line therapy consisted of the standard of care regimen docetaxel combined to trastuzumab and pertuzumab followed by surgery of the primary breast tumor and radiofrequency ablation of the unique metastatic hepatic lesion given an excellent response and the presence of oligometastatic disease.

Chemotherapy was stopped after 6 cycles of docetaxel and the patient was kept on maintenance therapy with trastuzumab and pertuzumab. Three months after the last cycle of chemotherapy, the patient developed numbness in the extremities, generalized depressed tendon reflexes, and hypoesthesia in the lower third of legs. At the clinical examination, pes cavus foot deformity, bilateral foot drop and generalized depressed tendon reflexes were detected.

After a complete anamnesis, the patient mentioned that many members of her family (her sister, her niece and her grand-father) were diagnosed with the CMT type IA disease. A genetic analysis of *PMP22* gene confirmed the diagnosis in our patient and conduction velocity studies demonstrated demyelinating abnormalities concordant with the diagnosis. In fact, multiple ligation-dependent probe amplification of the exons 1-5 of the *PMP22* gene located at 17p11.2 showed genomic duplication comprising the *PMP22* gene.

The patient continued her treatment for breast cancer based on targeted therapies (pertuzumab and trastuzumab) and hormonal therapy (letrozole) and is currently in complete remission. For her stable persistent neurologic deficits physiotherapy was prescribed for maintaining posture and balance, genetic counseling for the family members, namely her two sons, and avoidance of neurotoxic drugs.

DISCUSSION

Neurologic toxicities represent the second most frequent chemotherapy-induced side effect after hematologic toxicities. Vinca-alkaloids, taxanes and platinum-based agents are the most frequent drugs inducing peripheral neurotoxicity. These drugs are used in the treatment of ovarian, breast, lung, prostate, colon and hematological malignancies. Chemotherapy-induced neuropathy is usually dose-dependent. Patients with pre-existing neuropathic symptoms due to diabetes, hereditary neuropathies or earlier treatment with neurotoxic chemotherapy are probably more vulnerable to further development of chemotherapy-induced peripheral neuropathy[2,3].

We report, here, an interesting case of a hereditary CMT IA disease revealed after the administration of docetaxel in combination with anti-HER2 antibodies, in an advanced HER2-positive breast cancer. Our case represents the first case of CMT IA revealed after the administration of docetaxel. It is less likely that this disease was revealed by trastuzumab or pertuzumab, because it was not reported in the literature any CMT related to biologic agents.

A case of an aggravation of CMT after the administration of carboplatin and paclitaxel for ovarian cancer was reported before; the symptomatology resolved after the replacement of paclitaxel by docetaxel, considered as less neurotoxic 4. Thus, our case demonstrates that even less neurotoxic taxanes, as docetaxel, can sometimes reveal or worsen CMT neuropathies.

In some cases, CMT IA has been diagnosed after the administration of neurotoxic drugs including chemotherapeutical agents, mainly vincristine. The cases reported in the literature are summarized in Table 1.

A retrospective case series, in three families with known hereditary neuropathies treated with vincristine, concluded that vincristine in patients with 17p11.2-12 may lead to severe neurotoxicity from vincristine and that this drug should not be administered in patients with CMT1A[5]. On the other hand, a recent study of the Mayo Clinic investigated the association of non-CMT polyneuropathy with *CMT* genes in patients treated with paclitaxel. The results demonstrated a relationship between the *CMT* gene allelic variability and the susceptibility to chemotherapy-induced peripheral neuropathy[6].

This case stresses again the importance for on-

Table 1 Summary of all the reported cases in the literature of Charcot-Marie-Tooth revealed after administration of chemotherapy

Ref.	Patient characteristics	Drug	Malignancy	Signs and symptoms	Diagnosis
Uno *et al*[7], 1999	44 M	Vincristine	NHL	Rapid and marked weakening progressing to quadriplegia and bulbar palsy pes cavus (hollow foot)	Slower nerve conduction velocity 17p11.2-12 duplication
Martino *et al*[4], 2005	F	Paclitaxel/ Carboplatine	Ovarian cancer	Distal sensory and motor neuropathy; Unable to walk, write, or drive	Already diagnosed
Hildebrandt *et al*[8], 2000	52 F	Vincristine	NHL	Dysphagia, dysarthria, muscular weakness of both lower and upper extremities, areflexia, paraesthesia of the fingertips and bilateral sensory impairment of feet and lower legs	Peripheral axonal and demyelinating sensorimotor neuropathy 17p11.2 duplication
Graf *et al*[5], 1996	9 F	Vincristine	Acute lymphoblastic leukemia	Severe acquired weakness, areflexia and distal muscle atrophy	17p duplication
	18 F		Burkitt lymphoma	Pes cavus, distal muscle atrophy and weakness, stocking glove sensory deficits	17p duplication Slower nerve conducting velocity
	46 M		Testicular embryonal cell carcinoma	Foot drop, per cavus and areflexia, marked weakness	Slow motor nerve conduction velocity

NHL: Non-Hodgkin lymphoma; M: Male; F: Female.

cologists to perform a complete anamnesis on past personal and familial history, before the administration of a neurotoxic chemotherapeutical regimen. It is also crucial to perform a complete neurological examination before administrating neurotoxic chemotherapies to avoid the worsening of non-diagnosed peripheral neuropathies.

COMMENTS

Case characteristics

The patient presented numbness in the extremities, generalized depressed tendon reflexes, and hypoesthesia in the lower third of legs, 3 mo after the last cycle of chemotherapy.

Clinical findings

The clinical examination of the patient revealed pes cavus foot deformity, bilateral foot drop and generalized depressed tendon reflexes.

Differential diagnosis

Acute myelitis or Guillain-Barré syndrome are possible differential diagnosis.

Laboratory findings

A multiple ligation-dependant probe amplification of the exons 1-5 of the *PMP22* gene located at 17p11.2 showed genomic duplication comprising the *PMP22* gene.

Pathological diagnosis

Pathological diagnosis was not necessary.

Treatment

Physiotherapy was prescribed for maintaining posture and balance, genetic counseling for the family members, namely her two sons, and avoidance of neurotoxic drugs.

Experiences and lessons

Perform a complete anamnesis on past personal and familial history, before the administration of a neurotoxic chemotherapeutical regimen. Perform a complete neurological examination before administrating neurotoxic chemotherapies to avoid the worsening of non-diagnosed hereditary peripheral neuropathies.

Peer-review

This manuscript presents the first case of Charcot-Marie-Tooth disease identified after the administration of docetaxel in combination with anti-HER2 antibodies. This is an important and interesting report of a rare case.

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Contents

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MINIREVIEWS

Contents

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MINIREVIEWS

Epidemic of non-alcoholic fatty liver disease and hepatocellular carcinoma

Adnan Said, Aiman Ghufran

Adnan Said, Division of Gastroenterology and Hepatology, Department of Medicine, William S. Middleton VAMC, University of Wisconsin School of Medicine and Public Health, Madison, WI 53705, United States

Aiman Ghufran, Gastroenterology and Hepatology, Medical College of Wisconsin, Milwaukee, WI 53226, United States

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Correspondence to: Adnan Said, MD, MS, Associate Professor, Division of Gastroenterology and Hepatology, Department of Medicine, William S. Middleton VAMC, University of Wisconsin School of Medicine and Public Health, 4223 MFCB, 1685 Highland Avenue, Madison, WI 53705,
United States. axs@medicine.wisc.edu
Telephone: +1-608-2634034
Fax: +1-608-2655677

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Abstract

Non-alcoholic fatty liver disease (NAFLD) associated hepatocellular carcinoma (HCC) incidence is increasing worldwide, paralleling the obesity epidemic. Although most cases are associated with cirrhosis, HCC can occur without cirrhosis in NAFLD. Diabetes and obesity are associated risk factors for HCC in patients. Given the sheer magnitude of the underlying risk factors (diabetes, obesity, non-cirrhotic NAFLD) screening for HCC in the non-cirrhotic population is not recommended. Optimal screening strategies in NAFLD cirrhosis are not completely elucidated with Ultrasound having significant limitations in detection of liver lesions in the presence of obesity and steatosis. Consequently NAFLD-HCC is more often diagnosed at a later stage with larger tumors and reduced opportunities for curative treatments as opposed to HCC in other causes of cirrhosis. When HCC is found at a curative stage treatments including liver transplantation, resection and loco-regional therapies are associated with good results similar to that seen in HCV-HCC. Future strategies under study include the use of chemopreventive and antioxidant agents to reduce development of cirrhosis and non-alcoholic steatohepatitis (NASH). Strategies to reverse NASH *via* weight loss, control of associated conditions like diabetes are key strategies in reducing the increasing incidence of NASH-HCC. Novel therapeutic agents for NASH are in trials and if successful in achieving reversal of NASH will be an important strategy in reducing NAFLD-HCC.

Key words: Non-alcoholic fatty liver disease; Hepatocellular carcinoma; Screening; Epidemiology; Pathophysiology; Diagnosis; Liver transplant; Resection; Locoregional therapy; Treatment

Core tip: Non-alcoholic fatty liver disease (NAFLD) related hepatocellular carcinoma (HCC) is rapidly increasing worldwide. HCC in NAFLD is often detected at

a more advanced stage than in hepatitis C virus (HCV). Challenges include earlier recognition of cirrhosis in NAFLD to allow earlier screening for liver cancer. NAFLD also has a higher proportion of HCC occurring in the absence of cirrhosis. Given the sheer number of patients with non-cirrhotic NAFLD, screening for HCC in this population is not practical. Instead prevention and treatment of non-alcoholic steatohepatitis to prevent cirrhosis should be an important strategy. When NAFLD-HCC is found at a curative stage, results with liver transplant, resection and loco-regional therapy are similar to that seen in HCV-HCC.

Said A, Ghufran A. Epidemic of non-alcoholic fatty liver disease and hepatocellular carcinoma. *World J Clin Oncol* 2017; 8(6): 429-436 Available from: URL: http://www.wjgnet.com/2218-4333/full/v8/i6/429.htm DOI: http://dx.doi.org/10.5306/wjco.v8.i6.429

INTRODUCTION

Non-alcoholic fatty liver disease (NAFLD) has become the leading chronic liver disorder in the developed world, with a worldwide prevalence ranging from 6% to 35%[1]. The incidence of NAFLD is continuing to rise worldwide, paralleling the epidemic of metabolic syndrome worldwide. NAFLD is caused by an insulin-resistant state, occurring in the presence of diabetes, obesity, and metabolic syndrome[2].

EPIDEMIOLOGY

The incidence of hepatocellular carcinoma (HCC) in the United States and worldwide continues to rise, with an age-adjusted incidence rising from 1.5 to 6.7 per 100000 individuals in the past 30 years[3,4]. The most recent Surveillance, Epidemiology and End Results (SEER) registry data show that the rates for new liver and intrahepatic bile duct cancer cases have been rising on average 3.0% each year over between 2004 and 2013 (Figure 1)[5]. Most cases of HCC in the United States occur over age 50, with over 70% of cases occurring in men[4] and the ethnics groups with the highest incidence rates in the United States are Asian/Pacific Islanders and Hispanics (Table 1)[6].

In the United States, SEER registries 4929 cases of HCC between 2004-2009 were examined[7]. 14.1% of HCC were due to NAFLD and between 2004-2009 NAFLD-HCC showed a 9% annual increase. However, the rise in incidence now may be plateauing in the United States[8].

RISK FACTORS AND PATHOGENESIS

Cirrhosis is the most common underlying cause of HCC, with 80%-90% of patients diagnosed with HCC having underlying cirrhosis[9]. There is new data emerging to suggest that NAFLD may be an independent risk factor for HCC, even in the absence of cirrhosis[10-12] (Figure 2).

Diabetes and obesity are known independent risk factors for the development of HCC. There appears to be a common pathway *via* insulin resistance and its subsequent inflammatory cascade in the development of NASH and HCC. HCC is increased in patients with diabetes[13,14] as well as obesity[15]. In a prospective United States study of more than 900000 adults, overweight and obesity were associated with excess cancer mortality with an Odds ratio of 4.52 for liver cancer mortality in men and an Odds ratio of 1.68 in women. In a case control study of HCC, diabetes was associated with a 2-3 fold increased risk of HCC regardless of presence of other risk factors[8].

Genetic polymorphisms (I148M) in the gene encoding patatin-like phospholipase domain-containing protein 3 (PNPLA3) is a known risk factor for histologic steatosis as well as NASH, fibrosis and cirrhosis[16]. It has now also been shown to be an independent risk factor for development of HCC with a meta-analysis showing that PNPLA3 rs738409 SNP is associated with an Odds ratio of 1.40 for HCC in cirrhosis including NAFLD[17]. The common genetic mutations of hemochromatosis (C282Y and H63D) have been implicated in risk of developing NAFLD and HCC as well. In a recent meta-analysis a significantly increased risk of NAFLD and HCC was discovered. H63D polymorphism was associated with increased risk of developing non-cirrhotic HCC in the African population[18]. Putatively this is due to increased iron overload leading to hepatic inflammation, fibrosis and carcinogenesis.

Insulin resistance also leads to release of free fatty acids (FFA) and other reactive oxygen species that cause oxidative stress and inflammation. Trans-4-hydroxy-2-nonenal, a product of lipid peroxidation has been shown to cause mutations of the p53 tumor suppressor gene that is associated with more than half of human cancers including HCC[11]. The inflammation caused by oxidative stress leads to an increased release of inflammatory and inhibitory cytokines including tumor necrosis factor-α (TNF-α), interleukin-6 (IL-6), and nuclear factor kappa B (NF-κB)[19]. Presence of these chemical mediators leads to hepatocyte death, compensatory proliferation, and ultimately carcinogenesis.

Leptin is a pro-inflammatory adipokine that is elevated in patients with NAFLD[20]. Leptin is associated with increasing expression of pro-inflammatory cytokines like TNF-α and IL-6[21], and activation of Janus Kinase (JAK)[22]. It can cause tumor growth and has been associated with HCC recurrence after treatment[23]. Adiponectin is an anti-inflammatory adipokine that has been shown to inhibit angiogenesis *via* modulation of apoptosis in an animal model. Adiponectin deficiency in obese states has been linked to carcinogenesis[24]. It is specific to adipose tissue and is decreased in insulin-resistant states, and thus may potentially play a role in development of HCC.

HCC IN NAFLD WITH ADVANCED FIBROSIS

NAFLD associated HCC occurs in patients with cirrhosis

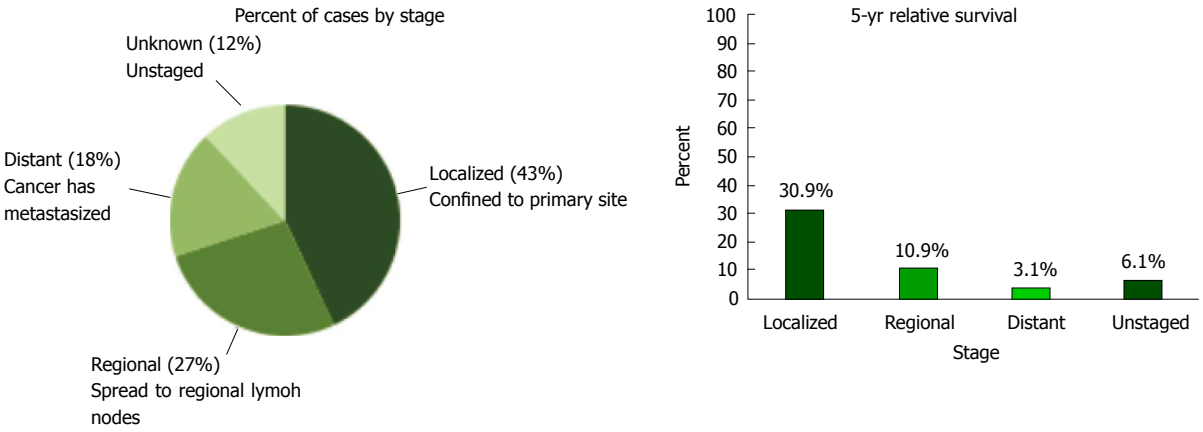

Figure 1 Percent of cases and 5-year relative survival by stage at diagnosis: Liver and intrahepatic bile duct cancer.

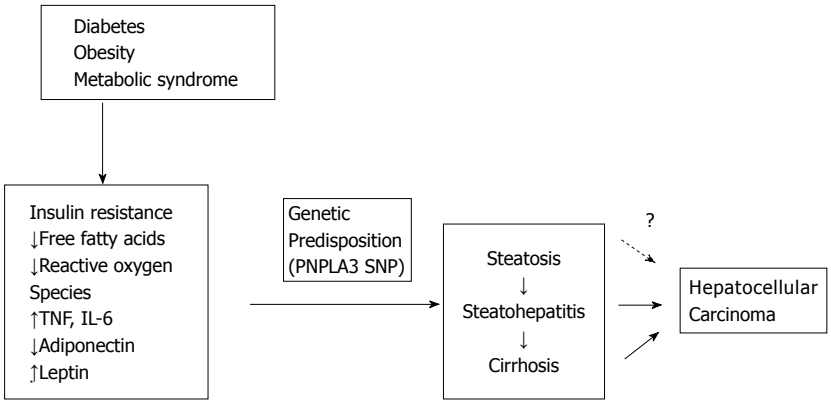

Figure 2 Development of hepatocellular carcinoma in non-alcoholic fatty liver disease.

at variably reported rates. A single center cohort study reported HCC at an annual incidence of 2.6%[25] compared to a 4% incidence in those with hepatitis C related cirrhosis. Other studies have reported overall incidence of HCC in NAFLD Cirrhosis from 2.4% at 7 years to 12.8% over 3 years[26].

Given the rising incidence of NAFLD, and the advances in curative options for Hepatitis C infection, NAFLD is expected to become the leading cause of HCC in developed nations[10]. Known risk factors for HCC in cirrhotic NAFLD include male gender, age over 70, and underlying diabetes and hypertension[27].

HCC IN NAFLD WITHOUT ADVANCED FIBROSIS

There is increasing evidence to suggest that NAFLD contributes to non-cirrhotic HCC as well[10]. Also, while the presence of hepatic steatohepatitis is an established risk factor for development of cirrhosis and HCC[28], there are case reports of HCC complicating underlying NAFLD in the absence of hepatitis or advanced fibrosis, making associations between steatosis, steatohepatitis, cirrhosis, and HCC complex[10].

While several studies have reported HCC in non-cirrhotic NAFLD the risk seems to be lower (0% to 3% over 20 years)[26]. Recent studies however have shown that non-cirrhotic HCC may be more common in NAFLD compared to other chronic liver diseases. Studies from multiple countries including United States, Japan and France of NAFLD-associated HCC have shown that a significant proportion of HCC occurs in the absence of cirrhosis in NAFLD. In a histologic analysis of resected NAFL-HCC from France the majority of patients did not have cirrhosis and 65% had stage 0-2 fibrosis[29]. In the study from Japan of 87 HCC patients only 51% had histologic cirrhosis and 28% had stage 1-2 fibrosis only[27]. In a United States veterans Study of 1500 HCC cases, 8% of the HCC cohort had NAFLD associated HCC and only 65% of the NAFLD-HCC cohort had cirrhosis (NAFLD had > 5 fold risk of HCC without cirrhosis compared to HCV related cirrhosis[30].

Several large-scale epidemiological studies have shown that there is a higher incidence of HCC in patients with obesity and diabetes, as well as poorer outcomes[10]. The relative risk of liver cancer was 117% for overweight subjects and 189% for the obese[31], while risk of mortality in men with a BMI > 35 kg/m^2 can be as high as 4.5 times that in men with a normal BMI[15]. Similarly, presence of diabetes alone increases the risk of development of HCC three-fold[8]. Other independent risk factors for HCC in NAFLD include

Table 1 Age-adjusted hepatocellular carcinoma incidence and liver cancer mortality rates per 100000 persons, 2006-2010[5]

Outcome	Age (yr)	All races		None-hispanic						Hispanic	
				White		Black		API			
		Rate	95%CI	Rate	95%CI	Rate	95%CI	Rate	95%CI	Rate	95%CI
HCC	Overall	5.9	(5.8-5.9)	4.2	(4.2-4.3)	7.5	(7.3-7.8)	11.7	(11.3-12.0)	9.5	(9.3-9.8)
Incidence	35-49	2.2	(2.1-2.3)	1.4	(1.3-1.5)	2.5	(2.2-2.8)	4.7	(4.3-5.2)	3.2	(2.9-3.4)
SEER 18	50-64	16.5	(16.2-16.8)	12.2	(11.9-12.6)	26.9	(25.8-28.1)	23.5	(22.4-24.7)	24.3	(23.3-25.3)
	> 65	22.3	(21.9-22.7)	16.0	(15.5-16.4)	22.4	(20.9-23.9)	54.7	(52.4-57.0)	40.5	(38.7-42.4)
Liver cancer	Overall	4.3	(4.3-4.3)	3.6	(3.5-3.6)	6.4	(6.3-6.6)	8.2	(7.9-8.4)	7.0	(6.9-7.2)
Mortality	35-49	1.2	(1.2-1.2)	0.9	(0.8-0.9)	2.0	(1.9-2.2)	2.8	(2.6-3.1)	1.4	(1.3-1.5)
US	50-64	9.7	(9.5-9.8)	7.7	(7.6-7.8)	18.6	(18.2-19.1)	13.0	(12.4-13.6)	13.5	(13.0-13.9)
	> 65	20.1	(19.9-20.3)	17.2	(17.0-17.5)	24.5	(23.7-25.3)	43.2	(41.6-44.8)	36.7	(35.6-37.8)

API: Asians and Pacific Islanders; HCC: Hepatocellular carcinoma; SEER: Surveillance, Epidemiology and End Results.

polymorphisms in the PNPLA3 gene (I148M)[32]. In the most recent met-analysis, HFE mutation (C282Y and H63D) was also shown to be associated with HCC risk in NAFLD populations including for the H63D mutation in non-cirrhotic African populations[18].

CLINICAL PRESENTATION AND DIAGNOSIS

In the US SEER registries 4929 cases of HCC between 2004-2009 were examined[7]. Fourteen point one percent were due to NAFLD. Patients with NAFLD-HCC were older, had more advanced tumor stage at presentation and had shorter survival time and were less likely to receive a liver transplant.

These findings are seen in non-United States centers as well. In a large retrospective cohort study from Germany, of 1119 patients with HCC, those with NASH-HCC were older, had higher metabolic complications but better liver function at presentation[33]. Resection was performed in only 17.8% and transplant in 4.4% of these patient and overall survival for NAFLD-HCC was lower than that for HCV-HCC.

An Italian multicenter observational study of NAFLD-HCC *vs* HCV-HCC was performed[34]. Compared to HCV-HCC, NAFLD-HCC was more again likely associated with larger tumors, more infiltrative tumors and was more likely to be detected outside surveillance. Survival was significantly shorter in NAFLD-HCC (25.5 mo *vs* HCV-HCC (33.7 mo) regardless of tumor stage. However analysis of patients with HCC in Milan criteria sent for curative treatments showed similar survival in NASH-HCC and HCV-HCC (38.6 mo *vs* 41 mo).

In a study from 2 centers in the United Kingdom, 275 HCV related HCC patients were compared with 212 NAFLD related HCC patients. Patients with NAFLD-HCC had lower rates of cirrhosis and were significantly older and had larger tumors than those with HCV-HCC. Those with NAFLD-HCC were less likely to receive curative therapy than HCV-HCC including liver transplant (21/212 of NAFLD-HCC) *vs* 80/275 of HCV-HCC. Despite this overall survival from diagnosis was similar for NAFLD-HCC (56% at 1year and 23% at 3 years) and HCV-HCC (58% at 1 year and 21% at 3 years)[35].

The reasons for these tumor stage differences are multi-factorial. The current AASLD Guidelines recommend that patients with cirrhosis undergo regular surveillance for HCC with ultrasound every 6 mo[36]. While this may suffice in most patients with cirrhosis, patients with NASH are often obese, thus limiting the diagnostic ability of ultrasound[37]. The ITALICA study group showed that HCC was significantly less likely to be diagnosed during surveillance in patients with cryptogenic cirrhosis compared to HCV patients, translating into a greater prevalence of advanced HCC stage and poor survival[38]. MRI, therefore, may offer a better enhanced surveillance for HCC but comes at a higher cost and reduced accessibility.

Underdiagnosis of cirrhosis is a common problem as it leads to lack of screening and potentially increased stage of HCC when diagnosed resulting in worse outcomes. In a large cohort of HCC patients (1201) in the VA, 24.6% had undiagnosed cirrhosis prior to HCC diagnosis and patients with NAFLD had higher odds of having undiagnosed cirrhosis (OR = 4.77)[39].

HCC TREATMENT IN NASH

Similar to other causes of HCC, NAFLD associated HCC occurs in the context of liver disease and liver function and portal hypertension are integral in multimodality assessment and treatment of HCC.

In addition to actual tumor stage, the severity of liver impairment also affects the available options for management of HCC. Thus, the most commonly used schema for management of HCC is Barcelona Clinic Liver Cancer (BCLC)[36,40] which incorporates both stage of the tumor as well as disease severity of underlying liver impairment (Figure 3)[36].

As per current SEER data, 42.9% of patients with HCC are diagnosed at the local stage, and the 5-year survival for localized liver and intrahepatic bile duct cancer is 30.9%[5]. Curative therapies include resection for early stage HCC with thermal ablative therapies also showing comparable results for single small tumors. Liver transplantation is a good option for patients with Milan (T2) criteria tumors who have complications of

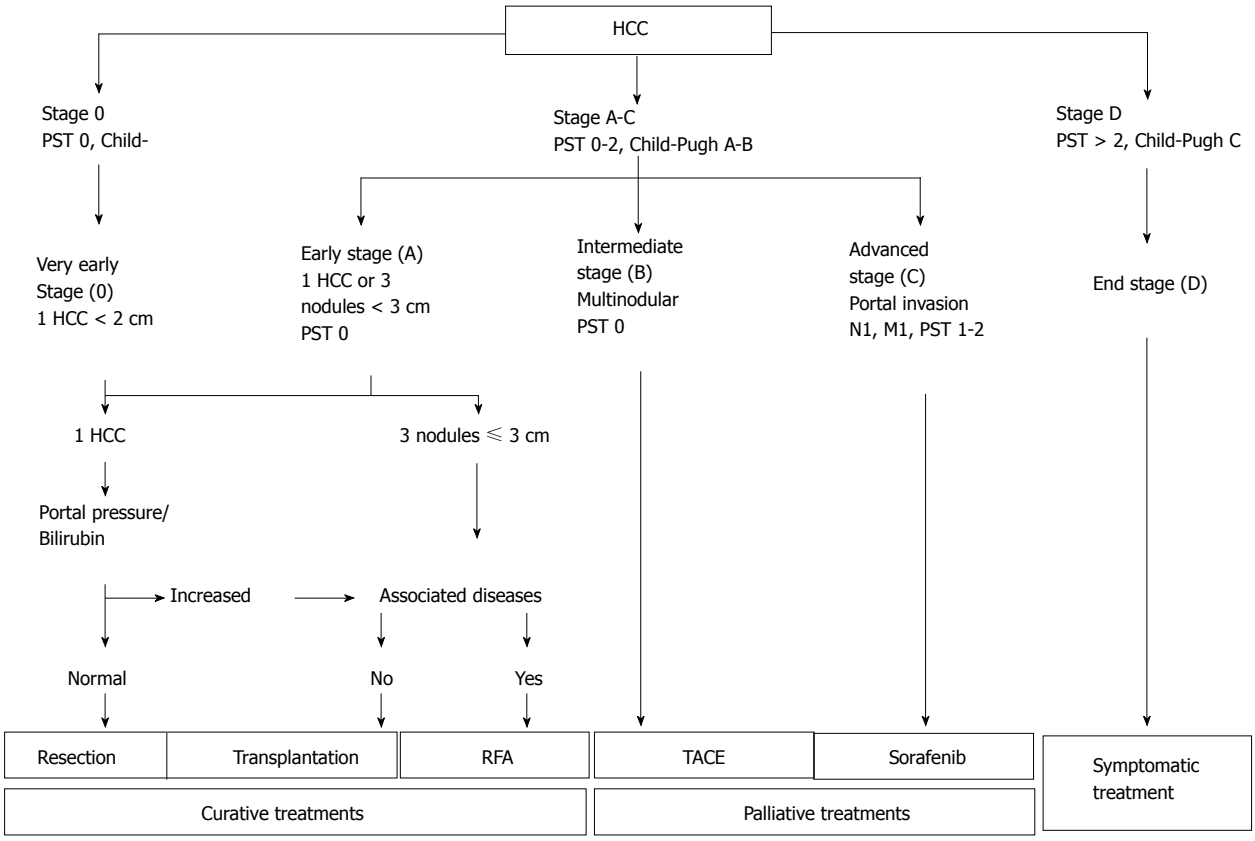

Figure 3 Barcelona clinic liver cancer staging system[36]. HCC: Hepatocellular carcinoma; TACE: Trans-arterial chemoembolization.

cirrhosis and portal hypertension[41].

Patients with larger tumors can be candidates for intra-arterial therapy including trans-arterial chemoembolization (TACE) and Y-90 radioembolization that are utilized with goals of tumor control and not always for potentially curative ends. Thus finding tumors that are small and limited to the liver offers patients the widest array of potential treatments and hope of cure[42].

Liver transplant and resection

With the growing epidemic of NAFLD, NAFLD associated HCC is the second most common indication for liver transplant (LT) for HCC in the United States after HCV since 2006, increasing 4 fold since 2002[43]. In this study, Wong *et al*[43] analyzed 10061 patients with HCC who underwent LT for HCC between 2002-2012. Since MELD system was implemented in 2002, HCC related liver transplants increased significantly from 3.3% (*n* = 143) of all LT in 2002 to 23.3% (*n* = 1336) in 2012. NASH related HCC also increased significantly from 8.3% of all HCC related LT in 2002 to 13.5% in 2012 and were the second leading reason for HCC related LT behind HCV.

In a study from 2 United Kingdom liver transplant centers between 2000 and 2014, 487 patients with HCC associated with NAFLD or HCV presented to the transplant centers. 275 had HCC secondary to HCV and 212 secondary to NAFLD[35]. Patients with NAFLD were significantly older than HCV patients at time of HCC diagnosis (69.6 years *vs* 58.6 years). Absence of cirrhosis was more common in NAFLD patients (13%) *vs* HCV patients (1%). The non-cirrhotic patients were more likely to be older, have DM and had larger tumor size (likely due to non-surveillance). NAFLD patients had significantly larger tumors at presentation and were less likely to receive liver transplant than HCV-HCC patients and were more likely to receive TACE. Overall survival was however similar between NAFLD and HCV HCC at 3 years from diagnosis (21% and 23%).

Outcomes for LT for NAFLD-HCC were compared in a US study with LT for HCC-HCV and HCC-ALD. Over a 50 mo median follow up there was no difference in tumor free survival and overall survival (curative treatments *vs* HCV, ALD)[44].

In an Italian study that compared resection in HCC-associated with metabolic syndrome with HCC-HCV outcomes were similar in 96 HCC-Metabolic Syndrome and 96 HCC-HCV patients after resection. All patients had Child A cirrhosis and operative mortality was 2.1% (similar between the 2 groups). Morbidity and liver failure rates were also similar and were impacted by cirrhosis need for major hepatectomy and MELD score but not by histologic steatohepatitis. Five year overall survival was better in HCC-Metabolic Syndrome *vs* HCC-HCV (65.6% *vs* 61.4%, $P = 0.03$)[45].

Reddy *et al*[44] reported 3-year survival after resection was better in NASH *vs* HCV-ALD patients (60.9% *vs* 36.2%) including on multivariable analysis. Therefore

curative treatments like resection are an acceptable treatment for NASH patients.

Locoregional therapies

Liver-directed therapy with percutaneous ablation is a frontline therapeutic option in patients with HCC in its early stage. These options include ablation by chemical agents (acetic acid or ethanol) or by heat (radiofrequency, microwaves, laser, or cryotherapy). The efficacy of these therapies is followed by contrast-enhanced CT scan, with lack of contrast uptake suggestive of adequate response[36]. NASH-HCC outcomes with ablation are reported to be as efficacious as HCV-HCC or ALD-HCC[44].

Non-curative options

TACE and Sorafenib are available non-curative options for HCC[36,40]. TACE may sometimes be employed to downstage a tumor prior to use of transplantation as a curative option.

Chemoprevention

Given the current limitation in treatment options, there is currently an interest in targeting the known molecular pathways as treatment options for HCC. NAFLD and obesity associated HCC has been linked to oxidative stress, hyperinsulinemia, and chronic inflammation. Addressing metabolic syndrome can be a preventative measure against development of HCC. These options include, but are not limited to, exercise, weight loss, and optimal control of diabetes, and hypertension, if present. In fact, one study showed a lower relative risk of developing HCC in vigorously active subjects compared to those with a sedentary lifestyle[46]. This reduction in risk was found to be independent of BMI.

In preliminary studies, dietary antioxidants like, vitamin C and E, selenium and coenzyme Q, vitamin D supplementation, and a Mediterranean diet have been shown to prevent hepatic carcinogenesis[47]. This is of particular interest given the known role of antioxidants in limiting and even reverting fibrosis in patients with NASH[48]. Similarly, the use of metformin, known to reduce insulin resistant and subsequent steatohepatitis, has been shown to be associated with reduced incidence of HCC in diabetic patients[47].

In animal models anti-inflammatory and anti-oxidant compounds like green tea, BCAA and acyclic retinoids have shown promise in preventing HCC. BCAA supplementation in cirrhosis is associated with improvements in insulin resistance and inhibition of IGF-1 and IGF-2 expression in the db/db obese mouse liver and reduced expression of liver cancers[49]. In Human study long term supplementation with BCAA reduced HCC in obese patients with cirrhosis[50]. Green tea extracts have been associated with beneficial effects on weight loss, insulin resistance and inflammatory cytokines in animal models[51]. In animal models beneficial effects on carcinogenesis have been reported as well by modulation of tyrosine kinase and Pi13/AKT pathways[52] and in reducing hepatic tumors in DEN treated db/db/mice. With green tea extracts no human data have been published showing efficacy. Acyclic Retinoids derivates of vitamin A exert their effect through nuclear receptors including RXRalpha, which is found in abundant supply in human liver. Supplementation of retinoids has shown beneficial effects in maintaining hepatocyte homeostasis in hepatocyte carcinogenesis[53]. In a long term human study acyclic retinoids reduced the chance of HCC recurrence and death by 40%[54]. Given the significant association of diabetes with HCC, metformin has been studied in cohort and case-control studies and a meta-analysis showed a significantly reduced risk of HCC with metformin use in diabetics[55].

CONCLUSION

In summary, there is indisputable evidence showing the increased risk of HCC in patients with NAFLD regardless of the presence of advanced fibrosis and steatohepatitis. Patients with HCC and NAFLD are increasing more rapidly than any other indication for liver transplantation. Patients with NAFLD are candidates for curative and non-curative therapies with encouraging results.

Diagnosing HCC in advanced stages of tumor or liver disease can render curative options futile and call for development of alternate guidelines for enhanced HCC surveillance in patients with metabolic syndrome. The current challenges include developing optimal surveillance options in targeted populations. There is also a huge potential in development of therapies targeting NASH and molecular pathways as preventive options for HCC in patients with cirrhosis in general and NAFLD in particular.

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MINIREVIEWS

Metronomic chemotherapy for non-metastatic triple negative breast cancer: Selection is the key

Connie Rabanal, Rossana Ruiz, Silvia Neciosup, Henry Gomez

Connie Rabanal, Rossana Ruiz, Silvia Neciosup, Henry Gomez, Department of Medical Oncology, Instituto Nacional de Enfermedades Neoplasicas, Lima 15038, Peru

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Correspondence to: Connie Rabanal, MD, Department of Medical Oncology, Instituto Nacional de Enfermedades Neoplasicas, Av. Angamos Este 2520 Surquillo, Lima 15038, Peru. crabanal@auna.pe
Telephone: +51-1-921139544

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Abstract

Triple negative breast cancer (TNBC) accounts for 15%-20% of all breast cancer, and is still defined as what it is not. Currently, TNBC is the only type of breast cancer for which there are no approved targeted therapies and maximum tolerated dose chemotherapy with taxanes and anthracycline-containing regimens is still the standard of care in both the neoadjuvant and adjuvant settings. In the last years, metronomic chemotherapy (MC) is being explored as an alternative to improve outcomes in TNBC. In the neoadjuvant setting, purely metronomic and hybrid approaches have been developed with the objective of increasing complete pathologic response (pCR) and prolonging disease free survival. These regimens proved to be very effective achieving pCR rates between 47%-60%, but at the cost of great toxicity. In the adjuvant setting, MC is used to intensify adjuvant chemotherapy and, more promisingly, as maintenance therapy for high-risk patients, especially those with no pCR after neoadjuvant chemotherapy. Considering the dismal prognosis of TNBC, any strategy that potentially improves outcomes, specially being the oral agents broadly available and inexpensive, should be considered and certainly warrants further exploration. Finally, the benefit of MC needs to be validated in properly designed clinical trials were the selection of the population is the key.

Key words: Metronomic chemotherapy; Triple negative breast cancer; Neoadjuvant; Adjuvant; Maintenance

Core tip: Triple negative breast cancer (TNBC) is the only type of breast cancer for which there are no approved targeted therapies. Metronomic chemotherapy (MC) is being explored as an alternative to improve outcomes in TNBC. In neoadjuvant setting, purely metronomic and hybrid approaches achieve complete pathologic response (pCR) rates between 47%-60%, but at the cost of great toxicity. In the adjuvant setting, MC is used to intensify adjuvant chemotherapy and, promisingly, as maintenance therapy for high-risk patients, especially those with no pCR. Considering the dismal prognosis of TNBC, any

strategy that improves outcomes, specially being broadly available and inexpensive, should be considered.

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INTRODUCTION

Triple negative breast cancer (TNBC) accounts for 15%-20% of all breast cancer cases and is still defined as what it is not[1]. This entity is a molecularly heterogeneous and generally aggressive disease with poor survival[2]. Currently, TNBC is the only type of breast cancer for which there are no approved targeted therapies and maximum tolerated dose (MTD) chemotherapy with taxanes and anthracycline-containing regimens is still the standard of care in both the neoadjuvant and adjuvant settings[3]. Nowadays, there is no evidence that prolonging treatment or escalating doses confers any benefit[4].

In the last years, aiming to improve responses in TNBC and because of the lack of target therapies, metronomic chemotherapy (MC) has being explored. In the neoadjuvant setting, purely metronomic and hybrid (approach which includes combined MTD chemotherapy with MC) neoadjuvant regimes, have been developed with the objective of increasing pathologic complete response (pCR) and prolonging disease free survival (DFS).

In the adjuvant setting, MC is used to intensify adjuvant chemotherapy and, more interestingly, as maintenance therapy for high-risk patients, especially those with no pCR after neoadjuvant chemotherapy.

This review outlines the rationale, preclinical data and relevant clinical trials of MC for TNBC as a promising alternative in selected populations, considering it economic viability for our health system care.

UNDERSTANDING METRONOMIC CHEMOTHERAPY

The term MC was first used by Hanahan in 2000, referring to the "close, regular administration of a chemotherapeutic drug for a long time with no extended drug-free breaks"[5]. It was originally conceived as a strategy to break resistance to chemotherapy by targeting the tumor vasculature instead of the tumor cells[5].

MTD-based conventional chemotherapy regimens aim to eliminate as many tumor cells as possible by causing direct or indirect damage to their DNA, and thus disrupting its replication in proliferating cells. Due to the low proliferation index of endothelial cells, conventional MTD chemotherapy causes very limited damage on them[6,7]. Moreover, as the antiangiogenic effect is not sustained, endothelial cells recover during the rest periods, supporting tumor regrowth and therefore contributing to tumor resistance. Using drugs at a low dose, decreases toxicity and allows continuous administration to overcome this effect[8]. It has also been reported that in mice with tumor resistance to MTD chemotherapy, exposure to the same drugs, at lower but frequent doses, can achieve a response[9].

One disadvantage of this regimen is the empiricism in finding the optimal "low dose" or "optimal biologic dose" (OBD)[10]. Shaked *et al*[11] have investigated pharmacodynamic cellular biomarkers for determining OBD of different metronomic regimens based in sustained declines in circulating VEGFR-2$^+$ endothelial progenitor cells induced by prolonged daily low dose metronomic chemotherapy.

In Table 1, we compare MTD chemotherapy *vs* MC. MC is considered as a multi-mechanism therapy.

Inhibition of angiogenesis

The benefit of MC is mainly attributed to its direct activity on the drug-sensitive tumor endothelial cells. MC has been shown to reduce the angiogenic potential by decreasing in levels and viability the sustained of bone marrow – derived endothelial progenitor cells, producing vessel normalization, increasing tumor perfusion and thrombospondin 1 (THBS-1) which is an antiangiogenic glycoprotein responsible of inhibiting the circulating endothelial cell[12,13].

In animal models, it has been demonstrated that low dose cyclophosphamide induces apoptosis in endothelial cells of the tumor microvasculature, compromising DNA repair processes, and therefore inducing a prolonged antiangiogenic effect[8]. Also, Browder *et al*[14] showed metronomic cyclophosphamide (CTX) was effective against drug-resistant lung and breast carcinoma cell lines.

Activation of immunity

It is a well-known fact that tumor cells escape from the immune system surveillance and that immuno-suppression caused by chemotherapy, contributes to tumor growth[15]. Nevertheless, it has been recently suggested that certain cytotoxic drugs such as cyclo-phosphamide, anthracyclines and taxanes may also have immuno-stimulatory properties, specifically due to their effect on regulatory T (T-reg) cells which are CD4$^+$CD25$^+$ lymphocytes enriched with tumor necrosis factor receptor (TNF) and cytotoxic T lymphocyte associated antigen 4 (CTLA4)[16].

T-reg cells inhibit immune responses depending on cytokines and on antigen-specific-dependent pro-cesses[17]. In particular, they suppress lymphocytes CD8$^+$, CD4$^+$ T helper and natural killer T cells[17]. It has been demonstrated that T-reg cells increase alongside tumor upstaging and their presence is associated to

Table 1 Comparing maximum tolerated dose chemotherapy *vs* metronomic chemotherapy

	Maximum tolerated dose chemotherapy (conventional)	Metronomic chemotherapy
Dose	High doses	Low doses or biologic optimal doses
Administration	Administered at defined intervals (3 weekly, weekly) determined by the recovery of bone marrow	Dosing frequency is continuous (weekly, every other day, daily)
Plasma concentration	Rise and fall of the plasma concentration of the drug	Sustained plasma concentration of the drug
Target	Proliferating tumor cells	Endothelial cells in the growing vasculature of the tumor
Toxicity	Acute and cumulative toxicity is a concern	Acute toxicity is rare. Cumulative toxicity is unknown, except for etoposide (related to leukemia)

poor response to treatment[18]. In comparison with tumors exposed to MTD regimens, those exposed to MC exhibit a markedly reduced number of T-reg cells[19]. Tanaka *et al*[20] analyzed the activity of 54 different drugs effect *in vitro* dendritic cells, concluding that vinblastine, etoposide and paclitaxel, administered in low doses, decreased the levels of T-reg cells and delayed tumor progression.

Induction for tumor dormancy

Tumor dormancy was defined by Willis in 1940s and redefined by Hadfield in the early 1950s as a temporary mitotic and growth arrest[21]. Dormant cells are present in the early phase of tumor progression or after completing treatment. In the early phase, epithelial pre-invasive lesions can undergo epithelial-mesenchymal transition, and then acquire metastatic growth capacity after long periods of dormancy[22]. After completing treatment, dormant tumor cells may be the source of tumor recurrence, suggesting that these could became refractory to conventional treatment[23,24]. Folkman *et al*[25] showed that metronomic activity induces tumor dormancy, being this the predominant mechanism involved in maintaining the avascular phase. So, when a tumor escapes from the immune surveillance, MC can inhibit tumor development and achieve a long-term control of the disease[26].

The "4D" Effect

Clinical studies demonstrated that a long exposure to one or more agents and deprivation of others, introducing break periods of MTD with MC, may increase treatment efficacy. This phenomenon is named 4D effect or drug-driven dependency/deprivation effect[27,28]. André *et al*[29] postulated that tumor cells become dependent on chemotherapeutic agents during long exposures and sudden withdrawal or replacement therapy may lead to cell death.

METRONOMIC CHEMOTHERAPY IN TBNC

Neoadjuvant setting

Specially in TNBC, neoadjuvant chemotherapy is effective in down staging the tumor, therefore allowing breast conserving procedures or surgery in initially irresectable tumors. Additionally, neoadjuvant chemotherapy permits an early evaluation of the effectiveness of systemic therapy *in vivo*. Achieving a pCR is a surrogate marker for prolonged DFS, and less local and distant recurrence[30,31].

For TNBC, MTD chemotherapy based in anthracyclines and taxanes is still the standard of care. The rate of pCR with this combination ranges between 20% and 39%[32]. In the most successful experience, von Minckwitz *et al*[33] reported a pCR of 39% in 509 patients treated with TAC (docetaxel/doxorubicin/cyclophosphamide). The rate of pCR has been reported to further increase with the addition of platinum salts. Nevertheless, an important proportion of patients would still have residual disease at the end of neoadjuvant treatment. In order to improve the results, several groups have tried to intensify the induction chemotherapy regimens by incorporating metronomic principles. These schemes use conventional drugs at metronomic doses or combine MTD chemotherapy with MC in a hybrid approach (Table 2).

Metronomic-only approach

Interestingly the studies presented below incorporate platinum salts to conventional drugs in a metronomic approach. It should be recalled, that although the GeparSixto results demonstrated that platinum salts increase responses, this practice is still not a standard for TNBC[33].

A small phase Ⅱ trial NCT00542191, recently presented at ASCO 2016, used weekly doxorubicin and daily oral cyclophosphamide followed by weekly paclitaxel and carboplatin as neoadjuvant treatment in 18 patients. The pCR rate was 47.6% with a 5-year Overall Survival (OS) of 90% for those who achieved a pCR *vs* 12.5% for those who did not. However, 62% of patient experienced grade (G) 3 or G4 neutropenia, 24% febrile neutropenia, 12 patients discontinued treatment due to related toxicities and 3 died before completing treatment[34]. A similar regimen was previously tested by Tiley in 2012, achieving a pCR of 46% (40% pCR, 6.6% CR with foci of ductal carcinoma in situ). Granulocyte colony stimulating factor was added for absolute neutrophil count (ANC) $\leqslant$ 1000. Main toxicities were related to mielosuppresion and two patients came off study due to prolonged neutropenia. Five patients had G4 neutropenia, 1 patient experienced G3 thrombocytopenia, and 1 developed G3 neuropathy[35]. Although their effectiveness, toxicity represented a major

Table 2 Neoadjuvant metronomic chemotherapy in triple negative breast cancer

	Ref.	Type of study	*n*	Patient characteristic	Regimens	pCR	Adverse events
Only MC	Hildebrand *et al*[34] 2016	Single arm phase Ⅱ	18	TNBC, ⩾ T2 T4: 5 patients Node +: 12 patients EC Ⅱ: 47.4% EC Ⅲ: 28.6%	Part 1 (12 wk) Weekly DX 24 mg/m^2 Ⅳ Daily CTX 60 mg/m^2 PO Followed by Part 2 (12 wk) Weekly PTX 80 mg/m^2 Ⅳ Weekly C 2AUC Ⅳ	47.60%	Neutropenia G3-G4: 62% Febrile neutropenia: 24%
	Tiley *et al*[35] 2012	Single arm phase Ⅱ	17	TNBC, T2-T4, N0-N1 Median age: 45 yr (25-83) Inflammatory breast cancer: 3	Part 1 (12 wk) Weekly DX 24 mg/m^2 Ⅳ Daily CTX 60 mg/m^2 PO Followed by Part 2 (12 wk) Weekly PTX 80 mg/m^2 Ⅳ Weekly C 2AUC Ⅳ	46.60%	Thrombocytopenia G3: 5% Neutropenia G4: 29% Neuropathy G3: 5%
	Ignatova *et al*[36] 2016	Single arm phase Ⅱ	40	TNBC cT2-4, N2-3, M0 Median age: 50 yr (27-69) Histologic grade 3: 33.3% Ki67 > 20%: 100%	Part 1 (9 wk) Weekly PTX 60 mg/mm^2 Ⅳ Weekly C 2AUC Ⅳ Then followed by Part 2 (9 wk) Weekly DX 25 mg/m^2 Ⅳ Daily CTX 50 mg bid PO Daily X 500 mg tid PO	60%	Neutropenia G3-4: 22.2% Mucositis 8.3% Hand-foot syndrome G3: 5.6%
Hybrid	Masuda *et al*[37] 2014	Single arm phase Ⅱ	40	ER < 10%, T2-T4, N0-N1 Median age 52 yr (33-69) N1: 40% ER < 10%: 17.5% EC I: 12.5% EC Ⅱ: 77.5% EC ⅢA: 10%	Part 1 (4 Cycles every 21 d) Day 1, 7, 14 PTX 80 mg/m^2 Ⅳ Daily CTX 50 mg PO Daily X 1200 mg PO Followed by Part 2 (4 Cycles every 21 d) Day 1 5-FU 500 mg/m^2 Ⅳ Day 1 E 100 mg/m^2 Ⅳ Day 1 CTX 500 mg/m^2 Ⅳ	47.50%	Neutropenia G3-4: 35% Hand foot syndrome G3-4: 8%
	Cancello *et al*[38] 2015	Single arm phase Ⅱ	34	ER ⩽ 10%, PR ⩽ 10%, Her2- Median age: 45 yr (31-64) Premenopausal: 73% EC Ⅱ: 35% EC Ⅲ: 67% Histologic grade 3: 82%	Part 1 (4 cycles every 21 d) Day 1 5-FU 200 mg/m^2 per day continuous Day 1, 2 E 25 mg/m^2 Ⅳ Day 1, P 60 mg/m^2 Ⅳ Followed by Part 2 (three cycles every 28 d) Day 1, 7, 14 PTX 90 mg/m^2 Daily CTX 50 mg/d	56%	Neutropenia G3-4: 38% Anemia G3-4: 3%

EC: Clinical stage; ER: Estrogen receptor; DX: Doxorubicin; CTX: Cyclophosphamide; PTX: Paclitaxel; C: Carboplatin; X: Capecitabine; 5-FU: 5-fluoracil; E: Epirubicin; P: Cisplatin; pCR: Pathologic response; TNBC: Triple negative breast cancer; MC: Metronomic chemotherapy.

limitation for both trials.

Ignatova *et al*[36], added capecitabine and carboplatin to an anthracycline and taxane metronomic regimen, achieving pCR in 60% of patients, the highest pCR rate reported to date with MC. Forty patients with locally advanced TNBC (cT2-T4 N2-3 M0) were treated with metronomic weekly paclitaxel plus carboplatin for 9 wk, followed by weekly doxorubicin, daily oral cyclophosphamide and capecitabine for another 9 wk. Dose limiting toxicities were neutropenia G3 (22%), mucositis G3 (8%) and hand-foot syndrome G3 (5.6%).

Hybrid approach: MTD plus MC

Masuda *et al*[37] conducted a phase II study that included 40 patients with TNBC or low hormonal receptor BC treated with 4 cycles of weekly paclitaxel plus daily oral cyclophosphamide and capecitabine, followed by 4 cycles of FEC (5-FU/epirubicine/cyclophosphamide) every 3 wk. Importantly, this regimen achieved a pCR rate of 47.5% and breast preservation in 72.7% of cases. Adverse events (AE) related were G3-4 neutropenia and hand-foot syndrome, in 35% and 8% of cases, respectively[37].

Cancello *et al*[38] evaluated the efficacy of a neo-adjuvant regimen in terms of Ki-67 variation, clinical response and toxicity in 34 patients with HER2-negative, ER and PR < 10% BC. Chemotherapy consisted of 4 rounds of ECF (epirubicine/cisplatin/5-FU) every 21 d followed by weekly paclitaxel every 28 d for 3 courses concomitantly with metronomic oral

cyclophosphamide. Importantly, response to treatment was obtained in 91% of patients and 56% achieved a pCR. Also, a 41% difference in the percentage of Ki-67 positive cells was found between the surgical specimens and the pretreatment tumor core biopsy for the entire population (95%CI: 30-51; $P < 0.0001$) *vs* 22% for those who did not achieve a pCR (95%CI: 7-38; $P = 0.0097$). AE of grade 3 or more included neutropenia in 38% and anemia in 3%. The authors concluded that neoadjuvant ECF regimen followed by weekly paclitaxel with metronomic cyclophosphamide is very effective in achieving high pCR rates and a significant reduction of Ki-67[38].

MC alone or in combination with MTD chemotherapy is effective in achieving high pCR rates. Nevertheless, it is important to point out that all the studies mentioned above but one, incorporate platinum salts as a part of the neoadjuvant regimen; therefore, their results should be compared against regimens that contain neoadjuvant platinum as well. Interestingly, the only trial that did not include platinum salts, also achieved a higher pCR rate than standard MTD chemotherapy. In all cases, toxicity is of concern. The addition of granulocyte stimulating factor or the use of intermittent metronomic schedules might reduce toxicity while maintaining effectivity. We believe that this approach warrants consideration in the younger population, which is able to better tolerate toxicity and should be given the opportunity to achieve a better pCR and therefore better outcomes. Bigger phase Ⅲ studies comparing MC *vs* MTD are needed.

ADJUVANT SETTING

Adjuvant chemotherapy in BC aims to eliminate minimal residual disease. The antiangiogenic and pro-immune properties of MC potentially induce tumor dormancy and eradicate residual cancer cells, becoming an option to improve outcomes in TNBC patients. Attempts to replace standard MTD chemotherapy with metronomic capecitabine have failed, resulting in inferior outcomes[39]. Recently, intensifying adjuvant chemotherapy or adding maintenance with metronomic methotrexate, cyclophosphamide or capecitabine have been tested with promising results (Table 3).

Intensification of adjuvant chemotherapy

Nasr *et al*[40] reported data on a small phase Ⅲ study that evaluated the role of metronomic methotrexate and cyclophosphamide after adjuvant therapy with anthracyclines, taxanes and carboplatin for stage Ⅱ or Ⅲ TNBC. One hundred fifty-eight patients were enrolled and randomized to 3 cycles of FEC-100 followed by 3 cycles of docetaxel and carboplatin followed by methotrexate and cyclophosphamide for 1 year or to 3 cycles of FEC-100 followed by 3 cycles of docetaxel without any further treatment. Although not starting from a standard of care due to the inclusion of carboplatin, this trial showed important benefits in median DFS (28 mo *vs* 24 mo, $P = 0.05$) and OS (37 mo *vs* 29 mo, $P = 0.04$) with the addition of carboplatin plus metronomic maintenance in a head-to-head design[40].

FinXX, a large randomized phase 3 clinical trial integrated capecitabine into standard adjuvant therapy. Women with axillary node-positive or greater than 20 mm node-negative BC of any histology were randomly assigned to receive either 3 cycles of docetaxel and capecitabine followed by 3 cycles of cyclophosphamide, epirubicin, and capecitabine ($n = 743$) or 3 cycles of docetaxel followed by 3 cycles of FEC ($n = 747$). The primary endpoint was recurrence-free survival (RFS), and it was not significantly different between the groups. However, in an exploratory analysis, adding capecitabine seemed to impact BC-specific survival (HR = 0.64; 95%CI: 0.44 to 0.95; $P = 0.027$) and RFS in women with TNBC, particularly those who had more than 3 metastatic axillary lymph nodes at the time of diagnosis[41].

As currently proposed, adding metronomic chemotherapy to MTD adjuvant regimens hasn't improved outcomes in TNBC. Nevertheless, selected high-risk patients might derive some benefit that needs further exploration.

Maintenance-only approach

The phase Ⅲ IBCSG Trial 22 enrolled 1086 women with triple negative or HER-2 positive BC with any nodal involvement. After adjuvant chemotherapy, patients were randomized to maintenance with continuous oral cyclophosphamide and weekly oral methotrexate for 1 year *vs* observation. After a median follow-up of 6.9 years, DFS was not significantly better for patients assigned to maintenance compared with those assigned to observation. Nevertheless, patients with TN, node-positive disease had a non-significant reduction of 7.9% in the absolute risk of relapse ($n = 340$; HR = 0.72; 95%CI: 0.49 to 1.05). In general, the metronomic part of the treatment was well tolerated with only 14% of patients experiencing a grade 3 or 4 treatment-related AE[42].

A different approach was evaluated in the CREATE-X study, presented at the 2015 San Antonio Breast Cancer Symposium. This phase 3 randomized clinical trial evaluated the role of capecitabine maintenance in 910 HER2-negative (TN and luminal) BC patients with residual disease defined as no pCR or node-positive disease, after neoadjuvant chemotherapy with anthracycline and/or taxanes. Thirty-one percent of patients had TNBC, 80% received sequential anthracyclines and taxanes, and approximately 60% had prior 5-FU. Patients were randomized to receive capecitabine 2 wk on and 1 wk off, for up to 8 cycles *vs* observation. Only 38% and 58% of patients completed 8 and 6 cycles of chemotherapy respectively. At 5 years, DFS (primary endpoint) was 74.1% with capecitabine maintenance compared to 67.7% in the control arm,

Table 3 Adjuvant metronomic chemotherapy in triple negative breast cancer

	Ref.	Study design	*n*	Regimens	Characteristics	Outcome	Adverse events
MTD plus MC	Nars *et al*[40] 2015	Phase Ⅲ	*n*: 158 A: 78	Arm A: Part 1 (3 cycles) Day 1 5FU 500 mg/m^2 PO Day 1 E 100 mg/m^2 Day 1 CTX 500 mg/m^2 Day 1-2 MTX 2.5 mg twice/d PO Part 2 (3 cycles) Day 1 T 80 mg/m^2 Day 1 Ca 5AUC Followed by MC × 1 yr Daily CTX 50 mg/d PO	Median age: 46 yr TNBC Stages II-Ⅲ Tumor size > 1.0 cm Positive or negative axillary lymph nodes; ECOG < 2	Median DFS = 2 Arm A: 28 mo Arm B: 24 mo *P* = 0.05 OS : Arm A: 37 mo Arm B: 29 mo *P* = 0.04	Arm A Neutropenia G3: 19% Neutropenia G4: 1.9% Febril neutropenia G3: 12% Nausea, vomiting G3: 12% Arm B: Neutropenia G3: 17% Febril Neutropenia G3: 9%
			B: 80	Arm B: Part 1 (3 cycles) Day 1 5FU 500 mg/m^2 PO Day 1 E 100 mg/m^2 Day 1 CTX 500 mg/m^2 Part 2 (3 cycles) Day 1 T 80 mg/m^2			
	FIN XX *et al*[41] 2011	Phase Ⅲ	A: 753	Arm A : Part 1 - every 3 wk for 3 cycles Day 1 T 60 mg/m^2 IV Day 1-15 X 900 mg/m^2 twice/d PO Followed Part 2 -every 3 wk for 3 cycles Day 1 CTX 600 mg/m^2 IV Day 1 E 75 mg/m^2 IV	Median age: 52 yr Luminal, TNBC, Her2 T1: 46%, T2: 47% 1-3 positive axillary nodes: 62% > 3 positive axillary nodes: 28% Grade 3: 42% ER negative: 24% Her 2 +: 19%	DFS 5 yr (*P* = 0.087) A: 86.6% B: 84.1% Subgroup: TNBC > 3 axillary nodes: HR, 0.64; 95%CI: 0.44 to 0.95 (*P* = 0.027)	6 deaths related to treatment Arm A: 4 patients Arm B: 2 patients Discontinued treatment Arm A: 24% Arm B: 3%
			B: 747	Arm B: Part 1 (every 3 wk x 3 cycles) Day 1 T 80 mg/m^2 IV Part 2 (every 3 wk x 3 cycles) Day 1 CTX 600 mg/m^2 IV Day 1 E 75 mg/m^2 IV Day 1 5FU 600 mg/m^2 IV			
Main-tenance	IBCSG Trial 22 Oct. 2016[42]	Phase Ⅲ	n: 1086 A: 542	Arm A: (every week for 1 yr) Daily CTX 50 mg/d PO Day 1-2 MTX 2.5 mg twice/d PO on	Median age: 51 yr TNBS, Her 2 Premenopausal: 45% Node positive disease 42%	6.9 yr OS: HR 0.84; 95%CI, 0.66 to 1.06; *P* = 0.14); TNBC: (*n* = 814; HR = 0.80; 95%CI: 0.60 to 1.06)	Arm A Grade 3-4 treatment related AE: 14% patients Hypertransaminasemia G3 G4: 7%
			B: 539	Arm B: Observation	Her2 +: 19%, only 52% received trastuzumab TNBC: 75% Tumor > 2 cm: 54% Grade 3: 84% 1-3 node +: 25% > 3 node +: 16% Prior anthracycline: 60% Prior anthracycline + taxane: 26.1%	TNBC, node-positive disease: *n* = 340 HR = 0.72; (95%CI: 0.49 to 1.05)	Leukopenia G3-G4 : 2% 2 patients with AML

	CREATE-X trial 2015[43]	Phase Ⅲ	*n*: 455	Arm A: (every 3 wk for 8 cycles) Day 1-14 X 1250 mg/m^2 twice/d Arm B: Observation	Luminal TBNC patients Prior: Neoadyuvant no pCR or node positive Anthracycline and/or taxane: 80% 5FU regimen: 60% Six cycles completed: 58% Eight cycles completed: 38%	5 yr DFS: (P = 0.00524). A: 74.1% B: 67.7% 30% reduction in risk 5 yr OS P < 0.01 A: 89.2% B: 83.9%	Arm A: HFS G3: 10.9%
Ongoing	CIBOMA/2004-01/ GEICAM 2003-11 trial 2010[45]	Phase Ⅲ	A: 207 B: 193	Arm A: every 3 wk for 8 cycles Day 1–14 X 1000 mg/m^2 per twice day PO Arm B: Observation	Median age: 51 yr TNBC Caucasian: 63.9% Postmenopausal: 68.2% Basal phenotype: 82% Neoadjuvant: 9.7% Adjuvant: 86.4% Complete 8 cycles: 77.3%	Ongoing	Arm A: HFS G3: 17.4% Diarrhea: 2.9% Fatigue: 1.9%
	ECOG – ACRIN Cancer Research Group EA 1131 trial[46]	Phase Ⅲ	Expected 562	Arm A: observation Arm B: Carboplatin / Cisplatin day 1 IV every 3 wk for 4 cycles Arm C: Capecitabine twice daily on days 1-14 every every 3 wk for 6 courses	TNBC Stage Ⅱ-Ⅲ Residual basal like disease after neoadjuvant chemotherapy	Ongoing	Ongoing

5FU: 5-Fluoracil; E: Epirubicin, Ca: Carboplatin; T: Docetaxel; CTX: Cyclophosphamide; MTX: Methotrexate; X: Capecitabine; AT: Anthracycline/taxane regimen; HFS: Hand-foot syndrome.

with a statistically significant 30% reduction in the risk of recurrence (one-sided P = 0.00524). Likewise, a statistically significant reduction in the risk of death was observed, with OS rates of 89.2% and 83.9%, respectively (one-sided P < 0.01)[43]. In the subgroup analysis, the benefit of adding capecitabine was even greater in the TNBC subgroup which achieved a 42% reduction in the risk of recurrence[43].

Despite the fact that both phase Ⅲ trials evaluated maintenance therapy for early BC, there exist remarkable differences on their design and target population (Table 4). The IBCSG trial 22 included hormone negative-receptor early BC patients, of whom only 26% received current standard chemotherapy with anthracyclines and taxanes. Moreover, only 59% of the HER 2 positive patients received anti HER 2 target agents. The varying treatments logically modified outcomes with statistical implications. Also, because all patients were recruited after adjuvant therapy, no risk groups were identified. Treatment non-adherence was also an issue as the study had a high incidence (13%) of not-started treatment in those assigned to CM maintenance.

On the other hand, the CREATE-X study included luminal and TNBC patients, of whom 80% received sequential anthracyclines and taxanes. Outstandingly, this trial very early recognized residual disease as a poor prognostic factor and considered the addition of capecitabine as maintenance aiming to improve DFS and OS. This study included a better selected but still heterogeneous population of luminal and TNBC patients. We believe that, as for luminal BC patients, pCR has not been correlated with outcomes, the positive results observed in both populations are produced by different mechanisms and mostly driven by the TNBC cases. A limitation of the CREATE-X study is the fact that these results were obtained in an only-Asian population, precluding their generalizability, particularly in terms of sensibility and tolerance which differs from those reported for the Caucasian population[44].

Residual disease after neoadjuvant chemotherapy is a biomarker of high risk. In this setting, further treatment seems to be beneficial, especially for TNBC. We believe that selecting the population for clinical trials through this or other biomarkers is key for designing further research initiatives.

Ongoing trials and future perspectives

Ongoing trials are exploring the role of MC in different settings. The CIBOMA/2004-01/GEICAM 2003-11 trial, added capecitabine as maintenance after standard chemotherapy exclusively for TNBC. Patients were randomized to receive standard anthracycline and/or taxane-containing chemotherapy or 4 cycles of doxorubicin-cyclophosphamide (for node-negative disease) as (neo)adjuvant treatment followed by 8 cycles of capecitabine at 1000 mg/m^2 twice a day, 14 d on and 7 d off, every 3 wk *vs* observation. The most frequent grade 3/4 capecitabine-related clinical AE were hand-foot

Table 4 Maintenance for triple negative breast cancer

	IBCSG Trial 22, Oct. 2016	CREATE-X trial, 2015
Study design	Phase Ⅲ	Phase Ⅲ
Accrual time	2000-2012	2007-2012
Number of patients	N: 1086	N: 910
	CM: 542	X: 455
	Obs: 539	Obs: 455
Setting	Prior adjuvant ± RT	Prior neoadyuvant ± RT
Study population	TNBC: 75%	Luminal or TNBC
	HER2+: 19%	No pCR o node positive
Previous treatment	A + CMF: 60%	A: 4.1%
	CMF: 16%	AT sequential: 81%
	AT sequential + CMF: 26%	AT concurrently: 13.6%
	H: 59% (of HER2+)	TC: 5%
Study treatment	C 50 mg/d PO Daily M 2.5 mg bid PO Days 1-2 *vs* Observation	X 1250 mg/m^2 twice/d PO Day 1-14 *vs* Observation
Time of treatment	Every week for 1 yr	Every 3 wk for 8 cycles (6 mo)
DFS	5 yr DFS:	5 yr DFS:
	CM: 78.1%	X: 74.1%
	Obs: 74%	Obs: 67.7%
	HR = 0.84 (95%CI: 0.66 to 1.06; P = 0.14)	HR (95%CI): 0.70 (0.53-0.93); P = 0.00524
	TNBC: n = 814; HR = 0.80; 95%CI: 0.60-1.06	30% reduction in risk
	TNBC, node-positive disease: n = 340; HR = 0.72; 95%CI: 0.49-1.05	
OS	No results	5 yr OS
		X: 89.2%
		Obs: 83.9%,
		P < 0.01
Adverse events	Hipertransaminasemia	X: HFS G3: 10.9%
	G3-G4: 7%	Neutropenia G3: 6.6%
	Leukopenia: 2%	Diarrhea G3: 3%
		Obs: Neutropenia 1.6%
		Diarrhea: 0.4%

C: Cyclophosmide; M: Methotrexate; X: Capecitabine; ER: Estrogen receptor; PR: Progesterone receptor; A: Anthracycline; F: 5Fluoracil; T: Taxane; TNBC: Triple negative breast cancer; H: Herceptin.

syndrome (17.4%), diarrhea (2.9%), and fatigue (1.9%). After 6 years of follow-up and with a small number of events, no differences in DFS have been detected so far. Disease-free survival (DFS) is still ongoing[45].

The phase Ⅲ ECOG-ACRIN Cancer Research Group - EA 1131 trial will define which treatment-if any- is more effective in prolonging DFS in patients with residual basal-like TNBC, following neoadjuvant chemotherapy. Five hundred sixty-two patients are expected to be included and randomized to receive further treatment with cisplatin/carboplatin, capecitabine or observation. This clinical trial is currently recruiting participants. The estimated primary completion date is on May 2019[46].

CONCLUSION

MC is a multi-mechanism therapy that due to its accessibility and affordability, stands as an attractive alternative or complement for a selected group of TNBC patients in both the neoadjuvant and adjuvant setting. In neoadjuvant regimens pCR rates obtained with MC are high, as well as it is toxicity. In the adjuvant setting, metronomic maintenance for patients with residual disease after neoadjuvant therapy seems to be feasible and effective in prolonging DFS and these results are encouraging.

Considering the dismal prognosis of TNBC, any strategy that potentially improves outcomes, specially being the oral agents broadly available and inexpensive, should be considered and certainly warrants further exploration. Finally, the benefit of MC needs to be validated in properly designed clinical trials were the selection of the population is the key.

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MINIREVIEWS

With increasing trends of prostate cancer in the Saudi Arabia and Arab World: Should we start screening programs?

Mostafa A Arafa, Danny M Rabah

Mostafa A Arafa, Danny M Rabah, Cancer Research Chair, College of Medicine, King Saud University, Riyadh 4536, Saudi Arabia

Danny M Rabah, Department of Surgery, College of Medicine, King Saud University, Riyadh 4536, Saudi Arabia

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Correspondence to: Mostafa A Arafa, MD, Professor of Epidemiology, Cancer Research Chair, College of Medicine, King Saud University, 3125 King Abdullah street, Riyadh 4536, Saudi Arabia. marafa@ksu.edu.sa
Telephone: +966-5-8129051

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Abstract

Incidence rate for prostate cancer in the Arab World is significantly lower than United States and Europe, it ranges from 5.5% to 39.2%. However, the incidence and the number of deaths is expected to increase. In Saudi Arabia, the crude incidence rate and age standardized incidence rate of prostate cancer are reported to be steadily increasing in between 2001-2008. Only two screening trials were attempted in 2001 and 2009 which yielded an incidence rate of 1.17% and 2.5% respectively. Men in the Arab world are sharing a common characteristic of poor knowledge and poor attitude towards prostate cancer examination and screening practices. They are ill-informed about the PSA test's strengths and drawbacks because the doctors are not talking to them about the importance of counselling. Men should be encouraged to do PSA testing before the age of 50 and till the age of 70 years. This could be achieved by enhancing their attitude and enriching the knowledge of the physicians towards PSA testing, harms and benefits, through shared decision making, which would increase men's knowledge scores, reduced their decisional conflict and promote greater involvement in decision making.

Key words: Prostate cancer; Incidence; Arab World; Screening

Core tip: Despite the very low incidence and the number of deaths from prostate cancer in the Arab World, it is expected to increase. In Saudi Arabia, the crude incidence rate and age standardized incidence rate of prostate cancer are reported to be steadily increasing in between 2001-2008. Men in the Arab world are characterized by poor knowledge and poor attitude towards prostate cancer examination and screening practices. We recommend against mass screening, but men should be encouraged to do PSA testing before the age of 50 and till the age of 70 years, through shared decision making.

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INTRODUCTION

Prostate cancer is representing a major public health problem in the developed world, the figures reported from Europe and United States indicated a higher age standardized incidence rate (ASIR) and mortality rate. The incidence varies more than 25-fold worldwide, where the rates are higher in Northern American, Australia, and northern Europe, because of the practice of PSA testing and biopsy which has become widespread in those areas. The reported mortality rates paralleled the incidence rates, with a considerable decrease in most of the countries except Eastern Europe[1,2].

The corresponding reported figures from the Arab World is much and significantly lower. The ASIR rate ranged from 39.2% in Lebanon to 5.5% in Saudi Arabia, while the data on mortality rates are not available[3] (Figure 1).

Access to the health care and accuracy of the cancer registries in the Middle East is affecting the ASIR of prostate cancer reported from such countries. However, GLOBOCAN, 2012 reported that prostate cancer incidence in the Middle East North Africa (MENA) region is expected to increase from 29377 new cases in 2012 to 38562 new prostate cancer cases in 2020 along with an increase in mortality from prostate cancer from 15422 prostate cancer deaths in 2012 to 19681 deaths in 2020[1]. In Saudi Arabia, the crude incidence rate and ASIR of prostate cancer are reported to be steadily increasing in between 2001-2008 and then after[4].

No screening programs were adopted in the Arab world except for breast cancer. The first screening trial for prostate cancer was attempted in Saudi Arabia in 2001 and yielded an incidence of 1.17%[5]. Nine years later Rabah reported an incidence of 2.5%, in a larger sample and in a different health facility, amongst the studied cohort; and 27% were metastatic[6]. In Saudi Arabia, Many confirmed cases of prostate cancer were diagnosed before the age of 50, in addition, the distribution of the PSA levels among the Saudi men was lower than other European countries[7].

The men in the Arab world are sharing a common characteristic of poor knowledge and poor attitude towards prostate cancer examination and screening practices[8]. Such poor behavior towards their health could be ascribed to their level of awareness, or different barriers which may prevent them from seeking early detection and diagnosis of prostate cancer, *i.e.*, mistrust of physicians, fear of diagnosis, fear of testing procedures, DRE threatens sexuality and others[9].

The men in the Arab world remain ill-informed about

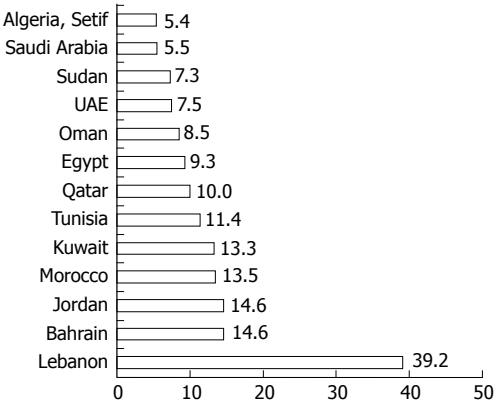

Figure 1 Age standardized incidence rate (%) of prostate cancer in the Arab World during the period 2010-2014.

the PSA test's strengths and drawbacks because the doctors are not talking to them about the importance of counseling. The results of a survey on the primary health care physicians in Saudi Arabia about their knowledge and behavior on prostate cancer counseling and screening indicated that nearly 55% were practicing counseling and they had a poor attitude and deficient knowledge towards counseling and referring patients[10]. The decision to provide screening would be influenced by factors related to the physicians, patients and screening guideline. Physicians who have good scientific evidence are more likely to practice informed decision making with their patients as they believe that men need to know about the Pros and Cons of PSA testing to make their decisions[10].

A recent review concluded that the evidence does not indicate that the benefits of using PSA for prostate cancer screening outweigh the harms[11]. In the same context, in our Arab countries, the incidence of prostate cancer is still low, but the trend is increasing in the last few years, however, we recommend against mass screening, but encourage men to do PSA testing before the age of 50 and till the age of 70 years. This could be achieved by enhancing their attitude and enriching the knowledge of physicians towards PSA testing, harms and benefits, through shared decision making, which would increase men's knowledge scores, reduced their decisional conflict and promote greater involvement in decision making.

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