ISSN 2218-4333 (online)

World Journal of *Clinical Oncology*

World J Clin Oncol 2016 December 10; 7(6): 425-440

Published by Baishideng Publishing Group Inc

WJCO World Journal of Clinical Oncology

A peer-reviewed, online, open-access journal of clinical oncology

Editorial Board

2015-2018

The *World Journal of Clinical Oncology* Editorial Board consists of 297 members, representing a team of worldwide experts in oncology. They are from 41 countries, including Argentina (1), Australia (6), Austria (2), Belgium (1), Brazil (1), Bulgaria (1), Canada (6), China (43), Cuba (1), Denmark (4), France (4), Germany (11), Greece (1), Hungary (1), India (7), Iran (2), Ireland (1), Israel (1), Italy (33), Japan (27), Malaysia (3), Netherlands (8), Norway (3), Peru (1), Poland (1), Portugal (2), Qatar (1), Romania (1), Russia (1), Saudi Arabia (3), Singapore (2), South Korea (12), Spain (11), Sri Lanka (1), Sweden (2), Switzerland (1), Syria (1), Turkey (7), United Kingdom (3), United States (78), and Viet Nam (1).

José Antonio Lopez-Guerrero, *Valencia*
Gracia Merino, *Leon*
Jordi Muntane, *Seville*
Ernest Nadal, *L'Hospitalet*
Amalia Palacios-Eito, *Cordoba*
Isabel T Rubio, *Barcelona*
Albert Selva-O'Callaghan, *Barcelona*

Sri Lanka

Kemal I Deen, *Dehiwela*

Sweden

Yihai Cao, *Stockholm*
Hong Xie, *Stockholm*

Switzerland

Nicolas C Buchs, *Geneva*

Syria

Roger von Moos, *Chur*

Turkey

Ahmet Altun, *Sivas*
Beste Atasoy, *Istanbul*
Ahmet Dirier, *Gaziantep*
Ozkan Kanat, *Bursa*
Serhan Kupeli, *Adana*
Kazim Sahin, *Elazig*
Isik G Yulug, *Ankara*

United Kingdom

Andrew Gaya, *London*
Konstantinos Lasithiotakis, *York*
Sebastian Oltean, *Bristol*

United States

ARM Ruhul Amin, *Atlanta*
Soley Bayraktar, *Ardmore*
Amer Beitinjaneh, *Charlottesville*
Maurizio Bocchetta, *Maywood*
Deliang Cao, *Springfield*
Daniel VT Catenacci, *Chicago*
Zhe-Sheng Chen, *Queens*
Guan Chen, *Milwaukee*
Duc Phuc Do, *Chicago*
Cathy Eng, *Houston*
Jeffrey M Farma, *Philadelphia*
Markus H Frank, *Boston*
Sidney Wang Fu, *Washington*
Mei R Fu, *New York*
Siqing Fu, *Houston*
Song Gao, *Houston*
Mamdooh Ghoneum, *Los Angeles*
Ruben Rene Gonzalez-Perez, *Atlanta*
Rachel Nicole Grisham, *New York*
Sanjay Gupta, *Cleveland*
Gerald M Higa, *Morgantown*
Chung-Tsen Hsueh, *Loma Linda*
GK Jayaprakasha, *College Station*
Johnny Kao, *West Islip*
Nimmi Singh Kapoor, *Orange*
Arianna L Kim, *New York*
Mark Alan Klein, *Minneapolis*
Sunil Krishnan, *Houston*
Melanie Haas Kucherlapati, *Boston*
Mahmoud N Kulaylat, *Buffalo*
Adeyinka O Laiyemo, *Washington*
Marie Catherine Lee, *Tampa*
James W Lillard, *Atlanta*
Shiaw-Yih Lin, *Houston*
Wei Liu, *Frederick*
Zhao-Jun Liu, *Miami*
Jirong Long, *Nashville*
Jianrong Lu, *Gainesville*
James L Mulshine, *Chicago*
Ronald B Natale, *Los Angeles*
Matthew E Nielsen, *Chapel Hill*
Kutluk Oktay, *Valhalla*
Chung S Park, *Fargo*
Tayebeh Pourmotabbed, *Memphis*
Raj Pruthi, *Chapel Hill*
Jay Dilip Raman, *Hershey*
Jianyu Rao, *Los Angeles*
Gaiane M Rauch, *Houston*
William C Reinhold, *Bethesda*
Monica Rizzo, *Atlanta*
Eben L Rosenthal, *Birmingham*
Joan J Ryoo, *Los Angeles*
Virgilio S Sacchini, *New York*
Neeraj K Saxena, *Baltimore*
Caner Saygin, *Cleveland*
Masood A Shammas, *Boston*
Amar B Singh, *Omaha*
Khalid Sossey-Alaoui, *Cleveland*
Lu-Zhe Sun, *San Antonio*
Weijing Sun, *Pittsburgh*
Viqar Syed, *Bethesda*
Li Tao, *Fremont*
Anish Thomas, *Bethesda*
Reid Thompson, *Philadelphia*
Shahid Umar, *Kansas City*
Huan N Vu, *Richmond*
Chong-Zhi Wang, *Chicago*
Bin Wang, *Chester*
Jin Wang, *Houston*
Guojun Wu, *Detroit*
Michiko Yamagata, *Waltham*
Wannian Yang, *Danville*
Eddy S Yang, *Birmingham*
Jennifer Yunyan Zhang, *Durham*
Bin Zhang, *New York*
Shaying Zhao, *Athens*
Jin-Rong Zhou, *Boston*

Viet Nam

Phuc Van Pham, *Ho Chi Minh*

WJCO World Journal of Clinical Oncology

Contents

Bimonthly Volume 7 Number 6 December 10, 2016

ORIGINAL ARTICLE

Basic Study

Retrospective Study

NAME OF JOURNAL
World Journal of Clinical Oncology

ISSN
ISSN 2218-4333 (online)

LAUNCH DATE
November 10, 2010

FREQUENCY
Bimonthly

Fang-Fang Ji, Vice Director
World Journal of Clinical Oncology
Baishideng Publishing Group Inc
8226 Regency Drive, Pleasanton, CA 94588, USA
Telephone: +1-925-2238242
Fax: +1-925-2238243
E-mail: editorialoffice@wjgnet.com
Help Desk: http://www.wjgnet.com/esps/helpdesk.aspx
http://www.wjgnet.com

PUBLISHER
Baishideng Publishing Group Inc
8226 Regency Drive,
Pleasanton, CA 94588, USA
Telephone: +1-925-2238242
Fax: +1-925-2238243
E-mail: bpgoffice@wjgnet.com
Help Desk: http://www.wjgnet.com/esps/helpdesk.aspx
http://www.wjgnet.com

PUBLICATION DATE
December 10, 2016

INSTRUCTIONS TO AUTHORS
http://www.wjgnet.com/bpg/gerinfo/204

ONLINE SUBMISSION
http://www.wjgnet.com/esps/

World Journal of Clinical Oncology

Submit a Manuscript: http://www.wjgnet.com/esps/
Help Desk: http://www.wjgnet.com/esps/helpdesk.aspx
DOI: 10.5306/wjco.v7.i6.425

World J Clin Oncol 2016 December 10; 7(6): 425-432
ISSN 2218-4333 (online)

ORIGINAL ARTICLE

Basic Study

Better to be alone than in bad company: The antagonistic effect of cisplatin and crizotinib combination therapy in non-small cell lung cancer

Nele Van Der Steen, Christophe Deben, Vanessa Deschoolmeester, An Wouters, Filip Lardon, Christian Rolfo, Paul Germonpré, Elisa Giovannetti, Godefridus J Peters, Patrick Pauwels

Nele Van Der Steen, Christophe Deben, Vanessa Deschoolmeester, An Wouters, Filip Lardon, Christian Rolfo, Paul Germonpré, Patrick Pauwels, Center for Oncological Research, University of Antwerp, 2610 Wilrijk, Belgium

Nele Van Der Steen, Christophe Deben, Vanessa Deschoolmeester, Patrick Pauwels, Department of Pathology, Antwerp University Hospital, 2650 Edegem, Belgium

Nele Van Der Steen, Elisa Giovannetti, Godefridus J Peters, Department of Medical Oncology, VU University Medical Center, 1081 HV Amsterdam, The Netherlands

Christian Rolfo, Department of Oncology, Antwerp University Hospital, 2650 Edegem, Belgium

Paul Germonpré, Department of Pneumology, AZ Maria Middelares, 9000 Ghent, Belgium

Elisa Giovannetti, Cancer Pharmacology Lab, AIRC Start-Up Unit, University of Pisa, 56124 Pisa, Italy

Author contributions: All the authors contributed to the manuscript.

Supported by Institute for Innovation, Science and Technology Flanders (IWT), NO: 121114.

Institutional review board statement: Not applicable.

Conflict-of-interest statement: To the best of our knowledge, no conflict of interest exists.

Manuscript source: Invited manuscript

Correspondence to: Elisa Giovannetti, MD, Department of Medical Oncology, VU University Medical Center, room 1.53, De Boelelaan 1117, 1081 HV Amsterdam, The Netherlands. elisa.giovannetti@gmail.com
Telephone: +31-20-4442630

Received: June 20, 2016
Peer-review started: June 20, 2016
First decision: August 11, 2016
Revised: September 29, 2016
Accepted: October 25, 2016
Article in press: October 27, 2016
Published online: December 10, 2016

Abstract

AIM

To investigate the potential benefit of combining the cMET inhibitor crizotinib and cisplatin we performed *in vitro* combination studies.

METHODS

We tested three different treatment schemes in four non-small cell lung cancer (NSCLC) cell lines with a different cMET/epidermal growth factor receptor genetic background by means of the sulforhodamine B assay and performed analysis with Calcusyn.

RESULTS

All treatment schemes showed an antagonistic effect in all cell lines, independent of the cMET status. Despite their different genetic backgrounds, all cell lines (EBC-1,

HCC827, H1975 and LUDLU-1) showed antagonistic combination indexes ranging from 1.3-2.7. These results were independent of the treatment schedule.

CONCLUSION
These results discourage further efforts to combine cMET inhibition with cisplatin chemotherapy in NSCLC.

Key words: Non-small cell lung cancer; Combination therapy; Cisplatin; Crizotinib; cMET

Core tip: Targeted therapies are a valuable treatment option in non-small cell lung cancer. Several therapies have now been approved like erlotinib and gefitinib for epidermal growth factor receptor - mutant patients and crizotinib for Anaplastic Lymphoma Kinase-rearranged patients. However, resistance against these therapies eventually occurs. Combination therapy might be able to overcome or delay this resistance. Here we investigate the combination of the cMET inhibitor crizotinib with cisplatin in a panel of non-small cell lung cancer (NSCLC) cell lines with different histological and genetic backgrounds. We show that this leads to strong antagonism in all of the used cell lines. Furthermore we also link these results to the earlier *in vitro* and clinical results of the combination of erlotinib/gefitinib with cisplatin based chemotherapy in NSCLC.

Van Der Steen N, Deben C, Deschoolmeester V, Wouters A, Lardon F, Rolfo C, Germonpré P, Giovannetti E, Peters GJ, Pauwels P. Better to be alone than in bad company: The antagonistic effect of cisplatin and crizotinib combination therapy in non-small cell lung cancer. *World J Clin Oncol* 2016; 7(6): 425-432 Available from: URL: http://www.wjgnet.com/2218-4333/full/v7/i6/425.htm DOI: http://dx.doi.org/10.5306/wjco.v7.i6.425

INTRODUCTION

During the last decade, targeted therapies have revolutionized the treatment for non-small cell lung cancer (NSCLC). Several epidermal growth factor receptor-tyrosine kinase inhibitors (EGFR-TKIs) have been approved for patients with sensitizing mutations in EGFR[1-3]. Furthermore, several cMET inhibitors are currently under development with promising clinical benefit[4,5]. However, only a small percentage of NSCLC patients are eligible for these treatments. Thus, for the majority of NSCLC patients, cisplatin based therapy remains the standard of care treatment in first or later lines, usually in combination with pemetrexed, gemcitabine or a taxane[6-9].

cMET, with its ligand hepatocyte growth factor (HGF), is known to be activated in many tumor types, including NSCLC[10], with cMET amplification recognized as a resistance mechanism during EGFR tyrosine kinase inhibition[11]. The cMET and EGFR signaling pathways are heavily intertwined[12,13], with EGFR activation being sufficient for downstream cMET phosphorylation. The mitogen activated protein kinase (MAPK) dependent activation of cMET by EGFR takes place at different regulatory levels, with cMET transcriptional upregulation, the elongation of cMET half-life and a decrease in cMET-ubiquitylation[12]. Upon binding of HGF, the cMET receptor dimerizes and cross-phosphorylation takes place. This ultimately leads to phosphorylation of the docking sites recruiting proteins involved in the signaling of MAPK cascades, phosphoinositide 3 kinase (PI3K), signal transducer and activator of transcription 3 (STAT3) and nuclear factor-κB (NF-κB). Thus activating many oncogenic processes such as migration, invasion, and angiogenesis[14]. Two main cMET aberrations have been described, which can be used to predict sensitivity to cMET therapies: Amplification of the *cMET* gene[4] and cMET exon 14 skipping[5,15].

Several small molecule inhibitors and monoclonal antibodies inhibiting cMET signaling are currently being investigated in several clinical trials[16]. One of these small molecule inhibitors is crizotinib, which was originally developed as a cMET inhibitor[17] but has been approved for treatment of anaplastic lymphoma kinase (ALK)-translocated NSCLC patients[18]. Currently, crizotinib is being investigated in several clinical trials (METROS trial and the NCT02499614) for the treatment of patients with cMET-dependent NSCLC and in other cancer types where patients carry a cMET amplification[16,19].

The combination of a cMET inhibitor and cisplatin has not been investigated in NSCLC patients to date. However, *in vitro* studies show contradictory results where the outcome is dependent on tumor type and origin. For example, addition of the cMET ligand HGF enhanced cisplatin resistance in seven different NSCLC cell lines. This was explained by the fact that HGF binding induces cMET signaling which led to activation of focal adhesion kinase (FAK). FAK, in turn, suppressed the apoptosis inducing factor (AIF), resulting in a decreased sensitivity to cisplatin[20]. Therefore, theoretically, inhibition of cMET could possibly result in sensitization towards cisplatin. However, another study in SW620 cells, a *KRAS* mutated colon cancer cell line, showed that conditioned knock-down of cMET did not influence cisplatin sensitivity[21]. In contrast, ovarian cancer cell lines were sensitized towards cisplatin with the addition of HGF[22], this was established to be linked to the p38-MAPK signaling of cMET[23]. HGF pretreatment of these cells decreased the transcription of protein phosphatase 2A, thus increasing the effect of cisplatin[24].

Given the contradictory results in previous studies, more studies were warranted. Therefore, we investigated whether a combination of these compounds could result in a synergistic treatment effect in NSCLC cell lines with different cMET and EGFR genetic backgrounds.

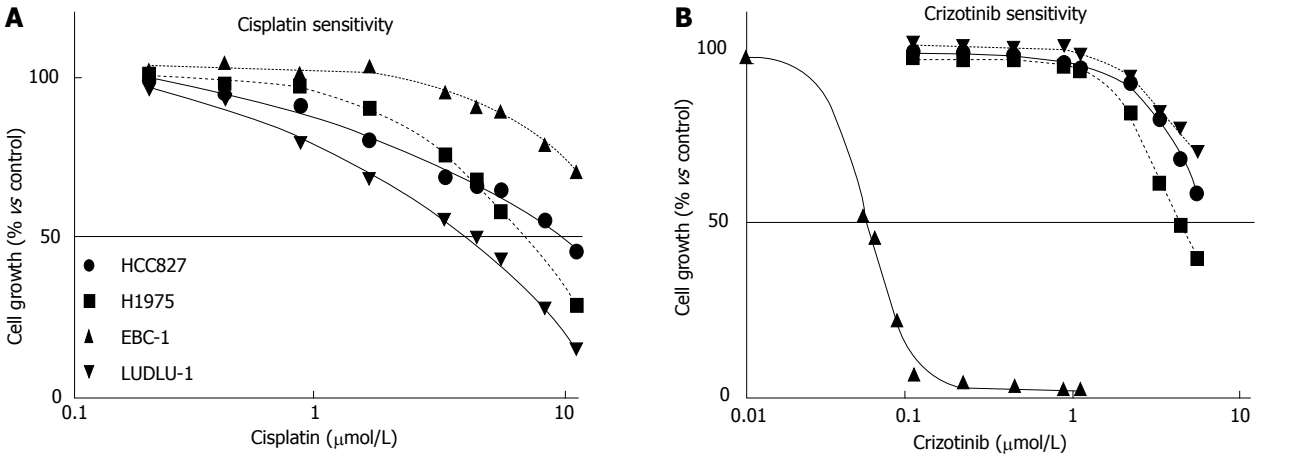

Figure 1 Sensitivity of several non-small cell lung cancer cell lines to cisplatin (A) and crizotinib (B) monotherapy. Cells were exposed to the drugs for 72 h. Cisplatin and crizotinib concentrations are depicted in μmol/L. Values are means of at least 3 separate experiments. The maximal SEM was ± 9%.

Table 1 Cell line properties and drug sensitivity

	HCC827	H1975	EBC-1	LUDLU
Properties				
Histology	Adeno	Adeno	Squamous	Squamous
EGFR-status	Exon 19 deletion	L858R + T790M	Wild-type	Wild-type
cMET-status	Wild-type	Wild-type	Amplification	Wild-type
Drug sensitivity (μmol/L, IC_{50} ± SEM)				
Cisplatin	8.39 ± 0.36	6.10 ± 0.07	16.52 ± 0.89	3.37 ± 0.19
Crizotinib	6.05 ± 0.11	4.00 ± 0.06	0.054 ± 0.002	8.12 ± 0.28

Cells were treated with cisplatin or crizotinib during 72 h. Drug sensitivity is given in μmol/L and given as IC_{50} ± SEM of 3 separate experiments. EGFR: Epidermal growth factor receptor.

MATERIALS AND METHODS

Cell lines and reagents

Four NSCLC cell lines were included in this study. The HCC827 and H1975 cell lines were purchased from the American Type Culture Collection (ATCC), the EBC-1 cell line from the Japanese Collection of Research Bioresources (JCRB, Japan) and the LUDLU-1 cell line from the European Collection of Authenticated Cell Cultures (ECACC) (Figure 1 and Table 1). The EBC-1 cell line was cultured in DMEM (Invitrogen, Merelbeke, Belgium) supplemented with 10% FBS, 1% penicillin/streptomycin, L-glutamine (2 mmol/L) and sodium pyruvate (1 mmol/L). The HCC 827, H1975 and LUDLU-1 cell lines were cultured in RPMI1640 (Invitrogen) supplemented with 10% FBS, 1% penicillin/streptomycin, L-glutamine (2 mmol/L) and sodium pyruvate (1 mmol/L). Cultures were incubated at 37 ℃ under an atmosphere of 5% CO_2. The HCC827 cell line harbors an exon 19 deletion in the *ErbB1* gene[25], while the H1975 cell line has L858R and T790M mutations in the *ErbB1* gene[26]. The EBC-1 cell line harbors a cMET amplification[27], while the LUDLU-1 is wild-type for both EGFR and cMET (Table 1). All cell lines were wild-type for ALK, free from mycoplasma contamination and STR profiles were checked.

Cisplatin and crizotinib were purchased from Selleckchem (Huissen, The Netherlands). Cisplatin was dissolved in a sterile 0.9% NaCl solution (Fisher Scientific, Aalst, Belgium), while crizotinib was dissolved in dimethylsulfoxide (DMSO). Both were diluted in phosphate buffered saline (PBS) to the desired concentrations.

Cell proliferation assay: Sulforhodamine B assay

Cells were harvested from exponential phase cultures by trypsinization (Trypsin-EDTA 0.05% with phenol red, Invitrogen, Merelbeke, Belgium), counted, seeded in sterile 96-well plates and allowed to attach before treatment. Optimal seeding densities for each cell line were determined to ensure exponential growth during a 5-d or 7-d assay. For the 5-d assay the EBC-1 and HCC827 were seeded at 4500 cell/well, H1975 at 3500 cell/well and the LUDLU-1 at 8000 cell/well. For the 7-d assay the EBC-1 and HCC827 were seeded at 1500 cell/well, the H1975 at 850 cell/well and the LUDLU-1 at 4000 cell/well. Cells were incubated with cisplatin alone (0-10 μmol/L for 72 h), crizotinib alone (0-5 μmol/L for 72 h) or with a combination of both. The combination used crizotinib at a fixed concentration (IC_{20} or IC_{40}), while a concentration range of cisplatin (0-10 μmol/L) was added. Cells treated with 0.1% diluted DMSO in the case of crizotinib or pure PBS in the case of cisplatin were used as controls. Three combination schedules

Table 2 Combination indexes for the different non-small cell lung cancer cell lines for the 3 treatment schemes

Drug scheme	HCC827		H1975		EBC-1		LUDLU-1	
	Criz	CI ± SEM	Criz	CI ± SEM	Criz	CI ± SEM	Criz	CI ± SEM
Cisplatin +	3 μmol/L	1.58 ± 0.10	3 μmol/L	1.94 ± 0.27	0.025 μmol/L	2.08 ± 0.49	3 μmol/L	2.65 ± 0.30
Crizotinib	5 μmol/L	1.54 ± 0.15	5 μmol/L	1.93 ± 0.19	0.05 μmol/L	1.42 ± 0.06	4 μmol/L	2.71 ± 0.14
Cisplatin →	3 μmol/L	1.74 ± 0.17	3 μmol/L	1.75 ± 0.30	0.025 μmol/L	2.29 ± 0.53	3 μmol/L	1.27 ± 0.13
Crizotinib	5 μmol/L	2.06 ± 0.30	5 μmol/L	1.96 ± 0.14	0.05 μmol/L	2.38 ± 0.56	4 μmol/L	1.34 ± 0.15
Crizotinib →	1 μmol/L	2.70 ± 0.37	1 μmol/L	1.58 ± 0.24	0.025 μmol/L	2.08 ± 0.49	2 μmol/L	1.74 ± 0.14
Cisplatin	2 μmol/L	2.42 ± 0.21	2 μmol/L	0.95 ± 0.03	0.05 μmol/L	1.42 ± 0.06	3 μmol/L	1.89 ± 0.17

Cells were treated with the indicated fixed concentration of crizotinib (IC_{20} and IC_{40}) either simultaneously for 72 h (indicated by "+"), or sequential with 72 h cisplatin preceding 72 h crizotinib or crizotinib preceding cisplatin (indicated by "→"). The simultaneous treatment of LUDLU-1 was performed 2 times, all other conditions were tested at least 3 times. Criz: Crizotinib; CI: Combination index; SEM: Standard error of mean.

were investigated: (1) simultaneous exposure to cisplatin and crizotinib for 72 h; (2) cisplatin for 72 h, followed by washing and crizotinib for 72 h; or (3) 72 h of crizotinib followed by washing and cisplatin for 72 h (Table 2). When crizotinib was used as first drug, the concentration was reduced in three out of the four cell lines, due to the toxic after-effect of this drug.

After treatment, growth inhibition was determined by the sulforhodamine B (SRB) assay, as previously described[28]. In short, the medium was discarded and the cells were fixed with ice cold 10% Trichloric acid (Fisher Scientific, Aalst, Belgium) solution for 1 h at 4 ℃. Next, the plates were washed 5 times with demineralized water. The cells were stained with 100 μL 0.1% SRB (Acros organics, Geel, Belgium) dissolved in 1% glacial acetic acid (Fisher Scientific, Aalst, Belgium) for at least 15 min and subsequently washed five times with 1% acetic acid to remove unbound stain. The plates were left to dry at room temperature and bound protein stain was solubilized with 100 μL 10 mmol/L unbuffered Tris base [tris (hydroxymethyl) aminomethane] (Fisher Scientific, Aalst, Belgium) and read at an optical density (OD) of 540 nm (IMark microplate absorbance reader, Biorad, Nazareth, Belgium)[29].

Statistical analysis

Each test was performed at least three times, unless otherwise stated. Results are presented as mean ± SEM.

To assess the IC_{50} value of cisplatin and crizotinib, WinNonlin software was used (Pharsight Corporation, Mountain View, CA, United States). To determine possible synergism between cisplatin and crizotinib, the combination index (CI) was calculated with the Calcusyn software of Biosoft. This program is based on the method of Chou *et al*[30,31] to assess whether a combination of two drugs results in an antagonistic effect (CI > 1.2), an additive effect (0.8 < CI < 1.2) or a synergistic effect (CI < 0.8). This method takes into account the fraction of affected cells of both monotherapies and compares this with the fraction of affected cells of the combination therapies.

RESULTS

The effects of cisplatin and crizotinib monotherapy were investigated in four NSCLC cell lines (Figure 1). LUDLU-1 cells were most sensitive to cisplatin, followed by the EGFR-mutated H1975 and HCC827 cell lines. As for the cMET amplified EBC-1 cell line, concentrations up to 10 μM cisplatin induced only 30% growth inhibition and the IC_{50} value was determined by extrapolation (Figure 1).

EBC-1 cells were 74-150 fold more sensitive to crizotinib than the other 3 cell lines, due to the presence of a cMET amplification in these cells. The IC_{50} values of the HCC827 and LUDLU-1 cell line were determined by extrapolation, with the LUDLU-1 being the most resistant to crizotinib (Figure 1 and Table 1). Based on these results, we decided to use the IC_{20} and IC_{40} values of crizotinib during combination treatment (Table 2).

Despite their different genetic backgrounds for cMET and EGFR, all cell lines showed strong antagonism (CI ranging from 1.3 to 2.7) when crizotinib and cisplatin were combined, which was independent of the used treatment schedule (Table 2). This antagonistic effect was visible for all growth inhibition rates of the cells (Figure 2). However, for one treatment condition, *i.e.*, crizotinib followed by cisplatin treatment in the H1975 cell line, an additive effect (CI = 1.0) could be detected. However, this combination only led to 40% growth inhibition at most and needs to be interpreted with caution.

DISCUSSION

Although both cisplatin and crizotinib are active drugs used in monotherapy for the treatment of various forms of NSCLC, the combination of both compounds was found to be antagonistic, independent of the genetic background of the investigated cell lines.

As described in literature, the high sensitivity of the EBC-1 cell line for crizotinib monotherapy can be explained by its cMET amplification, which is known to confer sensitivity to crizotinib and other cMET small molecule inhibitors[19]. In contrast, the EBC-1 cells were not sensitive to cisplatin, with an IC_{50} value around 16 μmol/L. Although

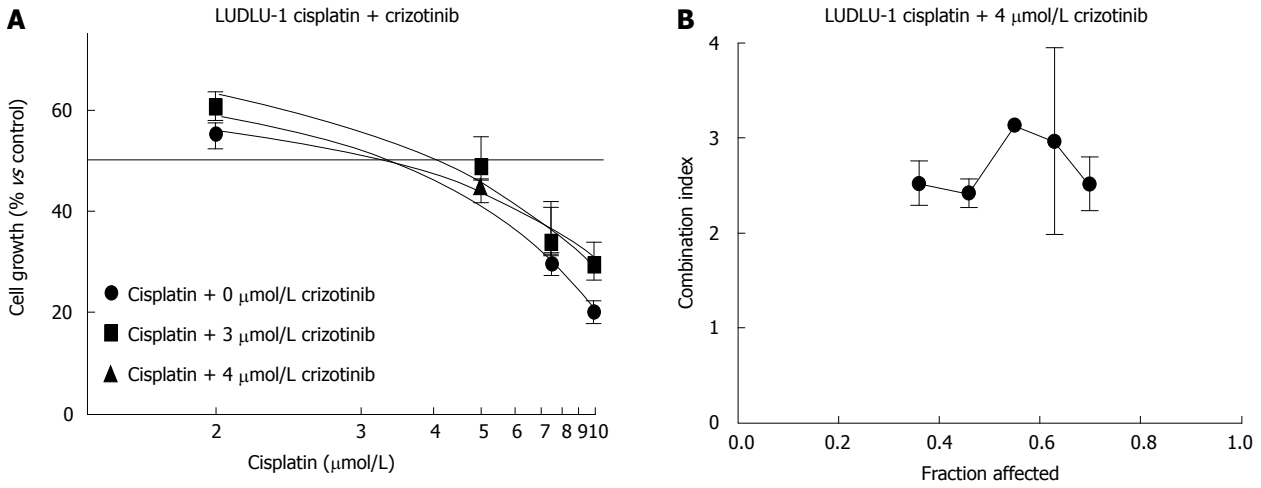

Figure 2 Evaluation of the combination of cisplatin and crizotinib in non-small cell lung cancer cell lines using the fraction affected combination index method. A combination index > 1.2 is antagonistic. A: Growth curves of LUDLU-1 for cisplatin and crizotinib simultaneous treatment during 72 h; B: Combination Index *vs* Fraction affected for the LUDLU-1 cell line, treated simultaneously during 72 h with cisplatin and 5 μmol/crizotinib.

we did not investigate common resistance mechanisms for cisplatin (such as transporters or DNA repair[32-34]) the cMET amplification might also explain the observed results, since cMET activation can induce cisplatin resistance in cell lines[20]. In contrast to the EBC-1 cells, the LUDLU-1 cells (WT EGFR, WT cMET) where the most sensitive to cisplatin but resistant to crizotinib.

When both therapies were combined, an antagonistic effect was observed in all cell lines, even in the cMET amplified EBC-1 cell line with high basal levels of cMET, independent of the treatment schedule. Previous studies suggested that the addition of HGF induced cisplatin resistance in NSCLC cell lines[20], since the activation of cMET would lead to decreased AIF levels. However, a cMET inhibitor combined with cisplatin had never been investigated previously.

Other TKIs have been known *in vitro* to synergize with chemotherapy, such as EGFR-inhibitors with platinum doublet chemotherapy[35-38], whereas clinical trials showed no substantial benefit when combining both drugs. Combinations of cisplatin with EGFR-TKIs, have been investigated extensively, both *in vitro* and *in vivo*. In wild-type EGFR (WT-EGFR) NSCLC cell lines, cisplatin may upregulate phosphorylated EGFR, thus sensitizing these cells to erlotinib; However, in NSCLC cell lines with sensitizing EGFR mutations, combining cisplatin with erlotinib treatment was found to be antagonistic[36]. Other studies showed that platinum analogs in combination with erlotinib led to synergistic cell death in EGFR-mutant NSCLC cell lines and xenografts[37,38]. Possible mechanisms for this synergy are a decrease in hypoxia-inducible factor 1α (HIF1α), a decrease in c-Myc or cell cycle effects[37], while also platinum-adduct formation by cisplatin was increased[38]. However, several clinical trials[8,9,39-41] combining cisplatin with EGFR-TKIs show no benefit in EGFR-WT or in EGFR-mutant patients. Furthermore, triple combinations of cisplatin, pemetrexed and gefitinib[39]; cisplatin, gemcitabine and erlotinib[40] or cisplatin, pemetrexed followed by gefitinib maintenance therapy[41] showed no or only a minor beneficial effect[42]. In contrast, studies investigating the dual combination of erlotinib and pemetrexed, showed synergism in NSCLC cell lines with different genetic backgrounds[35]. Several molecular mechanisms contributed to this synergism. Firstly, pemetrexed increased phosphorylated-EGFR, thus enhancing the effect of EGFR-blocking by erlotinib. Secondly, the combination of both drugs enhanced the reduction of Akt-phosphorylation, leading to increased apoptosis. Finally, the combination of both drugs also decreased the Thymidylate Synthase (TS) *in situ* activity[35], which has been correlated with increased pemetrexed sensitivity[43,44].

For many combination therapies no appropriate preclinical investigations were performed before starting clinical trials to determine whether synergism could be expected and what would be the most optimal treatment schedule. This also precludes proper patient selection. Possibly, the combination of both EGFR/cMET inhibitors with cisplatin and pemetrexed chemotherapy activates survival mechanisms that abrogate the benefit of inhibiting these receptor tyrosine kinases, although these mechanisms remain to be further investigated.

Given the intertwining of the EGFR and cMET signaling, we opted to test the same combination in EGFR mutant cell lines. These cell lines reflect the NSCLC patient populations with exon 19 deletion, L858R and T790M mutations in EGFR, cMET amplification, and different histological subtypes (adenocarcinoma and squamous cell carcinoma). Despite mimicking several clinical combinations *in vitro*, the results showed strong antagonism in all the tested treatment schemes.

In conclusion, we show that the combination of the cMET inhibitor crizotinib with cisplatin is moderately to strongly antagonistic in four NSCLC cell lines. This effect was independent of the cMET/EGFR genetic background, the histological subtype of the cells and the used treatment schedule. Our *in vitro* results suggest an antagonistic effect of combining cMET inhibition with

cisplatin in NSCLC, discouraging further development of this combination in an *in vivo* and/or clinical setting.

COMMENTS

Background

During the last decade, several targeted therapies have been developed for the treatment of lung cancer, inhibiting specific receptors in cancer patients. Given the small number of patients eligible for these therapies, cisplatin based therapy still remains the standard of care treatment for most non-small cell lung cancer (NSCLC) patients. The potential benefit of combining cisplatin with targeted therapies, predominantly against the epidermal growth factor receptor (EGFR), has proved to be disappointing. To investigate the potential benefit of combining cisplatin with crizotinib, the authors have performed *in vitro* studies on a panel of NSCLC lines with different genetic backgrounds.

Research frontiers

The combination of a cMET inhibitor and cisplatin has not been investigated in NSCLC patients to date. However, *in vitro* studies show contradictory results where the outcome is dependent on tumor type and origin. For example, addition of the cMET ligand hepatocyte growth factor (HGF) enhanced cisplatin resistance in seven different NSCLC cell lines.

Innovations and breakthroughs

In vitro studies show contradictory results where the outcome is dependent on tumor type and origin. For example, addition of the cMET ligand HGF enhanced cisplatin resistance in seven different NSCLC cell lines. However, another study in SW620 cells, a *KRAS* mutated colon cancer cell line, showed that conditioned knock-down of cMET did not influence cisplatin sensitivity. In contrast, ovarian cancer cell lines were sensitized towards cisplatin with the addition of HGF. HGF pretreatment of these cells decreased the transcription of protein phosphatase 2A, thus increasing the effect of cisplatin. Here the authors show that the combination of the cMET inhibitor crizotinib with cisplatin is moderately to strongly antagonistic in four NSCLC cell lines. This effect was independent of the cMET/EGFR genetic background, the histological subtype of the cells and the used treatment schedule.

Applications

The *in vitro* results suggest an antagonistic effect of combining cMET inhibition with cisplatin in NSCLC, discouraging further development of this combination in an *in vivo* and/or clinical setting.

Terminology

NSCLC: Non-small cell lung cancer; EGFR: Epidermal growth factor receptor, one of the known drivers of NSCLC.

Peer-review

This is an interesting work that will help to understand the molecular mechanism of resistance of EGFR inhibitors and the necessity of continuing search of new investigation for the treatment of such lethal disease that is NSCLC.

REFERENCES

1 **Mok TS**, D'arcangelo M, Califano R. Clinical outcomes with erlotinib in patients with epidermal growth factor receptor mutation. *Drugs* 2012; **72** Suppl 1: 3-10 [PMID: 22712792 DOI: 10.2165/1163014-S0-000000000-00000]

2 **Taron M**, Ichinose Y, Rosell R, Mok T, Massuti B, Zamora L, Mate JL, Manegold C, Ono M, Queralt C, Jahan T, Sanchez JJ, Sanchez-Ronco M, Hsue V, Jablons D, Sanchez JM, Moran T. Activating mutations in the tyrosine kinase domain of the epidermal growth factor receptor are associated with improved survival in gefitinib-treated chemorefractory lung adenocarcinomas. *Clin Cancer Res* 2005; **11**: 5878-5885 [PMID: 16115929 DOI: 10.1158/1078-0432.CCR-04-2618]

3 **Paez JG**, Jänne PA, Lee JC, Tracy S, Greulich H, Gabriel S, Herman P, Kaye FJ, Lindeman N, Boggon TJ, Naoki K, Sasaki H, Fujii Y, Eck MJ, Sellers WR, Johnson BE, Meyerson M. EGFR mutations in lung cancer: correlation with clinical response to gefitinib therapy. *Science* 2004; **304**: 1497-1500 [PMID: 15118125 DOI: 10.1126/science.1099314]

4 **Smolen GA**, Sordella R, Muir B, Mohapatra G, Barmettler A, Archibald H, Kim WJ, Okimoto RA, Bell DW, Sgroi DC, Christensen JG, Settleman J, Haber DA. Amplification of MET may identify a subset of cancers with extreme sensitivity to the selective tyrosine kinase inhibitor PHA-665752. *Proc Natl Acad Sci USA* 2006; **103**: 2316-2321 [PMID: 16461907 DOI: 10.1073/pnas.0508776103]

5 **Frampton GM**, Ali SM, Rosenzweig M, Chmielecki J, Lu X, Bauer TM, Akimov M, Bufill JA, Lee C, Jentz D, Hoover R, Ou SH, Salgia R, Brennan T, Chalmers ZR, Jaeger S, Huang A, Elvin JA, Erlich R, Fichtenholtz A, Gowen KA, Greenbowe J, Johnson A, Khaira D, McMahon C, Sanford EM, Roels S, White J, Greshock J, Schlegel R, Lipson D, Yelensky R, Morosini D, Ross JS, Collisson E, Peters M, Stephens PJ, Miller VA. Activation of MET via diverse exon 14 splicing alterations occurs in multiple tumor types and confers clinical sensitivity to MET inhibitors. *Cancer Discov* 2015; **5**: 850-859 [PMID: 25971938 DOI: 10.1158/2159-8290.CD-15-0285]

6 **Eberhardt WE**, De Ruysscher D, Weder W, Le Péchoux C, De Leyn P, Hoffmann H, Westeel V, Stahel R, Felip E, Peters S. 2nd ESMO Consensus Conference in Lung Cancer: locally advanced stage III non-small-cell lung cancer. *Ann Oncol* 2015; **26**: 1573-1588 [PMID: 25897013 DOI: 10.1093/annonc/mdv187]

7 **Besse B**, Adjei A, Baas P, Meldgaard P, Nicolson M, Paz-Ares L, Reck M, Smit EF, Syrigos K, Stahel R, Felip E, Peters S. 2nd ESMO Consensus Conference on Lung Cancer: non-small-cell lung cancer first-line/second and further lines of treatment in advanced disease. *Ann Oncol* 2014; **25**: 1475-1484 [PMID: 24669016 DOI: 10.1093/annonc/mdu123]

8 **Herbst RS**, Giaccone G, Schiller JH, Natale RB, Miller V, Manegold C, Scagliotti G, Rosell R, Oliff I, Reeves JA, Wolf MK, Krebs AD, Averbuch SD, Ochs JS, Grous J, Fandi A, Johnson DH. Gefitinib in combination with paclitaxel and carboplatin in advanced non-small-cell lung cancer: a phase III trial--INTACT 2. *J Clin Oncol* 2004; **22**: 785-794 [PMID: 14990633 DOI: 10.1200/JCO.2004.07.215]

9 **Giaccone G**, Herbst RS, Manegold C, Scagliotti G, Rosell R, Miller V, Natale RB, Schiller JH, Von Pawel J, Pluzanska A, Gatzemeier U, Grous J, Ochs JS, Averbuch SD, Wolf MK, Rennie P, Fandi A, Johnson DH. Gefitinib in combination with gemcitabine and cisplatin in advanced non-small-cell lung cancer: a phase III trial--INTACT 1. *J Clin Oncol* 2004; **22**: 777-784 [PMID: 14990632 DOI: 10.1200/JCO.2004.08.001]

10 **Gherardi E**, Birchmeier W, Birchmeier C, Vande Woude G. Targeting MET in cancer: rationale and progress. *Nat Rev Cancer* 2012; **12**: 89-103 [PMID: 22270953 DOI: 10.1038/nrc3205]

11 **Engelman JA**, Zejnullahu K, Mitsudomi T, Song Y, Hyland C, Park JO, Lindeman N, Gale CM, Zhao X, Christensen J, Kosaka T, Holmes AJ, Rogers AM, Cappuzzo F, Mok T, Lee C, Johnson BE, Cantley LC, Jänne PA. MET amplification leads to gefitinib resistance in lung cancer by activating ERBB3 signaling. *Science* 2007; **316**: 1039-1043 [PMID: 17463250 DOI: 10.1126/science.1141478]

12 **Breindel JL**, Haskins JW, Cowell EP, Zhao M, Nguyen DX, Stern DF. EGF receptor activates MET through MAPK to enhance non-small cell lung carcinoma invasion and brain metastasis. *Cancer Res* 2013; **73**: 5053-5065 [PMID: 23794705 DOI: 10.1158/0008-5472.CAN-12-3775]

13 **Guo A**, Villén J, Kornhauser J, Lee KA, Stokes MP, Rikova K, Possemato A, Nardone J, Innocenti G, Wetzel R, Wang Y, MacNeill J, Mitchell J, Gygi SP, Rush J, Polakiewicz RD, Comb MJ. Signaling networks assembled by oncogenic EGFR and c-Met. *Proc Natl Acad Sci USA* 2008; **105**: 692-697 [PMID: 18180459 DOI: 10.1073/pnas.0707270105]

14 **Van Der Steen N**, Pauwels P, Gil-Bazo I, Castañon E, Raez L, Cappuzzo F, Rolfo C. cMET in NSCLC: Can We Cut off the Head of the Hydra? From the Pathway to the Resistance. *Cancers* (Basel) 2015; **7**: 556-573 [PMID: 25815459 DOI: 10.3390/cancers7020556]

15 **Van Der Steen N**, Giovannetti E, Pauwels P, Peters GJ, Hong DS, Cappuzzo F, Hirsch FR, Rolfo C. cMET Exon 14 Skipping: From the Structure to the Clinic. *J Thorac Oncol* 2016; **11**: 1423-1432 [PMID: 27223456 DOI: 10.1016/j.jtho.2016.05.005]

16 **Garajová I**, Giovannetti E, Biasco G, Peters GJ. c-Met as a Target for Personalized Therapy. *Transl Oncogenomics* 2015; **7**: 13-31 [PMID: 26628860 DOI: 10.4137/TOGOG.S30534]

17 **Cui JJ**, Tran-Dubé M, Shen H, Nambu M, Kung PP, Pairish M, Jia L, Meng J, Funk L, Botrous I, McTigue M, Grodsky N, Ryan K, Padrique E, Alton G, Timofeevski S, Yamazaki S, Li Q, Zou H, Christensen J, Mroczkowski B, Bender S, Kania RS, Edwards MP. Structure based drug design of crizotinib (PF-02341066), a potent and selective dual inhibitor of mesenchymal-epithelial transition factor (c-MET) kinase and anaplastic lymphoma kinase (ALK). *J Med Chem* 2011; **54**: 6342-6363 [PMID: 21812414 DOI: 10.1021/jm2007613]

18 **Ou SH**, Bazhenova L, Camidge DR, Solomon BJ, Herman J, Kain T, Bang YJ, Kwak EL, Shaw AT, Salgia R, Maki RG, Clark JW, Wilner KD, Iafrate AJ. Rapid and dramatic radiographic and clinical response to an ALK inhibitor (crizotinib, PF02341066) in an ALK translocation-positive patient with non-small cell lung cancer. *J Thorac Oncol* 2010; **5**: 2044-2046 [PMID: 21102269 DOI: 10.1097/JTO.0b013e318200f9ff]

19 **Ou SH**, Kwak EL, Siwak-Tapp C, Dy J, Bergethon K, Clark JW, Camidge DR, Solomon BJ, Maki RG, Bang YJ, Kim DW, Christensen J, Tan W, Wilner KD, Salgia R, Iafrate AJ. Activity of crizotinib (PF02341066), a dual mesenchymal-epithelial transition (MET) and anaplastic lymphoma kinase (ALK) inhibitor, in a non-small cell lung cancer patient with de novo MET amplification. *J Thorac Oncol* 2011; **6**: 942-946 [PMID: 21623265 DOI: 10.1097/JTO.0b013e31821528d3]

20 **Chen JT**, Huang CY, Chiang YY, Chen WH, Chiou SH, Chen CY, Chow KC. HGF increases cisplatin resistance via down-regulation of AIF in lung cancer cells. *Am J Respir Cell Mol Biol* 2008; **38**: 559-565 [PMID: 18096875 DOI: 10.1165/rcmb.2007-0001OC]

21 **Li Y**, Wang J, Gao X, Han W, Zheng Y, Xu H, Zhang C, He Q, Zhang L, Li Z, Zhou D. c-Met targeting enhances the effect of irradiation and chemical agents against malignant colon cells harboring a KRAS mutation. *PLoS One* 2014; **9**: e113186 [PMID: 25427200 DOI: 10.1371/journal.pone.0113186]

22 **Rasola A**, Anguissola S, Ferrero N, Gramaglia D, Maffe A, Maggiora P, Comoglio PM, Di Renzo MF. Hepatocyte growth factor sensitizes human ovarian carcinoma cell lines to paclitaxel and cisplatin. *Cancer Res* 2004; **64**: 1744-1750 [PMID: 14996735 DOI: 10.1158/0008-5472.CAN-03-2383]

23 **Coltella N**, Rasola A, Nano E, Bardella C, Fassetta M, Filigheddu N, Graziani A, Comoglio PM, Di Renzo MF. p38 MAPK turns hepatocyte growth factor to a death signal that commits ovarian cancer cells to chemotherapy-induced apoptosis. *Int J Cancer* 2006; **118**: 2981-2990 [PMID: 16395709 DOI: 10.1002/ijc.21766]

24 **Olivero M**, Ruggiero T, Saviozzi S, Rasola A, Coltella N, Crispi S, Di Cunto F, Calogero R, Di Renzo MF. Genes regulated by hepatocyte growth factor as targets to sensitize ovarian cancer cells to cisplatin. *Mol Cancer Ther* 2006; **5**: 1126-1135 [PMID: 16731744 DOI: 10.1158/1535-7163.MCT-06-0013]

25 **Amann J**, Kalyankrishna S, Massion PP, Ohm JE, Girard L, Shigematsu H, Peyton M, Juroske D, Huang Y, Stuart Salmon J, Kim YH, Pollack JR, Yanagisawa K, Gazdar A, Minna JD, Kurie JM, Carbone DP. Aberrant epidermal growth factor receptor signaling and enhanced sensitivity to EGFR inhibitors in lung cancer. *Cancer Res* 2005; **65**: 226-235 [PMID: 15665299]

26 **Pao W**, Miller VA, Politi KA, Riely GJ, Somwar R, Zakowski MF, Kris MG, Varmus H. Acquired resistance of lung adenocarcinomas to gefitinib or erlotinib is associated with a second mutation in the EGFR kinase domain. *PLoS Med* 2005; **2**: e73 [PMID: 15737014 DOI: 10.1371/journal.pmed.0020073]

27 **Lutterbach B**, Zeng Q, Davis LJ, Hatch H, Hang G, Kohl NE, Gibbs JB, Pan BS. Lung cancer cell lines harboring MET gene amplification are dependent on Met for growth and survival. *Cancer Res* 2007; **67**: 2081-2088 [PMID: 17332337 DOI: 10.1158/0008-5472.CAN-06-3495]

28 **Pauwels B**, Korst AE, de Pooter CM, Pattyn GG, Lambrechts HA, Baay MF, Lardon F, Vermorken JB. Comparison of the sulforhodamine B assay and the clonogenic assay for in vitro chemoradiation studies. *Cancer Chemother Pharmacol* 2003; **51**: 221-226 [PMID: 12655440 DOI: 10.1007/s00280-002-0557-9]

29 **Vichai V**, Kirtikara K. Sulforhodamine B colorimetric assay for cytotoxicity screening. *Nat Protoc* 2006; **1**: 1112-1116 [PMID: 17406391 DOI: 10.1038/nprot.2006.179]

30 **Chou TC**, Talalay P. Quantitative analysis of dose-effect relationships: the combined effects of multiple drugs or enzyme inhibitors. *Adv Enzyme Regul* 1984; **22**: 27-55 [PMID: 6382953 DOI: 10.1016/0065-2571(84)90007-4]

31 **Bijnsdorp IV**, Giovannetti E, Peters GJ. Analysis of drug interactions. *Methods Mol Biol* 2011; **731**: 421-434 [PMID: 21516426 DOI: 10.1007/978-1-61779-080-5_34]

32 **Fink D**, Zheng H, Nebel S, Norris PS, Aebi S, Lin TP, Nehmé A, Christen RD, Haas M, MacLeod CL, Howell SB. In vitro and in vivo resistance to cisplatin in cells that have lost DNA mismatch repair. *Cancer Res* 1997; **57**: 1841-1845 [PMID: 9157971]

33 **Safaei R**, Larson BJ, Cheng TC, Gibson MA, Otani S, Naerdemann W, Howell SB. Abnormal lysosomal trafficking and enhanced exosomal export of cisplatin in drug-resistant human ovarian carcinoma cells. *Mol Cancer Ther* 2005; **4**: 1595-1604 [PMID: 16227410 DOI: 10.1158/1535-7163.MCT-05-0102]

34 **Samimi G**, Safaei R, Katano K, Holzer AK, Rochdi M, Tomioka M, Goodman M, Howell SB. Increased expression of the copper efflux transporter ATP7A mediates resistance to cisplatin, carboplatin, and oxaliplatin in ovarian cancer cells. *Clin Cancer Res* 2004; **10**: 4661-4669 [PMID: 15269138 DOI: 10.1158/1078-0432.CCR-04-0137]

35 **Giovannetti E**, Lemos C, Tekle C, Smid K, Nannizzi S, Rodriguez JA, Ricciardi S, Danesi R, Giaccone G, Peters GJ. Molecular mechanisms underlying the synergistic interaction of erlotinib, an epidermal growth factor receptor tyrosine kinase inhibitor, with the multitargeted antifolate pemetrexed in non-small-cell lung cancer cells. *Mol Pharmacol* 2008; **73**: 1290-1300 [PMID: 18187583 DOI: 10.1124/mol.107.042382]

36 **Van Schaeybroeck S**, Kyula J, Kelly DM, Karaiskou-McCaul A, Stokesberry SA, Van Cutsem E, Longley DB, Johnston PG. Chemotherapy-induced epidermal growth factor receptor activation determines response to combined gefitinib/chemotherapy treatment in non-small cell lung cancer cells. *Mol Cancer Ther* 2006; **5**: 1154-1165 [PMID: 16731747 DOI: 10.1158/1535-7163.MCT-05-0446]

37 **Lee JG**, Wu R. Erlotinib-cisplatin combination inhibits growth and angiogenesis through c-MYC and HIF-1α in EGFR-mutated lung cancer in vitro and in vivo. *Neoplasia* 2015; **17**: 190-200 [PMID: 25748238 DOI: 10.1016/j.neo.2014.12.008]

38 **Avan A**, Adema AD, Hoebe EK, Huijts CM, Avan A, Veal GJ, Ruijtenbeek R, Wosikowski K, Peters GJ. Modulation of signaling enhances the efficacy of the combination of satraplatin and erlotinib. *Curr Drug Targets* 2014; **15**: 1312-1321 [PMID: 25382189 DOI: 10.2174/1389450115666141107110321]

39 **Soria JC**, Wu YL, Nakagawa K, Kim SW, Yang JJ, Ahn MJ, Wang J, Yang JC, Lu Y, Atagi S, Ponce S, Lee DH, Liu Y, Yoh K, Zhou JY, Shi X, Webster A, Jiang H, Mok TS. Gefitinib plus chemotherapy versus placebo plus chemotherapy in EGFR-mutation-positive non-small-cell lung cancer after progression on first-line gefitinib (IMPRESS): a phase 3 randomised trial. *Lancet Oncol* 2015; **16**: 990-998 [PMID: 26159065 DOI: 10.1016/S1470-2045(15)00121-7]

40 **Wu YL**, Lee JS, Thongprasert S, Yu CJ, Zhang L, Ladrera G, Srimuninnimit V, Sriuranpong V, Sandoval-Tan J, Zhu Y, Liao M, Zhou C, Pan H, Lee V, Chen YM, Sun Y, Margono B, Fuerte F, Chang GC, Seetalarom K, Wang J, Cheng A, Syahruddin E, Qian X, Ho J, Kurnianda J, Liu HE, Jin K, Truman M, Bara I, Mok T. Intercalated combination of chemotherapy and erlotinib for patients with advanced stage non-small-cell lung cancer (FASTACT-2): a randomised, double-blind trial. *Lancet Oncol* 2013; **14**: 777-786 [PMID: 23782814 DOI: 10.1016/S1470-2045(13)70254-7]

41 **Yang JC**, Kang JH, Mok T, Ahn MJ, Srimuninnimit V, Lin CC, Kim DW, Tsai CM, Barraclough H, Altug S, Orlando M, Park K. First-line pemetrexed plus cisplatin followed by gefitinib maintenance therapy

versus gefitinib monotherapy in East Asian patients with locally advanced or metastatic non-squamous non-small cell lung cancer: a randomised, phase 3 trial. *Eur J Cancer* 2014; **50**: 2219-2230 [PMID: 24953333 DOI: 10.1016/j.ejca.2014.05.011]

42 **Gao G**, Ren S, Li A, Xu J, Xu Q, Su C, Guo J, Deng Q, Zhou C. Epidermal growth factor receptor-tyrosine kinase inhibitor therapy is effective as first-line treatment of advanced non-small-cell lung cancer with mutated EGFR: A meta-analysis from six phase III randomized controlled trials. *Int J Cancer* 2012; **131**: E822-E829 [PMID: 22161771 DOI: 10.1002/ijc.27396]

43 **Shih C**, Chen VJ, Gossett LS, Gates SB, MacKellar WC, Habeck LL, Shackelford KA, Mendelsohn LG, Soose DJ, Patel VF, Andis SL, Bewley JR, Rayl EA, Moroson BA, Beardsley GP, Kohler W, Ratnam M, Schultz RM. LY231514, a pyrrolo[2,3-d]pyrimidine-based antifolate that inhibits multiple folate-requiring enzymes. *Cancer Res* 1997; **57**: 1116-1123 [PMID: 9067281]

44 **Peters GJ**, Backus HH, Freemantle S, van Triest B, Codacci-Pisanelli G, van der Wilt CL, Smid K, Lunec J, Calvert AH, Marsh S, McLeod HL, Bloemena E, Meijer S, Jansen G, van Groeningen CJ, Pinedo HM. Induction of thymidylate synthase as a 5-fluorouracil resistance mechanism. *Biochim Biophys Acta* 2002; **1587**: 194-205 [PMID: 12084461 DOI: 10.1016/S0925-4439(02)00082-0]

P- Reviewer: Deng B, Kawai H, Mehdi I, Neninger E
S- Editor: Qiu S **L- Editor**: A **E- Editor**: Lu YJ

World Journal of Clinical Oncology

Submit a Manuscript: http://www.wjgnet.com/esps/
Help Desk: http://www.wjgnet.com/esps/helpdesk.aspx
DOI: 10.5306/wjco.v7.i6.433

World J Clin Oncol 2016 December 10; 7(6): 433-440
ISSN 2218-4333 (online)

ORIGINAL ARTICLE

Retrospective Study

Circulating cytokeratin-positive cells and tumor budding in colorectal cancer

Bruno Märkl, Narjes Wilhelms, Matthias Anthuber, Gerhard Schenkirsch, Günter Schlimok, Daniel Oruzio

Bruno Märkl, Narjes Wilhelms, Institute of Pathology, Klinikum Augsburg, 86156 Augsburg, Germany

Matthias Anthuber, Department of Visceral Surgery, Klinikum Augsburg, 86156 Augsburg, Germany

Gerhard Schenkirsch, Clinical and Population-Based Cancer Registry Augsburg, 86156 Augsburg, Germany

Günter Schlimok, Hematology and Oncology, Diakonissen-krankenhaus Augsburg, 86156 Augsburg, Germany

Daniel Oruzio, Onkologische Praxis MVZ, 86508 Rehling, Germany

Author contributions: Märkl B designed the study, collected the blood samples, performed the statistical analyses and drafted the manuscript; Wilhelms N collected the data, completed the follow-up data, was responsible for the graphics and revised the manuscript; Anthuber M was responsible for the surgical component and informed consent and provided analytical oversight; Schenkirsch G provided follow-up data and revised the manuscript; Schlimok G was involved in designing the study and provided analytical oversight; Oruzio D was responsible for the immunocytochemical analysis and revised the manuscript; all authors have read and approved the final version to be published.

Institutional review board statement: The study was reviewed and approved by the Ethikkomission der Landesärztekammer Bayern.

Informed consent statement: All study participants provided informed written consent prior to study enrollment.

Conflict-of-interest statement: All authors declare that no conflicting interests (including but not limited to commercial, personal, political, intellectual or religious interests) exist.

Data sharing statement: Technical appendix, statistical code, and dataset available from the corresponding author at bruno.maerkl@klinikum-augsburg.de. Participants consent was not obtained but the presented data are anonymized and risk of identification is low.

Manuscript source: Invited manuscript

Correspondence to: Dr. med. Bruno Märkl, MD, PhD, Institute of Pathology, Klinikum Augsburg, Stenglinstrasse 2, 86156 Augsburg, Germany. bruno.maerkl@klinikum-augsburg.de
Telephone: +49-821-4003199
Fax: +49-821-400173199

Received: August 3, 2016
Peer-review started: August 5, 2016
First decision: September 2, 2016
Revised: September 10, 2016
Accepted: October 17, 2016
Article in press: October 19, 2016
Published online: December 10, 2016

Abstract

AIM

To investigate whether circulating cytokeratin-positive (CK^+) cells in the mesenteric blood of resected colorectal specimens are prognostic and correlate with tumor budding.

METHODS

Fifty-six colorectal specimens were collected between 9/2007 and 7/2008. Blood from the mesenteric vein was drawn immediately after receiving the fresh and unfixed specimens in the pathology department. After separation of the mononuclear cells by Ficoll-Hypaque

density-gradient centrifugation, cytological smears were immunocytochemically stained for CK18. Tumor budding was evaluated on slides stained for pan-cytokeratin. The identification of ⩾ 30 buds/1.3 mm^2 was defined as high grade budding.

RESULTS

CK^+ cells and clusters were identified in 29 (48%) and 14 (25%) of the samples, respectively. Two cells were identified in one of three non-malignant cases. Clusters were found exclusively in malignant cases. The occurrence of CK^+ cells or clusters was not associated with any of the evaluated clinicopathological factors, including surgical technique and tumor budding. Moreover, the occurrence of CK^+ cells or clusters had no influence on the cancer-specific survival [75 mo (CI: 61; 88) *vs* 83 mo (CI: 72; 95) and 80 mo (CI: 63; 98) *vs* 79 mo (CI: 69; 89), respectively].

CONCLUSION

CK^+ cells and showed neither prognostic significance nor an association with tumor budding. It is very likely that CK18-staining is not specific enough to identify the relevant cells.

Key words: Colorectal cancer; Circulating cells; Tumor budding; Peripheral blood; Survival

Core tip: Blood from the mesenteric vein of 56 colorectal specimens was drawn and evaluated for CK18 positive epithelial cells (CK^+). CK^+ cells and clusters were identified in a high proportion of cases. However, these cells and clusters were not associated with any of the evaluated clinicopathological factors, including surgical technique and tumor budding. Moreover, the occurrence of CK^+ cells or clusters had no influence on the cancer specific survival. Immunocytochemical staining for CK18 does not seem to be a specific marker of mesenteric blood cells for prognostic identification of relevant circulating tumor cells.

Märkl B, Wilhelms N, Anthuber M, Schenkirsch G, Schlimok G, Oruzio D. Circulating cytokeratin-positive cells and tumor budding in colorectal cancer. *World J Clin Oncol* 2016; 7(6): 433-440 Available from: URL: http://www.wjgnet.com/2218-4333/full/v7/i6/433.htm DOI: http://dx.doi.org/10.5306/wjco.v7.i6.433

INTRODUCTION

Colorectal cancer is a leading cause of cancer-related death, with almost 50000 estimated deaths in the United States in 2016[1]. The prognosis and therapy strongly depend on the UICC tumor stage. Nevertheless, it is well known that a certain proportion of stage Ⅰ/Ⅱ cancers develop an aggressive clinical course. However, approximately 40% of stage Ⅲ cancers show a favorable outcome despite the occurrence of regional lymph node (LN) metastases[2]. Therefore, alternative or additional prognostic factors are necessary to improve both prognostic estimation and therapeutic stratification in colorectal cancer. The National Comprehensive Cancer Network (NCCN) defined risk factors in stage Ⅱ colorectal cancers that justify the administration of an adjuvant therapy[3]. Several attempts have been made to identify other staging strategies. A very sophisticated approach is the development of multigene assays that could be demonstrated to be prognostic in stage Ⅱ colorectal cancers[4,5]. However, due to the limited evidence concerning their clinical value, these tests were not recommended by the NCCN. The only molecular feature that garnered a recommendation is the microsatellite instability (MSI) status[3]. Very recently, MSI, which is caused by mismatch repair (MMR) deficiency, was demonstrated to be highly predictive for immunotherapy by PD-1 blockade[6,7] Since 2005, Pagès *et al*[8] focused on the host's immune response to the tumor. They developed an immune score based on the densities of $CD3^+$ and $CD8^+$ T-cells and showed that this score is independently prognostic. Currently, a large international multicenter study is ongoing to validate the prognostic role of the immunoscore[9]. A different approach is the detection, quantification and analysis of circulating tumor cells (CTC). These cells circulate in the blood stream or are found in the bone marrow and are believed to be a source of distant metastases. Based on our experiences handling and cannulating fresh colorectal specimens[10,11] for LN isolation, we hypothesized that the detection of epithelial cells in the venous blood of these specimens could be prognostic for the development of hematogenous tumor dissemination and progressive disease. Furthermore, we were interested in whether the occurrence of circulating CK^+ cells is associated with tumor budding. Therefore, we collected blood samples from these specimens and evaluated the occurrence of cytokeratin-positive (CK^+) cells. In this retrospective study we analyzed the prognostic role of these cells in colorectal cancer.

MATERIALS AND METHODS

Patients

Fifty-six colorectal cancer cases were collected between September 2007 and July 2008. We assumed a strong correlation between the detection of circulating CK^+ cells and the occurrence of distant metastases with lethal outcome. An absolute difference concerning lethal outcome of 50% with a power of 0.8 and with Alpha = 0.05 resulted in a calculated sample size of 19 cases in each group (proportions sample size test). Inclusion criteria were proven or suspected cancer, a curative intent and free resection margins. For the survival analysis, only malignant cases with a minimal survival time of 2 mo were included. Follow-up data were provided by the Clinical and Population-Based Cancer Registry of Augsburg. Additional data were acquired from

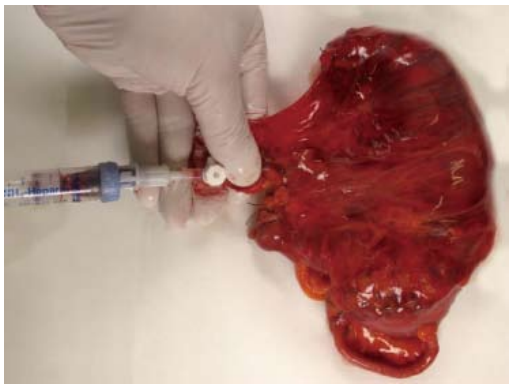

Figure 1 This image illustrates the blood draw from the mesenteric vein. A standard *i.v.* catheter is used to cannulate the mesenteric vein after removal of the clip.

clinical and laboratory information systems. Informed and written consent was obtained from all patients. The study was approved by the ethics committee of the Landesärztekammer Bayern. The study was performed according to the national rules.

Blood sample collection

Immediately after resection, colorectal specimens were delivered fresh to the in-house laboratory of the Institute of Pathology. The specimens were not opened to avoid contamination by epithelial cells from the mucosa. Manual manipulation was reduced to a minimum to reduce the chance of artificial tumor dissemination. After gentle cleaning, the specimens were placed on a clean board and the main vessels were clamped proximally. Then, the ligation or the clip that was placed by the surgeon was withdrawn. The venous vessel was then cannulated with a standard *i.v.*-catheter (17 Gauge, Braun, Melsungen, Germany). Zero point five milliliter to 8 mL (mean: 3.8 mL; SD: 2.6 mL) of venous blood was drawn using NH_4-heparin blood collection tubes (Sarstedt, Nürnbrecht, Germany) (Figure 1). Then, the blood sample was immediately stored until future use.

Blood sample preparation and immunocytochemistry

The protocol for preparing the cytological samples was initially established for the detection of CK^+ cells in bone marrow aspirates[12,13]. In brief, the mononuclear cells were separated by Ficoll-Hypaque density gradient centrifugation (density, 1.077 g/mole) at 900 × g for 30 min. The cells were then washed and centrifuged at 150 × g for 5 min. Approximately 1 × 10^6 cells were placed on each glass slide.

To detect epithelial cells within the peripheral blood, a monoclonal antibody against cytokeratin 18 [Clone CK18 (Clone CK2), 1: 100; Chemicon, Hofheim, Germany] was used. The reactions were developed with the alkaline phosphatase anti-alkaline phosphatase technique combined with a new fuchsin stain to indicate antibody binding, as previously described[12,13]. CK^+ cells and clusters were counted manually (Figure 2). For that all slides were screened by a very experienced technician.

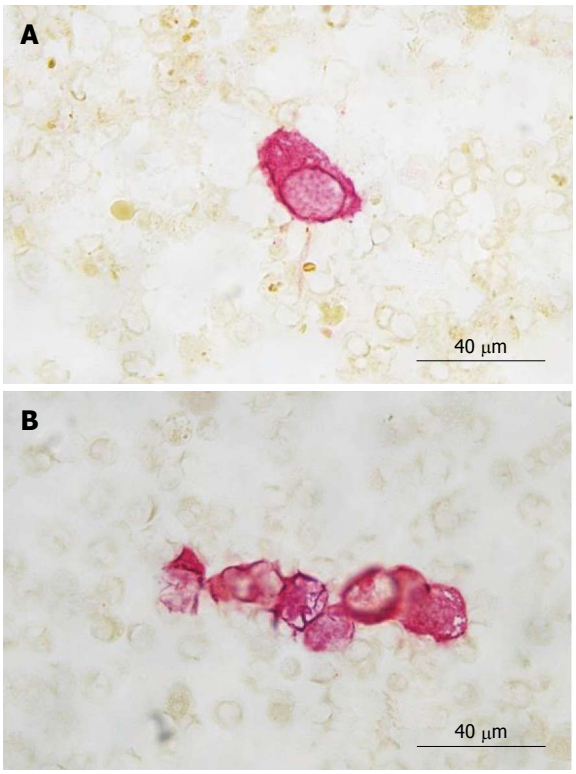

Figure 2 Cytokeratin 18 (Clone cytokeratin 2) cytochemistry. A: A single CK^+ cell is shown in this image; B: A CK^+ cell cluster is shown in this image. CK^+: Cytokeratin-positive.

All positive cases were confirmed by a hemato-oncologist (DO). Data concerning interobserver agreement between these two investigators are not available.

Histopathological evaluation, immunohistochemistry and tumor budding

Colorectal specimens were macroscopically evaluated after fixing overnight in 10% buffered formalin. LNs were dissected using the methylene-blue method[10,11]; samples from the resection margins, the tumor-region and other conspicuous areas were paraffin-embedded. The slides were stained with hematoxylin and eosin (HE) and evaluated by an experienced pathologist (BM). Based on the HE-morphology, slides were selected for further pan-cytokeratin staining which was performed to enable optimal evaluation of tumor budding. For this evaluation, monoclonal mouse antibody AE1/AE3 was used (dilution 1:50; DAKO). Immunoreactions were developed using a labelled streptavidin-biotin system (DAKO Real detection system). All reactions were performed on a Dako-Autostainer system (DAKO, Glostrup, Denmark).

Tumor budding was evaluated by one pathologist (BM). It was defined as detached single tumor cells or clusters of up to four cells. The cut-off for high-grade budding was adapted from Ueno *et al*[14] and defined as $\geqslant$ 30 buds/20 × magnification (= 1.3 mm^2).

Statistical analysis

Metric data were compared using the Mann-Whitney rank sum test. Tabulated data were analyzed with the

Table 1 Clinicopathological data

	Complete collective *n* = 56	CK^+ cell negative *n* = 27	CK^+ cell positive *n* = 29	*P*-value	CK^+ cell cluster negative *n* = 42	CK^+ cell cluster positive *n* = 14	*P*-value
Mean age ± SD	70 ± 13	71 ± 11	69 ± 11	0.844	71 ± 12	66 ± 13	0.167
Gender (M:F)	1:1.5	1:1.7	1:1.4	1.0	1:2	1:0.75	0.538
Laparoscopic surgery	15 (27%)	5 (19%)	10 (34%)		10 (24%)	5 (36%)	
Open surgery	41 (73%)	22 (81%)	19 (66%)	0.223	32 (76%)	9 (64%)	0.489
Right colon	21 (38%)	10 (37%)	11 (38%)		16 (38%)	5 (36%)	
Left colon	29 (52%)	13 (48%)	16 (55%)	0.927	21 (50%)	8 (57%)	0.979
Rectum	6 (11%)	4 (15%)	2 (7%)	0.414[1]	5 (12%)	1 (7%)	1.0[a]
Mean LN count ± SD	32 ± 19	29 ± 16	35 ± 21	0.219	30 ± 16	36 ± 25	0.961
LN positivity	20 (36%)	11 (41%)	9 (31%)	0.632	16 (38%)	4 (29%)	0.747
Low grade	33 (59%)	17 (63%)	22 (76%)		28 (67%)	11 (79%)	
High grade	20 (36%)	8 (30%)	6 (21%)	0.576	11 (26%)	3 (21%)	0.735
Non-malignant	3 (5%)	2 (7%)	1 (3%)	n.c.	3 (7%)	0 (0%)	n.c.
pT1/2	16 (29%)	7 (26%)	9 (31%)		11 (26%)	5 (36%)	
pT3/4	37 (66%)	18 (67%)	19 (66%)	0.977	28 (67%)	9 (64%)	0.736
Mean budding ± SD	21 ± 27	20 ± 23	22 ± 30	0.957	19 ± 20	21 ± 26	0.663
High grade budding	16 (29%)	6 (22%)	10 (34%)	0.472	10 (24%)	6 (43%)	0.190
Distant metastases	11 (20%)	5 (19%)	6 (21%)	1.0	8 (19%)	3 (21%)	1.0

[1]Rectum *vs* colon. CK^+: Cytokeratin positive; SD: Standard deviation; LN: Lymph node; n.c.: Not calculated.

χ^2 test or Fisher's exact test depending on the expected frequency of the observations. Mean values are given ± 1 standard deviation (SD). Linear regression analysis was performed to calculate correlations between metric data. For the survival analyses, Kaplan-Meier curves were calculated and log-rank tests were performed. ROC analyses were performed to determine the optimized cut-offs. The calculation of the follow-up time was performed according to Schemper and Smith[15]. A *P* value < 0.05 was considered significant. All calculations were performed using the statistics package SigmaPlot 13.0 (Systat, Richmond, VA, United States). The statistical methods of this study were reviewed by Bruno Märkl.

RESULTS

Patients

Fifty-six patients were consecutively collected within 10 mo between 2007 and 2008. The patient characteristics are summarized in Table 1. The mean and median follow-up times were 74 (95%CI: 68; 79 mo) and 80 mo (CI: 77; 83 mo), respectively.

CK^+ cells and clusters and their relation to clinicopathological characteristics

CK^+ cells were found in 29 (52%) cases with a mean number of 12 ± 14 cells/10^6 cells. One of these cases was non-malignant with two detected CK^+ cells. CK^+ cell clusters were detected in 14 (25%) cases. The mean number of clusters in positive cases was 3 ± 3 clusters/10^6 cells. No clusters were found in non-malignant cases (Figure 2). There was a strong correlation between CK^+ cells and clusters (R = 0.727; $P < 0.001$). Clusters were always accompanied with single CK^+ cells.

None of the evaluated clinicopathological features (age, gender, location, LN count, grading, T-stage, metastases) showed an association with the occurrence of CK^+ cells or clusters (Table 1). In particular, neither CK^+ cells nor CK^+ clusters showed an association with tumor budding (R = 0.180; $P = 0.185$ and R = 0.0637; $P = 0.647$, respectively). The surgical technique (open *vs* laparoscopic technique) did not influence the occurrence of CK+ cells or clusters (Table 1).

Survival analysis

Forty-eight cases met the inclusion criteria for the cancer-specific survival (CSS) analysis. The CSS analysis revealed no significant differences between cases with or without CK^+ cells or clusters (Figure 3A and B). Despite the lack of significance, the Kaplan-Meier curve for CK^+ cells discriminated between CK^+ positive and negative cases with mean CSS times of 75 mo (CI: 61; 88) *vs* 83 mo (CI: 72; 95) (Figure 3A), respectively. The outcome of CK^+ cluster positive and negative cases was identical, with mean survival times of 80 mo (CI: 63; 98) *vs* 79 mo (CI: 69; 89) (Figure 3B), respectively. A non-significant trend towards an adverse outcome was found in cases with high-grade tumor budding, with a mean survival time of 71 mo (CI: 53; 89 mo) *vs* 83 mo (CI: 73; 93 mo) ($P = 0.187$, Figure 3C), respectively. ROC analysis identified a certain cut-off that was not positive, *i.e.*, did not reveal a threshold with areas under the curve of 0.51 and 0.55 for CK^+ cells and clusters, respectively.

DISCUSSION

In this study, we investigated the prognostic role of circulating CK^+ cells and clusters obtained from the mesenteric blood of colorectal specimens. It was our hypothesis that the venous blood from these specimens should be enriched in circulating CK^+ positive cells originating from the tumor. We used a technique that was well established for the detection of CK^+ cells in the bone marrow of breast, prostate, lung and colorectal cancer

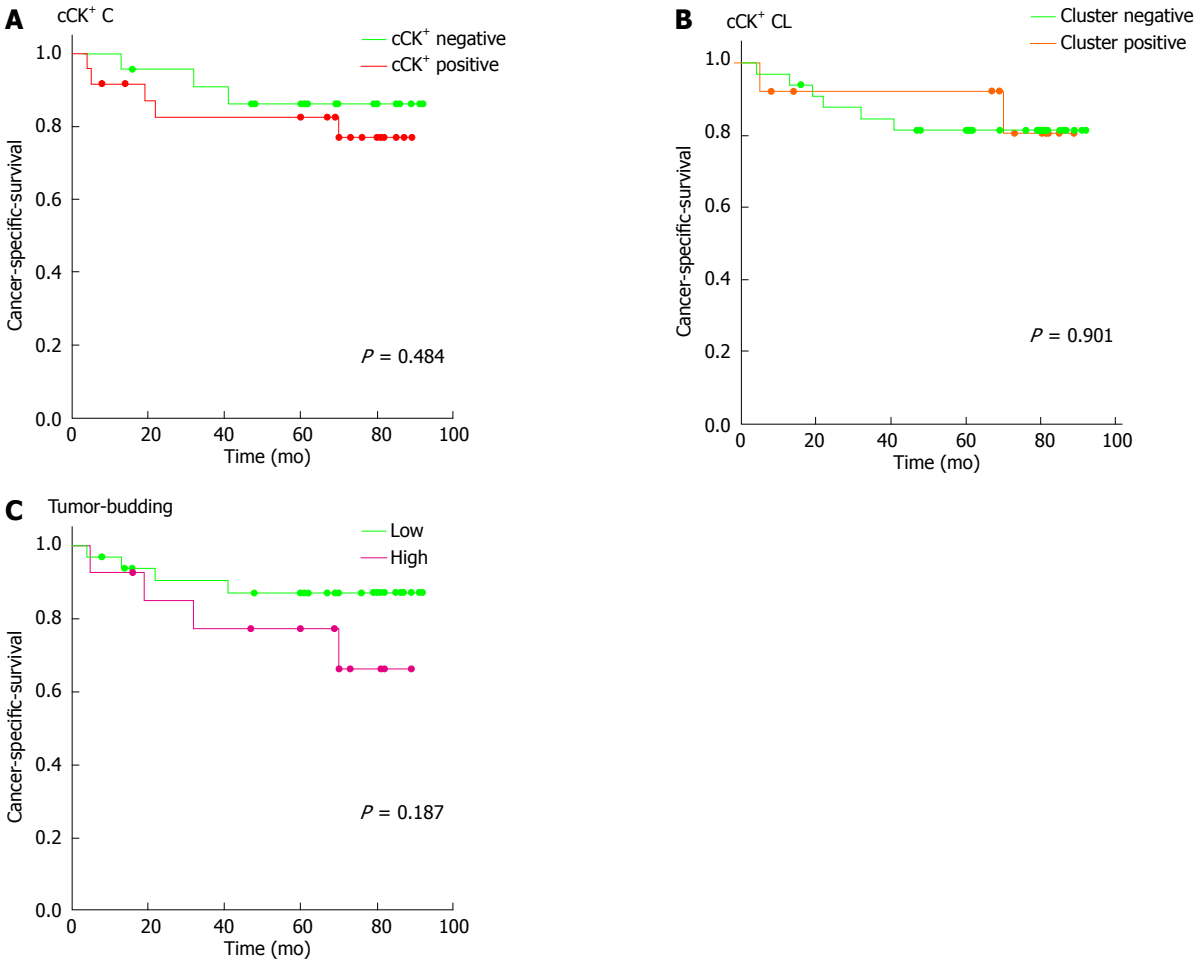

Figure 3 Cancer specific survival. A: Circulating CK$^+$ cell negative *vs* positive; B: Circulating CK$^+$ cell cluster negative *vs* positive; C: Tumor budding negative/low grade *vs* high grade. CK$^+$: Cytokeratin-positive.

patients. Using this method, the detection of cytokeratin-positive cells in the bone marrow could be demonstrated to be prognostic[12,13,16,17].

In this study, we found circulating CK$^+$ cells and clusters in the mesenteric blood in a high proportion of cases (52% and 27%, respectively). This positive rate is within the range published in the literature (Table 1). However, it must be noted that only Leather *et al*[18] used immunocytochemistry to detect circulating epithelial cells in the mesenteric blood. In all other identified studies, molecular or flow cytometry techniques were used[19-35]. By using case numbers, we calculated a mean positivity rate in these studies of 43%. When we restricted this calculation to studies that also included stage Ⅳ cases, the mean positivity rate was 55%. We detected 2 CK$^+$ cells/10^6 cells in one non-malignant case with diverticulitis. The phenomenon of circulating epithelial cells in the blood in the absence of a malignant tumor has been found by other authors. Pantel *et al*[36] reported the detection of CK$^+$ cells in benign colon diseases using two different commercial tests in 11.3% and 18.9% of cases, respectively. In summary, this indicates that the results generated with our immunocytochemical method are comparable to other techniques and are valid.

Despite using an obviously sensitive method, we could not confirm our hypothesis of circulating epithelial cells in the mesenteric blood being prognostic markers of colorectal cancer that correlate with tumor budding. This study is limited by a relatively small case number (n = 56) and is therefore underpowered to detect effects that are possibly smaller than expected. We presumed that the prognostic effect of CK$^+$ cells was at least as strong as node positivity. Indeed, nodal status revealed a good discrimination with regards to cancer specific survival with a P value of 0.058 (data not shown). The strengths of this study are the long follow-up time and the precise evaluation of histological features including an immunohistochemical tumor budding assessment.

Tumor budding is a well investigated prognostic parameter in gastrointestinal cancers. Despite considerable limitations due to the lack of a generally accepted definition and only moderate interobserver agreement, it has been shown in many studies[37,38]. It is believed to be an expression of the epithelial-mesenchymal transition (EMT), which is an important initial step in cancer progression[39]. None of the studies shown in Table 2 investigated the possible relationship between the phenomenon of tumor cell isolation at the invasion front of colorectal cancers and

Table 2 Literature: Circulating tumor cells in the mesenteric blood

Ref.	*n*	Year	Method	Material	Stages	%positive	Prognostic relevance
Leather *et al*[18]	42	1993	ICC	Mesenteric and peripheral blood	Ⅰ, Ⅱ, Ⅲ, Ⅳ	15	n.a.
Nakamori *et al*[19]	35	1997	PCR	Mesenteric and peripheral blood	Ⅰ, Ⅱ, Ⅲ, Ⅳ	26	uv predictive for recurrence
Luo *et al*[20]	54	1999	PCR	Mesenteric blood	Ⅰ, Ⅱ, Ⅲ, Ⅳ	76	Predictive for metastases
Taniguchi *et al*[22]	53	2000	PCR	Mesenteric and peripheral blood	Ⅰ, Ⅱ, Ⅲ	68	uv survival
Yamaguchi *et al*[23]	52	2000	PCR	Mesenteric blood	Ⅰ, Ⅱ, Ⅲ, Ⅳ	44	mv survival
Iinuma *et al*[21]	23	2000	MACS	Mesenteric blood	Ⅰ, Ⅱ, Ⅲ, Ⅳ	39	uv survival
Fujita *et al*[25]	35	2001	PCR	Mesenteric blood	Ⅰ, Ⅱ, Ⅲ	29	uv recurrence/survival
Etoh *et al*[24]	24	2001	PCR	Mesenteric blood	Ⅰ, Ⅱ, Ⅲ, Ⅳ	29	uv recurrence/survival
Guller *et al*[26]	39	2002	PCR	Mesenteric and peripheral blood	Ⅰ, Ⅱ, Ⅲ	8[1]/28[2]	[3]
Tien *et al*[27]	58	2002	PCR	Mesenteric and peripheral blood	Ⅱ, Ⅲ, Ⅳ	45[4]	n.a.
Akashi *et al*[28]	80	2003	PCR	Mesenteric blood	Ⅰ, Ⅱ, Ⅲ	44	uv metastatic disease; mv no
Nozawa *et al*[29]	41	2003	RTA	Mesenteric and peripheral blood	Ⅰ, Ⅱ, Ⅲ, Ⅳ	37	uv predictive for metastatic disease
Sunouchi *et al*[30]	37	2003	PCR	Mesenteric blood	Ⅰ, Ⅱ, Ⅲ, Ⅳ	43	uv survival
Zhang *et al*[32]	58	2005	PCR	Bone marrow, portal blood, peripheral blood	Ⅰ, Ⅱ, Ⅲ, Ⅳ	74	correlation with stage - no outcome analysis
Sadahiro *et al*[31]	100	2005	PCR	Mesenteric and peripheral blood	Ⅰ, Ⅱ, Ⅲ	45[5]/48[6]	no
Kanellos *et al*[34]	108	2006	PCR	Mesenteric blood	Ⅰ, Ⅱ, Ⅲ	11	uv metastatic disease/survival
Iinuma *et al*[33]	167	2006	PCR	Mesenteric and peripheral blood	Ⅰ, Ⅱ, Ⅲ, Ⅳ	10/34[7]	mv survival
Tseng *et al*[35]	135	2015	FACS	Mesenteric	Ⅰ, Ⅱ, Ⅲ	68	mv survival

[1]Blood; [2]Blood and peritoneal fluid; [3]No separate evaluation for blood samples; [4]Multiple measurements; [5]Mesenteric blood; [6]Peripheral blood; [7]Mesenteric. n.a.: Not available; uv: Uni-variable; mv: Multi-variable; ICC: Immunocytochemistry; PCR: Polymerase chain reaction; MACS: Magnetic activated cell sorting; FACS: Fluorescence activated cell sorting.

the occurrence of CTCs in the blood. Moreover, a literature search within the Medline, Embase and Google Scholar databases did not reveal an investigation that addressed this topic. Cao *et al*[40] postulated in a review that EMT leads to tumor budding and subsequent blood vessel invasion. However, this is not supported by other references. To us, it seemed quite obvious that a correlation between these two factors exists. However, we were not able to confirm this hypothesis. We could not identify a correlation between tumor budding and circulating CK^+ cells and could not confirm that a combination of tumor budding and CK^+ cells was prognostic. Tumor budding alone discriminated clearly between two prognostic groups (Figure 3C). However, significance was likely not achieved due to the small sample number.

The data concerning the prognostic significance of CTCs and disseminated tumor cells (DTCs) are conflicting[41]. However, there is growing evidence that CTC/DTCs are of prognostic significance. Two commercial tests based on immunomagnetic separation targeting EpCAM (BerEp4) are currently available. They have proven to be prognostic, particularly in the metastatic stage of different cancers including colorectal cancer[42,43]. Two meta-analyses addressed this topic. Katsuno *et al*[44] restricted their analysis to molecularly detected CTCs in mesenteric blood and included 9 studies. They found a favorable outcome in patients negative for CTCs [hazard ratio (HR) 0.4-0.08][44]. Rahbari *et al*[45] included 36 studies with a total 3094 patients. They also identified a prognostic effect of CTCs. However, stratification according to the sampling compartment revealed that CTCs of peripheral blood were prognostic but those of the mesenteric bone marrow blood were not[45]. Similarly, our study found that the identification of CK^+ cells or clusters had no prognostic effect. In addition, the approach using ROC analyses to identify a certain cut-off of cells which might be prognostic failed.

CTCs seem to comprise different cell types of neoplastic and non-neoplastic origin. Moreover, it is very likely that cells derived from cancer have different potential to escape from immunogenic destruction and to establish tumor growth at a distant site. Depending on the compartment, cells may undergo a change in their phenotype[40,41,46]. As mentioned before, EMT is a hallmark process in cancer progression and is associated with impaired outcome[46,47]. Cells undergoing EMT lose their epithelial phenotype and gain mesenchymal features. The use of methods optimized for the detection of epithelial cells is prone to fail in the detection of all CTCs. Moreover, these methods may fail to detect the most relevant cells[48]. Currently, the most interesting cells in this context are cells with stem-cell features. The realization of a fast, exact and cost effective technical method to detect these cells is likely the most promising approach.

In this study, we hypothesized that the immunocytochemical detection of CK^+ cell in the mesenteric blood of colorectal cancer specimens correlates with tumor budding and could serve as an easy to determine prognostic factor. Drawing the blood after resection would avoid delay and additional risk during the operation. None of these hypotheses could be confirmed in our study. Given the current literature, peripheral blood and not mesenteric blood is the optimal material for the detection of CTCs. More sophisticated techniques including molecular approaches are relatively expensive and their availability is limited. Nevertheless, they have the potential to detect exactly the cells which are most likely to be relevant to the clinical

course of the disease. Immunocytochemical detection seems to be less specific and is not favorable.

ACKNOWLEDGMENTS

The authors are thankful to Carmen Sailer for her excellent technical assistance.

COMMENTS

Background

Colorectal cancer is one of the most common cancers in men and women. Its prognosis depends mainly on the (UICC-) tumor stage. However, it is also known that certain proportion of cancers with otherwise favorable features and low stages show an aggressive clinical course while locally advanced cancers so not relapse. It is accepted that the detection of circulating tumor cells has the potential to improve the prognosis estimation not only in colorectal cancer.

Research frontiers

The main topic in the research field of circulating tumor cells is the influence of the different compartments (peripheral blood, mesenteric blood or bone marrow) on the clinical significance of the detected cells. Other important questions are the methods for the assessment and the type of cells (*e.g.*, stem cells) which are most informative to predict the outcome.

Innovations and breakthroughs

The innovation of this study is the evaluation of the blood draw from resected specimens. A direct correlation with tumor budding as a source for the circulating tumor cells is also a new approach.

Applications

Because the authors' hypotheses could not be confirmed, the main conclusions are that mesenteric blood is probably not the best compartment for the identification of the relevant cells and more sophisticated methods may be superior over immunocytochemistry. Molecular techniques are more sensitive in detecting cells with a high potential to serve as the origin for distant metastases.

Terminology

Circulating tumor cells are cells that lost its cohesion to the primary tumor mass and achieved access to the vascular system including the bone marrow.

Peer-review

This is an interesting manuscript which appears to add to the existing body of literature around this subject. The design is clear.

REFERENCES

1 **Society AC**. Cancer Facts & Figures 2016. Atlanta: American Cancer Society, 2016
2 **The National Cancer Registration Service EO**. Bowel cancer survival statistics. Available from: URL: http://www.cancerresearchuk.org/health-professional/cancer-statistics/statistics-by-cancer-type/bowel-cancer/survival#one
3 **NCCN**. NCCN Clinical Practice Guidelines in Oncology (NCCN Guidelines). Colon Cancer (Version 2.2015). USA: NCCN, 2015
4 **Kopetz S**, Tabernero J, Rosenberg R, Jiang ZQ, Moreno V, Bachleitner-Hofmann T, Lanza G, Stork-Sloots L, Maru D, Simon I, Capellà G, Salazar R. Genomic classifier ColoPrint predicts recurrence in stage II colorectal cancer patients more accurately than clinical factors. *Oncologist* 2015; **20**: 127-133 [PMID: 25561511 DOI: 10.1634/theoncologist.2014-0325]
5 **Gray RG**, Quirke P, Handley K, Lopatin M, Magill L, Baehner FL, Beaumont C, Clark-Langone KM, Yoshizawa CN, Lee M, Watson D, Shak S, Kerr DJ. Validation study of a quantitative multigene reverse transcriptase-polymerase chain reaction assay for assessment of recurrence risk in patients with stage II colon cancer. *J Clin Oncol* 2011; **29**: 4611-4619 [PMID: 22067390 DOI: 10.1200/JCO.2010.32.8732]
6 **Diaz LA**, Le DT. PD-1 Blockade in Tumors with Mismatch-Repair Deficiency. *N Engl J Med* 2015; **373**: 1979 [PMID: 26559582 DOI: 10.1056/NEJMc1510353]
7 **Dudley JC**, Lin MT, Le DT, Eshleman JR. Microsatellite Instability as a Biomarker for PD-1 Blockade. *Clin Cancer Res* 2016; **22**: 813-820 [PMID: 26880610 DOI: 10.1158/1078-0432.CCR-15-1678]
8 **Pagès F**, Galon J, Dieu-Nosjean MC, Tartour E, Sautès-Fridman C, Fridman WH. Immune infiltration in human tumors: a prognostic factor that should not be ignored. *Oncogene* 2010; **29**: 1093-1102 [PMID: 19946335]
9 **Galon J**, Mlecnik B, Bindea G, Angell HK, Berger A, Lagorce C, Lugli A, Zlobec I, Hartmann A, Bifulco C, Nagtegaal ID, Palmqvist R, Masucci GV, Botti G, Tatangelo F, Delrio P, Maio M, Laghi L, Grizzi F, Asslaber M, D'Arrigo C, Vidal-Vanaclocha F, Zavadova E, Chouchane L, Ohashi PS, Hafezi-Bakhtiari S, Wouters BG, Roehrl M, Nguyen L, Kawakami Y, Hazama S, Okuno K, Ogino S, Gibbs P, Waring P, Sato N, Torigoe T, Itoh K, Patel PS, Shukla SN, Wang Y, Kopetz S, Sinicrope FA, Scripcariu V, Ascierto PA, Marincola FM, Fox BA, Pagès F. Towards the introduction of the 'Immunoscore' in the classification of malignant tumours. *J Pathol* 2014; **232**: 199-209 [PMID: 24122236 DOI: 10.1002/path.4287]
10 **Kerwel TG**, Spatz J, Anthuber M, Wünsch K, Arnholdt H, Märkl B. Injecting methylene blue into the inferior mesenteric artery assures an adequate lymph node harvest and eliminates pathologist variability in nodal staging for rectal cancer. *Dis Colon Rectum* 2009; **52**: 935-941 [PMID: 19502859 DOI: 10.1007/DCR.0b013e31819f28c9]
11 **Märkl B**, Kerwel TG, Jähnig HG, Oruzio D, Arnholdt HM, Schöler C, Anthuber M, Spatz H. Methylene blue-assisted lymph node dissection in colon specimens: a prospective, randomized study. *Am J Clin Pathol* 2008; **130**: 913-919 [PMID: 19019768]
12 **Braun S**, Pantel K, Müller P, Janni W, Hepp F, Kentenich CR, Gastroph S, Wischnik A, Dimpfl T, Kindermann G, Riethmüller G, Schlimok G. Cytokeratin-positive cells in the bone marrow and survival of patients with stage I, II, or III breast cancer. *N Engl J Med* 2000; **342**: 525-533 [PMID: 10684910 DOI: 10.1056/NEJM200002243420801]
13 **Pantel K**, Schlimok G, Angstwurm M, Weckermann D, Schmaus W, Gath H, Passlick B, Izbicki JR, Riethmüller G. Methodological analysis of immunocytochemical screening for disseminated epithelial tumor cells in bone marrow. *J Hematother* 1994; **3**: 165-173 [PMID: 7530132 DOI: 10.1089/scd.1.1994.3.165]
14 **Ueno H**, Murphy J, Jass JR, Mochizuki H, Talbot IC. Tumour 'budding' as an index to estimate the potential of aggressiveness in rectal cancer. *Histopathology* 2002; **40**: 127-132 [PMID: 11952856]
15 **Schemper M**, Smith TL. A note on quantifying follow-up in studies of failure time. *Control Clin Trials* 1996; **17**: 343-346 [PMID: 8889347]
16 **Lindemann F**, Schlimok G, Dirschedl P, Witte J, Riethmüller G. Prognostic significance of micrometastatic tumour cells in bone marrow of colorectal cancer patients. *Lancet* 1992; **340**: 685-689 [PMID: 1381801]
17 **Weckermann D**, Polzer B, Ragg T, Blana A, Schlimok G, Arnholdt H, Bertz S, Harzmann R, Klein CA. Perioperative activation of disseminated tumor cells in bone marrow of patients with prostate cancer. *J Clin Oncol* 2009; **27**: 1549-1556 [PMID: 19237635 DOI: 10.1200/JCO.2008.17.0563]
18 **Leather AJ**, Gallegos NC, Kocjan G, Savage F, Smales CS, Hu W, Boulos PB, Northover JM, Phillips RK. Detection and enumeration of circulating tumour cells in colorectal cancer. *Br J Surg* 1993; **80**: 777-780 [PMID: 7687189]
19 **Nakamori S**, Kameyama M, Furukawa H, Takeda O, Sugai S, Imaoka S, Nakamura Y. Genetic detection of colorectal cancer cells in circulation and lymph nodes. *Dis Colon Rectum* 1997; **40**: S29-S36 [PMID: 9378009]
20 **Luo C**, Li S. [The detection and its clinical significance of cancer cells in portal vein blood of patients with colorectal carcinoma]. *Zhonghua Waike Zazhi* 1999; **37**: 214-215 [PMID: 11829822]
21 **Iinuma H**, Okinaga K, Adachi M, Suda K, Sekine T, Sakagawa K, Baba Y, Tamura J, Kumagai H, Ida A. Detection of tumor cells in blood using CD45 magnetic cell separation followed by nested mutant allele-specific amplification of p53 and K-ras genes in patients with colorectal cancer. *Int J Cancer* 2000; **89**: 337-344 [PMID: 10956407]

22 **Taniguchi T**, Makino M, Suzuki K, Kaibara N. Prognostic significance of reverse transcriptase-polymerase chain reaction measurement of carcinoembryonic antigen mRNA levels in tumor drainage blood and peripheral blood of patients with colorectal carcinoma. *Cancer* 2000; **89**: 970-976 [PMID: 10964326]

23 **Yamaguchi K**, Takagi Y, Aoki S, Futamura M, Saji S. Significant detection of circulating cancer cells in the blood by reverse transcriptase-polymerase chain reaction during colorectal cancer resection. *Ann Surg* 2000; **232**: 58-65 [PMID: 10862196]

24 **Etoh T**, Ueo H, Inoue H, Sato K, Utsunomiya T, Barnard GF, Kitano S, Mori M. Clinical significance of K-Ras mutations in intraoperative tumor drainage blood from patients with colorectal carcinoma. *Ann Surg Oncol* 2001; **8**: 407-412 [PMID: 11407514]

25 **Fujita S**, Kudo N, Akasu T, Moriya Y. Detection of cytokeratin 19 and 20 mRNA in peripheral and mesenteric blood from colorectal cancer patients and their prognosis. *Int J Colorectal Dis* 2001; **16**: 141-146 [PMID: 11459287]

26 **Guller U**, Zajac P, Schnider A, Bösch B, Vorburger S, Zuber M, Spagnoli GC, Oertli D, Maurer R, Metzger U, Harder F, Heberer M, Marti WR. Disseminated single tumor cells as detected by real-time quantitative polymerase chain reaction represent a prognostic factor in patients undergoing surgery for colorectal cancer. *Ann Surg* 2002; **236**: 768-775; discussion 775-776 [PMID: 12454515 DOI: 10.1097/01.SLA.0000036267.30107.B9]

27 **Tien YW**, Lee PH, Wang SM, Hsu SM, Chang KJ. Simultaneous detection of colonic epithelial cells in portal venous and peripheral blood during colorectal cancer surgery. *Dis Colon Rectum* 2002; **45**: 23-29 [PMID: 11786759]

28 **Akashi A**, Komuta K, Haraguchi M, Ueda T, Okudaira S, Furui J, Kanematsu T. Carcinoembryonic antigen mRNA in the mesenteric vein is not a predictor of hepatic metastasis in patients with resectable colorectal cancer: a long-term study. *Dis Colon Rectum* 2003; **46**: 1653-1658 [PMID: 14668591 DOI: 10.1097/01.DCR.0000098926.23792.58]

29 **Nozawa H**, Watanabe T, Ohnishi T, Tada T, Tsurita G, Sasaki S, Kitayama J, Nagawa H. Detection of cancer cells in mesenteric vein and peripheral vessels by measuring telomerase activity in patients with colorectal cancer. *Surgery* 2003; **134**: 791-798 [PMID: 14639358 DOI: 10.1016/s0039]

30 **Sunouchi K**, Machinami R, Mori M, Namiki K, Hattori S, Murata Y, Tsuchiya T, Mizuno H, Tadokoro M. Clinical impact of carcinoembryonic antigen messenger ribonucleic acid expression in tumor-draining vein blood on postoperative liver metastasis in patients with colorectal carcinoma: a prospective, cohort study. *Dis Colon Rectum* 2003; **46**: 467-473 [PMID: 12682539 DOI: 10.1097/01.DCR.0000059664.63723.A8]

31 **Sadahiro S**, Suzuki T, Ishikawa K, Saguchi T, Maeda Y, Yasuda S, Makuuchi H, Yurimoto S, Murayama C. Detection of carcinoembryonic antigen messenger RNA-expressing cells in portal and peripheral blood during surgery does not influence relapse in colorectal cancer. *Ann Surg Oncol* 2005; **12**: 988-994 [PMID: 16244799 DOI: 10.1245/ASO.2005.03.565]

32 **Zhang XW**, Yang HY, Fan P, Yang L, Chen GY. Detection of micrometastasis in peripheral blood by multi-sampling in patients with colorectal cancer. *World J Gastroenterol* 2005; **11**: 436-438 [PMID: 15637763 DOI: 10.3748/wjg.v11.i3.436]

33 **Iinuma H**, Okinaga K, Egami H, Mimori K, Hayashi N, Nishida K, Adachi M, Mori M, Sasako M. Usefulness and clinical significance of quantitative real-time RT-PCR to detect isolated tumor cells in the peripheral blood and tumor drainage blood of patients with colorectal cancer. *Int J Oncol* 2006; **28**: 297-306 [PMID: 16391782]

34 **Kanellos I**, Zacharakis E, Kanellos D, Pramateftakis MG, Tsahalis T, Altsitsiadis E, Betsis D. Prognostic significance of CEA levels and detection of CEA mRNA in draining venous blood in patients with colorectal cancer. *J Surg Oncol* 2006; **94**: 3-8 [PMID: 16788936 DOI: 10.1002/jso.20549]

35 **Tseng JY**, Yang CY, Yang SH, Lin JK, Lin CH, Jiang JK. Circulating CD133(+)/ESA(+) cells in colorectal cancer patients. *J Surg Res* 2015; **199**: 362-370 [PMID: 26119272 DOI: 10.1016/j.jss.2015.05.057]

36 **Pantel K**, Denève E, Nocca D, Coffy A, Vendrell JP, Maudelonde T, Riethdorf S, Alix-Panabières C. Circulating epithelial cells in patients with benign colon diseases. *Clin Chem* 2012; **58**: 936-940 [PMID: 22205690 DOI: 10.1373/clinchem.2011.175570]

37 **Puppa G**, Senore C, Sheahan K, Vieth M, Lugli A, Zlobec I, Pecori S, Wang LM, Langner C, Mitomi H, Nakamura T, Watanabe M, Ueno H, Chasle J, Conley SA, Herlin P, Lauwers GY, Risio M. Diagnostic reproducibility of tumour budding in colorectal cancer: a multicentre, multinational study using virtual microscopy. *Histopathology* 2012; **61**: 562-575 [PMID: 22765314 DOI: 10.1111/j.1365-2559.2012.04270.x]

38 **Märkl B**, Arnholdt HM. Prognostic significance of tumor budding in gastrointestinal tumors. *Expert Rev Anticancer Ther* 2011; **11**: 1521-1533 [PMID: 21999126 DOI: 10.1586/era.11.156]

39 **Brabletz T**, Jung A, Reu S, Porzner M, Hlubek F, Kunz-Schughart LA, Knuechel R, Kirchner T. Variable beta-catenin expression in colorectal cancers indicates tumor progression driven by the tumor environment. *Proc Natl Acad Sci USA* 2001; **98**: 10356-10361 [PMID: 11526241 DOI: 10.1073/pnas.171610498]

40 **Cao H**, Xu E, Liu H, Wan L, Lai M. Epithelial-mesenchymal transition in colorectal cancer metastasis: A system review. *Pathol Res Pract* 2015; **211**: 557-569 [PMID: 26092594 DOI: 10.1016/j.prp.2015.05.010]

41 **Hardingham JE**, Grover P, Winter M, Hewett PJ, Price TJ, Thierry B. Detection and Clinical Significance of Circulating Tumor Cells in Colorectal Cancer--20 Years of Progress. *Mol Med* 2015; **21** Suppl 1: S25-S31 [PMID: 26605644 DOI: 10.2119/molmed.2015.00149]

42 **Hardingham JE**, Kotasek D, Farmer B, Butler RN, Mi JX, Sage RE, Dobrovic A. Immunobead-PCR: a technique for the detection of circulating tumor cells using immunomagnetic beads and the polymerase chain reaction. *Cancer Res* 1993; **53**: 3455-3458 [PMID: 8101760]

43 **Cohen SJ**, Punt CJ, Iannotti N, Saidman BH, Sabbath KD, Gabrail NY, Picus J, Morse M, Mitchell E, Miller MC, Doyle GV, Tissing H, Terstappen LW, Meropol NJ. Relationship of circulating tumor cells to tumor response, progression-free survival, and overall survival in patients with metastatic colorectal cancer. *J Clin Oncol* 2008; **26**: 3213-3221 [PMID: 18591556 DOI: 10.1200/JCO.2007.15.8923]

44 **Katsuno H**, Zacharakis E, Aziz O, Rao C, Deeba S, Paraskeva P, Ziprin P, Athanasiou T, Darzi A. Does the presence of circulating tumor cells in the venous drainage of curative colorectal cancer resections determine prognosis? A meta-analysis. *Ann Surg Oncol* 2008; **15**: 3083-3091 [PMID: 18787906 DOI: 10.1245/s10434-008-0131-8]

45 **Rahbari NN**, Aigner M, Thorlund K, Mollberg N, Motschall E, Jensen K, Diener MK, Büchler MW, Koch M, Weitz J. Meta-analysis shows that detection of circulating tumor cells indicates poor prognosis in patients with colorectal cancer. *Gastroenterology* 2010; **138**: 1714-1726 [PMID: 20100481 DOI: 10.1053/j.gastro.2010.01.008]

46 **Lim SH**, Becker TM, Chua W, Ng WL, de Souza P, Spring KJ. Circulating tumour cells and the epithelial mesenchymal transition in colorectal cancer. *J Clin Pathol* 2014; **67**: 848-853 [PMID: 25008452 DOI: 10.1136/jclinpath-2014-202499]

47 **Guinney J**, Dienstmann R, Wang X, de Reyniès A, Schlicker A, Soneson C, Marisa L, Roepman P, Nyamundanda G, Angelino P, Bot BM, Morris JS, Simon IM, Gerster S, Fessler E, De Sousa E Melo F, Missiaglia E, Ramay H, Barras D, Homicsko K, Maru D, Manyam GC, Broom B, Boige V, Perez-Villamil B, Laderas T, Salazar R, Gray JW, Hanahan D, Tabernero J, Bernards R, Friend SH, Laurent-Puig P, Medema JP, Sadanandam A, Wessels L, Delorenzi M, Kopetz S, Vermeulen L, Tejpar S. The consensus molecular subtypes of colorectal cancer. *Nat Med* 2015; **21**: 1350-1356 [PMID: 26457759 DOI: 10.1038/nm.3967]

48 **Grover PK**, Cummins AG, Price TJ, Roberts-Thomson IC, Hardingham JE. Circulating tumour cells: the evolving concept and the inadequacy of their enrichment by EpCAM-based methodology for basic and clinical cancer research. *Ann Oncol* 2014; **25**: 1506-1516 [PMID: 24651410 DOI: 10.1093/annonc/mdu018]

P- Reviewer: Horne J, Lewitowicz P, Shimada Y **S- Editor**: Song XX
L- Editor: A **E- Editor**: Lu YJ

Published by **Baishideng Publishing Group Inc**
8226 Regency Drive, Pleasanton, CA 94588, USA
Telephone: +1-925-223-8242
Fax: +1-925-223-8243
E-mail: bpgoffice@wjgnet.com
Help Desk: http://www.wjgnet.com/esps/helpdesk.aspx
http://www.wjgnet.com