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EDITORIAL

# New insights into tumor dormancy: Targeting DNA repair pathways

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## Abstract

Over the past few decades, major strides have advanced the techniques for early detection and treatment of cancer. However, metastatic tumor growth still accounts for the majority of cancer-related deaths worldwide. In fact, breast cancers are notorious for relapsing years or decades after the initial clinical treatment, and this relapse can vary according to the type of breast cancer. In estrogen receptor-positive breast cancers, late tumor relapses frequently occur whereas relapses in estrogen receptor-negative cancers or triple negative tumors arise early resulting in a higher mortality risk. One of the main causes of metastasis is tumor dormancy in which cancer cells remain concealed, asymptomatic, and untraceable over a prolonged period of time. Under certain conditions, dormant cells can re-enter into the cell cycle and resume proliferation leading to recurrence. However, the molecular and cellular regulators underlying this transition remain poorly understood. To date, three mechanisms have been identified to trigger tumor dormancy including cellular, angiogenic, and immunologic dormancies. In addition, recent studies have suggested that DNA repair mechanisms may contribute to the survival of dormant cancer cells. In this article, we summarize the recent experimental and clinical evidence governing cancer dormancy. In addition, we will discuss the role of DNA repair mechanisms in promoting the survival of dormant cells. This information provides mechanistic insight to explain why recurrence occurs, and strategies that may enhance therapeutic approaches to prevent disease recurrence.

**Key words:** Quiescence; Homologous recombination; Non-homologous end joining; Tumor dormancy; DNA repair



**Core tip:** One of the main causes of metastasis is tumor dormancy in which cancer cells remain concealed, asymptomatic, and untraceable over a prolonged period of time. Recent studies have suggested that DNA repair mechanisms may contribute to the survival of dormant

cancer cells. Under certain conditions, dormant cells can re-enter into the cell cycle and resume proliferation leading to recurrence. Understanding the molecular and cellular regulators underlying the transition from tumor dormancy to metastatic disease may provide insight into how recurrence occurs and also discover strategies that may enhance therapeutic approaches to prevent metastatic cancer.

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## INTRODUCTION

Metastatic tumor growth can account for the majority of cancer-related deaths worldwide[1]. In fact, nearly 30% of breast cancers will relapse years or decades after the initial treatment[2-4]. Different subtypes of breast cancer display different recurrence behaviors. For examples, late tumor relapses frequently occur in estrogen receptor-positive (ER+) breast cancers whereas relapses in estrogen receptor-negative breast cancers or triple negative breast tumors arise early resulting in a higher mortality risk[2,5]. Tumor dormancy, one of the main causes of metastasis, occurs when disseminated tumor cells remain concealed, asymptomatic, and untraceable over a prolonged period of time. Cancer cells can become dormant at the onset of disease or after the initial therapeutic treatment, and can remain dormant for years or even decades after the first treatment[6]. Dormant cells can be characterized by exhibiting slow growth rates, having the ability to escape frontline treatment and the host's immune system, and demonstrating the capability to self-renew. Multiple studies have shown that many cancers such as breast and prostate cancers, melanoma, B-cell lymphoma, leukemia, and carcinoma contain dormant cancer cells[7-15]. Therefore, it is important to understand the molecular mechanisms that govern the transition of dormant cells into metastatic disease.

To date, three mechanisms have been identified to trigger tumor dormancy including cellular, angiogenic, and immunologic dormancies (Figure 1)[16]. Cellular dormancy is characterized as a state in which cells are quiescent and halted in the G0 phase of the cell cycle (Figure 1). The microenvironment of tumors can prompt cancer cells to enter into cellular dormancy like hypoxic environments, which is associated with malignancies, and causes cancer cell proliferation to decrease[17]. Under certain circumstances such as the addition of growth factor, cytokines, nutrients or chemical agents, dormant cells can re-enter into the cell cycle and resume proliferation. Many cancer therapeutic treatments target the cell cycle which permits the cells to enter into quiescence. This allows the cancer cells to escape treatment subsequently leading to disease recurrence[16,18-20]. Once dormant cancer cells exit G0 arrest, a second mechanism termed angiogenic dormancy can limit the tumor size by preventing angiogenesis and therefore the tumor cannot obtain the nutrients required for continual growth. These cells can maintain a balance between proliferation and apoptosis resulting in the inability to detect the tumor[6,16] (Figure 1). The immune system can also contribute to cancer cell dormancy by maintaining a balance between clearance and proliferation[16] (Figure 1). During immunologic dormancy, DTCs can be eliminated or they can stay in an equilibrium state and, over time, environmental factors and genomic instability can cause the cells to exit the equilibrium state resulting in tumor growth and recurrence[21].

The precise molecular mechanism in which cancer cells enter and exit dormancy remains to be elucidated. One mechanism that plays a major role in cancer growth is the DNA repair pathways, and recently, studies indicate that the DNA repair pathways can lead to tumor dormancy[15,22]. Therefore, it may be possible to target dormant cancer cells through these pathways. Below, we will discuss the current understanding of the three mechanism of tumor dormancy and the role of double-strand breaks (DSBs) DNA repair pathways in dormant cancer cells. This information may improve the development of relevant study models and enhance therapeutic approaches to prevent disease recurrence.

## CELLULAR DORMANCY

Cellular dormancy or quiescence is a process that occurs naturally in normal adult stem cells such as hemopoietic and spermatogonial stem cells. These stem cells serve as a source for self-renewal and maintenance of tissues throughout a person's lifetime. However, in a heterogeneous cancer cell population, dormancy can be disadvantageous because cancer cells can evade treatments leading to metastatic recurrence[16,18-20] (Figure 1).

Several studies have demonstrated that the expression of the cellular proliferation, Ki-67, and apoptotic markers are significantly diminished in patients with clinical dormancy[23-27]. In addition, positive Ki-67 expression was correlated with breast cancer recurrence and poor prognosis[28]. The stepwise progression of the cell cycle is regulated by cyclins and cyclin-dependent kinases (CDKs). In particular, cellular quiescence is controlled either directly or indirectly by these regulators. Within the microenvironment, the interactions between the CDK inhibitors, p27 (Kip1) and p21 (Cip1, Waf1), maintain a balance between proliferative and dormant hematopoietic stem cells[29]. Recently, Fitzgerald *et al*[30] (2015) demonstrated that treatment of head and neck squamous cell carcinoma patients with radiation resulted in cellular quiescence *via* the upregulation of p21. In addition, the DREAM complex which consist of a Retinoblastoma (Rb)-like pocket protein, E2F, and

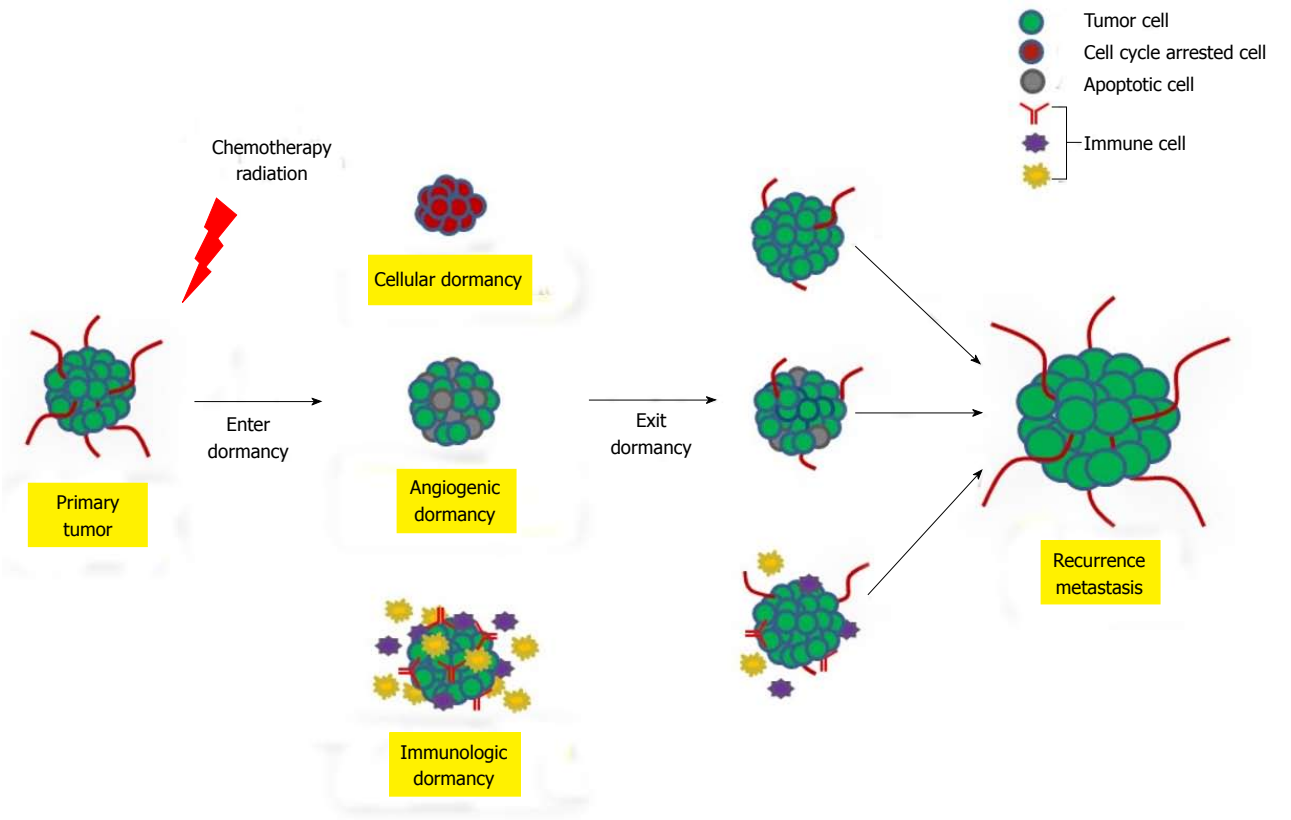


**Figure 1 Mechanisms of human tumor dormancy.** Schematic depicting three mechanisms that lead to tumor dormancy after the initial clinical treatment. Tumor dormancy can result from cell cycle arrest (cellular dormancy), tumor size limitation due to a lack of functional blood vessels (angiogenic dormancy), or immunosurveillance (immunologic dormancy). Figure adapted from Almog[16] (2010) and Wang and Lin[6] (2013).

mutilvulval class B (MuvB) proteins, is a critical regulator of cell cycle arrest[31]. The MuvB protein is known to recruit, bind, and direct transcription regulators to the promoter of key cell cycle genes during various stages within the cell cycle[32]. During dormancy, MuvB binds to all of the components of the DREAM complex and represses the transcription of all cell cycle-dependent genes[32-34]. Disruption of various components of the DREAM complex results in the inability to repress the cell-cycle dependent genes and subsequently the cells re-enter the cell cycle[35,36]. Quiescence is also established by the dual specificity tyrosine phosphorylation-regulated kinase (DYRK). This protein activates the DREAM complex by phosphorylating a MuvB subunit, LIN52, which promotes the interaction of MutB with the other core components of the DREAM complex[31]. An isoform of DYRK, DYRK1B, can stabilize p27 (Kip1) which increases the turnover of cyclin D consequently inhibiting cell from entering into the cell cycle[37,38]. CDK4 and CDK6 inactivate the tumor suppressor, Rb, subsequently allowing cells to enter into the cell cycle. By pharmaceutically blocking these kinases, Rb-cells can exit the cell cycle and enter into a dormant state[39]. These results clearly demonstrate the need for balance between the DREAM and proliferative complexes in order to maintain cells in a quiescent state.

Mis-regulation of cell cycle proteins can result in tumor formation, dormancy, and recurrence. Prostate cancer, breast cancer, and renal cell carcinoma are linked to the loss of p27 (Kip1)[40-42]. In addition, reduction in p27 (Kip1) is used as a strong prognostic marker for recurrence and poor outcomes in renal cell carcinoma patients[42]. Loss of p53, the upstream regulator of p21, was correlated with drug resistance and recurrence in colorectal cancer[43]. Overexpression of cyclin D is associated with recurrence of multiple neoplasms including breast, lymphomas, prostate, and non-small cell lung cancers[44-46]. Overexpression of cyclin D1 can occur *via* a multitude of different mechanisms including genetic rearrangements, amplification of the gene locus, oncogenic signaling, and mutation in the gene that result in the inability to degrade the protein[44]. Recently, Kim *et al*[47] (2014) reported that overexpression of the cell cycle regulators CDK4, CDK6, pRB, and cyclin D1 was correlated with the recurrence of atypical meningioma. Furthermore, some evidence suggested that overexpression of CDK4 may be connected to nasopharyngeal carcinoma tumor aggression and serve as a diagnostic biomarker[48]. Clearly, these results demonstrate the importance in controlling the cell cycle and how aberrant regulation may lead to tumor recurrence and poor prognosis.

## ANGIOGENIC DORMANCY

The majority of tumors require the recruitment of blood vessels to support continual growth. When tumors fail to establish a sufficient vasculature, then they enter into

a state of avascular or angiogenic dormancy (Figure 1). Tumor dormancy *via* angiogenesis requires the interaction between the microenvironment and cell cycle regulators including p21, p27, Myc, urokinase receptor (u-PAR), extracellular regulated kinase (ERK), and p38[49]. Blockage of the metastasis-associated u-PAR, intergrins, focal adhesion kinase or epithelial growth factor receptor can result in tumor suppression and induction of tumor dormancy[49]. U-PAR can also regulate tumor dormancy by favoring p38 activation over ERK activation[50]. In addition, the activation of the PI3K/c-Myc pathway controls the level of thrombospondin (TSP), a vital factor of tumor dormancy[16]. Troyanovsky *et al*[51] (2001) also discovered that the expression of angiostatin can control tumor dormancy by suppressing tumor growth, and one mediator of angiostatin, angiomotin, was highly elevated in dormant cells.

The transition from avascular tumor to a highly vascularized tumor is termed the "angiogenic switch"[16,21]. Balancing the pro-angiogenic and anti-angiogenic factors is vital in regulating the angiogenic switch. Satchi-Fainaro *et al*[52] (2012) discovered that dormant glioblastoma cells express high levels of anti-angiogenic factors including TSP, angiomotin, and insulin-like growth factor binding protein 5, and low levels of pro-angiogenic proteins (endothelial cell-specific marker 1 and epithelial growth factor receptor). Furthermore, TSP-1 and endothelial-derived perlecan were found to maintain breast cancer cells in a dormant state therefore suppressing tumor growth[53,54]. Another key protein that plays a role in controlling the switch from dormancy to tumor growth is heat shock protein 27 (HSP27)[55]. Decreased expression of HSP27 in breast cancer cells resulted in reduced cell proliferation and migration caused by lower levels of secreted vascular endothelial growth factor (VEGF) and basic fibroblast growth factor, known pro-angiogenic factors[55]. Recently, the hypoxia inducible factor, HIF-2$\alpha$, was shown to promote angiogenesis in hepatocellular carcinoma[56]. HIF-2$\alpha$ increased plasminogen activator inhibitor 1 which lowered active plasmin concentrations resulting in increased angiogenesis[56].

The formation of dormant cell niches can be controlled by the microenvironment. Several proteins such as latent transforming growth factor $\beta$ (TGF-$\beta$) binding protein (LTBP), bone morphogenetic protein 7 (BMP7), and osteopontin (OPN) all influence the establishment of quiescent cell niches[57-59]. Overexpression of LTBP in nasopharyngeal carcinoma induced cancer cell dormancy and reduced VEGF expression thus inhibiting the migration and angiogenesis of tumor cells[57]. BMP7, a member of the TGF-$\beta$ superfamily, signaling facilitates the balance between dormant prostate cancer cells and metastasis[58]. Administration of BMP7 in mice significantly reduced tumor growth whereas inhibition of BMP7, *via* the secreted antagonist COCO, resulted in metastasis[58,59]. Leukemic dormancy occurs within bone marrow niches and is influenced by the expression of OPN[14]. Acute lymphobaslic leukemia blasts express high levels of the OPN receptor, VLA-4, which permits the cells to adhere to stroma-derived OPN secreted by osteoblasts within the bone marrow niche[14]. This interaction drives leukemia blast into dormancy and this causes the cells to escape chemotherapy and/or radiation treatment[14]. In addition, antibody neutralization of OPN resulted in leukemia blast to exit dormancy and re-enter the cell cycle[14]. Taken together, these data support the notion that communication between cancer cells and cells associated with the tumor microenvironment is important for controlling the transition between dormancy and angiogenesis.

## IMMUNOLOGIC DORMANCY

Tumor dormancy can be established by preserving equilibrium between immune response and tumor cells (Figure 1). The mechanism of how tumor cells enter and exit immunologic dormancy is not well understood. The immune system can control dormancy *via* three different methods including elimination, equilibrium, and escape. The innate and adaptive immune systems work together to detect and eliminate transformed cancer cell prior to the host becoming clinically symptomatic. If the tumor cells are not completely eliminated, then the host's immunity can restrict tumor growth resulting in the continuance of cells within a dormant state. Over time, the tumor cells can adapt to the immune environment causing cells to exit dormancy leading to recurrence[60-62] and tumor metastasis (Figure 1). For example, DTC can reduce T-cell activation which weakens the cytotoxic T-lymphocyte response thus cells escape apoptosis[63]. Direct tumor immunosuppression can mediate the escape from dormancy by driving the overexpression of B7 homolog 1 (B7-H1) which inhibits T-cell activation and the cytotoxic T lymphocyte (CTL) response[63]. In addition, cancer cells can escape tumor dormancy by inhibiting antigen presentation and by methylating cytokine signaling 1 thus leading to resistance to CTL-induced apoptosis[63]. Furthermore, loss of $CD4^+$ or $CD8^+$ T-cells can result in tumor cell dormancy escape[64]. Several cell types within the immune system can indirectly regulate the escape from dormancy by secreting proteins that promote angiogenesis. Interleukin 23, produced by macrophages, suppresses anti-tumor effectors responses, whereas interleukin 12 represses tumor growth[65,66]. The glycoprotein, macrophage stimulating 1 (MS1) can bind to its receptor, MS1 receptor (MST1R), thus suppressing antitumor immune response and promoting cell proliferation, survival, and chemotaxis. The loss of MST1R increases antitumor $CD8^+$ T-cell responses resulting in higher levels of secreted tumor necrosis factor $\alpha$ subsequently leading to the inability of micrometastatic cancer cells to generate macrometastases[67,68]. In addition, myeloid-derived suppressor cells, regulatory T-cells, and tumor-associated macrophages can also indirectly promote tumor cells to escape dormancy[63]. These cells can secrete mitogens and proangiogenic molecules which

promote cell proliferation, angiogenesis and immunosupression causing the cells to exit dormancy[63]. These results demonstrate the importance in controlling the immune system to prevent tumor recurrence and metastasis.

Genomic instability may facilitate the escape of dormant cancer cells from immunological dormancy. Over time, if cancers cells do not have the capability to repair their DNA, they can accumulate mutations allowing the cells to evade anti-tumor immunity leading to recurrence. Therefore, understanding how DNA repair mechanism function in dormant cells may lead to new developments to detect and treat dormant cancer cells.

## DNA REPAIR MECHANISMS

Many cancer drugs induce high levels of DNA lesions both single-stranded (SSB) and double-stranded, which results in the death of proliferating cells. Mechanism involved in SSB and DSBs break repair significantly affect the cancer cells ability to evade radiation and chemotherapy treatments. SSBs are repair through the base excision repair pathway. The damaged base is recognized and excised by DNA glycosylases which generates abasic sites. PARP1 and PARP2 proteins sense the SSB and recruit other factors such as XRCC1 to the damaged region[69]. Loss of heterozygosity of OGG1, a DNA glycosylase, is associated with papillary thyroid cancer[70].

DSBs are considered to be the most toxic form of DNA lesions[71-73]. When DNA lesions occur, cells can utilize DNA damage repair pathways to restore the DNA and maintain the genomic integrity of the cell. Two of the major DSBs repair pathways are homologous recombination (HR) and non-homologous end jointing (NHEJ). HR utilizes the DNA sequence from the homologous sister chromatid to repair the DSBs, and occurs predominately in the S and G2 phases of the cell cycle. HR is a major mechanism to ensure the high fidelity of genetic information and because this process uses the homologous sequence as a template, it is considered to be a more error-free repair pathway. Once the HR process is initiated, the DSB is resected to create a 3' overhang that becomes coated with ssDNA-binding protein RPA. Once this filament is formed, RPA is replaced by RAD51 in an ATM/CHK2/BRCA1/BRCA2/PALB2-dependent manner[69]. RAD51 is a key HR repair protein with recombinase activity. One of the main functions of RAD51 is to invade the sister chromatid and identify the template sequence, and reduced RAD51 expression is associated with decreased HR activities[74].

In contrast to HR pathway, NHEJ takes place throughout the cell cycle and involves the direct ligation of broken ends without the need of homologous templates which results in more errors being incorporated within the DNA sequence[75]. Upon initiation of NHEJ, Ku70 and Ku80 form heterodimers that detect and bind the DNA ends. The Ku proteins will then recruit the catalytic subunit, DNA-Protein Kinase (DNA-PK). This step is required for XRCC4 and Lig4-mediated rejoining of the damaged DNA ends during NHEJ[69]. DNA-PK complex acts as a molecular sensor for NHEJ repair[76,77], and cells lacking DNA-PK function fail to show proper NHEJ[78-84]. Additionally, PARP1 may compete with Ku protein to bind the DSB ends resulting in an alternative NHEJ pathway.

Many cancers have abnormalities in the DNA repair pathways, therefore several therapeutics have been developed to exploit these defects. The NHEJ catalytic subunit, DNA-PK, is considered to be up-regulated in radiation-resistant glioblastoma and prostate cancers[85,86]. Recently, clinical trials have shown that inhibitors of DNA-PK have increased the sensitivity of cancer cells to DNA damaging agents however these drugs have been avoided due to the toxicity to normal cells[87]. Small molecular inhibitors of DNA ligase Ⅳ, which is involved in NHEJ, have also been used to decrease cell proliferation and increase the tumor inhibitory effect of chemotherapeutics that cause DSBs[88]. The mis-regulation of genes associated with HR, RAD51, BRCA1, ERCC1, APE1, and PARP1, are also observed in various cancers and are associated with resistance to chemotherapies[87]. Specifically, mutations in BRCA1, BRCA2, ATM, CHEK2, and RAD50 have been identified in several cancers including lung, ovarian, pancreatic, and leukemia[69]. Besides drugs that target RAD51, currently there are very little therapeutics that target other proteins involved in HR[87]. Alternatively, targeting the alternative NHEK pathway *via* PARP1 inhibitors have been used to treat BRCA1 or BRCA2-defected cancers[69].

DNA repair pathways have been shown to play a vital role in the survival of dormant cancer cells after the initial therapeutic treatments. In hepatocellular carcinoma, the stem cell population switches from actively dividing to dormant after the first round of chemotherapy, which allows for the survival of malignant cells[89,90]. The dormant cells contain less DSBs after chemotherapy treatment, and Nishikawa *et al*[15,22] (2012) demonstrated that these cells activated the NHEJ pathway to repair the DNA damage[15,22]. Furthermore, our unpublished data indicates that the NHEJ pathway is important in facilitating DSBs repair in ER+ dormant breast cancer cells after exposure to chemotherapy or radiation. In addition, we discovered that when these cells were treated with chemotherapeutics and exited dormancy, genomic instability increased leading to more aggressive phenotypes and chemotherapy resistance (Lin, unpublished data).

HR may also be involved in DNA repair of dormant cancer cells. The human Fanconi anemia monoubiquitination pathway has been implicated in promoting DNA repair *via* HR[91]. Recently, defects in this pathway resulted in the accumulation of DNA damage causing hematopoietic stem cells to exit their dormant state. The repeated activation of the hematopoietic stem cells out of their quiescent state can lead to the complete

collapse of the hematopoietic system triggering diseases such as Fanconi anemia and leukemia[92].

## CONCLUSION

One of the most difficult clinical challenges that we face today is the effective treatment of malignant diseases due to the inability to detect dormant cancer cells[93]. Recently, Kim *et al*[94] (2012) established a dormancy gene signature in ER+ breast cancer cells. When two of these genes, BHLHE41 and NR2F1, are knocked-down in the breast cancer cells, *in vivo* cell growth increased[94]. While these data are promising in identifying dormant cells, it has yet to be used diagnostically. Therefore, it is important to continue investigating the mechanism that control cancer dormancy. Targeting pathways involved in cellular, angiogeneic or immunologic dormancy may provide a way to detect dormant cells as well as treating metastatic cancer.

A possible mechanism to target dormant cancer cells is through the DNA repair pathways, and recent studies have suggested that DNA repair mechanisms may contribute to the survival of dormant cancer cells. In particular, the NHEJ pathway may cause a high frequency of spontaneous mutagenesis subsequently resulting in genomic instability and tumor progression[75]. However, more studies need to be performed to determine if other DNA repair mechanism facilitate the maintenance and survival of dormant cells. In addition, these pathways are not intrinsic to dormant cancer cells. Therefore, understanding the mechanisms of how dormancy is involved in recurrence is urgent for the prevention of secondary tumors. Several advancements have been made to characterized dormant cancer cells, however, to date, there is a lack of suitable model systems to detect and maintain cells in a dormant state. Development of *in vivo* and *in vitro* model systems are imperative to identify key molecular determinants of dormancy, which may lead to strategies for detecting and eliminating dormant cancer cells thus preventing recurrence and reducing cancer mortality.

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EDITORIAL

# Significant methodologic variations in calculating renal function changes following kidney tumor surgery: A quality reporting issue?

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## Abstract

Renal tumor surgery places patients at increased risk for chronic kidney disease (CKD). Accurate quantification of kidney function changes before and after surgery is essential to determine the magnitude of decline attributable to an index procedure. Current literature, however, highlights heterogeneity and inconsistencies in measurement techniques thereby contributing to ambiguity amongst studies. Further efforts are necessary to standardize reporting of kidney function outcomes related to renal surgery.

**Key words:** Radical nephrectomy; Partial nephrectomy; Nephroureterectomy; Glomerular filtration rate; Chronic kidney disease



**Core tip:** Accurate assessment of renal function changes following kidney tumor surgery is essential for quantifying the degree of decline attributable to an index procedure. Current studies, however, demonstrate significant heterogeneity in the timing and calculated formulas used for determining kidney function changes. These variations in methodology significantly confound interpretations regarding the impact of surgical technique on global renal function. Standardization of the reporting process is essential to more accurately characterize and potentially modify aspects of surgical care that can benefit from improvement.

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## INTRODUCTION

Studies indicate that kidney tumor surgeries including

**Table 1 Data from the 99 studies in contemporary literature reporting renal function outcomes related to renal surgery *n* (%)**

| | | | | | |
|---|---|---|---|---|---|
| No. patients per study | | | | | |
| Mean | | | 308 | | |
| Range | | | 7-2402 | | |
| Preoperative serum Cr collection – months prior to surgery | | | | | |
| | < 1 mo | 1-2 mo | | Unspecified | |
| Studies | 11 (11) | 1 (1) | | 87 (88) | |
| Postoperative serum Cr collection – months after surgery | | | | | |
| | < 3 mo | 3-12 mo | > 12 mo | Unspecified | Multiple |
| Studies | 5 (5) | 9 (9) | 4 (4) | 17 (17) | 64 (65) |
| Method for estimating renal function | | | | | |
| | MDRD | CKD-EPI | Other | None | |
| Studies | 66 (67) | 8 (8) | 20 (20) | 5 (5) | |

MDRD: Modification of diet in renal disease; CKD-EPI: Chronic kidney disease epidemiology collaboration.

radical nephrectomy (RN), partial nephrectomy (PN), and radical nephroureterectomy (RNU) place patients at risk for declining renal function. For example, in 2006, Huang *et al*[1] demonstrated that patients undergoing RN for kidney tumors had a significantly increased risk of developing subsequent chronic kidney disease (CKD). Furthermore, these authors observed that this risk of CKD following nephrectomy in cancer patients is greater than that for donor nephrectomy and suggested that this may be attributable to baseline kidney dysfunction. Therefore, accurate and reproducible assessment of kidney function before and after kidney tumor surgery is essential to determine the magnitude of decline attributable to an index procedure. In this regard, we suspect that current reporting of kidney function changes following a surgical procedure may be heterogenous and inconsistent in the literature. To better investigate this issue, we reviewed the contemporary literature and evaluated the methodologies currently used and adequacy of reporting.

## LITERATURE STUDY

The PubMed database was queried to identify studies that evaluated changes in renal function after RN, PN and RNU. We included all articles that evaluated both pre- and post-operative renal function based on estimated glomerular filtration rate (eGFR) and serum creatinine concentration. Data regarding the number of patients included in the study, the time frame for obtaining the pre- and post-operative serum creatinine levels, and the methodology for estimating renal function were collected.

## RESULTS

Data collected from 99 articles were included in the analysis (Table 1). The mean number of patients included in these studies was 308, ranging from 7 to 2402. In 100% of the studies, there was a single pre-operative creatinine serving as the baseline value, although 88% of the articles failed to specify the timing prior to surgery. Following surgery, 65% of studies reported multiple creatinine measurements at various time points while 17% failed to specify timing of collection. The Modification of Diet in Renal Disease (MDRD) (67%) and CKD Epidemiology Collaboration (CKD-EPI) (8%) equations were most commonly used for eGFR calculations. Nonetheless, 20% of studies used other methodologies including renal scintigraphy, Cockroft-Gault equation, Mayo Clinic Quadratic equation, or combinations of these different methods. Five percent of studies did not calculate an eGFR and relied solely on serum creatinine values.

## DISCUSSION

This analysis highlights that there exist significant methodological variations in calculating renal function related to kidney surgery in the contemporary literature. In particular, there is poor reporting of timing of serum creatinine collections as well as variability in methods used to estimate renal function. Serum creatinine concentration alone is a poor estimate of kidney function because it is affected by several factors including age, gender, ethnicity, muscle mass, creatinine secretion, and extrarenal excretion[2]. Furthermore, these factors can be affected by medications, hydration status, diet, certain disease states, and exercise[3]. Thus, there is a relatively wide range of normal serum creatinine levels as well as individual variability and these characteristics render it a poor predictor of early decline in renal function. Moreover, there is concomitant loss of both renal function and muscle mass in the elderly, so serum creatinine level may give the impression of normal renal function when the GFR is in fact low[2]. Many patients undergoing surgery for renal tumors are generally older and accordingly are an especially poor population for using serum creatinine level alone for estimating renal function.

Kidney function is better approximated using the estimated GFR, which is determined using the serum creatinine concentration and several other variables such as age, gender, and race. The two equations used most commonly in the contemporary literature are the MDRD study equation and the CKD-EPI equation. The MDRD study equation has been shown to be more accurate and precise than the Cockroft-Gault equation for those with a GFR less than approximately 90 mL/min per 1.73 $m^2$. However, there are questions about its validity for persons without renal disease, persons > 70 years old, and patients with serious comorbid conditions[1,4]. The CKD-EPI equation was developed to overcome some of the shortcomings of the MDRD equation and be more applicable to the general population. It was found to be more accurate than the MDRD Study equation and have lower bias, especially in persons with an eGFR greater than 60 mL/min per 1.73 $m^2$, thus reducing that rate of false-positive diagnoses of stage 3 CKD[5]. This was further highlighted by a study by Clark *et al*[6], where it

was found that for patients with two functioning kidneys who underwent PN, the CKD-EPI equation provides slightly higher eGFRs compared to the MDRD equation at baseline and follow-up. However, there was no significant difference between the two equations when calculating the percent change of eGFR pre- and post-operatively[6].

This study highlights the methodological variation in the contemporary literature for determining renal function related to kidney surgery. The collection of serum creatinine levels was nonhomogeneous between studies, with variable numbers of measurements and poorly reported time frames. Additionally, there is utilization of multiple methods for estimating renal function, further confounding interpretation of the data. Such ambiguity amongst studies renders comparison of outcomes highly problematic. Further investigation is warranted to better standardize the reporting of kidney function outcomes related to renal surgery.

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EDITORIAL

# Modulators of alternative splicing as novel therapeutics in cancer

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## Abstract

Alternative splicing (AS), the process of removing introns from pre-mRNA and re-arrangement of exons to give several types of mature transcripts, has been described more than 40 years ago. However, until recently, it has not been clear how extensive it is. Genome-wide studies have now conclusively shown that more than 90% of genes are alternatively spliced in humans. This makes AS one of the main drivers of proteomic diversity and, consequently, determinant of cellular function repertoire. Unsurprisingly, given its extent, numerous splice isoforms have been described to be associated with several diseases including cancer. Many of them have antagonistic functions, *e.g.*, pro- and anti-angiogenic or pro- and anti-apoptotic. Additionally several splice factors have been recently described to have oncogene or tumour suppressors activities, like SF3B1 which is frequently mutated in myelodysplastic syndromes. Beside the implications for cancer pathogenesis, de-regulated AS is recognized as one of the novel areas of cell biology where therapeutic manipulations may be designed. This editorial discusses the possibilities of manipulation of AS for therapeutic benefit in cancer. Approaches involving the use of oligonucleotides as well as small molecule splicing modulators are presented as well as thoughts on how specificity might be accomplished in splicing therapeutics.

**Key words:** Novel cancer therapeutics; Splicing switching oligonucleotides; Alternative splicing; Small molecules; Splicing modulators



**Core tip:** Genome-wide studies have recently shown that more than 90% of genes are alternatively spliced in humans. This makes alternative splicing (AS) one of the main drivers of proteomic diversity. Numerous splice isoforms have been described to be associated with cancer. Additionally several splice factors have been shown to have oncogene or tumour suppressors activities. Beside the implications for cancer pathogenesis, de-regulated AS is recognized as one of the novel areas of cell biology where therapeutic manipulations may be designed. This editorial discusses the possibilities of manipulation of AS for therapeutic benefit in cancer.

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## INTRODUCTION

In the last years we have seen a plethora of anticancer agents that try to acquire more specific and targeted treatment in comparison with the conventional chemo- and radiotherapies used in the clinic. While it is highly unlikely they will be able to be used as mono-therapies on a large scale in oncology - due to the inherent problem of developing resistant clones as exemplified by the B-Raf inhibitor vemurafenib in melanoma[1], they have certainly proved very useful in combination therapies or as adjuvants that can improve overall survival in association with conventional therapies or reduce the doses used in chemo- and radiotherapies and therefore decrease side-effects.

Most of targeted anti-cancer drugs approved in clinical practice today are targeting receptor tyrosine kinases or cytoplasmic signalling molecules. However, since cancer cells are different from normal cells in virtually any property and function from DNA repair to regulating apoptosis or metabolism, theoretically drugs that hamper tumour growth may be designed at any level of gene regulation – transcriptional, post-transcriptional or post-translational. Indeed, recent years have produced intense research on potential new drugs (some already in trials or in the clinic) that are based on epigenetic modulation[2], DNA repair[3] or microRNAs[4] to name a few.

One level that has not been explored so far is represented by modulation of alternative splicing (AS).

## AS

Splicing is the removal of introns during processing of pre-mRNA. Through AS the composition of the mature RNA may be changed through exon skipping, mutually exclusive exons, intron retention or 3' and 5' alternative splice sites[5]. AS has emerged in the post-genomic era as the main driver of proteome diversity with at least 94% of multi-exon genes being alternatively spliced in humans[6,7]. AS is one of the main control mechanisms for cell phenotype, and a process deregulated in disease. There are over 2000 splicing mutations known, involving 303 genes and implicated in 370 diseases[8]. Therefore it has become essential to study how this process is regulated, and how it can become deregulated in disease.

While the disease most commonly linked to deregulation of AS in several genes is cancer[9], there are many in-depth reports of pathogenic splice variants in diseases ranging from neuromuscular disorders[10] to diabetes[11] or cardiomyopathies[12].

## AS IN CANCER - ASSOCIATED NOISE OR CAUSALITY?

An increasing amount of literature in the last years shows involvement of splicing in cancer and an incredible number of splice variants have been described to be associated with tumour progression - for recent reviews see[9,13,14]. For example, epidermal growth factor receptor, which is mutated in several cancers, has a splice variant that is missing exon 4 and is highly expressed in several cancers; this exon deletion makes the protein constitutively active[15]. K-Ras has two alternate exons - 4A and 4B - and depending on their inclusion/exclusion there is a strong differential association with various forms or localization of colon cancer[16]. The tumour suppressor p53 has two splice isoforms p53beta and p53gamma that result from two alternate exons; these isoforms modulate the activity of the main isoform and the way it regulates apoptosis in various contexts[17]. Finally, another notable example is the well-studied tumour suppressor retinoblastoma protein for which more than 15% of the mutations described in various cancers are related to splicing[18,19].

The main question that arises - especially having a therapeutic purpose in mind - are these modifications simply by-products of the oncogenic process or do they drive pathogenesis of cancer? While inevitably some splice variants are "associated noise" similar to physiology, there is compelling evidence for "pathogenic" AS in cancer.

Firstly, similar with mutations in transcription factors that denote many of them as oncogenes, there are mutations of spliceosome components or splice factors - *e.g.,* SF3B1 in myelodysplastic syndromes[20].

Secondly, there is clear evidence of splicing-specific variants that may be induced by signalling in the cancer cell environment and result in acquired functions for the cancer cells that helps their pathogenic evolution. For example, while normal cells/tissues generally have a high level of the anti-angiogenic vascular endothelial growth factor A (VEGF-A) isoforms $VEGF_{165}b$, this is lost in cancers, with expression of predominantly pro-angiogenic $VEGF_{165}a$, which maintains a state of high and chaotic neovascularization in tumours[21]. However, no mutation has been identified so far that could account for this shift in the ratio of the two splice isoforms which is highly likely due to changes in the microenvironment during step-wise progression of the oncogenic process.

Finally, recent years have clearly shown that defective splicing contributes to one of the most challenging problems in oncology - acquired resistance to treatments. While there are numerous examples[22] we want to point-out the well-known case of Vemurafenib. Patients treated with this drug invariably develop resistance. While several mechanisms have been described, in about a third of cases this occurs through faulty AS that results in truncated B-Raf which do not have the Ras-binding domain[23].

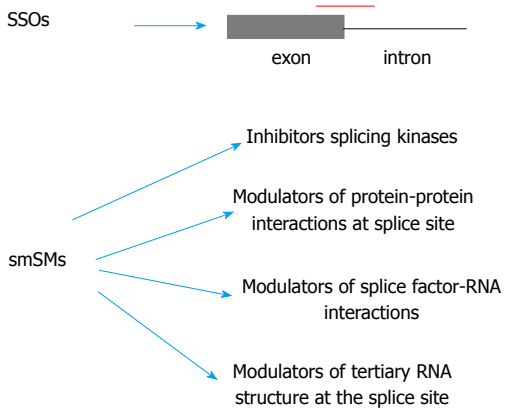


**Figure 1 Possible ways to modulate alternative splicing for therapeutic purposes.** smSMs: Small molecule splicing modulators; SSOs: Splicing-switching oligonucleotides.

## THERAPEUTIC MANIPULATION OF SPLICING

Can we modify splicing and use it as a new level where therapeutic interventions may be designed? While there is no drug in the clinic that modifies splicing yet, there are certainly extremely exciting developments in the past few years. The general idea is to try and switch the splicing of a certain isoform that has been identified as deleterious and promoting the oncogenic process in functional studies towards a beneficial isoform.

The strategy most used so far involves anti-sense oligos (ASO) or splicing-switching oligos (SSOs). The general principle is to design ASOs that bind either exon-intron junctions or regulatory sequences like enhancers or silencers in introns or exons, therefore affecting the splice outcome of the targeted event. So far SSOs have been proved very promising, with several of them in clinical trials, *e.g.,* for Duchenne muscular dystrophy or spinal muscular atrophy[24].

There is a growing number of small-molecule splicing modulators (smSM) that have been shown to affect splicing. An interesting example is amiloride. This is a long-time used diuretic with the main mechanism of action through effects on the ion pumps in the renal tubules. However, it has been found in a screen to potently affect splicing of several genes involved in apoptosis and further-on to be able to decrease tumour growth in animal models[25]. Recently a class of small molecule compounds that inhibit SRPK1, a major regulator of AS through SR-protein phosphorylation, has been shown to inhibit VEGF splicing and angiogenesis in a model of ocular neovascularization[26] as well as melanoma xenografts growth[27] and orthotopic prostate cancer mouse models[28].

Potentially, other types of molecules could be involved in splicing modulation, like chemicals that affect splice factor/RNA interactions or molecules that affect directly the tertiary structure of a particular splice junction (Figure 1).

## WILL SPLICING MODULATORS BE SPECIFIC?

Specificity is highly unlikely to be an important problem for SSOs, which are designed to bind on defined RNA sequences, though potential problems with delivery and toxicity might still be challenging.

SmSMs could potentially affect several other splice events regulated by the same splicing kinase or splicing factor intended to be modulating - however, the key issue is whether the manipulation of the intended targeted splice event is dominant functionally in the system/cell line of interest (*i.e.,* the other splice events affected do not result in major unintended modifications in cell properties).

It is interesting to point-out a recent paper reporting the development of smSMs of the SMN splicing and attenuation of spinal muscular atrophy[10]. The compounds were found in a screen using a splicing reporter that mimicked the endogenous splicing event. When an RNA-seq analysis was performed to assess specificity it was found that very few splice junctions are affected, therefore proving that specificity in splicing therapeutics using small molecules may be accomplished.

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EDITORIAL

# New findings on thymic epithelial tumors: Something is changing

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## Abstract

Thymic epithelial tumors (TETs) are uncommon neoplasms with a wide range of anatomical, clinical, histological and molecular malignant entities. To date the management of TETs within clinical practice is based on a multimodal therapeutic strategy including surgery, chemotherapy and radiotherapy with a multidisciplinary approach and prognostic evaluation is mainly based on Masaoka staging and World Health Organization classification. Therefore novel strategies are needed, especially for refractory and/or recurrent TETs and for thymic carcinomas that present a poor prognosis. Personalized approaches are currely being developed and molecular targets are emerging from recent integrated genomic analyses. Targeted therapy will represent an important treatment option for TETs with an aggressive histology. To date, data indicate that vascular endothelial growth factor molecules, insulin-like growth factor 1 receptor, cyclin-dependent kinases and mammalian target of rapamycin may be potentially useful as targeted biological therapies.

**Key words:** Thymic epithelial tumors; Thymoma; Thymic carcinoma; Targeted therapy; Programmed cell death-1



**Core tip:** Thymic epithelial tumors (TETs) are uncommon neoplasms with a wide range of anatomical, clinical, histological and molecular malignant entities. To date the management of TETs within clinical practice is based on a multimodal therapeutic strategy including surgery, chemotherapy and radiotherapy with a multidisciplinary approach and prognostic evaluation is mainly based on Masaoka staging and World Health Organization classification. Targeted therapy will represent an important treatment option for TETs with an aggressive histology.

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## INTRODUCTION

Thymic epithelial tumors (TETs) are uncommon neoplasms with a wide range of anatomical, clinical, histological and molecular malignant entities[1,2].

## AVAILABLE TREATMENTS

To date the management of TETs within clinical practice is based on a multimodal therapeutic strategy including surgery, chemotherapy and radiotherapy with a multidisciplinary approach and prognostic evaluation is mainly based on Masaoka staging and World Health Organization classification.

## NEW EVIDENCES

Therefore novel strategies are needed, especially for refractory and/or recurrent TETs and for thymic carcinomas (TC) that present a poor prognosis. Personalized approaches are currently being developed and molecular targets are emerging from recent integrated genomic analyses[3-5].

However where does research aim and what could we expect for the future in this setting?

We believe that targeted therapy will represent an important treatment option for TETs with an aggressive histology.

To date, data indicate that vascular endothelial growth factor molecules, insulin-like growth factor 1 receptor (IGF1R), cyclin-dependent kinases (CDK) and mammalian target of rapamycin may be potentially useful as targeted biological therapies.

In this regard, Thomas *et al*[6] in non-randomized phase Ⅱ trial demonstrated efficacy of sunitinib in patients with pre-treated TC.

As IGF1R overexpression is a poor prognostic factor, Rajan *et al*[7] recently reported that Cituxumumab, an IGF1-R directed monoclonal antibody, could produce a promising 90% disease control rate in refractory thymomas.

Therefore, Besse *et al*[8] have initiated a single-arm Phase Ⅱ study with Milciclib, an CDK inhibitor, in advanced TC/B3 thymomas based on good overall response rate, observed in a phase Ⅰ study.

Also Zucali *et al*[9] conducted a single arm, single-stage, open label, multicentre phase Ⅱ trial with everolimus in pre-treated TETs and TC patients. Out of 35 enrolled patients, 71.4% achieved disease control with a median PFS was 12.1 mo, while median OS was 24.0 mo.

The main aim of ongoing trials and new studies is to increase knowledge about etiology and genetic alterations involved in various types of TETs, leading to development and use of biological therapies that will be particularly useful for managing of refractory, recurrent tumors and for TC.

Additionally, STAT3 and PD-L1 protein expression level, both involved in bad prognosis, may have vital importance to evaluate the prognosis of TETs, especially precise for the highly malignant TETs.

## CONCLUSION

In our opinion, further investigations on these genes could increase our knowledge about molecular mechanisms responsible for the TETS heterogeneity, about tumor interactions with adjacent healthy tissue and as regard its variegated response to treatments, to guarantee the development of new promising therapies[10,11].

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EDITORIAL

# Is there still a place for docetaxel rechallenge in prostate cancer?

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## Abstract

Three-weekly docetaxel plus prednisone is the standard first-line cytotoxic treatment for patients with metastatic castrate-resistant prostate cancer (mCRPC). Today, several new treatment options are available for patients with tumor progression after first-line docetaxel: Abiraterone, enzalutamide, cabazitaxel, sipuleucel-T immunotherapy, and the radionuclide radium-223. However, despite the evolving scenario in CRPC treatment, the optimal sequencing of the innovative therapies remains unclear. The reintroduction of docetaxel at the occurrence of disease progression after a drug holiday (docetaxel rechallenge) was often proposed, and this chemotherapeutic agent showed to maintain antitumor activity in mCRPC patients. Docetaxel rechallenge may still constitute a valid treatment option mainly for patients with favorable response to first-line docetaxel, at least > 3 mo progression-free interval, age less than 75 years, good performance status, and acceptable docetaxel toxicity. The risk of cumulative toxicity must be evaluated, since sensory neuropathy, nail disorders and fatigue might occur on docetaxel rechallenge.

**Key words:** Abiraterone acetate; Docetaxel; Prostate cancer; Prostate-specific antigen; Rechallenge



**Core tip:** New treatment options are currently available for metastatic castrate-resistant prostate cancer (mCRPC) patients after first-line chemotherapy with docetaxel. The actual role of docetaxel rechallenge in the evolving scenario of mCRPC treatment is discussed in this editorial.

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## INTRODUCTION

From 2004, three-weekly docetaxel plus prednisone is the standard first-line cytotoxic treatment for patients with metastatic castrate-resistant prostate cancer (mCRPC)[1,2]. In TAX 327 trial, which compared 3-weekly docetaxel plus prednisone *vs* mitoxantrone plus prednisone, 45% of patients receiving docetaxel and prednisone achieved ⩾ 50% prostatic-specific antigen (PSA) reduction, and the median duration of PSA response was 7.7 mo. The patients received a maximum of 8-10 docetaxel cycles, and more that one third of them discontinued chemotherapy without evidence of disease progression. At 4-year follow-up, 3-weekly docetaxel plus prednisone maintained a statistically significant advantage in overall survival (OS) compared to mitoxantrone plus prednisone (19.2 mo *vs* 16.3 mo, $P = 0.004$)[3].

The reintroduction of docetaxel at the occurrence of disease progression after a drug holiday was often proposed in mCRPC patients, and the drug showed to maintain antitumor activity[4-6]. The truly docetaxel rechallenge consists in the reintroduction of the drug in patients responding to first-line docetaxel who discontinued chemotherapy without evidence of disease progression. Although significant advantages in terms of OS were not demonstrated, all studies reported > 25% PSA response on docetaxel rechallenge in patients achieving an initial good response to first-line treatment with the same drug[4-7].

## SUGGESTED ELIGIBILITY CRITERIA FOR DOCETAXEL RECHALLENGE

Docetaxel has been the first drug to report a survival benefit for mCRPC patients. Although these men are usually elderly and with concomitant comorbidities, some of them still have an acceptable performance status and might be proposed for another treatment after docetaxel failure. Today several new treatment options are available for patients with tumor progression after first-line docetaxel: abiraterone, enzalutamide, cabazitaxel, sipuleucel-T immunotherapy, and the radionuclide radium-223[8-12].

Abiraterone acetate, a selective irreversible inhibitor of cytochrome P-450c17, prolonged OS in chemotherapy-naïve or docetaxel-pretreated patients[8,13]. Enzalutamide, a novel androgen receptor signaling inhibitor, significantly prolonged OS and improved quality of life compared to placebo in men with post-docetaxel CRPC[9]. Enzalutamide was recently approved also in pre-docetaxel patients[14].

Cabazitaxel, a second-generation taxane, achieved a statistically significant improvement in OS when added to prednisone *vs* mitoxantrone plus prednisone in mCRPC patients[10]. Sipuleucel-T, an active cellular immunotherapy, prolonged OS among asymptomatic mCRPC patients[11], and Radium-223, which has high bone affinity, improved OS and time to first skeletal-related event[12]. Despite the availability of these new agents in mCRPC patients, their optimal sequencing remains unclear[15].

The possibility of a docetaxel rechallenge has been largely limited by the introduction of abiraterone, enzalutamide and cabazitaxel in the treatment of CRPC patients. Neverthless, it must be considered that the reintroduction of docetaxel can reduce the possibility to administer to the patients one of the new available treatment options. However, a docetaxel rechallenge therapy may be a cheaper option considering the budget impact on health plans of new anticancer agents[16]. Furthermore, the situation is actually complicated by recent trials which might led to early prescription of docetaxel in combination with androgen-deprivation therapy, or for the new indications of abiraterone and enzalutamide in pre-docetaxel patients[13,14,17]. In this setting, some clinical reports suggested a cross-resistance when first-line chemotherapy with docetaxel was administered after the new hormonal agent abiraterone, while there were very few experiences about docetaxel rechallenge after failure to abiraterone or other agents[18-20].

The results of the ongoing randomized phase Ⅱ study CANTATA (EudraCT 2012-003835-40) comparing cabazitaxel with docetaxel rechallenge will add useful informations about the role of docetaxel rechallenge in the mCRPC new agents-era.

Docetaxel rechallenge may still have a role in mCRPC, but a carefull selection of patients has to be performed. Most studies reported that ⩾ 50% PSA response to first-line docetaxel was the main predictive factor for the favorable outcome on the reintroduction of the same drug. A progression free-interval (PFI) of > 6 mo after first-line docetaxel was associated with high fequency of good PSA responses and symptomatic responses on docetaxel rechallenge in a large retrospective study, and encouraging 20.4 mo median OS was reported[21]. Another study described a longer median PFS (6.3 mo *vs* 3.4 mo) and median OS (19.4 mo *vs* 12.8 mo) with docetaxel rechallenge in mCRPC patients progressing at > 3 mo after the last docetaxel cycle with respect to those progressing within 3 mo[22]. In a study of 46 patients with CRPC rechallenged with docetaxel, the PSA response was 66%, and the median OS was 32 mo. In this study a docetaxel rechallenge was safely repeated sveral times, and the good responders had a median PFI of 6 mo[7].

On the other hand, it was reported that PFI < 3 mo was associated with no benefit from docetaxel rechallenge, probably because of early development of complex mechanisms of resistance to the drug[23].

Available findings indicate that docetaxel rechallenge might still constitute a valid treatment option, and some eligibility criteria may be suggested: good response to first-line docetaxel, at least > 3 mo PFI, age less than 75 years, and acceptable docetaxel toxicity (Table

**Table 1 Main eligibility criteria for docetaxel rechallenge in metastatic castrate-resistant prostate cancer patients**

| |
|---|
| PFI > 3-6 mo |
| > 50% PSA response to first-line docetaxel |
| No cumulative docetaxel-toxicity |
| Age < 75 yr |
| ECOG PS 0-1 |

PFI: Progression free-interval; PSA: Prostatic-specific antigen.

1). On the other hand, very elderly patients and/or men with worsened performance status could benefit from less aggressive treatment options. Furthermore, since the chemotherapy agent cabazitaxel shows low incidence of severe sensory neuropathy, this drug may be a valid treatment choice for patients who exhibit unacceptable toxicity to docetaxel[10].

Another intriguing treatment strategy, especially for patients with PFI 3-6 mo, might be to combine docetaxel rechallenge with another agent which might help to overcome the resistance to docetaxel. Among chemotherapeutic agents which were investigated, epirubicin resulted feasible and tolerable when combined with docetaxel on a weekly schedule[24]. A randomized phase Ⅱ study suggested an advantage in PSA response, PFS, and OS for the combination of docetaxel and epirubicin compared with docetaxel alone in advanced CRPC patients[25]. In a recent clinical study, our research team reported encouraging results with rechallenge of docetaxel combined with weekly epirubicin in 26 men with advanced CRPC following progression on docetaxel and abiraterone acetate, with PSA response in 26.9% of patients, 4.4 mo PFS, and 10.7 median OS[26]. Among the subjects who were symptomatic at baseline, pain was reduced in 9 patients (38.1%) with a significant decrease in analgesic use. The weekly epirubicin/doxorubicin treatment was well tolerated: grade 3 neutropenia occurred in 19.2% of patients, and no grade 4 toxicity or congestive heart failure was observed.

These encouraging results may also suggest that abiraterone treatment after docetaxel failure does not reduce the efficacy of a delayed docetaxel rechallenge. Larger studies should be performed to investigate if epirubicin or other agents may play a role in restoring the sensitivity and reversing the resistance to docetaxel in patients who were previously poor-responders to the same drug.

Despite the addition of a drug to docetaxel rechallenge might led to overcome the resistance to docetaxel, the risk of eventual increase in the occurrence of adverse events must be considered, too[27-29]. Moreover, sensory neuropathy, nail disorders and fatigue might occur on docetaxel rechallenge[6,7,21].

Though the feasibility and activity of docetaxel rechallenge in mCRPC patients have been demonstrated in several studies before the new agents-era, very few data are available about the reintroduction of the drug in heavily pretreated subjects. It might hypothesized that in mCRPC patients with PFI 3-6 mo a delayed rechallenge by intercalation of a non-docetaxel treatment might be effective, with possible restoring of sensitivity to the drug. In this setting, in other tumors such as relapsed ovarian cancer, the PFI prolongation by intercalation by an effective non-platinum regimen resulted in survival advantage with subsequent platinum-based regimens[30,31].

Another interesting point is that docetaxel rechallenge on weekly schedule might be offered, especially for mCRPC patients with some degree of toxicity during 3-weekly docetaxel. Neverthless, a few small experiences suggested that weekly docetaxel schedule might be effective in patients not-responding to first-line 3-weekly docetaxel[7,32]. In conclusion, as we all await additional studies to clarify the optimal sequencing of the new available agents in mCRPC, docetaxel rechallenge may have still a role for well selected patients.

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EDITORIAL

# Current and future treatment of anaplastic lymphoma kinase-rearranged cancer

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## Abstract

Aberrant forms of the anaplastic lymphoma kinase (ALK) are involved in the pathogenesis of several types of cancer, including anaplastic large cell lymphoma, non-small-cell lung cancer (NSCLC), inflammatory myofibroblastic tumors, colorectal cancer, neuroblastoma and others. In general, the ALK catalytic domain is rearranged and fused to a dimerization domain encoded by an unrelated gene. Less frequently, full-length ALK is activated by point mutations. The common theme is unregulated firing of ALK downstream signalling, leading to uncontrolled cell division and increased cell survival. ALK-driven tumors can be treated with Crizotinib, an orally available dual ALK/MET inhibitor, currently approved for advanced ALK-positive NSCLCs. Crizotinib-treated patients achieve high response rates, with an excellent toxicity profile. However, drug-resistant disease often develops, particularly in NSCLC patients. The processes leading to drug resistance include both ALK-dependent (point mutations or gene amplification), as well as ALK-independent mechanisms, which are here briefly discussed. Recently, Ceritinib has been approved for Crizotinib-refractory NSCLC, further extending patients' survival, but resistance again emerged. Novel ALK kinase inhibitors are currently under clinical development, showing great promise for improved efficacy in drug-resistance disease. It is opinion of the author that drug-resistance is likely to arise under any treatment, due to intrinsic heterogeneity and adaptability of cancer. To prevent or delay this phenomenon, we need to treat less advanced disease, with drugs that are rapidly effective in order not to allow enough time for tumor evolution, and we want to have more and more drugs with non-overlapping resistance profiles, for subsequent lines of targeted therapy. Finally, the use of drug combinations may exponentially decrease the chances of resistance.

**Key words:** Anaplastic lymphoma kinase tyrosine kinase receptor; Protein kinase inhibitors; Drug resistance; Crizotinib; Drug combinations



**Core tip:** In this Editorial article, I discuss the issue of anaplastic lymphoma kinase (ALK) driven cancer and its specific treatment with selective ALK tyrosine kinase inhibitors. The problem of acquired drug resis-

tance is shortly reviewed and clinical data with novel investigational ALK inhibitors are presented. The possibility of specific combination therapies is briefly discussed.

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## INTRODUCTION

This year marks the 55th anniversary since the first specific oncogenic alteration was described[1]. It took over 40 years since the initial observation of the Philadelphia chromosome, to bring a concrete benefit to the patients carrying such abnormality[2]. However, the discovery of imatinib was not simply the end of a medical problem, but it represented the beginning of a new era in cancer therapy. Personalized medicine is now a reality. Curiously, about the time when imatinib was described for the first time, a new fusion oncogene was identified in a subset of non-Hodgkin lymphoma patients and its catalytic portion was named after the disease, anaplastic lymphoma kinase (ALK)[3]. It is astounding to think that this time it took only 12 years before a specific treatment was administered to ALK+ patients (NCT00585195; study start 2006)[4].

ALK is a receptor tyrosine kinase whose expression is normally restricted to the developing neuronal tissue. When activated, two ALK molecules dimerize and trans-phosphorylate on specific tyrosine residues, thus triggering downstream signaling, which includes the Ras/MAPK, PI3K/AKT, Cdc42/Rac and JAK/STAT pathways[5]. Aberrant activation of ALK kinase is oncogenic and it is found in several cancers, including anaplastic large-cell lymphoma (ALCL), non-small cell lung cancer (NSCLC), inflammatory myofibroblastic tumor, neuroblastoma, as well as thyroid, colorectal and breast cancer[5]. In most cases, constitutive ALK activation is caused by chromosomal rearrangements that lead to expression of fusion oncoproteins comprising an amino-terminal dimerization region derived from different 5'-fusion partners (NPM1, EML4, KIF5B, and many others)[6] and a carboxy-terminal kinase domain derived from the ALK gene. These fusion proteins are aberrantly expressed in tissues where ALK is not normally expressed, and constitutively activated by means of the dimerization domain, with no need of ligand. In neuroblastoma, full-length ALK is activated by point mutations in its kinase domain, that are thought to force the kinase fold into a permanently active conformation[7].

The extremely low physiological expression level of ALK in normal cells, together with the demonstrated driving oncogenic role in tumor cells, make ALK fusion proteins a perfect therapeutic target.

## CRIZOTINIB, FIRST-IN-CLASS ALK INHIBITOR

Preclinical data clearly supported the use of ALK inhibitors in ALK-driven malignancies[8]. Translation of these data to the clinic led to accelerated approval of Crizotinib (PF-02341066, Xalkori™, Pfizer Inc.) an ALK/MET inhibitor launched in 2011, which is currently the front-line therapy for ALK+ NSCLC[4,9]. In particular, phase Ⅲ trials showed a significant advantage of Crizotinib *vs* chemotherapy in terms of progression-free survival (PFS) and response rate (RR), both in chemotherapy-pretreated and naïve patients[10,11]. Although most trials only evaluated Crizotinib in NSCLC patients, clinical reports on its use in other tumors indicated that all ALK+ cancers may be effectively treated with ALK inhibitors[12-14]. Notably, in contrast to short-lived responses in NSCLC (PFS is usually < 1 year), approximately half of ALCL patients who achieve a complete remission (CR) stay disease-free for prolonged periods, up to > 3 years at data cutoff[14]. This may relate to the ability of Crizotinib to eradicate tumor-propagating ALCL cells[15]. Importantly, Crizotinib has limited side effects, usually mild and reversible. Most common adverse events include nausea, emesis, fatigue, diarrhea and visual disturbances. Grade 3 elevations in alanine and aspartate aminotransferases were observed in a small fraction on patients. In addition, QTc prolongation was observed in 2.7% of patients across various clinical trials. Few cases of esophageal ulceration, regressed upon drug discontinuation, were also reported.

## RESISTANCE TO CRIZOTINIB

Despite impressive efficacy, resistance to Crizotinib is a major hurdle, leading to treatment failure in most NSCLC patients. Several mechanisms of drug-resistance have been described. Approximately one-third of the patients develop a clone that carries point mutations in the ALK kinase domain, which render the enzyme refractory to inhibition by Crizotinib[16,17]. In other cases, activation of bypass signaling pathways allow the cells to grow independently of ALK[17,18]. While point mutations are generally considered to pre-exist in a very small subclone that is selected by the drug and expands under treatment, bypass signaling is thought to be an adaptive mechanism. In some patients, amplification of non-mutated fusion gene leads to resistance, simply by gene dosage[17]. These cases may be treated by a drug increase, or, as suggested by preclinical evidence of drug-dependency in cells with oncogenic signal overflow, by a drug holiday[19] (and our unpublished data).

Point mutations have been extensively studied both *in vitro* and *in vivo*. Similarly to most first-generation inhibitors, Crizotinib causes the selection of cells harboring a mutated gatekeeper residue[20], in this case a Leu to Met substitution at position 1196 of ALK. The gatekeeper is a key residue that controls access to the

active site. When it is replaced by bulkier aminoacids, as is the case of the L1196M mutant of ALK, it can cause steric clash with the drug, impeding inhibitor binding. Drugs that are not affected by the aminoacid change, or more potent inhibitors that are still clinically active despite an affinity loss, are needed to overcome such mutants. In our laboratory, we observed the selection of a L1196Q mutant in NPM-ALK+ cells *in vitro*, under Crizotinib treatment[21]. In addition to gatekeeper mutants, several other mutations have been described in patients, as well as in preclinical models[14,17,22-24], spanning the ALK kinase domain from the region immediately aminoterminal to the αC helix, to the DFG motif. Some mutants directly affect drug binding, while others are believed to alter the kinetics or the conformational equilibrium of the kinase, causing a shift towards a more active conformation.

## NEXT-GENERATION ALK INHIBITORS

Resistance to Crizotinib has fostered the search of novel, second-generation ALK inhibitors that may overcome resistant clones. Ceritinib (LDK378, Zykadia™; Novartis, Switzerland) showed great efficacy in ALK+ NSCLC patients, both Crizotinib-resistant and naïve[25], with limited side effects, in a phase Ⅰ trial, leading to fast-track approval by FDA in 2014. More trials are ongoing, but the message is that resistant patients can be effectively treated, thus further extending overall survival (OS) of these patients. Interestingly, patients that had no prior Crizotinib display a much better PFS curve compared to Crizotinib-resistant/intolerant individuals (50% *vs* 25% remain progression-free after 24 mo) although the data were still immature at cutoff. RRs in TKI naïve patients are similar with Crizotinib and Ceritinib, however a direct comparison between the two drugs in first-line treatment has not been done yet. Moreover, whether sequential or combined treatment will yield better outcomes is not known. The combined OS of Crizotinib-Ceritinib sequential therapy was shown to be 49.4 mo in a recent retrospective analysis of metastatic NSCLC[26]. As a comparison, OS from metastatic diagnosis in a comparable group of ALK-wild-type controls were approximately 24 mo[9]. Unfortunately, Ceritinib-resistant mutants do arise under treatment[27]. In particular, substitutions at F1174 and G1202 residues have been observed in lung cancer patients progressing on Ceritinib.

Alectinib (CH5424802, Alecensa™) co-developed by Roche and Chugai, demonstrated impressive efficacy in EML4-ALK+ NSCLC patients: phase Ⅰ-Ⅱ studies reported 93% RR in TKI-naïve patients and 55% in patients who had progressed on Crizotinib, including brain metastases[28,29], with mostly mild (grade 1-2) side effects. Updated results from the AF-001JP study confirmed 93.5% RR including 19.6% CRs. Follow-up indicated a 2-year PFS of 76% (median PFS not reached at median follow-up > 30 mo)[30]. The drug is now approved for NSCLC patients in Japan. Once again, however, resistance occurs, although at lower frequency compared with Crizotinib. Mutations at I1171, F1174 and G1202 were observed in various analyses[31,32].

Preliminary phase Ⅰ-Ⅱ results with Brigatinib (AP26113, ARIAD Pharmaceuticals) were recently presented at AACR 2015[33]. The compound showed pan-ALK inhibitory activity. An interesting analysis showed that all clinically reported ALK mutants are sensitive to Brigatinib concentrations that are well below the determined mean plasma levels, indicating that the drug may be able to overcome all mutants and possible prevent or limit resistance. Again, the G1202R mutation appears to be somewhat borderline, suggesting that this mutant might be expected to emerge under Brigatinib therapy. Indeed, preclinical work indicates that although G1202R mutant xenografts responded to Brigatinib better than to other ALK inhibitors, yet no regression was achieved. In an *in vitro* assessment of NPM-ALK and EML4-ALK mutants sensitivity to clinically relevant inhibitors, we noted that G1202R is the most intractable mutant of all[34]. Only the new compound PF-06463922 was able to inhibit this mutant at low nanomolar doses[35]. PF-06463922 is a very potent and selective ALK/ROS1 inhibitor undergoing phase Ⅰ evaluation, with a very large therapeutic window[36]. Indeed, recent preclinical *in vivo* data demonstrate potent PF-06463922 activity against G1202R mutant xenografts, as well as other mutations[37].

The analysis of all clinical data available so far with second-generation ALK inhibitors highlights an interesting phenomenon: in most trials, RRs in Crizotinib-resistant patients were higher than expected based on the frequency of ALK-dependent resistance, which overall accounts for approximately 30%-40% of cases. If the numbers are correct, we have to postulate that the new drugs are able to kill ALK-independent resistance. This may occur by inhibition of bypass pathways (for example Brigatinib is a potent EGFR inhibitor, Ceritinib blocks IGF1R). If this is the case, then the reciprocal occurrence may also be true, with Crizotinib effectively blocking second-generation inhibitors-resistant tumors possibly driven by MET activation.

## COMBINATION THERAPY

The problem of drug-resistance is really a major issue in cancer. Even very effective targeted therapies eventually fail, especially in highly heterogeneous diseases such as NSCLC. Knowing the cause of resistance helps in designing new and more effective drugs, which however in turn select for additional, perhaps more aggressive resistant clones. One alternative path to tackle such problem may be represented by combination therapies, since it is statistically more difficult for a cancer to acquire simultaneous resistance to more than a drug. However, combinations need to be rationally designed based on deep knowledge of the tumor biology, and thoroughly validated. For instance, combining ALK inhibition with anti-CD30 therapy (Brentuximab vedotin, Adcetris®,

INN) may have synergistic efficacy in NPM-ALK+/CD30+ ALCL. Similarly, ALK inhibitors may be combined with blockers of bypass pathways, or downstream effectors such as mTOR or PI3K inhibitors. Although attractive, these strategies have yet to be fully explored and validated in relevant models. Nevertheless, they may represent a logic way to try and eradicate the disease.

## CONCLUSION

In conclusion, a new era has opened for ALK+ cancer patients. Although it is difficult to foresee definitive cure, due to the tremendous ability of advanced tumors to adapt to a new environment, we can extend life expectancy of these patients significantly, with at least the aim to make it a chronic disease. Although only few patients have been described, it seems that at least for ALK+ lymphoma this goal is not very far[14].

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EDITORIAL

# Cancer screening: Between appropriateness and effectiveness

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## Abstract

Two similar words, effectiveness and efficacy, have comparable insight and nearly describe analogous meaning for a screening test, yet clear understanding and perception of their diverse meanings will help clarify the basis of the differing conclusions about whether screening tests for different cancers reduce morbidity and mortality. Screening test may not be effective even when it sounds to be efficacious, on the other hand it should be efficacious when the test is effective.

**Key words:** Mortality; Screening test; Effectiveness; Efficacy; Cancer; Early detection



**Core tip:** Screening test should take account of heterogeneity among cancers. The effectiveness of any screening test should be evaluated on the basis of "whether it does more good than harm". Health professionals should be aware that such tests should outweigh the potential harm of investigating healthy people and consider the effect of intervening in apparently symptomless people.

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## INTRODUCTION

Screening is the probable identification of an unrecognized disease or defect by means of examinations, tests or any other procedures that can be practically and effectively applied. There are different aspects that should be considered upon the implementation of any screening procedure: specificity, sensitivity, positive and negative predictive values and acceptability. The likelihood that a positive screening test, predictive value positive, will give a correct result largely depends on the disease prevalence within the community. The lower the prevalence rate, the less the effectiveness of any screening health program even with the best screening tests[1].

The success of any screening program relies on a number of crucial factors, *i.e.*, the target disease or

cancer under screening should be highly prevalent and of public health importance, which is indicated by high morbidity or mortality, the treatment should be available and effective for decreasing morbidity and mortality, the screening test should be inexpensive and feasible, and the procedure itself must be convenient and virtually free of discomfort or risk[2].

When adopting an effective screening program, two major objectives should be considered: (1) a high level of case detection at an early stage when treatment can be more effective and before developing signs and symptoms, and a reasonably low level of false positive results; and (2) identification of risk factors which increase the probability of developing the disease and getting use of this knowledge to prevent or reduce the disease prevalence by changing these risk factors. Different criteria should be met for a screening test and the disease under screening to fulfill the previous objectives: The test should be competent of detecting a high percentage of disease in its preclinical state, hence the development of the disease from latent to affirmed condition should be amply understood, it has to be secure and cost-effective (the cost of case-detection including diagnosis and treatment should be economically balanced in relation to available expenditure), and it should lead to noticeably improved health outcomes on the basis of a continuing process and not once and for all projects[3].

Two similar words, Effectiveness and Efficacy, have comparable insight and nearly describe analogous meaning for a screening test, yet clear understanding and perception of their diverse meanings will help clarify the basis of the differing conclusions about whether screening tests for different cancers reduce morbidity and mortality. Screening test may not be effective even when it sounds to be efficacious, on the other hand it should be efficacious when the test is effective**.**

The most frequent method for appraising the effectiveness of a screening program is to compare the survival among cases detected as a result of screening with the survival of cases detected because of the occurrence of signs and symptoms.

Two contradicting results have emerged from the largest two longitudinal studies; The European Randomized Study of Screening for Prostate Cancer (ERSPC) reported that there was a 20% lower death rate from prostate cancer among men who were assigned to be screened in comparison to men not assigned to be screened, yet, screening itself carried a high risk for over-diagnosis[4]. On the other hand, the trial from the United States (Prostate, Lung, Colorectal and Ovarian Cancer Screening Trial) declared that examination of the prostate and screening with a PSA cutoff of 4 ng/mL did not decrease the death rate from such cancer[5].

Screening programs themselves may have an effect on health and healthcare, which may in turn significantly impinge on the effectiveness of the programs. Whereas several screening methods have been shown to be effective in reducing the mortality of breast, cervical, colorectal and oral cancers, recommendations for liver, prostate and stomach cancer screening based on effectiveness, harm *vs* benefit and cost-effectiveness consideration are not clear or strong.

Many factors should be considered for determining the effectiveness of a cancer screening program, *i.e.*, quality adjusted life years (QALY), balance between costs and benefits, interval of screening and age at which screening should be conducted. The reported results from the ERSPC trial concluded that prostate cancer screening would be cost-effective when it is limited to few screens in subjects between 55 and 60 years of age, while it is less cost-effective when screening is conducted in subjects beyond 63 years of age because of loss of QALYs due to over-diagnosis[6].

In general, screening tests should take account of heterogeneity among cancers. The effectiveness of any screening test should be evaluated on the basis of "whether it does more good than harm". Health professionals should be aware that such screening tests should outweigh the potential harm of investigating healthy people and to consider the effect of intervening in apparently symptomless people.

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REVIEW

# *Helicobacter pylori* and microRNAs: Relation with innate immunity and progression of preneoplastic conditions

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## Abstract

The accepted paradigm for intestinal-type gastric cancer pathogenesis is a multistep progression from chronic gastritis induced by *Helicobacter pylori* (*H. pylori*) to gastric atrophy, intestinal metaplasia, dysplasia and ultimately gastric cancer. The genetic and molecular mechanisms underlying disease progression are still not completely understood as only a fraction of colonized individuals ever develop neoplasia suggesting that bacterial, host and environmental factors are involved. MicroRNAs are noncoding RNAs that may influence *H. pylori*-related pathology through the regulation of the transcription and expression of various genes, playing an important role in inflammation, cell proliferation, apoptosis and differentiation. Indeed, *H. pylori* have been shown to modify microRNA expression in the gastric mucosa and microRNAs are involved in the immune host response to the bacteria and in the regulation of the inflammatory response. MicroRNAs have a key role in the regulation of inflammatory pathways and *H. pylori* may influence inflammation-mediated gastric carcinogenesis possibly through DNA methylation and epigenetic silencing of tumor suppressor microRNAs. Furthermore, microRNAs influenced by *H. pylori* also have been found to be involved in cell cycle regulation, apoptosis and epithelial-mesenchymal transition. Altogether, microRNAs seem to have an important role in the progression from gastritis to preneoplastic conditions and neoplastic lesions and since each microRNA can control the expression of hundreds to thousands of genes, knowledge of microRNAs target genes and their functions are of paramount importance. In this article

we present a comprehensive review about the role of microRNAs in *H. pylori* gastric carcinogenesis, identifying the microRNAs downregulated and upregulated in the infection and clarifying their biological role in the link between immune host response, inflammation, DNA methylation and gastric carcinogenesis.

**Key words:** *Helicobacter pylori*; MicroRNA; Gastric cancer; Inflammation; DNA methylation; Preneoplastic conditions; Stomach neoplasms; Immune response



**Core tip:** *Helicobacter pylori* (*H. pylori*) are involved in the progression of gastric preneoplastic conditions and gastric carcinogenesis although the clear genetic and molecular mechanisms are not completely clear. MicroRNAs may have an important role in the development of *H. pylori* mediated pathology since they can alter the expression of hundreds to thousands of genes. In this article we present a comprehensive review about the microRNAs that are altered in *H. pylori* infection and the biological consequences of this alteration, linking the inflammatory and immune host response with the progression of preneoplastic conditions and gastric carcinogenesis.

Libânio D, Dinis-Ribeiro M, Pimentel-Nunes P. *Helicobacter pylori* and microRNAs: Relation with innate immunity and progression of preneoplastic conditions. *World J Clin Oncol* 2015; 6(5): 111-132 Available from: URL: http://www.wjgnet.com/2218-4333/full/v6/i5/111.htm DOI: http://dx.doi.org/10.5306/wjco.v6.i5.111

## INTRODUCTION

Gastric cancer (GC) is the fourth most common cancer and the second leading cause of cancer-related death[1]. *Helicobacter pylori* (*H. pylori*), a microaerophilic gram-negative bacteria that colonizes the gastric epithelium of over 50% of the world's population, has been identified as a definite (type Ⅰ) carcinogen by the World Health Organization and is thought to contribute for approximately 75% of GCs[2].

The accepted paradigm for the pathogenesis of intestinal-type GC is a multistep progression from inflammation/chronic gastritis induced by *H. pylori* to gastric atrophy, intestinal metaplasia, dysplasia and ultimately adenocarcinoma, as first suggested by Correa[3]. *H. pylori* are responsible for the initial stages of gastritis and atrophy and contributes to the progression to preneoplastic conditions/lesions and ultimately GC, but the molecular mechanisms underlying disease progression are still not completely understood. Besides, only a fraction of colonized individuals ever develop neoplasia, suggesting that strain-specific bacterial virulence factors, host responses and environmental factors may influence cancer risk.

MicroRNAs (miRNAs) are noncoding RNAs with 18-24 nucleotides which can cause mRNA degradation or translational inhibition, influencing the transcription and expression of various genes and playing an important role in inflammation, cell proliferation, apoptosis and differentiation. The biogenesis of miRNAs is initiated in the nucleus by the RNase Ⅲ enzyme Drosha[4]. Drosha and its cofactor Pasha (DGCR8) cleave primary miRNA transcripts generating precursor miRNAs of about 60 nucleotides (pre-miRNA) which are subsequently transported out of the nucleus to the cytoplasm for further processing into mature miRNA by Dicer, a cytoplasmic RNase Ⅲ[5,6]. Mature miRNAs are single-stranded RNA, 18-24 nucleotides long, which down-regulate specific gene products by translational repression of their target mRNAs *via* direct binding to 3' untranslated regions (3'-UTR) or by directing mRNA degradation *via* binding to perfectly complementary sequences[7].

Over one thousand microRNAs have been identified and each miRNA may regulate the expression of hundreds to thousands of target genes and it is estimated that 30%-92% of human genes are regulated by miRNA[8]. Identification of these target genes is critical to understand the biological role of each miRNA since miRNAs can influence the expression of tumor suppressor genes and oncogenes and thus are involved in proliferation and apoptosis, possibly contributing to initiation and progression of malignancy. In gastrointestinal cancers some miRNAs are downregulated suggesting that these downregulated miRNAs act as tumor suppressors (*e.g.,* mir-15b and mir-16, which target anti-apoptotic Bcl-2, are downregulated in GC)[9]. On the other hand, some miRNA are overexpressed in gastrointestinal cancers, suggesting their role as oncogenes (*e.g.,* miR-155, which represses expression of pro-apoptotic TP53INP1, is overexpressed in mucosa-associated lymphoid tissue lymphoma)[10].

*H. pylori* can affect the expression of various miRNAs which may induce epigenetic deregulation of oncogenes and tumor suppressor genes and may represent the bridge between *H. pylori*-gastritis and GC[11,12]. *H. pylori* possess a set of virulence factors necessary to successfully colonize the gastric mucosa and establish chronic infection. The vacuolating cytotoxin (VacA) exhibits vacuolating activity and is coded by the gene *vacA*, which is present in all *H. pylori* strains. VacA can induce apoptosis of host cells and suppress proliferation of T and B-lymphocytes, contributing to the ability of *H. pylori* to establish chronic infection through deregulation of the host immune response[13,14]. Besides, VacA can induce radical oxygen species (ROS) production and mitochondrial DNA mutation in gastric epithelial cells.

Another bacterial virulence factor is the *cag* pathogenicity island (cagPAI) which is present in about 60% of *H. pylori* strains and is associated with an increased risk of severe gastritis, ulcer disease and GC[15]. CagA can affect epithelial cells by several mechanisms and may contribute to GC development[16]. CagA was

associated with the epithelial tight-junction scaffolding protein ZO-1 and the transmembrane protein junctional adhesion molecule which modify the composition and function of the apical-junctional complex and disrupt junction-mediated functions[17].

cagPAI also encodes a bacterial type Ⅳ secretion system (T4SS), which translocates CagA into host cells that subsequently affects multiple pathways that alter host cell morphology, signaling and inflammatory responses[17,18]. Once inside the epithelial cell CagA is phosphorylated at tyrosine residues by the epithelial cell c-Src protein and Lyn kinases, and phosphorylated CagA then activates the Src homology-2 domain-containing tyrosine phosphatase, which activates the Erk1/2 pathway, deregulates the phosphatase activity and induces epithelial gastric cell proliferation and transformation[19].

CagA was shown to enhance NF-κB pathway through interaction with TNF-receptor associated factor 6 (TRAF6) and TFG-B-activating kinase-1[20], to activate activator protein-1 (AP-1), PI3K (which leads to B-catenin and NF-κB activation), NFAT and to induce higher levels of interleukin-8 (IL-8)[21,22]. Methylation of MGMT DNA repair gene was also associated with CagA in chronic gastritis, suggesting its role in epigenetic regulation[23]. Other effects of CagA involve interference with proteasome-mediated degradation of the tumor suppressor RUNX3 and TP53[24].

These bacterial factors contribute to adherence, persistence, host immune modulation and virulence. MiRNAs are host factors that may contribute to influence GC risk as each miRNA can potentially control hundreds to thousands of target genes and miRNA deregulation was associated with immune and inflammatory disorders and various malignancies. *H. pylori* have been demonstrated to modulate expression of miRNAs which may further contribute to *H. pylori*-related diseases[14]. However, the true role of miRNA deregulation in the tumorigenesis is not perfectly clear.

In this review we aim to summarize the available evidence concerning the role of microRNAs in gastric carcinogenesis through *H. pylori* infection, inflammation, DNA methylation and progression of preneoplastic conditions.

## *H. PYLORI*, IMMUNE HOST RESPONSES AND INFLAMMATION

Inflammation has long been recognized as a key factor in the development of many types of cancers. *H. pylori* induce chronic gastric inflammation which is the strongest known risk factor for development of atrophic gastritis, metaplasia, dysplasia, and ultimately GC through the accumulation of mutations, epigenetic modifications and deregulation of cell function. The chronic nature of *H. pylori*-gastritis is critical to the carcinogenic potential of this infection, resulting in a long-term interaction between the bacteria, inflammatory mediators and gastric epithelial and stem cells. Indeed, the preneoplastic gastric epithelial changes have been shown to carry numerous genomic, epigenetic and functional abnormalities than can also be detected in cancer tissues[25-28].

Host defense against pathogens requires appropriate innate immune responses, as excessive or inappropriate activation of the immune system can be deleterious. *H. pylori* infection elicits both humoral and cellular immune responses[29]. Host cells recognize invading pathogens and/or their secreted effectors/pathogen associated molecular patterns (PAMPs) through pathogen recognition molecules known as Toll-like receptors (TLRs) and NOD-like receptors, located on the cell membrane and in the cytoplasm, respectively, which subsequently activate adaptor proteins and transcription factors such as the NF-κB and AP-1[30].

Gastric epithelial cells constitute the first line of defense against *H. pylori*. In these cells, the innate immune response is characterized by NOD1-dependent activation of the NF-κB pathway in response to *H. pylori* peptidoglycan which is injected into the host cell cytoplasm *via* the T4SS[31]. NF-κB activation promotes cellular signaling changes and activation of adaptor proteins and transcription factors which mediate the release of cytokines that promote the recruitment of polynuclear cells and the activation of macrophages, dendritic cells (DCs) and mucosa infiltrating lymphocytes which take part in the innate and adaptive immune responses to the bacteria.

The bacteria also interacts with DCs, either in the gut lumen (where mucosal DCs insert dendrites through the tight junctions of the epithelial barrier) or within Peyer's patches in the small intestine (where resident DCs phagocytose bacteria), which may direct the nature of the adaptive immune responses[32]. Myeloid cells (monocyte/macrophage and DCs) constitute the second line of defense, sensing *H. pylori* components *via* TLR2, TLR4, TLR5 or NOD1 signaling. TLRs in the cell membrane of DCs trigger a signaling cascade in the host cell responsible for the initiation of the immune host response and lead to the secretion of proinflammatory cytokines such as IL-1B, IL-6, and TNF-α in order to establish T and B lymphocyte-mediated adaptive immunity[24,33,34]. Indeed, TNF-α contributes to monocyte maturation, IL-6 supports the transition between the early stages of the infection and the sustained mononuclear influx into the infected gastric mucosa, and IL-1B contributes to NF-κB pathway activation in myeloid cells[35].

NF-κB can be activated by *H. pylori* through proinflammatory mediators (*e.g.*, cytokines) and through TLR activation by PAMPs[20]. It has been proposed that *H. pylori* peptidoglycan (injected in the gastric epithelial cell *via* T4SS) activates NF-κB *via* NOD1, which then activates MAPKs in both the NF-κB and AP1 pathways, inducing NF-κB activity and leading to cytokine release namely IL-8[31,36,37]. In macrophages and DCs, the TLR family members TLR2, TLR5, TLR4 and TLR9

are involved in response to *H. pylori* infection[34], but discussion is ongoing as wether *H. pylori* LPS signals *via* TLR4 (a common receptor for Gram-negative enterobacterial LPS) or *via* TLR2 (the main receptor for G+ bacteria lipoteichoic acid), because *H. pylori* LPS lacks distinct features of the prototypical LPS[38]. When activated by bacterial LPS, TLR4 may recruit MyD88 and IRAK which subsequently activates NF-κB[39].

DCs also stimulate the production of IL-17 by lymphoid cells and release IL-23, a major cytokine involved in the induction and maintenance of Th17 responses, leading to a Th17 response against *H. pylori* which can affect the development of *H. pylori* gastritis[34,40,41]. Infection with cagPAI+ strains was associated with an increased production of IL-23[35]. However, an imbalance of the Th17/Treg axis may lead to suppressed Th17 and ineffective bacterial eradication, suggesting that DCs may also play a role in *H. pylori* immune escape through directing a Treg-skewed DC-induced helper T-cell differentiation[42].

Altogether, the mediators released by epithelial cells, macrophages and DCs activate T-lymphocytes with a predominant Th1 response, regulatory T-lymphocytes (Treg), B-lymphocytes which mature into mucosal plasma cells, and neutrophils which actively phagocytize *H. pylori*[24].

Despite the strong immune response, *H. pylori* is not cleared and produces a chronic inflammatory status which requires evasion from the immune system. Although *H. pylori* is generally considered an extracellular microorganism, some evidence supports that at least a subset of *H. pylori* has an intraepithelial location and that a minor fraction of *H. pylori* resides inside gastric epithelial cells, which may represent the site of residence for persistent infection[43]. Autophagy is suggested as an immune innate response against *H. pylori*, decreasing its survival, and it was shown that *H. pylori* can induce autophagy in gastric epithelial cells despite still being capable to replicate in these cells[44,45].

The progressive damage of gastric glands leads to mucosal atrophy and intestinal metaplasia which constitutes an environment with increased risk for the development of dysplasia and cancer. Mucosal atrophy in the gastric body and fundus lead to hypochlorhydria, which may further contribute to the overgrowth of other bacteria that can convert nitrites to carcinogenic nitroso-N-compounds and thus increase the carcinogenic activity in the gastric mucosa[46].

Gastrokine-1 (GKN1) is a protein present in gastric mucosal cells that protects the antral mucosa and promotes healing by facilitating restitution and proliferation after injury and may also play an important role in mucosal inflammation since its expression suppresses activation of NF-κB by inhibiting the degradation and phosphorylation of IkB and inactivating IKKalfa/beta[47,48]. Decreased GKN1 expression has been reported in *H. pylori*-infected patients and it was demonstrated a progressive decrease from chronic gastritis to atrophy and intestinal metaplasia[49]. Remarkably, in the latter study GKN1 was undetectable in tumoral tissues and was expressed in non-tumoral tissues, suggesting that GKN1 plays an important role in mucosal defense, and that its gene acts as a tumor suppressor[50]. More recently, Yoon *et al*[51] demonstrated that CagA reduces GKN1 expression and that GKN1 transfection suppresses the carcinogenic effects of CagA. GKN1 may also influence cytokine production, NF-κB pathway and COX-2 expression[52].

### *Inflammation and carcinogenesis*

Chronic inflammation plays an important role in the development of various cancers, including gastric adenocarcinoma, hepatocellular carcinoma associated with hepatitis B and C, immunoproliferative small intestinal disease associated with Campylobacter jejuni and cancer associated with ulcerative colitis. In fact, up to 25% of all cancers are thought to be associated with chronic inflammation, regardless of the presence or absence of infection[53].

The inflammatory milieu caused by chronic *H. pylori* infection contributes to carcinogenesis through activation of downstream targets that regulate cell cycle progression, proliferation, and apoptosis. NF-κB is a key regulator of inflammation and other cellular cascades and was identified as a molecular bridge between inflammation and cancer, since improper NF-κB activation transactivates several target genes harboring inflammatory (*e.g.,* COX2, iNOS, TNF-α), anti-apoptotic [*e.g.,* cIAP1 and 2, x-linked inhibitor of apoptosis (XIAP), Bcl-2, Bcl-3, Bcl-xL], cell cycle regulatory (*e.g.,* cyclin D1) and proangiogenic (*e.g.,* VEGF, angiopoietin) functions, and/or down-regulates pro-apoptotic genes (*e.g.,* p53, Bax, Bad)[54].

Other inflammatory mediators released from epithelial, mesenchymal and immune cells like proinflammatory cytokines, growth factors, ROS and reactive nitrogen species (RNS) can also promote cell proliferation, migration, angiogenesis and invasion through a stepwise accumulation of genetic and epigenetic alterations. Among these, cytokines play key roles in the inflammatory process, and IL-1B, IL-6, and TNF-α have been implicated in cancer development. Interleukin-1B and TNF-α induce NF-κB activation, which promotes cell growth/proliferation, suppresses apoptosis of epithelial cells and stimulates the production of growth factors and cytokines such as epidermal growth factor, IL-6, COX2 and ROS[55]. IL-6 activates STAT3 (signal transducer and activator of transcription 3), enhancing cell growth and growth factor production[56]. Besides, IL-6 promotes COX-2 induction and increases ROS production[57]. COX-2 subsequently enhances cell growth and angiogenesis while ROS can modify protein function[24].

TLRs may also lead to the production of inflammatory cytokines through AP-1 and NF-κB dependent transcription, playing a role in carcinogenesis through the activation of NF-κB and COX2[58-60]. In fact, incr-

easing levels of TLR2, 4 and 5 and decreasing levels of TLR inhibitors (PPARy and TOLLIP) were demonstrated through the spectrum of gastric carcinogenesis in our previous studies, suggesting that increasing TLR expression is associated with the progression of preneoplastic lesions[61,62].

The intricate balance between pro- and anti-inflammatory cytokines in chronic inflammation may mediate the outcome of *H. pylori* infection by affecting cell proliferation and apoptosis and various immune regulators take part in this regulation. An important role for miRNAs in modulating both the innate and adaptive immune responses has been suggested in various studies[63,64]. In the next section we will summarize the evidence regarding the role of miRNAs in the regulation of innate and adaptive immunity and inflammation.

### *MicroRNAs involved in the host immune response to H. pylori*

The first miRNA found to be influenced by *H. pylori* infection was miR-21. miR-21 was found to be over-expressed in both *H. pylori*-infected tissues and in GC[65,66]. NF-κB and IL-6 activate AP-1 and STAT3 respectively which are able to induce miR-21 and could explain miR-21 upregulation during *H. pylori* infection. Matsushima *et al*[11] characterized miRNA expression in *H. pylori*-infected human gastric mucosa and found 30 miRNAs significantly decreased in *H. pylori*-positive patients. Eight miRNAs enabled discrimination of *H. pylori* status with acceptable accuracy - miR-204 was the most decreased miRNA in *H. pylori*-infected followed by miR-455, miR-141, miR-203, let-7f, and miR-200a, whereas miRNA-223 was the only to be significantly increased. Gastritis scores of activity and chronic inflammation according to the updated Sydney system correlated significantly with the expression levels of diverse miRNAs. miR-223 expression was significantly increased in *H. pylori* -infected gastric mucosa and correlated positively with the degree of neutrophil infiltration (activity scores). miR-375 and miR-200c were inversely correlated with chronic inflammation and *H. pylori* density scores, respectively. On the other hand, in this study no significant correlation was found between miRNA expression and the degree of glandular atrophy and intestinal metaplasia. Expression levels of some miRNAs, including let-7 family, were significantly altered following infection with CagA(+) strains but not with CagA(-), suggesting that cagA might be involved in the regulatory processes of some miRNAs.

The differential expression of various miRNAs in *H. pylori*-positive gastric human tissues and *H. pylori* -negative controls was also examined in another study and significant correlations between 17 miRNAs, chronic gastritis and the level of the pro-inflammatory cytokines IL-1B, IL-6, IL-8 and TNF-α were found. However, that correlation disappeared in the presence of gastric atrophy and was inverse, for IL-6 and IL-8, in intestinal metaplasia[67]. Levels of miR-103, miR-375 and miR-200a were negatively correlated with IL-6, IL-8 and TNF-α, respectively. Let-7b was also found to be inversely correlated with IL-1b levels[67].

*H. pylori* CagA(+) was shown to decrease let-7 expression in the gastric epithelium and let-7 family expression levels have been shown to be negatively associated with histological scores for activity, chronic inflammation and *H. pylori* density[11,68]. Specifically, let-7b was significantly decreased in *H. pylori* -gastritis patients in a CagA-dependent manner and TLR4 3'UTR mRNA was shown to be a target for let-7b and thus let-7b can negatively regulate TLR4 expression post-transcriptionally[69]. Indeed, Teng *et al*[69] demonstrated that let-7b inhibition lead to increased TLR4 protein levels, activation of NF-κB and increased expression of COX-2 and CyclinD1, suggesting that *H. pylori* infection upregulates TLR4 expression and its downstream genes by downregulating let-7b expression. Furthermore, let-7b overexpression was associated with MyD88 downregulation and inhibition of NF-κB activity. Thus, decreased let-7b expression in *H. pylori* infection may promote inflammatory responses that contribute to the progression of gastric preneoplastic conditions. Let-7 was also found participate in cell differentiation, proliferation and apoptosis control and to be downregulated in several cancers including GC, suggesting that it acts as a tumor suppressor miRNA[70]. miR-7 was also found to be significantly decreased in both gastritis and gastric tumors in a mouse model, and in human GC the expression of miR-7 was inversely correlated with the levels of IL-1B and TNF-α, suggesting that miR-7 downregulation is related to the severity of inflammatory responses and possibly linked with gastric tumorigenesis[71]. In this regard, *in vitro* experiments showed that CagA significantly attenuates let-7 expression and enhances c-Myc, DNA methyltransferase 3B (DNMT3B) and Enhancer of Zeste homologue 2 (EZH2) expression, leading to Ras oncoprotein pathway activation with no associated inflammation[72].

miR-451 is also downregulated in both *H. pylori* infection and GC and targets macrophage migration inhibitory factor (MIF) and an inverse correlation was found between miR-451 and MIF expression in GC, suggesting that miR-451 functions as a tumor suppressor by silencing MIF expression, leading to a proliferative and anti-apoptotic phenotype[73].

Early in the acute phase of the infection *H. pylori* induces strong inflammatory responses and a transitory hypochlorhydria through repression of gastric $H^+$, $K^+$/ATP-ase which further facilitates gastric *H. pylori* colonization. NF-κB possesses binding regions in the $H^{+}/K^{+}$ promoter and have been shown to repress its transcriptional activity[74]. CagA protein and peptidoglycan-dependent mobilization of NF-κB were also implied in $H^{+}/K^{+}\alpha$ repression. miR-1289 is upregulated in *H. pylori* CagA infection and miR-1289 overexpression was found to attenuate $H^{+}/K^{+}\alpha$ expression through targeting $H^{+}/K^{+}\alpha$ 3' UTR and thus repressing mRNA translation[75].

*H. pylori* may also deregulate miRNA expression

to evade host defenses and successfully persist in the gastric niche. TLRs on the membrane of monocytes/DCs recognize and bind to PAMPs and then trigger downstream signaling pathways to initiate inflammatory responses. MiRNAs may regulate the tightly controlled TLR signaling and the downstream expression of genes and molecules in order to fine-tune the innate immune response and prevent overwhelming inflammation[76]. miR-146a and miR-155 were found to be upregulated by *H. pylori* (independently of cagPAI status) and may regulate the acute inflammatory response in myeloid cells and/or lymphocytes after pathogen recognition by TLR contributing to a negative regulation of the proinflammatory immune response[35]. TLR signaling activation and inflammatory cytokines such as TNF-α and IL-1B have also been shown to upregulate miR-146 and miR-155 during *H. pylori* infection[77,78].

miR-146 was found to be rapidly upregulated after LPS stimulation and after *H. pylori* infection in a CagA-independent and in a NF-κB-dependent manner through TLR signaling[79-81]. MiR-146a role was further explored and it was found that miR-146a targets and silences the TLR-signaling adaptor molecules interleukin-1 receptor-associated kinase (IRAK1) and TNF receptor-associated factor 6 (TRAF6) resulting in a negative-feedback loop regulation of TLR, NF-κB pathway and the downstream proinflammatory signaling in response to bacterial products, thus avoiding the overproduction of proinflammatory IL-1B and TNF-α cytokines[79-82]. As a result, the expression of key elements of the proinflammatory innate and adaptive immune responses like IL-1B, IL-8, TNF-α, growth related oncogene alpha, and macrophage inflammatory protein is negatively regulated by miR-146a overexpression in *H. pylori* infection[80], suggesting that this single miRNA plays an important role in the control of the inflammatory response to *H. pylori*, possibly restraining the tissue damage observed in patients with gastritis. Additionally, miR-146a overexpression was found to post-transcriptionally decrease prostaglandin endoperoxide synthase 2 expression[83], an enzyme responsible for the production of prostaglandin E2 which has been associated with *H. pylori* infection and infiltration of inflammatory cells to the gastric mucosa[84].

miR-155 is induced during both bacterial and viral infections in myeloid cells through activation of TLR-signaling pathways and also *via* a TLR-independent component that results partly from the activation of MyD88/Trif-independent PAMP receptors by T4SS[77,85]. *H. pylori* was found to upregulate miR-155 expression also *via* a NF-κB- and AP-1-dependent manner and significantly higher miR-155 levels were found in *H. pylori*-positive patients as compared with *H. pylori*-negative controls[86,87]. miR-155 was then found to regulate inflammation by targeting and decreasing myeloid differentiation primary response protein 88 (MyD88) protein levels which subsequently results in decreased NF-κB activation and thus in decreased release of proinflammatory cytokines like IL-8 and GRO-a, suggesting that miR-155 overexpression during *H. pylori* infection is also involved in the negative feedback regulation of the host inflammatory response through attenuating NF-κB activity[86,87]. Ceppi *et al*[88] showed that miR-155 modulates the TLR/IL-1 signaling pathway by targeting TAB2, an important signaling molecule that facilitates the activation of TRAF6 and NF-κB. Other gene transcripts of the NF-κB pathway like KK-epsilon (IKK), SMAD2 and Fas-associated Death Domains (FADD) were also described as miR-155 targets in one study[86].

Besides this role in the negative feedback regulation of the immune host response to *H. pylori*, miR-155 seems to be important in adaptive immunity contributing to the development of regulatory T cells (Treg), Th17 differentiation, induction of IL-17 and thus to the control of *H. pylori* infection.

*H. pylori* infection results in a predominantly T-cell mediated immunity rather than humoral immunity, with Th1 and Th17 responses which increase the production of IL-1B, TNF-α and IL-8[64]. Th17 cell differentiation is promoted by TNF-α and IL-6 while Th1 responses are triggered by IL-12 and INF-gamma[89]. MiR-155 deficient mice showed decreased production of IFN-γ and IL-17, impaired pathogen-specific Th1 and Th17 responses and fail to control *H. pylori* infection suggesting that miR-155 expression is required for the Th17/Th1 differentiation[90]. Interestingly, miR-155 deficient mice developed less severe infection-induced immunopathology such as severe chronic atrophic gastritis, epithelial hyperplasia and intestinal metaplasia.

Cholera toxin B subunit (CTB-UE), a multi-epitope vaccine composed by the cholera toxin B subunit and copies of B and Th cell epitopes from *H. pylori* urease A and B, showed a good therapeutic effect on *H. pylori* infection in a mice model which was closely related to the immune response mediated by miR-155 upregulation[91]. Indeed, CTB-UE vaccination significantly upregulated miR-155 expression which was associated with the induction of an immune response biased towards Th1 cells. In this experiment, miR-155 overexpression was also associated with decreased IL-17 production, maybe by inhibition of Th17 response, suggesting that CTB-UE could relieve *H. pylori* induced gastric inflammatory reaction *via* miR-155 upregulation[92].

Tang *et al*[93] found that autophagy is decreased in patients with chronic *H. pylori* infection and that miR-30b is upregulated during *H. pylori* infection. In their experiment mir-30b expression compromised autophagy and increased bacterial survival and replication through targeting BECN1 and ATG12, although there were inconsistent results concerning autophagy between *in vivo* and *in vitro* infections, suggesting that *H. pylori*-mediated autophagic processes may be complex and that many factors *in vivo* may be involved in autophagy inhibition[93].

Together these data suggest that *H. pylori* deregulates host miRNA expression to manipulate the host inflammatory immune response, which may promote bacterial survival and persistence within the gastric

mucosa. Besides, as these miRNAs have established roles in carcinogenesis as well as innate immunity, they could serve as an important link between *H. pylori*-induced inflammation and carcinogenesis. The previous findings suggest that microRNAs play an important role in the fine-tuning of both innate and adaptive immune responses and that miRNA deregulation may contribute to both *H. pylori* persistence and to *H. pylori* -mediated pathology.

## MICRORNAS AND DNA METHYLATION - THE BRIDGE BETWEEN INFLAMMATION AND CANCER?

Gastric carcinogenesis involves gradual accumulation of various genetic and epigenetic alterations leading to oncogene activation and loss of tumor suppressor gene function. Genetic alterations, such as p53, KRAS, PIK3CA and MLL mutations, as well as PIK3CA, C-MET, ERBB4 and CD44 amplifications are frequently found in GC, suggesting that may be key tumorigenic events[94].

In cancers arising in inflammatory environments, mutagenesis and epigenetic deregulation are the main mechanisms driving epithelial cells in the direction of cancer. Increased mutation burden of the epithelial genome occurs through both the increased occurrence of mutations due to direct damage of DNA (*e.g.,* ROS, RNS) and deficient repair of mutations prior to DNA replication (reduced function of MGM and MMR genes). *H. pylori* infection leads to chronic inflammation, accumulation of ROS and oxidative DNA damage in the gastric mucosa and was also associated with methylation and silencing of a number of genes through aberrant DNA methylation in the gastric mucosa, which may contribute to gastric carcinogenesis through the silencing of tumor suppressor genes[95-97]. Indeed, several inflammatory mediators, such as TNF-$\alpha$, IL-1B and ROS were implicated in aberrant DNA methylation during gastric carcinogenesis and a growing body of evidence suggests that, in addition to genetic alterations, epigenetic changes are also involved in the initiation and progression of GC[24,98,99]. Aberrant methylation of promoter CpG islands was also demonstrated in non-neoplastic tissues with *H. pylori* gastritis and CpG methylation has been shown to be partially reversible after *H. pylori* eradication further supporting the role of *H. pylori* and inflammatory mediators in epigenetic regulation[23,27,100,101].

Therefore, DNA methylation seems to be an important epigenetic process that occurs during malignant transformation and the rate of gene methylation is considered to be correlated with an increased risk of GC[102,103]. DNA methylation is regulated by a family of DNMT and includes global hypermethylation and hypermethylation of CpG islands confined to the regulatory regions of human genes. Methylation of CpG islands in promoter regions causes silencing of the downstream gene, whereas methylation in the coding region is usually associated with increased gene transcription. Thus, cancers display regional hypermethylation of promoter regions and global hypomethylation. The extensive epigenetic alteration in the background mucosa that gives rise to dysplasia and cancers represents an epigenetic field defect in inflammation and infection associated cancers. CpG methylation occurs early in gastric carcinogenesis, affecting genes such as MLH1, p14, p15, p16, CDKN2A, CDH1 - E-cadherin, LOX, APC, RUNX3, thrombospondin-1, tissue inhibitor of metalloproteinase 3, COX-2, and MGMT[26,96,98,104,105].

Several reports describe that binding of transcription factors to the promoter regions of specific miRNA genes activate the transcription of pre-miRNAs, thus increasing the expression of mature miRNAs. As an example, increased expression of c-Myc leads to the activation of miR-17-92 cluster by binding to its regulatory region[106]. On the other hand, intronic miRNAs are coordinately expressed with their host gene mRNA, while some miRNAs are located at cancer-associated genomic regions frequently involved in chromosomal abnormalities that may affect the differential expression of miRNAs. DNA methylation and histone modification, epigenetic changes that play critical roles in chromatin remodeling and regulation of gene expression may also influence the expression of some miRNAs genes by epigenetic alterations in their promoter regions. *H. pylori* infection was found to lead to ubiquitination and reduction of Drosha protein levels in GC cells and treatment of GC cells with a proteasome inhibitor (MG132) was associated with preservation of Drosha protein levels despite *H. pylori* infection, suggesting that *H. pylori* infection enhances the ubiquitin-proteasome pathway and may lead to downregulation of miRNAs by influencing Drosha expression post-transcriptionally[107].

Several tumor-suppressor miRNAs, including miR-124a, miR-137, miR-193a and miR-127 were reported to be silenced by aberrant DNA methylation of their promoter CpG islands in cancers[96]. *H. pylori* long-term colonization may induce epigenetic modification of gastric mucosal genes, including on the promoter regions of tumor suppressor miRNAs, which cannot be completely reversed only by bacterial eradication and thus miRNA silencing by aberrant DNA methylation is probably involved in gastric carcinogenesis[108]. Indeed, several miRNAs such as miR-210, miR-375 and miR-124-a1/a2/a3 were shown to have reduced expression in the gastric epithelium of chronically *H. pylori* -infected gastric mucosa due to DNA methylation[96,109]. Epigenetic silencing of let-7 with subsequent Ras pathway activation was also demonstrated after CagA transfection through enhancement of c-myc and DNMT3B and attenuation of miR-6a and miR-101 expression[110].

Higher levels of miRNA gene methylation were also found in noncancerous gastric mucosa of GC patients as compared with *H. pylori*-negative mucosa, suggesting that miRNA silencing is involved in the formation of a field defect for GC[96]. miR-124a (downregulated in *H. pylori*-infection) was found to down-regulate CDK6, an

**Table 1 MicroRNAs reduced by DNA methylation in *Helicobacter pylori* infection**

| MicroRNA | Targets | Consequences/associations |
|---|---|---|
| miR-210 | STMN1 | Aberrant proliferation |
| | DIMT1 | Increased *H. pylori* content, atrophy and neutrophil and mononuclear infiltration |
| miR-375 | MDM2 | p53 inhibition |
| | JAK1/STAT3 | JAK1/STAT3 activation and neoplastic transformation |
| | 14-3-3 | Bcl binding and cell survival |
| | PDK1 | PI3K/Akt pathway |
| miR-124 | CDK6 | Cell cycle progression |
| Let-7a | c-myc and DNMT3B | Ras pathway activation |
| miR-34 | Bcl-2 | Apoptosis inhibition |
| miR-10b | MAPs | Microtubule-associated protein oncogene |
| miR-185 | DNMT1 and EZH2 | Proliferation and EMT |
| | | LNM and poorer prognosis |
| miR-490-3p | Cyclin B1 | EMT; proliferation; colony formation; migration; invasion |
| | SMARCD1 | Metastasis and poorer survival |
| | | Decreased through the spectrum of gastric carcinogenesis |

STMN1: Stathmin/oncoprotein 18; DIMT1: DIM1 dimethyladenosine transferase 1 homolog (*S. cerevesiae*); MDM2: Mouse double minute 2 homolog/E3 ubiquitin-protein ligase Mdm2; JAK1: Janus kinase 1; STAT3: Signal transducer and activator of transcription 3; PDK1: Phosphoinositide-dependent kinase-1; CDK6: Cyclin-dependent kinase 6; DNMT3B: DNA (cytosine-5-)-methyltransferase 3 beta; Bcl-2: B-cell lymphoma 2; MAPs: Microtubule-associated proteins; DNMT1: DNA (cytosine-5)-methyltransferase 1; EZH2: Enhancer of zeste homolog 2; EMT: Epithelial-mesenchymal transition; LNM: Lymph node metastasis; SMARCD1: SWI/SNF-related matrix-associated actin-dependent regulator of chromatin subfamily D member 1.

oncogene involved in cell cycle progression, suggesting that miR-124a is involved in gastric carcinogenesis[111]. miR-34b and miR-34c (tumor suppressor miRNAs) and miR-10b (a miRNA that targets the microtubule-associated protein oncogene) were also found to be epigenetically silenced in GC due to hypermethylation of the neighboring CpG islands[112,113]. In the latter study, treatment with demethylating agents decreased miR-10b methylation and restored its expression, suggesting that modulation of miR-10b may represent a therapeutic approach for treating GC[113].

CpG island hypermethylation was also associated with decreased miR-210 in *H. pylori* -positive gastric mucosa, and miR-210 downregulation was associated with STMN1 upregulation, possibly leading to aberrant proliferation of gastric epithelial cells during chronic *H. pylori* infection[109]. In this study, miR-210 decreased in parallel with increased grades of neutrophil and mononuclear cell infiltration, atrophy and *H. pylori* content suggesting that miR-210 methylation is associated with disease progression of *H. pylori*-mediated gastric lesions. Besides, decreased miR-210 levels were lower in tumor tissues than in normal mucosa and 10 oncogenes were found to be strongly suppressed by miR-210, namely STMN1 (oncoprotein 18) and demethyladenosine transferase-1 (DIMT1). STMN1 and DIMT1 upregulation was also demonstrated in *H. pylori*-positive human stomachs.

GKN1 is thought to function as an hypomethylating agent and to exert its antiproliferative effects through downregulation of DNMT1 and EZH2, a histone methyltransferase involved in proliferation and epithelial-mesenchymal transition (EMT) promotion (by interacting with Snail and suppressing E-cadherin expression)[50,52,114]. Indeed, inactivation of DNMT1 and EZH2 in GC cells suppressed cell growth through G0/G1 and G2/M cell-cycle arrest, suggesting that GKN1 acts as a tumor suppressor through the regulation of epigenetic regulatory components and EMT-related proteins. Interestingly, expression of DNMT1 and c-myc was also positively associated with *H. pylori* CagA protein and methylation status, strongly supporting the view that GKN1 may play an important role in epigenetic regulation[115]. GKN1 was also found to upregulate miR-185 and was positively correlated with miR-185 expression and inversely correlated with DNMT1 and EZH2 expression. DNMT1 and EZH2 were found as targets of miR-185, suggesting that miR-185 inhibits cell growth by inducing cell-cycle arrest through the inactivation of DNMT1 and EZH2[114]. Accordingly, miR-185 downregulation was demonstrated in GC and lower miR-185 levels were associated with lymph node metastasis (LNM) and poorer prognosis[116].

The above results highlight the role of DNA methylation as a mechanism for epigenetic silencing of miRNA genes during chronic inflammation. Table 1 summarizes the microRNAs that were found to be reduced by DNA methylation in *H. pylori* infection and its target genes. Since aberrant DNA methylation has also been reported in other chronic inflammatory diseases that are causative for cancers, it seems that similar inflammation-induced DNA methylation leading to miRNA gene silencing can be an underlying tumorigenic mechanism associated with GC.

## GASTRIC PRENEOPLASTIC CONDITIONS AND GASTRIC CARCINOGENESIS - THE ROLE OF MICRORNAS

From the early stages of *H. pylori* gastritis, the infection and associated inflammation lead to epithelial cell

mutations, epigenetic, microRNA and gene expression changes, genomic instability, altered cellular signaling, and imbalance of proliferation and apoptosis of gastric epithelial cells, driving the progression from gastritis to pre-neoplastic and neoplastic lesions[26]. Shiotani *et al*[117] found a higher expression of oncogenic miRNAs (miR-17/92, miR-106b-93-25, miR-21, miR-194 and miR-196) in metaplastic intestinal mucosa compared with non-intestinal metaplastic mucosa and that *H. pylori* eradication improves miRNA deregulation in the gastric mucosa but not in metaplastic glands, suggesting that *H. pylori* long-term colonization induces epigenetic modifications not completely reversible by *H. pylori* eradication alone. Wang *et al*[118] also analyzed miRNA expression patterns in *H. pylori*-related gastritis and gastric intestinal metaplasia and found 20 differentially expressed miRNAs (DEMs), including 12 up-regulated and 8 down-regulated, and the top 5 DEMs were miR-486p, miR-645, miR-624, miR-504, and hsa-miR-106b. Lower expression of miR-106b and miR-204 was also found in *H. pylori*-positive gastric mucosa, suggesting that the downregulation of these miRNAs is associated with *H. pylori*-related chronic gastritis[11].

miR-106b was implicated in TGF-β and MAPK signaling pathways and miR-204 was related with calcium and neurotrophic signaling pathways and axon guidance[118]. In another study miR-204 was linked to the down-regulation of sirtuin 1 (SIRT1) and to the reversion of SIRT1-induced EMT and invasion in GC cells[119]. miR-106b was associated with suppression of TGF-β-induced cell cycle arrest and promotion of GC development in a previous study[120]. The frequency and extent of miR-106a (a miRNA overexpressed in GC) expression gradually increased during the transition from atypical hyperplasia to advanced carcinoma and had already positive signals in early precancerous lesions but negative signals in normal gastric mucosal epithelial cells, suggesting that the early changes of miR-106a potentially can become biomarkers for the early detection of GC[121]. miR-106a is upregulated in GC and targets retinoblastoma protein (RB1), a tumor suppressor protein that inhibits transcription factors of the E2F family[65]. miR-106a, upregulated in GC, was correlated with lymphatic and distant metastasis[65,122].

miR-320, a tumor suppressor miRNA downregulated in various solid tumors, targets Mcl-1 anti-apoptotic factor expression and miR-320 downregulation by *H. pylori* was demonstrated in a CagA-dependent manner. Furthermore, Mcl-1 expression levels were found to increase in parallel with the severity of neoplastic lesions (nonatrophic gastritis, intestinal metaplasia, or adenocarcinoma), Mcl-1 overexpression was associated with chemotherapeutic resistance and relapse of tumors and Mcl-1 depletion was found to promote apoptosis in cancer cells[123]. These findings suggest that *H. pylori* CagA suppresses miR-320 and upregulates Mcl-1 leading to inhibition of apoptosis and increasing the risk for GC. miR-101 and miR-515-5p are also downregulated in *H. pylori*-positive tissues and in GC and their downregulation was associated with an anti-apoptotic phenotype by targeting Mcl-1, leading to Mcl-1 overexpression[11,108,124]. Recently, Zhou *et al*[124] found that miR-101 also strongly reduces the expression of SOCS2 oncogene in GC cells and that miR-101 levels were inversely correlated with SOCS2 expression, suggesting that miR-101 acts as a growth-suppressive miRNA in *H. pylori*-related GC. CagA also attenuated miR-101 expression, which in turn further attenuated let-7 expression by histone and DNA methylation[72].

Another miRNA implicated in the progression of gastric preneoplastic conditions is miR-490-3p whose expression is progressively downregulated in gastritis, intestinal metaplasia and adenocarcinoma during *H. pylori* infection[125]. Hypermethylation of the promoter region of miR-490-3p was demonstrated in human GC tissues as well as miR-490-3p growth and metastasis suppressive effects (inducing G2/M and intra-S phase arrest and downregulating cyclin B1) through directly targeting SMARCD1 (a SWI/SNF chromatin remodelling complex subunit). Indeed, SMARCD1 was found to be markedly upregulated in GC and its higher expression was associated with poorer patients' survival independent of TNM staging. These findings suggest that *H. pylori* silences miR-490-3p expression by hypermethylation, which subsequently activates SMARCD1 conferring malignant phenotypes, mechanistically linking *H. pylori*, chromatin remodeling and gastric carcinogenesis[125]. It was also shown that miR-490-3p upregulates epithelial markers (*i.e.,* syndecan-1 and zo-1), downregulates mesenchymal markers (*i.e.,* fibronectin and vimentin) and inhibits colony formation, growth, cell migration and invasiveness, supporting the role of this miRNA in inhibiting EMT.

Forkhead box M1 (FoxM1), a key positive cell-cycle regulator is also implied in the pathogenesis of several types of cancers and was found to be increasingly overexpressed through the spectrum of gastric carcinogenesis. Feng *et al*[126] showed that mRNA expression of FoxM1 gradually increased from gastritis to cancer as compared with noncancerous tissues (6.7% of the cells in noncancerous gastric tissues, 21.7% in gastritis, 36.4% in AG/IM and 89.2% in GC). *H. pylori* CagA(+) infection was shown to reduce $P27^{Kip1}$ expression (a tumor suppressor which negative regulates cell-cycle) and was associated with FoxM1 upregulation and increased cell proliferation, alterations partially reversed by knockdown of FoxM1, suggesting that FoxM1 mediates the inhibition of $P27^{Kip1}$ induced by *H. pylori*. miR-370 directly targets FoxM1 gene reducing FoxM1 activity. Accordingly, expression of miR-370 gradually decreased from superficial gastritis, atrophic gastritis/IM to GC samples. Together these findings suggest that the miR-370-FoxM1 pathway is involved in the progression of *H. pylori*-induced gastritis to GC by affecting $P27^{Kip1}$ expression. The FoxM1 overexpression may reduce $P27^{Kip1}$ and thus increase cell proliferation and promotion of gastric carcinogenesis. Furthermore, transcription of

$P27^{Kip1}$ was inhibited by CagA *via* PI3K/Akt pathway in another study[127]. However, Lo *et al*[128] found that miR-370 was overexpressed in GC tissues and in plasma of GC patients and higher miR-370 levels were associated with LNM and higher clinical stage. TGF-β receptor Ⅱ was identified as a target for miR-370 in this study and an inverse correlation was found between mir-370 and TFG-B-RII in GC tissues.

miR-584 and miR-1290 upregulation was also demonstrated after CagA transfection, with subsequent downregulation of Foxa1 expression and promotion of EMT *in vitro*[110]. It was also shown that mice over-expressing miR-584 and miR-1290 developed gastric intestinal metaplasia after a long follow-up, suggesting a role for these miRNAs in the progression of preneoplastic conditions induced by *H. pylori*.

GKN1, a protein involved in mucosal defense and in the regulation of inflammatory pathways, was found to be decreased in *H. pylori*-infected mucosa and a progressive decrease from chronic gastritis to atrophy and intestinal metaplasia was demonstrated[49,50]. In non-neoplastic mucosal samples of patients with sporadic GC, GKN1 levels were able to predict gastric mucosal atrophy and intestinal metaplasia risk with an AUC value of 0.865 and 0.973, respectively, implicating GKN1 as an important player in gastric mucosal inflammation and a marker of the progression of gastric carcinogenesis[115]. GKN1 was found to upregulate miR-185 which targets DNMT1 and EZH2 expression and thus reduces DNA methylation.

Finally, the existence of various metaplastic processes has been recognized, including goblet cell intestinal metaplasia and spasmolytic-polypeptide-expressing metaplasia (SPEM)[129,130]. CD44 is a major adhesion molecule and receptor for hyaluronic acid that can coordinate normal and metaplastic gastric epithelial progenitor cell proliferation under conditions of parietal cell loss and is a putative gastric stem cell marker[131]. CD44v, a variant of CD44, was shown to interact with xCT (a glutamate-cystine transporter) and to contribute to ROS defense in cancer cells[132]. Inflammatory response to *H. pylori* infection leads to increased expression of CD44 and CD44v9 in the gastric mucosa; CD44v9 was found to be overexpressed in SPEM in mice models and CD44 ablation significantly attenuated SPEM development by suppressing the proliferation of metaplastic cells at the base of their gastric glands[133]. Ishimoto *et al*[134] recently showed that CD44v9 expression in gastric mucosal cells is correlated with *H. pylori* infection and that there is an association between CD44v9 expression in the gastric mucosa adjacent to tumor and in tumor cells, suggesting that the development of GC CD44v9+ is associated with *de novo* expression in the mucosa adjacent to the tumor. It was shown that *H. pylori* infection is associated with increasing number of myeloperoxidase inflammatory cells in the gastric mucosa leading to ROS accumulation which can induce miR-328-mediated CD44 overexpression, suggesting a role for miR-328 in *de novo* expression of CD44[134]. The authors concluded that CD44v expression was regulated by miR-328 suppression and it is possible that CD44v promotes the survival and proliferation of metaplastic cells which give rise to SPEM.

*In vitro* studies have also shown that miR-296-5p attenuates CDX1 anti-growth effects partly through ERK1/2 activation[135]. Indeed, GC tissues presented loss of CDX1 when compared with adjacent IM tissues and miR-296-5p was inversely correlated with CDX1, suggesting that the miR-296-5p-CDX1-ERK1/2 may be important to the progression of IM to GC and may provide therapeutic targets for the treatment of GC[135].

## *H. PYLORI* RELATED MICRORNAS AND EMT, CELL-CYCLE AND APOPTOSIS

The deregulation of cell cycle progression and increased cellular proliferation are hallmarks of malignancies. Cell cycle progression requires coordinated expression of cyclins, which results in sequential activation of cyclin-dependent kinases (CDKs). miRNA deregulation can promote cell cycle progression by upregulating cyclin expression and/or down-regulating CDK inhibitors expression (p15, 16, 18, 19, 21, 27, 28, 57)[14]. *H. pylori* may possibly exert its carcinogenic effects partly by modulating cyclins, CDKs and CDK inhibitors and deregulation of host miRNAs may affect the regulation of cell cycle and increase the propensity for gastric transformation[136].

Cellular transformation is also characterized by increased cellular proliferation and evasion of apoptosis. Apoptosis can be dependent on either the intrinsic or extrinsic pathways. Extrinsic apoptosis pathway is initiated through the activation of pro-apoptotic death receptors located in the cell surface by ligands like TNF. Ligand binding induces receptor clustering and the recruitment of the adaptor protein FADD, leading to induction of caspases and ultimately cell-death. The intrinsic apoptosis pathway is initiated within cells and hinges on the balance between pro-apoptotic (*e.g.,* Bax, Bak, Bim, BNIP3L, and Bid) and anti-apoptotic (*e.g.,* Bcl-2, Bcl-xL, and Mcl-1) proteins. MicroRNAs seem to play a role in apoptosis regulation by altering the expression of pro-apoptotic and anti-apoptotic factors.

A large number of microRNAs have been associated with the development and progression of GC, some being indicated as potential biomarkers for early diagnosis in patients at risk and others implicated as prognostic factors. In this review we summarize the evidence about microRNAs associated with both *H. pylori* and GC cancer, as recent reviews focused on the topic of microRNAs and GC in general.

The pro-inflammatory miR-21 was found to be overexpressed in *H. pylori* infection and was associated with decreased apoptosis, increased proliferation and invasion, suggesting that miR-21 may be important in the development of GC[66]. Indeed, miR-21 was found to negatively regulate RECK, a tumor suppressor gene

and suppressor of metastasis and angiogenesis that modulates matrix metalloproteases (MMPs) and is decreased in GC samples. Other tumor suppressors have been identified as miR-21 targets, such as PTEN (phosphatase and tensin homolog - a negative regulator of the Pi3K/Akt signaling pathway)[137,138] and actin-binding protein[139]. miR-222 is also upregulated in *H. pylori*-infected gastric mucosa and GC, and ectopic expression of miR-222 was found to promote cell proliferation and colony formation[140]. RECK was identified as a target for miR-222 and an inverse correlation between miR-222 levels and RECK was found suggesting that *H. pylori* may function as an initiator in carcinogenesis by upregulating miR-222, leading to RECK inhibition and thus promoting proliferation[140].

MiR-146a is involved in the regulation of innate immunity and inflammatory response to *H. pylori*, acting as a controller of the inflammatory response through the modulation of TLRs and cytokine signaling pathways and by reducing NF-κB activity through negative regulation of IRAK1 and TRAF6[79,80]. It is also well established that TLR2, 4, 5 and 9 are involved in *H. pylori* recognition[62,141] and that NF-κB is a key molecule in inflammation-cancer link[142]. miR-146a upregulation was found in *H. pylori*-positive gastric mucosa and in GC tissues as compared with matched non-tumor adjacent tissues[143]. In this study miR-146a was found to inhibit apoptosis by decreasing levels of SMAD4 (SMAD family member 4 - identified as a direct target of miR-146a), suggesting that miR-146a plays a role in the development of GC. Another study also found miR-146a upregulation in a GC mice model but identified caspase recruitment domain-containing protein 10 (CARD10) and COP9 signalosome complex subunit 8 (COPS8) as miR-146a targets. CARD10 and COPS8 were found to be involved in NF-κB activation, suggesting that miR-146a inhibits NF-κB activation thus reducing the expression of NF-κB -regulated tumor-promoting cytokines and growth factors and suggesting that in fact miR-146a have tumor suppressing properties[144]. Further supporting that miR-146a acts as a tumor suppressor, Hou *et al*[145] found decreased expression of miR-146a in 84% (36/43) of GC tissue samples and lower miR-146a expression was significantly associated with increased tumor size, poor differentiation and poorer overall survival. In fact, in these study miR-146a inhibited cell proliferation and promoted apoptosis in GC cell lines[145]. Accordingly, miR-146a was associated with suppression of invasion and metastasis in GC cells and in a mice model through targeting L1 cell adhesion molecule[146]. Lower expression levels of miR-146a were also found in GC tissues as compared with corresponding noncancerous tissue, and lower miR-146a levels were significantly associated with LNM, venous invasion and poorer overall survival[147]. Inhibition of migration and invasion through downregulation of EGFR and IRAK1 expression were attributed to miR-146 in the previous study. Pro-apoptotic effects of miR-146a through COX-2 inhibition were also shown in human GC cells and miR-146a density was positively correlated with apoptosis rates in *H. pylori*-positive GC tissues and negatively correlated with LNM among *H. pylori*-positive GC patients[148]. The previous findings were confirmed in a recent miRNA PCR array where it was found that miR-146a-5p is downregulated in GC patients, and low-expression of mir-124-3p, mir-146a-5p, mir-155-5p and mir-335-5p was significantly associated with LNM, lymphatic invasion, venous invasion and poor differentiation[149]. In a different study miR-155 was found to target SMAD2 and FADD, reducing their expression and leading to the downregulation of caspases and inhibition of apoptosis, thus suggesting an oncogenic potential for this microRNA[86].

In addition to microbial and environmental factors, there are a number of host factors that may contribute to gastric carcinogenesis namely single-nucleotide polymorphisms (SNPs) in inflammation-related miRNA, since only a small proportion of infected patients ultimately develop GC. Some studies have demonstrated that rs2910164 SNPs in miR-146a precursor can reduce mature miR-146a production which may modify the inflammatory process and miR-146a SNPs are the most extensively studied polymorphisms regarding increased susceptibility to GC[150,151]. However, some inconsistencies were found in the literature. Indeed, Okubo *et al*[152] found that the rs2910164 CC genotype is associated with significantly increased susceptibility to GC (OR = 1.30; 95%CI: 1.02-1.66, $P = 0.03$) and Song *et al*[153] reported that miR-146a rs2910164 CC carriers had a significantly increased risk of IM (OR = 1.42, 95%CI: 1.03-1.97) and dysplasia (OR = 1.54, 95%CI: 1.05-2.25) as compared with GG carriers and when stratified the analysis by *H. pylori* infection status found that rs2910164 C allele was associated with an increased risk of IM and dysplasia only among individuals with *H. pylori* (CC *vs* GG: OR = 1.53, 95%CI: 1.12-2.08, $P < 0.05$), suggesting that mir-146a rs2910164 polymorphism might promote the occurrence of IM and dysplasia jointly with *H. pylori* infection.

However, Zeng *et al*[154] found that subjects with GG and GC genotypes had a 58% increased risk of GC (adjusted OR = 1.58; 95%CI: 1.11-2.20, $P < 0.01$) and another Japanese study revealed the combined effect of miR-146a rs2910164 G/G and TLR4 +3725 C allele on the increased risk of severe gastric atrophy among the *H. pylori*-infected Japanese subjects[155]. Besides, in an European population various gene polymorphisms including miR-146a (G>C rs2910164) were not associated with the presence of high risk atrophic gastritis or GC[156]. Nevertheless, three recently published meta-analysis concluded that miR-146a rs2910164 GG or GC polymorphisms are associated with increased susceptibility to GC, especially in Asian population[157-159].

*H. pylori* CagA(+) was shown to decrease let-7 expression in the gastric epithelium and let-7 family expression levels have been shown to be negatively

associated with histological scores for activity, chronic inflammation and *H. pylori* density[11,68]. The let-7 family acts as tumor suppressors and its target genes are oncogenes such as Ras, c-myc and HMGA2 (high mobility group A2)[160,161]. Indeed, miR-7 is downregulated in GC and it has been shown that pre-miR-7 transfection into GC cells suppresses cell proliferation and colony formation, while let-7b knockdown was associated with growth promotion, migration and invasion[71,162]. Lower levels of let-7b were also found in *H. pylori* -infected and in GC tissues and collagen triple helix repeat containing 1 was found to be its direct target[162]. Let-7d downregulation was also associated with oncogene overexpression contributing to carcinogenesis.

*H. pylori* induces an invasive phenotype in epithelial cells that resembles EMT through the disruption of cell-cell junction and loss of apical-basolateral polarity mediated by the interaction of CagA with several junction proteins like ZO-1, JAM and E-cadherin[18,163]. *H. pylori* CagA is also associated with B-catenin release from E-cadherin and subsequent activation of Wnt/B-catenin signaling pathway, and deregulation of B-catenin seems to play a crucial role in GI cancers[164]. *H. pylori* CagA transfect into gastric epithelial cells results in miR-584 and miR-1290 upregulation, *via* NF-κB and Erk1/2 respectively[110]. miR-1290 was also implied in miR-584 activation. Foxa1 and Smad2 were identified as targets of miR-584 and miR-1290 and knockdown of Foxa1 was shown to promote EMT in GC cell lines. Overexpression of miR-584 and/or miR-1290 was also associated with decreased E-cadherin levels, suggesting that Foxa1 downregulation by miR-584 and miR-1290 promotes EMT. Overexpression of miR-584 and miR-1290 was also associated with the development of intestinal metaplasia through interference with cell differentiation and remodeling of gastric mucosa[110].

The miR-200 family (miR-200a,b,c, miR-141, miR-429) was also associated with epithelial differentiation and suppression of EMT in several types of cancers by inhibition of ZEB 1 and 2 (Zinc-finger E-box Binding homeobox 1 and 2 - transcriptional repressors of E-cadherin)[165,166]. In GC low miR-200b expression was associated with tumor size, LNM and lymphatic invasion and a strong correlation was found between miR-200b, ZEB2 and E-cadherin mRNA, *i.e.,* in cells overexpressing miR-200b ZEB2 mRNA levels were lower and E-cadherin expression levels were increased, which was associated with significantly reduced cellular proliferation, and inhibition of cellular migration and invasion, suggesting that miR-200b is a tumor suppressor miRNA[167]. ZEB2 also represses cyclin D1 transcription, a cyclin that promotes G1/S transition and is induced *via* AP-I in gastric epithelial cells during *H. pylori* infection and under CagA dependence[168]. The above findings suggest a role for miR-200 family and ZEB repression in the EMT-like phenotype in *H. pylori*-infected cells. miR-141, decreased in *H. pylori* -infected gastric tissue[11] targets fibroblast growth factor receptor (FGFR), and overexpression of miR-141 leads to decreased FGFR2 expression and inhibition of proliferation[169].

MiR-375 repression and B-catenin-activating mutation also was described in hepatocellular adenoma and carcinoma[170]. Ye *at al*[171] demonstrated that *H. pylori* LPS deregulates miR-375 and miR-106b expression in gastric epithelial cells and that downregulation of miR-375 was associated with increased expression of MDM2 (E3 ubiquitin-protein ligase Mdm2), an important negative regulator of the p53 tumor suppressor. *H. pylori* LPS also enhanced the tyrosine phosphorylation of JAK1, JAK2 and STAT3, and JAK1 and STAT3 were found as target genes of miR-106b, suggesting that *H. pylori* LPS may enhance JAK/STAT3 pathway *via* inhibition of miR-375 and miR-106b. These findings were confirmed in a recent study where it was found that *H. pylori* infection downregulates miR-375, which targets JAK2/STAT3. In these study, gain-of-function and loss-of-function experiments have shown that decreased miR-375 expression mimics the oncogenic effects of the JAK2/STAT3 pathway (which promotes neoplastic transformation by affecting the expression of Bcl-2 and TWIST1) and that treatment with siRNAs targeting JAK2 prevents proliferation and migration even in response to *H. pylori* infection[172]. In accordance with these findings, another study showed miR-375 downregulation in GC and miR-375 was found to reduce cell viability by targeting 14-3-3 zeta, an anti-apoptotic protein that promotes cell survival by binding to Bad, a pro-apoptotic protein[173]. PDK1 (3-phosphoinositide dependent protein kinase), a kinase that directly phosphorylates Akt and thereby regulates the PI3K/Akt signaling pathway was also identified as a direct target of miR-375.

TGF-β is involved in mucosal immunity and in the control of the physiological turnover of epithelial cells, and the downstream effectors of TFGB-dependent cell cycle arrest and apoptosis are the CDK inhibitor $p21^{CIP1/WAF1}$ and the pro-apoptotic factor Bim, respectively. miR-25, miR-93, miR-106b, and miR-130 inhibit apoptosis by preventing the expression of the pro-apoptotic protein, Bim[174]. The miR-106b-25 cluster (miR-106b, miR-93 and miR-25) was demonstrated to be abnormally upregulated in GC and it was associated with decreased response of gastric cells to TGF-β by interfering with the expression of p21 and Bim, affecting both cell cycle and apoptosis[120,175]. Indeed, miR-106b-25 cluster was found to silence $p21^{CIP1/WAF1}$, E2F1 and the proapoptotic factor Bim leading to a decreased response of gastric cells to the TGFb tumor-suppressor activity and to impairment of p21 tumor suppressor activities[120,174]. MiR-25 was also found to target and negatively influence Bim and the CDK inhibitors p27 and p57[176].

miR-130b and miR-301a are both upregulated in GC and may contribute to tumorigenesis and invasion by downregulation of Runx3 expression[177]. Overexpression of miR-130b in GC was demonstrated and it is believed to contribute to suppression of Bim in TGF-β media-

ted apoptosis by targeting RUNX3, a known tumor suppressor silenced by promoter hypermethylation in GC[178,179]. mir-301a was also reported to be upregulated in GC, and directly downregulates Runx3 expression[180]. Together these findings suggest that overexpression of these oncogenic miRNAs results in activation of CDK2 (promoting G1/S phase progression) and in impairment of the TGF-β mediated tumor suppressor pathways that may be critical steps in the development of gastric tumors.

miR-524-5p was also found to suppress cancer cell proliferation and invasion by downregulating Jagged-1 and Hes-1, two key components of the Notch signaling pathway[181] and it was suggested that miR-524-5p may also be involved in GC by regulating cell cycle and TGF-β signaling pathway[118]. miR-449, a tumor suppressor miRNA both downregulated in *H. pylori*-infected gastric mucosa and in GC, targets cyclin E2 and geminin (promoters of G1/S and M/G1 cell cycle progression), suggesting that miR-449 downregulation may be important in cell cycle progression and proliferation[182]. miR-449 was also found to target Met, geminin, and SIRT1, proto-oncogenes that may be related with proliferation, angiogenesis, invasion and metastasis[182].

miR-203 expression was found to be lower in *H. pylori*-positive tissues (both tumoral and non-tumoral) and in GC cell lines and miR-203 was found to directly target CASK (calcium/calmodulin-dependent serine protein kinase, a cytoskeletal protein overexpressed in various cancers)[183]. Indeed, CASK expression was found to be significantly higher in *H. pylori*-positive cells and was inversely correlated with miR-203 levels. Furthermore, miR-203 transfection could inhibit cell growth, colony formation and cell invasion, suggesting its potential tumor suppressor role in *H. pylori*-induced GC[183].

mir-29a is also significantly downregulated in GC and it targets p42.3 which regulates G2/M progression and promotes cell cycle progression and proliferation[184,185]. miR-29c is a tumor-suppressor miRNA significantly downregulated in GC tissues compared with non-tumoral gastric mucosa[186]. Treatment with celecoxib, a selective COX-2 inhibitor, significantly activates miR-29c expression suppressing anti-apoptotic Mcl-1[108,187]. This pathway could be one of the mechanisms of the chemopreventive effects of selective COX-2 inhibitors and suggesting that selective iCOX-2 may be a clinical option for the treatment of GC *via* restoration of mir-29c.

miR-181b is increased early after *H. pylori* infection, returns to normal levels early after *H. pylori* treatment (72h) and is upregulated in GC[188]. Timp3 (tissue inhibitor of MMP-3 and a pro-apoptotic factor), was identified as a direct target of miR-181 and miR-181b overexpression was associated with inhibition of apoptosis, cell proliferation, invasion and migration in GC cells. Timp3 downregulation in esophageal and GC has been linked with epigenetic changes namely gene methylation[189,190]. Together these data suggest that *H. pylori* infection can promote gastric carcinogenesis through miR-181b upregulation which leads to decreasing Timp3 levels, promoting proliferation, migration and invasion.

miR-223 is also overexpressed in GC and was suggested as an useful serum biomarker for its detection. Significantly higher levels of miR-223 were found in *H. pylori* -infected GC patients and in healthy controls with *H. pylori* infection (*vs* those without)[191]. In another study, Li *et al*[192] found that miR-223 was associated with migration and invasion through downregulation of erythrocyte membrane protein band 4.1-like3 (EPB-41L3). Besides, miR-223 upregulation was associated with higher proliferation, colony formation, migration and invasion of *H. pylori*-positive GC cells[193]. mir-27a has been identified as an oncogenic miRNA in GC by targeting the tumor suppressor prohibitin and FOXO1 (forkhead box protein O1), which may protect cells against oxidative stress[194-196].

Bcl-2 superfamily are a group of anti-apoptotic proteins whose expression can be regulated by tumor suppressor miRNAs (*e.g.,* miR-15b, miR-16, miR-34, miR-181b, miR-181c, and miR-497). These miRNA clusters are downregulated in GC cells leading to increased expression of Bcl-2 and inhibition of apoptosis[197]. In *H. pylori*-infected gastric mucosa miR-200bc/429 cluster is downregulated increasing expression of Bcl-2 and XIAP and thus inhibiting apoptosis[194,195,198].

Another tumor suppressor miRNA, mir-218 is significantly decreased in both *H. pylori*-infected mucosa and in GC tissues[199]. MiR-218 was shown to induce apoptosis in GC cells through direct targeting of epidermal growth factor receptor-co-amplified and overexpressed protein (ECOP) leading to inhibition of NF-κB transcriptional activation and inhibition of COX-2 transcription, leading to an apoptotic response[199]. miR-218 downregulation in GC cells was also correlated with increased metastasis and invasion through SLIT/ROBO1 signaling pathway upregulation[65,199,200]. Thus it seems that downregulation of miR-218 in GC cause ECOP overexpression, activation of NF-κB activity and COX-2 transcription, ultimately inhibiting apoptosis and inducing cell proliferation[199]. Tables 2 and 3 summarize the microRNAs that have been found to have a role in *H. pylori* -related gastric carcinogenesis. MicroRNAs overexpressed in GC generally target and repress tumor suppressor genes functioning as oncogenic miRNAs (Table 2), while tumor suppressor miRNAs that target and repress oncogenes are downregulated in GC (Table 3).

## EFFECTS OF *H. PYLORI* ERADICATION ON MICRORNAS

The effect of *H. pylori* eradication on reducing GC incidence is believed to be related to the risk existing at the time of eradication therapy[201]. A systematic review suggested that atrophic gastritis can undergo regression within one or two years after successful eradication of *H.*

**Table 2 Potential oncogenic microRNAs**

| MicroRNA | *H. pylori* | GC | Targets | Consequences/associations |
|---|---|---|---|---|
| miR-21 | ↑ | ↑ | RECK | Decreased apoptosis; cell proliferation, invasion |
| | | | PTEN | MMP stimulation |
| | | | ABP | PI3K/Akt signaling pathway activation |
| miR-106a | | | RB1 | E2F transcription; lymphatic and distant metastasis |
| miR-584 | ↑ | | Foxa1 | EMT promotion; decreased E-cadherin |
| miR-1290 | | | SMAD2 | Cell differentiation and remodeling; IM development |
| miR-296-5p | | ↑ | CDX1 | Erk1/2 activation; growth promotion |
| miR-222 | ↑ | ↑ | RECK | Proliferation |
| miR-223 | ↑ | ↑ | EPB41L3 | Migration and invasion |
| miR-106b-25 cluster | | ↑ | $p21^{CIP1/WAF1}$ | Decreased response to TGF-B |
| | | | Bim | Cell cycle progression; inhibition of apoptosis |
| | | | E2F1 | |
| miR-130b | | ↑ | RUNX3 \| Bim | Proliferation (CDK2 activation) and invasion |
| miR-301a | | | RUNX3 | Apoptosis inhibition |
| miR-181b | ↑ | ↑ | Timp3 | Inhibition of apoptosis, cell proliferation, invasion and migration |
| miR-27a | ↑ | ↑ | FoxO1 | Increased oxidative stress |
| | | | Prohibitin | |

*H. pylori*: *Helicobacter pylori*; GC: Gastric cancer; RECK: Reversion-inducing cysteine-rich protein with Kazal motifs; PTEN: Phosphatase and tensin homolog; ABP: Androgen-binding protein; MMP: Matrix metalloproteinase; PI3K: Phosphoinositide 3-kinase; E2F: E2F family; Foxa1: Forkhead box protein A1; SMAD2: Mothers against decapentaplegic homolog 2; EMT: Epithelial-mesenchymal transition; IM: Intestinal metaplasia; CDX1: Caudal type homeobox 1; Erk: Extracellular-signal-regulated kinases; EPB41L3: Erythrocyte membrane protein band 4.1-like 3; p21: Cyclin-dependent kinase inhibitor 1; Bim: Bim gene (Bcl-2 family member); TGF-B: Transforming growth factor beta; RUNX3: Runt-related transcription factor 3; Timp3: TIMP Metallopeptidase Inhibitor 3; FoxO1: Forkhead box protein O1.

*pylori*[202].

However regression of atrophic gastritis after *H. pylori* eradication seems to depend on the size and topographical distribution of atrophy, with a subsequent meta-analysis suggesting that gastric atrophic changes could only be reversible in cases located in the corpus but not in the antrum[203]. The presence of IM is a less reversible stage than atrophy alone, with meta-analysis suggesting that eradication at the IM stage is less effective and more likely to progress[203]. Lower *H. pylori* colonization of areas with IM may explain why the advantage of eradication is more limited at this stage. However, even if *H. pylori* eradication can't regress intestinal metaplasia, it may be beneficial in decreasing cancer risk in patients with widespread IM, as suggested in a Japanese multicenter study which showed that incidence of new cancers was reduced by one-third among those with *H. pylori* eradication compared with those without eradication therapy[204]. Despite this, GC still arises in the setting of IM even following *H. pylori* eradication and evidence concerning the ability of *H. pylori* eradication to reduce the risk of cancer in cases of widespread IM is lacking, though it seems to reduce progression.

Several studies recently assessed the potential benefits of *H. pylori* eradication on the miRNA deregulation and methylation status of the gastric mucosa. Indeed, aberrant methylation and methylation levels of CDH1 are reported to decrease after *H. pylori* eradication, suggesting that DNA methylation in gastric mucosa decreases when *H. pylori* is eradicated[101]. However, Ando *et al*[96] found that methylation levels of miR-124 were not decreased in individuals with past infection when compared to patients with current infection, suggesting that aberrant methylation induced in set cells may persist even after *H. pylori* eradication.

Shiotani *et al*[117] evaluated the expression of 21 miRNAs in gastric biopsies before and after *H. pylori* eradication in patients with history of endoscopically resected early GC and non-cancer controls and found that the expression of oncogenic miRNAs was significantly higher in the intestinal metaplastic glands than in the non-intestinal metaplastic glands, irrespective of *H. pylori* eradication. In neither group *H. pylori* eradication significantly changed any miRNA expression in the intestinal metaplastic glands, despite a beneficial effect of *H. pylori* eradication was seen in the control group where eradication decreased miR-223 expression and let-7d expression increased. The authors then concluded that *H. pylori* eradication improved miRNA deregulation but not in intestinal metaplastic glands[117], further supporting the clinical finding that intestinal metaplasia is a less reversible stage in the gastric carcinogenesis.

In another study by Shiotani *et al*[205], expression of serum miRNAs was evaluated in patients with history of endoscopically resected EGC and age and sex matched controls, before and one year after *H. pylori* eradication and it was found *H. pylori* eradication significantly decreased miR-106b levels and increased let-7d only in the control group.

Altogether these findings suggest that despite *H. pylori* eradication seems to be of benefit in the improvement of miRNA deregulation, some underlying processes may continue to promote tissue damage and contribute to the progression of the gastric carcinogenesis.

**Table 3 Potential tumor suppressor microRNAs**

| MicroRNA | *H. pylori* | GC | Targets | Consequences/associations |
|---|---|---|---|---|
| miR-185 | ↓ | | DNMT1 and EZH2 | DNA methylation; proliferation; EMT; LNM; poor prognosis |
| miR-204 | ↓ | | SIRT1 | EMT; invasion |
| miR-106b | ↓ | | | Proliferation (TGF-B induced cell cycle arrest suppression) |
| miR-320 | ↓ | ↓ | Mcl-1 | Apoptosis inhibition; progression of prenoplastic conditions<br>Relapse of tumors; chemoterapeutic resistance |
| miR-101,<br>miR-515-5p | ↓ | ↓ | Mcl-1<br>SOC2; DNMT1 | Apoptosis inhibition<br>Let-7 attenuation |
| miR-490-3p | ↓ | ↓ | Cyclin B1<br>SMARCD1 | EMT; proliferation; colony formation; migration; invasion<br>Metastasis and poorer survival<br>Decreased through the spectrum of gastric carcinogenesis |
| miR-370 | ↓ | ↓ | FoxM1 | ↓p27 expression; cell cycle progression and proliferation<br>Decreased through the spectrum of gastric carcinogenesis |
| miR-328 | ↓ | | CD44v9 | Survival and proliferation of metaplastic cells |
| Let-7 | ↓ | ↓ | Ras<br>c-myc<br>HMGA2<br>Cthrc1 | Cell proliferation and colony formation<br><br>Migration and invasion |
| miR-200,<br>miR-141,<br>miR-429 | ↓ | ↓ | ZEB1/2<br><br>Cyclin D1<br>Bcl-2\|XIAP | Epithelial differentiation; EMT suppression<br>Decreased E-cadherin, inhibition of migration and invasion<br>Proliferation<br>Apoptosis inhibition<br>Tumor size, lymphatic invasion and LNM |
| miR-141 | ↓ | | FGFR2 | Proliferation |
| miR-375 | ↓ | ↓ | MDM2<br>JAK2/STAT3<br>3/3/2014<br>PDK1 | p53 inactivation<br>Neoplastic transformation; proliferation and migration<br>Inhibition of apoptosis<br>PI3K/Akt signaling pathway activation |
| miR-524-5p | | ↓ | Jagged-1; Hes-1 | Cell proliferation and invasion |
| miR-449 | ↓ | ↓ | Cyclin E2\|Met<br>Gemini\|SIRT1 | Proliferation, angiogenesis, invasion and metastasis |
| miR-203 | ↓ | ↓ | CASK | Cell growth, colony formation and cell invasion |
| miR-29a<br>miR-29c | | ↓ | p42.3; Mcl-1 | Cell cycle progression and proliferation |
| miR-15b, 16, 34, 181b, 497 | | ↓ | Bcl-2 | Apoptosis inhibition |
| miR-218 | ↓ | ↓ | ECOP<br>SLIT/ROBO1 | Activation of NF-κB and increased COX-2; apoptosis inhibition<br>Invasion and metastasis |

*H. pylori*: *Helicobacter pylori*; GC: Gastric cancer; DNMT1: DNA (cytosine-5)-methyltransferase 1; EZH2: Enhancer of zeste homolog 2; EMT: Epithelial-mesenchymal transition; LNM: Lymph node metastasis; SIRT1: Sirtuin 1; TGF-B: Transforming growth factor beta; Mcl1: Myeloid cell leukemia 1; SOC2: Suppressor of clear homolog; SMARCD1: SWI/SNF-related matrix-associated actin-dependent regulator of chromatin subfamily D member 1; FoxM1: Forkhead box protein M1; HMGA2: High-mobility group AT-hook 2; Cthrc1: Collagen triple helix repeat containing 1; ZEB1/2: Zinc finger E-box binding homeobox 1/2; XIAP: X-linked inhibitor of apoptosis protein; FGFR2: Fibroblast growth factor receptor 2; MDM2: Mouse double minute 2 homolog; JAK1: Janus kinase 1; STAT3: Signal transducer and activator of transcription 3; PDK1: Phosphoinositide-dependent kinase-1; Hes-1: Hairy and enhancer of split-1; CASK: Calcium/calmodulin-dependent serine protein kinase; ECOP: EGFR-coamplified and overexpressed protein; NF-κB: Nuclear factor kappa B; COX-2: Cyclooxygenase-2.

## CONCLUSION

*H. pylori* infection is a key factor in gastric carcinogenesis and influences inflammation, proliferation, cell cycle progression and apoptosis, differentiation, migration and invasion. Chronic *H. pylori* gastritis results from both innate and adaptive immune responses that seem to be tightly regulated by miRNA. The inflammatory milieu within the gastric mucosa contributes do DNA methylation of tumor suppressor genes and to the accumulation of both genetic and epigenetic alterations in gastric epithelial cells, contributing to the progression of gastric carcinogenesis. Several studies implicate miRNA in DNA methylation and in the regulation of several inflammatory and neoplastic pathways including in GC. However, each miRNA can control the expression of hundreds to thousands of genes, making difficult to unravel all the processes under miRNA control and thus we are just beginning to understand the genetic and molecular mechanisms underlying the process of gastric carcinogenesis. Nevertheless, the existing studies allow us to understand the importance of these small non-coding nucleotides and to link inflammatory pathways to neoplastic transformation at a genetic level, despite some studies come from animal models and some inconsistencies exist in the literature concerning the function of some miRNAs.

Further studies are undoubtedly needed to continue to improve our knowledge about miRNA functions in *H. pylori* -related GC, both at a genetic and at a clinical level in order to bring miRNAs to clinical practice as markers of disease and as prognostic markers and one day epigenetic therapy may have a role in the treatment of patients with preneoplastic conditions after *H.*

*pylori* eradication and GC *via* downregulation of onco-miRNAs and activation of tumor suppressor miRNAs. Given the data summarized in this review, we believe that let-7, miR-106 family, miR-146a, miR-155, miR-181b, miR-223 and miR-375 are the miRNAs most consistently reported to have important roles in gastric *H. pylori*-related carcinogenesis and thus we suggest that these miRNAs deserve greater attention in clinical studies to found if they can be used as disease markers. Future studies on this topic should focus on both miRNA serum and tissue levels in patients in different stages of gastric carcinogenesis (not infected with *H. pylori*, chronic *H. pylori* gastritis, atrophic gastritis, intestinal metaplasia, dysplasia, invasive carcinoma and metastatic carcinoma). Furthermore, we believe that the modulation of miRNAs by *H. pylori* eradication and chemoprevention with COX-2 should also deserve attention in future studies.

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# Colon cancer and the epidermal growth factor receptor: Current treatment paradigms, the importance of diet, and the role of chemoprevention

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## Abstract

Colorectal cancer represents the third most common and the second deadliest type of cancer for both men and women in the United States claiming over 50000 lives in 2014. The 5-year survival rate for patients diagnosed with metastatic colon and rectal cancer is < 15%. Early detection and more effective treatments are urgently needed to reduce morbidity and mortality of patients afflicted with this disease. Here we will review the risk factors and current treatment paradigms for colorectal cancer, with an emphasis on the role of chemoprevention as they relate to epidermal growth factor receptor (EGFR) blockade. We will discuss how various EGFR ligands are upregulated in the presence of Western diets high in saturated and N-6 polyunsaturated fats. We will also outline the various mechanisms of EGFR inhibition that are induced by naturally occurring chemopreventative agents such as ginseng, green tea, and curcumin. Finally, we will discuss the current role of targeted chemotherapy in colon cancer and outline the limitations of our current treatment options, describing mechanisms of resistance and escape.

**Key words:** Chemoprevention; Colon cancer; Epidermal growth factor receptor; Western diet; Curcumin; Green tea; Ginseng



**Core tip:** This review article will summarize the risk factors and current treatment paradigms for colorectal cancer, with an emphasis on the role of targeted chemotherapy and chemoprevention as they relate to epidermal growth factor receptor (EGFR) blockade. It will include an overview of the structure and function of EGFR as well as intracellular pathways regulated by its activity. It will discuss how various EGFR ligands are upregulated in the presence of Western diets that are high in saturated and N-6 unsaturated fat, and will outline the various mechanisms of EGFR inhibition observed with several naturally occurring

chemopreventative agents including ginseng, green tea, and curcumin.

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## INTRODUCTION

A total of 1665540 new cancer cases and 585720 cancer deaths were projected to occur in the United States in 2014. Of these, colon and rectal cancer (CRC) will account for 8% of new cases, representing the third most common and the second deadliest type of cancer for both men and women[1], claiming over 50000 lives in 2014[1,2]. The 5-year survival rate for patients diagnosed with metastatic CRC is < 15%[1]. Early detection and treatment is crucial for the improvement in morbidity and mortality of patients afflicted with this disease.

Overexpression of epidermal growth factor receptor (EGFR) is common in many tumors. Specifically in CRC, EGFR is estimated to be overexpressed in 60%-80% of tumors, and is associated with a poor prognosis[2]. For these reasons EGFR has been targeted as a locus for treatment with small molecule inhibitors and monoclonal antibodies, with the latter playing a role in the treatment of metastatic disease. This review article will discuss risk factors and current treatment modalities for colorectal cancer and examine the roles of chemotherapy and chemoprevention.

## RISK FACTORS FOR COLORECTAL CANCER

Many factors have been identified contributing to the risk of colon cancer. These risk factors are believed to increase the rate at which genetic mutations occur in various oncogenes and tumor suppressor genes, and/or result in growth-promoting epigenetic modifications. Generally, these factors can be classified into the following categories: germline genetic mutations, environmental exposures, personal or family history of CRC, associated diseases, and demographic considerations.

There are several germline genetic mutations that greatly increase the incidence of colon cancer through distinct molecular mechanisms. The two syndromes that account for most of the hereditary diseases are Lynch syndrome, and familial adenomatous polyposis (FAP) syndrome. Recent estimates indicate that Lynch syndrome accounts for approximately 3% of CRC cases, while FAP syndrome contributes an additional 0.01%[3,4]. Lynch syndrome is caused by mutations in one or more of the DNA mismatch repair genes *MLH1*, *MSH2*, *MSH6*, *PMS2*, and *EPCAM*. The two most common forms of FAP syndrome are a result of a germline mutation in the APC gene. Other germline - inherited colorectal cancer syndromes include MUTYH-associated polyposis, Cowden syndrome, Peutz-Jeghers syndrome, and juvenile polyposis syndrome.

Environmental exposures associated with an increased risk of CRC include a history of abdominal radiation, smoking, alcohol use, and diet[5-8]. Of particular interest with respect to the EGFR receptor is the role of a high fat Western diet, which has been shown to promote the development of experimental colon cancer *via* an EGFR-mediated mechanism. The role of this pathway will be discussed in detail later.

Personal history of CRC or large adenomatous polyps (> 1 cm) or polyps with villous features increase the risk of colorectal cancer[9]. Family history of colon cancer or adenomatous polyps confers an increased risk of disease, even if these histories do not meet the criteria for the syndromes discussed above. US guidelines reflect this increased risk, with the ACG recommending earlier screening if a single first-degree relative was diagnosed with CRC or had an advanced adenoma diagnosed at age < 60 years or if two first-degree relatives were diagnosed with CRC or advanced adenomas[10].

Disease states associated with an increased incidence of colon cancer include IBD (both ulcerative colitis and Crohn's disease), diabetes, and obesity. As with many cancers, risk for CRC increases with age. CRC incidence is approximately equal in males and females, although there is an increased incidence and higher mortality rate among African Americans and an increased mortality among men. Recent studies suggest that testosterone effects in males rather the protective effects of estrogens in females account for increased male risk[11].

## APPROACH TO CRC MANAGEMENT

The management of CRC includes screening, staging, and treatment with surgery, chemotherapy, and/or radiation. As more than 20% of patients with CRC will present with metastatic disease with a 5 year survival rate < 15%[1], prevention is critical in colorectal cancer. Colorectal cancer prevention is primarily based on screening methods, which include stool tests, radiographic imaging, and colonoscopy to identify adenomatous polyps, a precursor lesion for colon cancer. Colonic polyps may be identified through these screening methods and then may be removed during colonoscopy. Colorectal cancer, once diagnosed, is defined as either colon or rectal cancer based on the anatomical location of the lesion, with the rectum being defined as the region extending from the transitional mucosa of the anal dentate line to the sigmoid colon at the peritoneal reflection. Recent studies of CRC suggest that tumors arising in the proximal and distal colon have different

molecular phenotypes with different prognostic outcomes. Interestingly, rectal cancers and tumors in the distal colon share many molecular features[12].

Upon diagnosis of CRC, staging is primarily accomplished through CT (with certain situations calling for additional PET-CT) of the chest, abdomen, and pelvis, using the TMN system, with the goal of identifying tumors appropriate for resection. If amenable to resection, the tumor is removed. Pathological staging and subsequent assessment of high-risk features for systemic recurrence are performed to help guide the utility of adjuvant chemotherapy with 5-FU based chemotherapies. In this regard, determining the presence of nodal disease is of particular importance. For metastatic disease, assessment of *RAS* gene status (KRAS/NRAS) and BRAF status (if KRAS is WT) determines whether or not the tumor is likely to respond to anti-EGFR monoclonal antibodies such as panitumumab and cetuximab. The rationale for this treatment paradigm and the specific pathways involved will be discussed later. In addition to genetic testing for individuals with CRC at younger ages or with CRC positive family history, search for metastatic lesions must be pursued to determine if patients are likely to benefit from resection of isolated metastasis. The timing of colectomy with resection of metastasis, and the use of various 5-FU based chemotherapeutics as neoadjuvant forms of chemotherapy such as FOLFOX, FOLFIRI, and CapeOX, along with bevacizumab, panitumumab, or cetuximab, depend on the individual patient and tumor characteristics. If resection of metastatic disease is impossible, neoadjuvant chemotherapy should be administered first if there is no imminent risk of obstruction or significant bleeding. In addition, the patient should undergo periodic re-assessment regarding the resectability of metastatic lesions[13].

For rectal cancer, endorectal ultrasound is important to assess the presence of LN involvement. In clinical T1-T2 node negative rectal cancer, surgical management should be pursued with a pathological assessment of TMN stage. High grade T lesions or node positive disease should be treated with adjuvant chemotherapy and radiation. In advanced clinical stage disease (T3 or higher or any node positive disease), neoadjuvant chemoradiation should be offered with adjuvant chemotherapy. The chemotherapeutics recommended in rectal cancer include the 5-FU based agents with oxaliplatin. In metastatic disease, there is a role for panitumumab and cetuximab if the tumors are KRAS/NRAS WT. As with colon cancer, the goal in metastatic rectal cancer is to periodically reassess the potential for resection of metastases. Treatment regimens for rectal *vs* colon cancer share many similarities, with the major difference being the use of radiation therapy for rectal cancer as outlined above[13]. There is, however, some data suggesting a benefit for adjuvant RT in colon cancer in select patients with high-risk features for local recurrence[14].

## EGFR PATHWAYS IN COLORECTAL CANCER

EGFR was one of the first targets to be exploited in cancer treatment. The receptor also known as HER (human EGF receptor) or c-erbB1, is a 170-kDa transmembrane protein with intrinsic protein tyrosine kinase activity. EGFR is one of four members of the c-erb subfamily of receptor protein tyrosine kinases. Two cysteine-rich domains comprise the ligand-binding region on the extracellular aspect of the cell. A single alpha-helical transmembrane domain connects the ligand-binding region to the intracellular receptor, which is comprised of three domains. One domain serves as a site for feedback attenuation by PKC and erk MAP kinases, another is a tyrosine kinase domain, and the third is a carboxy-terminal tail. EGFR is present on all epithelial and stromal cells, and is expressed on many glial and smooth muscle cells as well. It is a multifunctional receptor that plays a key role in cell division and apoptosis, cell differentiation and dedifferentiation, migration, and organogenesis[15]. EGFR executes these functions by activation of multiple signaling pathways including PLC-gamma-1, RAS-RAF-MEK-MAPKs, phosphatidylinositol-3 kinase and Akt, Src, the stress-activated protein kinases, PAK-JNKK-JNK, and the signal transducers and activators of transcription. Binding of a diverse array of ligands (EGF, TGF, amphiregulin, heparin-binding EGF, betacellulin, or epiregulin) induces receptor homodimerization or heterdimerization with other ErBb2 members (Figure 1).

EGFR ligands are released from membrane bound proligand forms by membrane bound metalloprotease enzymes of the ADAM family. ADAM17 is a key enzyme regulating release of EGFR ligands: EGF, amphiregulin, and heparin-binding EGF[16].

When liganded, the EGFR undergoes autophosphorylation *in trans* in the cytoplasmic kinase domain. Phosphorylated tyrosine residues function as docking sites that are recognized by adapter or effector proteins that contain src homology 2 domains or protein tyrosine binding domains. EGFR signal responses are cell-type specific and modulated by the specific activating EGFR ligand, the particular homo or heterodimeric ErbB partners formed and the availability of downstream effector pathways[17].

EGFR is expressed in 60%-80% of CRCs[2]. The mechanisms by which EGFR promotes tumorigenesis are diverse and involve both cell cycle dysregulation and the promotion of factors that aid in tumor survival. Studies in other tumors have dissected some of the mechanisms involved. In breast cancer cells, increased levels of EGFR have been associated with increased proliferative and angiogenic activity. Increased proliferation and angiogenesis are thought to be induced TGF, which correlated with increased mitotic activity. EGFR ligands TGF$\alpha$ and EGF have also been shown to function as chemoattractants for endothelial cells, with TGF$\alpha$

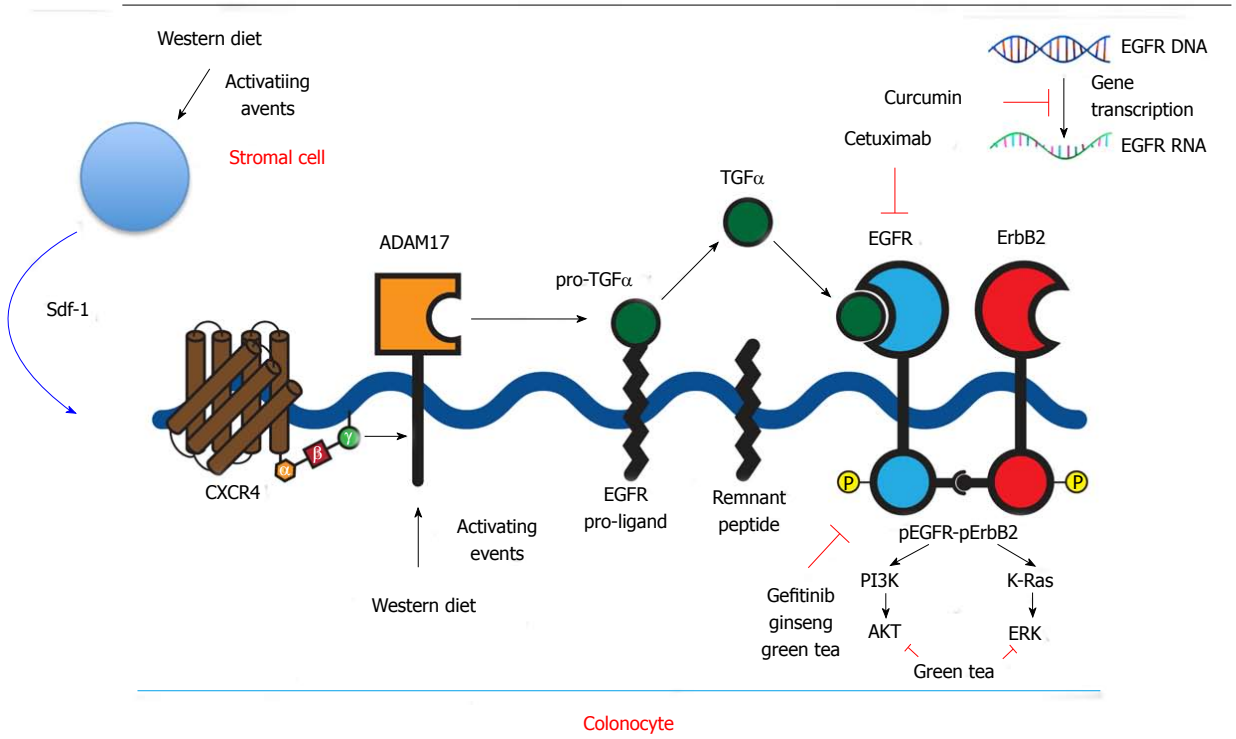


**Figure 1 Epidermal growth factor receptor pathways, western diet, chemoprevention, synthetic inhibitors.** EGFR: Epidermal growth factor receptor; CXCR4: C-X-C chemokine receptor type 4; TGF: Transforming growth factor; PI3K: Phosphatidylinositol-3 kinase; ERK: Extracellular regulated protein kinases.

additionally promoting the expression of VEGF[18-20]. EGFR overexpression blocks apoptosis through various mechanisms - in prostate cancer, the Ras/Raf/MEK cascade and the Rac/PAK1 signaling pathway have been implicated in the inactivation of the proapoptotic protein BAD that is inhibited by phosphorylation[21]. In breast cancer EGF and amphiregulin upregulated the expression of certain matrix metalloproteinases implicated in tumor progression and metastasis even in the presence of EGFR inhibition that blocked cell proliferation, suggesting that low levels of EGFR activation may promote MMP9 induction[22]. Finally, microRNAs have been shown to mediate EGFR effects on tumorigenesis. Specifically, miRNA-143 and -145 have been demonstrated to be downregulated when mice with wild type EGFR are fed a western diet high in fat, with increased expression of RAS and MYC implicated as some of the several important G1 regulators mediating this oncogenic effect. Colon cancers seen in EGFR mutant specimens demonstrated an increase in these same miRNAs without an increase in RAS and MYC activity, suggesting an alternate pathway of tumorigenesis in these tumors[23].

## EGFR, DIET, AND CHEMOPREVENTION

There is a strong association between Western diet and the incidence of colorectal cancer. This association was initially observed in the late 1960s, in epidemiological studies of the incidence of colon cancer in Japanese-American emigrants over the course of two generations following their adoption of a Western style diet, high in animal fat and red meat[24]. This association has been investigated in the azoxymethane (AOM) model of colon cancer that mimics many of the clinical, histological and molecular features of sporadic human colon cancer. AOM causes O6 methylation of DNA guanine bases resulting in activating mutations in K-ras and CTNNB1 (which codes for β-catenin)[25]. In this model, EGFR is required for tumor promotion by Western diet[26,27]. To demonstrate EGFR requirement, mice with wild type Egfr and mice homozygous for loss-of-function Waved-2 Egfr mutations were fed standard *vs* high-fat diets and cancer was induced by treating with AOM, followed by tumor promoting dextran sulfate sodium. The Waved-2 Egfr lacks 90% of wild type receptor kinase activity. The Egfr wild type mice in the high-fat group had a significantly higher tumor incidence compared to mice on standard diet but this tumor promoting effect of high fat diet did not occur in mice with mutant Egfr[7]. The proto-oncogenes CTNNB1, MYC, CNND1, and PTGS2 and the EGFR ligand TGFα were also found to be expressed at significantly higher levels in tumors from Egfr wild type mice treated with the high fat diet compared to tumors from mice with mutant Egfr[7].

In more recent preliminary studies we showed that Western diet increases ADAM17 expression and up-regulates EGFR ligands TGF-α and amphiregulin[28]. Stroma-derived factor 1 alpha (Sdf1α) was also increased by WD. Sdf1α is a ligand for the G-protein coupled receptor CXCR4. In other preliminary colon cancer studies we showed that Sdf1α induces the activation of EGFR (EGFR transactivation) by stimulating ADAM17

(Figure 1). ADAM17 is increased in human colon cancer that likely contributes to increases in EGFR ligands and signals observed in these tumors[29]. This mechanism of ligand-driven EGFR signals contrasts with activating EGFR mutations or gene amplification seen in other cancers such as brain and lung cancer[30].

CTNNB1 codes for β-catenin which is an integral part of the cell cytoskeleton as well as an important transcription factor in colonic tumorigenesis, which regulates many key tumor-promoting genes including *MYC*, *CCND1*, and *PTGS2*[31-33]. EGFR is an upstream regulator of β-catenin causing deacetylation that blocks β-catenin degradation and leads to nuclear localization of this molecule[34]. Nuclear localization was increased in all tumors. MYC was also expressed in all tumors and was highest in the mice with wild type Egfr fed a Western diet. CCND1 codes for cyclin D1 that controls G1-> S cell cycle progression and its expression was greater in mice with wild type Egfr compared to those with mutant Egfr. PTGS2 codes for Cox-2 that is also linked to Egfr status, with Cox-2 being 7-8 fold higher in mice with wild type Egfr fed a Western diet compared to standard diet. This finding is of particular interest as prior studies have demonstrated that K-Ras and β-catenin are required to induce Cox-2 in colon cancer cells, underscoring the importance of the EGFR-Kras-Cox-2 signaling cascade. Finally, the expression of the EGFR ligand TGFα was shown to correlate with tumor burden in both genotypes, with a stronger association with the wild type Egfr noted[7].

In addition to EGFR other factors have been implicated in high-fat diet promoted tumorigenesis, including increases in colonic secondary bile acids, elevations of serum insulin, insulin-like growth factor which can also stimulate EGFR through various mechanisms, and diet-induced changes in the microbiome[35-38].

In the study showing EGFR was required for Western diet to promote tumorigenesis, mice fed a Western diet exhibited weight gain, increased visceral fat and insulin resistance, consistent with the development of a metabolic syndrome, which is also implicated in colon cancer causation[7].

### Ginseng

The high morbidity and mortality rates of late stage presentation of colon cancer have prompted more investigation into preventative strategies. Ginseng as a chemopreventive agent has been shown to decrease the incidence of various forms of cancer in case control and prospective cohort studies[39-41]. Several studies have demonstrated the anti-tumor effects of ginseng extract, focusing on the diverse group of biologically active chemical structures called ginsenosides, glycosides with dammarane skeletons with varying sugar types, numbers, and linkage positions. Several have been isolated and administered to mice, resulting in statistically significant decreases in lung tumor incidence and reduced growth of colon tumor xenografts. Several mechanisms have been implicated in the anti-tumorigenic properties of ginseng including antioxidant, anti-proliferative, pro-apoptotic and anti-inflammatory actions of ginseng and more recently EGFR inhibitory effects have been identified[42-45]. Additionally, in a mouse model of colitis-associated colon cancer, American ginseng was shown to inhibit inflammation and suppress EGFR signaling, effects that are postulated to contribute to ginseng's anti tumorigenic properties[46] (Figure 1).

In studies of mice treated with a combination of Western diet alone or WD plus ginseng, colonic mucosal EGFR signals were noted to be increased in the Western diet group and Ginseng inhibited these increases. Ginseng also appears to inhibit tumorigenesis through other mechanisms, including the induction of apoptosis. Ginseng's anti tumor effects likely require ginseng metabolite activation by the colonic microbiome as several biologically active metabolites of ginsenosides are synthesized by gut microbes. One metabolite in particular, 20-O-b-(D-glucopyranosyl)-20(S)-protopanaxadiol or compound K, was shown to suppress growth of colon tumor xenografts[47].

### Green tea

Several other naturally occurring products have been studied as potential chemopreventative agents and been shown to inhibit EGFR signals. A bioactive green tea polyphenol, epigallocatechin-3-gallate (EGCG), has been shown to selectively inhibit EGF-dependent signaling in cervical cancer cells, leading to growth cessation and cell apoptosis. The mechanism of this selective inhibition was shown to involve suppression of EGFR-induced ERK1/2 (aka MAPK1 and 3) and AKT activation as well as direct suppression of ERK and AKT[48] (Figure 1). These kinases have been implicated in cell cycle progression; ERK1/2 signals both activation of the intrinsic or extrinsic apoptotic pathway depending on the ligand and cell type, and AKT has been shown to regulate cell proliferation and survival, with constitutive up-regulation of activated AKT demonstrated in many types of human cancers[49-51]. The importance of these cellular pathways is underscored by the observation that only selective kinases downstream of EGFR were inhibited, but not others. Increasing concentrations of EGCG exerted both short term reversible effects on cell cycle progression and long term cellular changes with increased rates of apoptosis[49].

### Curcumin

Another naturally occurring substance that has drawn the attention of the scientific community is curcumin, the yellow pigment of tumeric found in curry. It is produced by the rhizome of the plant tumeric and has been safely consumed and utilized for its medicinal properties for centuries. This substance has been shown to inhibit the growth of cancer cells by suppressing gene expression of cyclinD1 and EGFR[52]. Recent studies have demonstrated that curcumin inhibits binding of the transcription factor

EGR-1 to the EGFR promoter as well as suppresing *EGR*-1 gene expression through the ERK signal pathway, thereby suppressing *EGR-1* transactivation activity[53] (Figure 1). Of note, the concentrations required to achieve this growth suppression *in vitro*, are much higher than those normally achieved in blood and tissue *in vivo* following curcumin ingestion, but for colon cancer prevention colonic luminal concentrations may be more relevant. Recent developments of more stable curcumin analogues may also increase the efficacy of this compound[54].

## EGFR AS A CHEMOTHERAPEUTIC TARGET

With the potential central role of EGFR in tumorigenesis, several groups have successfully developed neutralizing antibodies or kinase inhibitors. Of particular interest are the monoclonal antibodies cetuximab and panitumaumab, as well as the small molecule inhibitors gefitinib and erlotinib.

Cetuximab and panitumumab act by binding the extracellular domain of EGFR and thereby inhibiting ligand-dependent activation and receptor dimerization. Cetuximab also may induce an immune response by antibody-dependent cell-mediated cytotoxicity[55-58] (Figure 1). In colon cancer, cetuximab is currently FDA approved for EGFR-positive metastatic disease in patients who cannot tolerate irinotecan-based therapy, or in combination with oxaliplatin, irinotecan, and 5-FU. These recommendations are based on a 2009 study that examined the use of cetuximab as a first-line treatment with FOLFOX, with assessment of tumor response in KRAS wildtype *vs* KRAS mutant tumors. Tumors with KRAS mutations resulting in constitutively active GTP-binding protein were shown to be resistant to EGFR inhibitors[59,60]. This trial confirmed previous findings and demonstrated significant differences between tumor response and risk of disease progression in the KRAS mutant and KRAS WT groups with the addition of cetuximab, though a difference of progression-free survival was not detected[61]. Panitumumab is also used in metastatic CRC and also requires WT KRAS for efficacy[62]. More recently a study suggested that tumors with KRAS mutations in codon 13 may remain susceptible to Cetuximab, whereas those with KRAS codon 12 mutations did not[63].

Small molecule EGFR receptor tyrosine kinase inhibitors, gefitinib and erlotinib are not used in the treatment of CRC. Gefitinib was initially approved for third-line treatment of patients with non-small cell lung cancer (NSCLC) based on preliminary small clinical trials but later studies demonstrated conflicting results of its efficacy[64]. A phase Ⅱ RCT of FOLFIRI *vs* gefitinib plus FOLFIRI did not show any benefit and demonstrated high toxicity[65]. There have since been studies looking at the efficacy of gefitinib in select groups of patients, initially based on demographic considerations such as non-smokers, Asians, and women, and later based on specific activating mutations[66,67], underscoring the importance of careful patient selection in maximizing the success of these targeted agents. Erlotinib is currently approved for second-line treatment of patients with locally advanced or metastatic NSCLC and first-line treatment for patients with locally advanced, unresectable, or metastatic pancreatic cancer in combination with gemcitabine[55]. Recent studies have looked at the combination of cetuximab and erlotinib in the treatment of chemotherapy-refractory metastatic CRC with promising results. These studies demonstrated improvement in response rates and progression free survival in patients with tumors having wild type EGFR compared to failures in the patients with tumors having mutant EGFR[68].

These studies point to the importance of assessing the mutation status of EGFR and KRAS when using EGFR targeted therapies. It should be noted that there are many other factors that determine a given patient's initial and subsequent response to therapy. This is highlighted by the fact that KRAS mutations only account for approximately 30%-40% of nonresponsive patients[60,69]. Mutations in other downstream signaling molecules such as BRAF have been shown to correlate with unresponsiveness to cetuximab and panitumumab[70]. Raf proteins are principal downstream effectors of KRAS in the RAS-RAF-MEK-MAPKs signaling cascade. They are activated directly by KRAS and serve to phosphorylate and activate the downstream kinase MEK, which phosphorylates ERK leading to numerous Ras-induced cellular responses[71,72]. Specifically, BRAF has a higher affinity for MEK leading to stronger MEK stimulation than A-Raf or c-Raf, and plays a critical role in promoting cell survival by activating the MAPK pathway[73]. The prognostic significance of these mutations with respect to survival is less clear, with some data indicating that gender may a role how these mutations affect tumor virulence. In one prospective cohort study, BRAF mutations were associated with a reduced cancer specific survival in men, particularly in lymph node positive disease, when compared to women. Additionally in microsatellite stable tumors, BRAF was found to be an independent predictor of poor prognosis in men[74]. The exact mechanisms of how gender may interact with BRAF mutation status are not yet clear. However, even when adjusting for BRAF mutant tumors to assess nonresponders to cetuximab and panitumumab, approximately 41% of nonresponders are left unaccounted for, suggesting the presence of other unknown mechanisms of resistance[70].

Responses even in selected groups of patients with wild type EGFR, KRAS, and BRAF alleles is not uniform, and all patients will ultimately develop acquired resistance to targeted therapy with monoclonal antibodies. Increased ERBB2 signaling has been shown to be one such mediator in resistant clones of previously cetuximab-sensitive cell lines *via* the up-regulation of ERK1/2 signaling[75]. This can occur through the amplification of ERBB2 itself or through the overexpression of hereguglin,

one of the ERBB3 ligands. Increased c-Met signaling may be another mechanism for EGFR antibody resistance[76,77]. Importantly, restoration of sensitivity to cetuximab has been demonstrated with the application of interfering RNA or small molecule inhibitors such as gefitinib, suggesting a potential valuable role of these small molecule kinase inhibitors in restoring efficacy of EGFR targeted therapies.

## CONCLUSION

We have reviewed the risk factors and current treatment paradigm for colorectal cancer, with an emphasis on the role of targeted chemotherapy and chemoprevention as they relate to EGFR blockade. The complex interplay between other growth promoting pathways that cross talk with EGFR and downstream EGFR effectors that can be driven by activating mutations make strategies that target EGFR vulnerable to several escape mechanisms. The role of Western diet and the exciting field of chemoprevention offer opportunities to target EGFR signaling cascade which plays a critical role in tumor promotion and progression. Future development of anti-EGFR directed nanoparticles restricted to the gut that could inhibit over active EGFR signals might hold promise to safely reduce colorectal cancer risk.

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# Portal vein embolization effect on colorectal cancer liver metastasis progression: Lessons learned

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## Abstract

Colorectal liver metastasis (CRLM) is the major cause of death in patients diagnosed with colorectal cancer. The gold standard treatment of CRLM is surgical resection. Yet, in the past, more than half of these patients were deemed unresectable due to the inadequate future liver remnant (FLR). The introduction of efficient portal vein embolization (PVE) preoperatively allowed more resections of metastasis in CRLM patients by stimulating adequate liver hypertrophy. However, several experimental and clinical studies reported tumor progression after PVE which critically influences the subsequent management of these patients. The underlying pathophysiological mechanism of tumor progression post-PVE is still not fully understood. In spite of the adverse effects of PVE, it remains a potentially curative procedure in patients who would remain otherwise unresectable because of the insufficient FLR. Currently, the challenge is to halt tumor proliferation following PVE in patients who require this technique. This could potentially be achieved by either attempting to suppress the underlying oncologic stimulus or by inhibiting tumor growth once observed after PVE, without jeopardizing liver regeneration. More research is still required to better identify patients at risk of experiencing tumor growth post-PVE.

**Key words:** Tumor growth; Portal vein embolization; Future liver remnant; Colorectal liver metastases; Liver resection; Prevention; Liver hypertrophy



**Core tip:** This article discusses the effect of portal vein embolization (PVE) on colorectal liver metastasis (CRLM) growth and the suggested methods of prevention. In addition to presenting the various experimental and clinical studies emphasizing the suggested tumoral enhancing effect of PVE, this article highlights the concept

of reversal of chemotherapy response, a potential effect occurring after PVE. This observation may impact significantly subsequent patients' management as it may affect the resectability state of patients. Moreover, potential methods to prevent tumor growth are discussed in this article, indicating the need for further research in this field and highlighting the complex interaction between CRLM and liver regeneration milieu.

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## INTRODUCTION

The most common site for colorectal cancer (CRC) metastasis is the liver which occurs in approximately 50% of patients during their disease course[1,2]. The 5-year survival rate of patients with local CRC is 90.3%, yet, survival drops ominously to 12.5% when remote metastases ensue in these patients[3]. In fact, colorectal liver metastasis (CRLM) is the leading cause of death in CRC patients with an overall median survival of 6-12 mo if not treated[4]. Surgical resection remains the gold standard and potentially curative treatment for CRLM[2,4]. In the past, only 15%-20% of patients with CRLM were candidates for liver resection because of insufficient future liver remnant (FLR), which puts patients at risk of hepatic dysfunction and post-operative morbidity and mortality[5-8]. Therefore, to increase resectability rate, the effective and relatively safe preoperative portal vein embolization (PVE) technique was introduced aiming to maximize the remnant liver volume before major hepatectomy[9]. The experience with preoperative PVE was first described more than 20 years ago by Makuuchi *et al*[10] in patients with hilar cholangiocarcinoma to induce ipsilateral hepatic atrophy and contralateral residual liver hypertrophy. The authors reported no major complications or liver failure in the 14 patients included in the study[10]. This successful and relatively safe technique allowed more liver metastases removal[6,9]. In addition to being recommended prior to major hepatectomy when the preoperative FLR is insufficient (< 25% of total liver volume), PVE is also part of the two-stage hepatectomy strategy, with the PVE being performed before the second stage resection, thereby facilitating resection in patients with bilateral CRLM[11]. Figure 1 provides a general overview of the clinical settings in which PVE is utilized.

An estimated FLR of less than 25% in patients with normal livers is a general indication for PVE prior to intended hepatectomy[12]. However, several studies reported the possible complications of PVE, namely inadequate FLR growth, higher disease recurrence and tumor growth acceleration in both embolized and non-embolized liver lobes[13-24]. Notably, rapid tumor progression following PVE remains a major concern for clinicians as it critically influences the clinical outcome and overall survival of CRLM patients[12,13]. As a matter of fact, local or distal tumor progression post-PVE may even lead to unresectable disease in a proportion of patients as observed in some studies[19,24]. Studies reporting tumor progression post PVE are summarized in Table 1. The exact mechanisms stimulating the hepatic atrophy-hypertrophy complex along with increased tumor volume post-PVE remains unclear. However, three mechanisms have been suggested to explain this occurrence: up-regulation of cytokines and growth factors stimulated by liver regeneration, compensatory increase in hepatic arterial blood perfusion and evoked cellular host response promoting local tumor growth[25].

The increasing body of evidence emphasizing the superior contribution of PVE to the observed tumoral growth triggered interest during the past decade. Hoekstra *et al*[26] examined the relationship between PVE and enhanced tumor growth in a rabbit model, which mirrored the clinical setting of CRLM patients. They concluded that a higher tumor growth rate occurred in the PVE group compared to non-PVE cohort group; however there was no significant difference between both groups in terms of markers of liver regeneration (IL-6, tumor necrosis factor alpha, growth factor hepatic growth factors and TGFB 1). Additionally, Maggiori *et al*[27] observed the same tumoral enhancing effect of PVE and ligation in their experimental rat model. Interestingly, this study showed that PVE increased tumor growth in the contralateral nonoccluded liver while decreasing it in the occluded liver portion. Similar results of diminished tumor volume in the embolized liver were also reported in another experimental animal study[28]. An additional experimental study conducted in an *in-vivo* rabbit model reported similar results of augmented tumor growth in the nonembolized liver whereas no effect was seen in the embolized liver[29]. Moreover, multiple clinical studies described concordant observations of tumor progression and higher recurrence rates in CRLM patients undergoing PVE[13-24]. A clinical study conducted by Pamecha *et al*[23] in 2009 was the first to correlate post-PVE tumor volumes measured by imaging with proliferative activity of cancer-cells observed on immunohistochemistry in two matched comparative groups. The authors confirmed the increased tumor growth rate was related to the proliferative activity post-PVE. Taking together all these experimental and clinical studies provides substantial evidence that PVE may play a critical role in promoting tumor growth.

Simoneau *et al*[13] attempted to further investigate this in one of the largest published observational studies. A total of 109 patients were included in the PVE group *vs* 11 patients in the no-PVE control group. Patients in the PVE group were further subdivided into bevacizumab group and non-bevacizumab group so as to evaluate the effect of pre-embolization chemothe-

**Table 1 Summary of several studies describing the effect of portal vein embolization on tumor progression**

| Ref. | No. of CRLM patients undergoing PVE | Percentage change in tumor volume and/or TGR and/or percentage of patients developing tumor progression after PVE |
|---|---|---|
| Simoneau *et al*[13] | $n$ = 109 | 33.4% increase in TV in the right lobe ($P$ < 0.001) and 49.9% increase in TV in the left lobe ($P$ = 0.022) post-PVE |
| Elias *et al*[14] | $n$ = 48 | 60% to 970% increase in TV post-PVE |
| Kokudo *et al*[15] | $n$ = 18 | +20.8% ($P$ = 0.016) increase in TV and 18.5% ($P$ = 0.014) increase in percent tumor volume post-PVE |
| Mueller *et al*[19] | $n$ = 53 | 80.9% ($n$ = 17/53) of patients were unresectable due to tumor progression post-PVE |
| Pamecha *et al*[21] | $n$ = 36 | 33% ($n$ = 12/36) of patients had tumor progression post-PVE |
| Hoekstra *et al*[22] | $n$ = 28 | 25% ($n$ = 7/28) of patients developed new lesions in FLR and 42% of patients ($n$ = 8/19) had tumor recurrence in the liver on follow up post-PVE |
| Pamecha *et al*[23] | $n$ = 22 | TGR post-PVE was 0.36 ± 0.68 mL/d (-1) ($P$ = 0.06) |
| Lindner *et al*[24] | $n$ = 19 | 21% of patients developed tumor progression post-PVE |

CRLM: Colorectal liver metastasis; PVE: Portal vein embolization; TV: Tumor volume; TGR: Tumor growth rate.

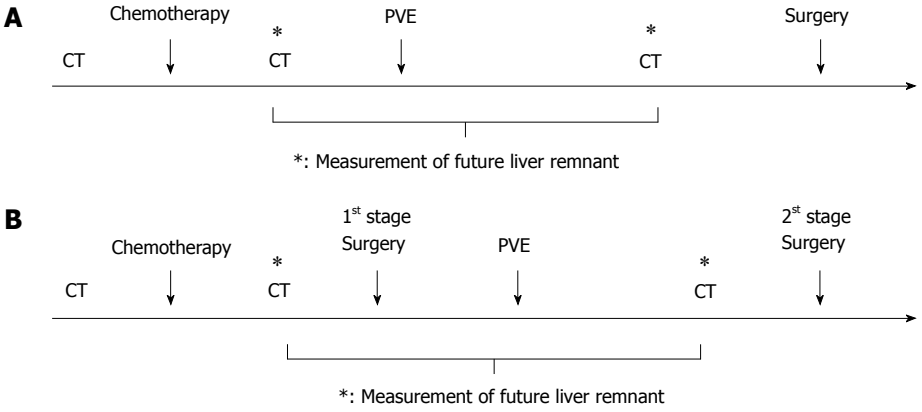


**Figure 1 Overview of the clinical settings in which portal vein embolization is used in colorectal liver metastasis patients: Before hepatectomy (A) before 2nd stage surgery in the two-staged hepatectomy strategy (B).** PVE: Portal vein embolization; CT: Computed tomography.

rapy given concurrently with bevacizumab on tumor progression and liver regeneration. Pre-embolization chemotherapy combined with anti-angiogenic therapy did not compromise liver regeneration as both groups had similar degrees of hepatic hypertrophy[13]. The study also showed a positive tumor growth rate (+0.07 $cm^3$/d) in the PVE group compared to a negative growth rate (-0.06 $cm^3$/d) in the control (no PVE) group ($P$ < 0.001), suggesting that PVE may be associated with tumor progression in some patients despite an initial response to chemotherapy. The results of the authors thereby introduced of the hypothesis that PVE may, in some instances, stimulate tumor growth and actually reverse the chemotherapeutic response. This suggests that the effect of PVE may overcome the downsizing chemotherapy effect in a subset of patients, who may be more susceptible to progress after such a stimulus. Overall, the data derived from these observational studies on PVE and tumor growth raise some questions that not only have significant clinical value in the management of CRLM patients, but also shed some light on the complexity of liver metastasis biology, progression and resistance to therapy.

Understanding the mechanisms of liver regeneration and tumor growth post-PVE and identifying common factors stimulating both pathways may help to develop methods to inhibit tumor growth. Liver regeneration is regulated at the molecular level by a wide variety of growth factors and cytokines, such as tumor necrosis factor, interleukin-6, hepatocyte growth factor (HGF), transforming growth factor (TGF), vascular endothelial growth factor and epidermal growth factor[30]. Current evidence suggests that the up-regulation of these factors is common to stimulation of tumor pathways, and this was suggested as a possible theory explaining tumor growth after PVE[25,31]. Notably, it was postulated in one experimental study that HGF may be a key regulator, as it is a key factor for both hepatocyte regeneration and cancer cells proliferation. The investigators have observed a significant increased serum HGF after PVE compared to controls[29]. Thus, it has been suggested that the administration of anti-inflammatories or growth factor inhibitors at the time of PVE could potentially help in inhibiting tumor progression. To date, this still remains a theoretical concept as no targeted therapy that would prevent tumor progression without compromising liver hypertrophy has been demonstrated in clinical studies.

In another perspective, some investigators have focused on clinical strategies that would limit post-PVE

tumor progression. Several approaches have been suggested in literature although a general consensus is still lacking. A preoperative period of 2-4 wk was suggested by Abdalla *et al*[12] to allow for adequate hepatic regeneration, while minimizing the time between PVE and resection is also recommended to reduce risk of tumor progression during the interval between end of chemotherapy and the procedure[22,24-25,32]. In addition, transarterial chemoembolization pre- and post-PVE was shown to be effective in preventing tumor growth in patients with hepatocellular carcinoma. Although its use for CRLM patients has not been reported, such an intervention may be a potentially promising strategy[25]. In addition, radio-embolization in the hepatic artery (for example with Yttrium-90), one of the current modalities of treatment of CRLM, may also hypothetically decrease the risk of tumor progression by decreasing the tumor arterial blood supply, with minimal effect to the normal adjacent liver parenchyma, but presently there is no evidence supporting its use post-PVE[25,33]. Lastly, the use of systemic therapy (neoadjuvant or adjuvant chemotherapy) is now widely used in the management of these patients[25,34]. In fact, chemotherapy may protect against tumor progression post-PVE without disturbing liver hypertrophy especially in patients who initially respond adequately[6,25,35]. Fischer *et al*[6] reported in an observational study that the administration of chemotherapy after embolization significantly reduced the rate of progression. Whether systemic or loco-regional therapy, many existing strategies have been and continue to be investigated as potential strategies to diminish tumor progression after embolization.

Despite the potential adverse effects of PVE, it remains an essential procedure done in the preoperative setting prior to major hepatectomy, allowing for resectability in patients who otherwise would remain unresectable due to insufficient FLR. More research is required to better stratify patients and identify those at increased risk of developing tumor growth post-PVE. Further research should focus on identifying tumors more responsive to a stimulatory environment and more prone to progress, to provide insight on the complex tumor biology of colorectal hepatic metastasis and to promote the development of personalized treatment strategies.

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ORIGINAL ARTICLE

Observational Study

# Lay perceptions of breast cancer in Western Kenya

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## Abstract

**AIM:** To explore lay perceptions of causes, severity, presenting symptoms and treatment of breast cancer.

**METHODS:** In October-November 2012, we recruited men and women (18 years and older) from households and health facilities in three different parts of Western Kenya, chosen for variations in their documented burdens of breast cancer. A standardized and validated tool,

the breast cancer awareness measure (BCAM), was administered in face-to-face interviews. Survey domains covered included socio-demographics, opinions about causes, symptoms, severity, and treatment of breast cancer. Descriptive analyses were done on quantitative data while open-ended answers were coded, and emerging themes were integrated into larger categories in a qualitative analysis. The open-ended questions had been added to the standard BCAM for the purposes of learning as much as the investigators could about underlying lay beliefs and perceptions.

**RESULTS:** Most respondents were female, middle-aged (mean age 36.9 years), married, and poorly educated. Misconceptions and lack of knowledge about causes of breast cancer were reported. The following (in order of higher to lower prevalence) were cited as potential causes of the condition: Genetic factors or heredity ($n$ = 193, 12.3%); types of food consumed ($n$ = 187, 11.9%); witchcraft and curses ($n$ = 108, 6.9%); some family planning methods ($n$ = 56, 3.6%); and use of alcohol and tobacco ($n$ = 46, 2.9%). When asked what they thought of breast cancer's severity, the most popular response was "it is a killer disease" ($n$ = 266, 19.7%) a lethal condition about which little or nothing can be done. While opinions about presenting symptoms and signs of breast cancer were able to be elicited, such as an increase in breast size and painful breasts, early-stage symptoms and signs were not widely recognized. Some respondents (14%) were ignorant of available treatment altogether while others felt breast cancer treatment is both dangerous and expensive. A minority reported alternative medicine as providing relief to patients.

**CONCLUSION:** The impoverished knowledge in these surveys suggests that lay education as well as better screening and treatment should be part of breast cancer control in Kenya.

**Key words:** Breast cancer; Health education; Cancer control; Lay health beliefs



**Core tip:** A survey of women's knowledge and beliefs about breast cancer causes, presentation, and treatment in Western Kenya uncovered significant ignorance and misperceptions. Effective approaches will be needed to remediate this situation if Kenyan national aspirations for breast cancer control are to succeed.

Naanyu V, Asirwa CF, Wachira J, Busakhala N, Kisuya J, Otieno G, Keter A, Mwangi A, Omenge OE, Inui T. Lay perceptions of breast cancer in Western Kenya. *World J Clin Oncol* 2015; 6(5): 147-155 Available from: URL: http://www.wjgnet.com/2218-4333/full/v6/i5/147.htm DOI: http://dx.doi.org/10.5306/wjco.v6.i5.147

## INTRODUCTION

Breast cancer has become a significant cause of morbidity and mortality globally. Developing countries are especially affected and are increasingly reporting more cases worldwide. In many developing countries, breast cancer care is not a priority for there are many other health priorities and limited health budgets. Consequently, these nations offer minimal attention to cancer, even while it is becoming a leading cause of death[1]. They also do not have organized data registries, thus they lack reliable data on breast cancer incidence, mortality, survival, and stage of presentation[2,3].

Factors associated with increased breast cancer incidence include increased life expectancy, reduction in competing risk of mortality from infections, change in reproductive patterns, and changes in lifestyles[4-6]. To compound the difficulties imposed by its rising incidence, breast cancer patients in developing countries enroll late for treatment. This delay has been associated with several factors. First, low levels of community and even health providers' awareness of breast cancer results in widespread ignorance about the problem. Second, many patients encounter barriers as they attempt to access appropriate treatment. Third, some find it extremely frustrating to access health care systems in some regions. Alternative health belief models and associated traditional, complementary health care systems persist. Lastly, breast cancer early detection programs are scarce[7-11].

In East Africa, the breast cancer incidence rate estimate is 19.3 per 100000 women[12]. Breast cancer is the most prevalent cancer among Kenyan women, and constitutes a major public health problem[13,14]. Although definite data are lacking for Kenya, estimates indicate that breast cancer accounts for about 23% of all cancers in the country[15]. Unfortunately, Kenya has not developed a comprehensive cancer surveillance system and there is no national population-based cancer registry[15]. Without representative data, a data-based and discerning national profile of the health burden of breast cancer is unattainable. Lack of routinely collected data hampers public policy response to the problem.

According to the Kenya Medical Research Institute (KEMRI), about 80% of reported cases of cancer are diagnosed at advanced stages, when very little can be achieved in terms of curative treatment[15]. Perhaps in response, the Kenyan government has launched a National Cancer Control Strategy that prioritizes cancer prevention and control. This strategic plan covers the period 2011 to 2016 and proposes a strategic foundation for cancer control and prevention, outlines a vision and mission, and recommends specific interventions and objectives as suitable for Kenya. Ultimately, the strategic plan aims to reduce the number of people who develop and die of cancer while ensuring a better quality of life for those still affected by the disease[15].

Since low public awareness and/or negative beliefs

about breast cancer have been noted as a contributor to potentially preventable deaths in breast cancer programs, we undertook a project to explore breast cancer awareness, knowledge and practices among men and women of Western Kenya in order to provide information that will guide subsequent prevention and treatment efforts. This particular paper reports descriptive data from the project, focusing especially on lay beliefs that emerged about causes, severity, presenting symptoms and treatment of breast cancer.

## MATERIALS AND METHODS

A cross-sectional study was conducted by a research team from the Academic Model Providing Access to Healthcare (AMPATH) program in Eldoret. AMPATH is a collaboration of Moi University School of Medicine, Moi Teaching and Referral Hospital (MTRH), the Kenyan Ministry of Health, and a consortium of North American Universities[16]. This project was embedded in the AMPATH Oncology Institute (AOI) and was supported by the Walther Cancer Foundation of Indianapolis, indiana United States data were collected in three communities served by AMPATH, including Turbo, Mosoriot and Kapsokwony between October-November 2012. The study sites were chosen on the basis of unpublished data from the Eldoret Cancer Registry to represent counties with high, and low burdens of breast cancer. Within the Cancer Registry, the largest number of breast cancer cases come from Uasin Gishu County (45%) where Turbo is located. Mosoriot community is in Nandi County and contributes 5% of breast cancer cases to the registry, while Mount Elgon provides the lowest number of cases to the registry (0.2%) and includes the community of Kapsokwony. The ethnic composition of these three counties taken together is reasonably representative of the ethnic communities of the whole AMPATH service area population of Western Kenya.

The study surveyed women (18 years and older) who voluntarily presented to their respective health facilities for special breast screening days as well as general community members living in the near vicinity of the health center. Ethical approval for the survey was obtained from the MTRH Institutional Research and Ethics Committee as well as the Indiana University Institutional Review Board.

The study survey instrument was in large part a standardized and validated survey questionnaire, one developed for assessment of breast cancer awareness in United Kingdom - the Breast Cancer Awareness Measure (BCAM)[17]. BCAM items were written to characterize beliefs in seven domains: knowledge of symptoms of breast cancer; women's confidence, skills and behavior in detecting a breast change; anticipated delay in contacting the doctor on discovering a symptom; barriers to seeking medical help; knowledge of age-related and lifetime risk of breast cancer; knowledge of any breast screening programs. For this study we modified the BCAM to include items of particular relevance in this Kenyan setting and added open-format, free-text inquiries about breast cancer. These questions were two in number: (1) "What are some beliefs, opinions, or traditions you have heard from others about breast cancer?" (in Kiswahili, Ni baadhi ya maoni ama tamaduni zipi ambazo umewahi kusikia kutoka kwa watu wengine kuhusu saratani ya matiti?); and (2) "In your opinion, what are some of the early warning signs of breast cancer, the ways in which one may know first that s/he has this condition?" (Kwanza habisa, kwa maoni yako ni, dalili gani za mapema zinazotahadharisha kuhusiana na saratani ya matiti? Yani njia ambzao mtu anaweza kutambua mapema kuwa anaugua huu ongonjwa?). The resultant tool was translated to Kiswahili, the national language, and was tested for understandability and completeness in three 1-2 h focus group discussions (FGDs) prior to fielding the survey. The FGDs included men and women who were > 18 years of age, drawn from those attending outpatient clinics for non-cancer related conditions. Individuals with current or previous diagnosis of cancer were excluded from the validation activity.

In the community and health center surveys, trained research assistants sought written consent and administered the validated semi-structured tool that facilitated collection of data on several topics. The socio-demographic tool was structured, while opinions about causes, symptoms, severity, and treatment of breast cancer were captured as free-text responses to the open-ended queries added to the BCAM. Responses to these questions were recorded verbatim and translated into English as necessary. These data were then coded, and emerging themes were identified, pooled and integrated into larger categories. To assure reliability of coding, independent coding and identification of themes were conducted by three investigators with negotiation of any identified differences. Descriptive analyses were done on quantitative data using Statistical Analysis System version 9.3 and STATA version 11.0. Each coded statement was viewed as a variable, and each respondent could have multiple responses to a single question. Tables 1 and 2 report frequency/percentage for each coded statement type, summarizing statements from a total of 1335 study participants in the three communities. In reporting these data we have pooled responses from all surveyed participants - those interviewed in the health centers and those interviewed in the communities served by the health centers - because in preliminary analyses the distribution of opinions from these two samples were not different.

## RESULTS

### *Participant characteristics*

This study enrolled a total of 1335 participants, 481 participants from Kapsokwony, 277 from Mosoriot and 577 from Turbo. Five-hundred and ninety-four of the participants were surveyed at the health centers and the remainder in community households in the near vicinity.

**Table 1 Lay opinions about causes of breast cancer in Western Kenya**

| Perceived cause | No. of coded statements (%) | | | |
|---|---|---|---|---|
| | Kapsokwony 597 (38%) | Mosoriot 297 (19%) | Turbo 672 (43%) | Total Opinions 1566 (100%) |
| Hereditary | 91 (15.2) | 33 (11.1) | 69 (10.3) | 193 (12.3) |
| Food consumed | 60 (10.1) | 38 (12.8) | 89 (13.2) | 187 (11.9) |
| Witchcraft and curses | 63 (10.6) | 7 (2.4) | 38 (5.7) | 108 (6.9) |
| Family planning methods | 23 (3.9) | 8 (2.7) | 25 (3.7) | 56 (3.6) |
| Alcohol and tobacco consumption | 18 (3.0) | 2 (0.7) | 26 (3.9) | 46 (2.9) |
| Breastfeeding | 12 (2.0) | 8 (2.7) | 8 (1.2) | 28 (1.8) |
| Not breastfeeding | 9 (1.5) | 6 (2.0) | 9 (13) | 24 (1.5) |
| Exposure to toxic substances | 11 (1.8) | 4 (1.3) | 7 (1.0) | 22 (1.4) |
| HIV and other sexual diseases | 6 (1.0) | 0 | 3 (0.4) | 9 (0.6) |
| Environmental changes | 2 (0.3) | 2 (0.7) | 4 (0.6) | 8 (0.5) |
| Radiation and vibrations | 7 (1.2) | 0 | 1 (0.1) | 8 (0.5) |
| Type of clothing | 2 (0.3) | 0 | 4 (0.6) | 6 (0.4) |
| Low sexual encounters | 1 (0.2) | 0 | 1 (0.1) | 2 (0.1) |
| Early sexual encounter | 2 (0.3) | 0 | 0 | 2 (0.1) |
| High number of sexual encounters | 1 (0.2) | 0 | 1 (0.1) | 2 (0.1) |
| Others[1] | 25 (4.2) | 5 (1.7) | 13 (1.9) | 43 (2.7) |
| No opinions expressed[2] | 264 (44.2) | 184 (61.9) | 374 (55.7) | 822 (52.5) |

[1]Others include: Becoming rich/wealthy, depression, dirt in the body, bacterial infection, injuries, traditional medicine not properly administered, not "having children", man sucking on breasts during pregnancy, male circumcision, fate, insect bites, lack of physical activity, and having big breasts; [2]N in this row = number of respondents expressing no opinions.

**Table 2 Lay perceptions of severity and symptoms/signs of breast cancer in Western Kenya**

| Perception | No. coded statements (%) | | | |
|---|---|---|---|---|
| | Kapsokwony 489 (36%) | Mosoriot 280 (21%) | Turbo 580 (43%) | Total Perceptions 1349 (100%) |
| Severity | | | | |
| Killer disease | 105 (21.5) | 45 (16.1) | 116 (20.0) | 266 (19.7) |
| Breasts are removed | 10 (2.0) | 3 (1.1) | 12 (2.1) | 25 (1.9) |
| Curable if detected early | 10 (2.0) | 6 (2.1) | 2 (0.3) | 18 (1.3) |
| A disease like any other | 5 (1.0) | 6 (2.1) | 3 (0.5) | 14 (1.0) |
| It does not exist | 3 (0.6) | 1 (0.4) | 0 | 4 (0.3) |
| Spreads to rest of the body | 2 (0.4) | 1 (0.4) | 0 | 3 (0.2) |
| Cancer eats away the breast | 0 | 1 (0.4) | 0 | 1 (0.1) |
| Don't know | 354 (72.4) | 217 (77.5) | 447 (77.1) | 1018 (75.5) |
| Symptoms/signs | | | | |
| | 1166 (40%) | 552 (19%) | 1192 (41%) | 2910 (100%) |
| Changes in breast size | 207 (17.8) | 109 (19.7) | 266 (22.3) | 582 (20.0) |
| Pain, tingle or tenderness of the breast | 195 (16.7) | 88 (15.9) | 243 (20.4) | 526 (18.1) |
| Lump in breast | 165 (14.2) | 72 (13.0) | 129 (10.9) | 366 (12.6) |
| Discharge from the breast | 163 (13.9) | 58 (10.5) | 131 (11.0) | 352 (12.1) |
| Wound on the breast | 92 (7.9) | 39 (7.1) | 90 (7.6) | 221 (7.6) |
| Itching | 80 (6.9) | 17 (3.1) | 68 (5.7) | 165 (5.7) |
| Change in breast skin color | 44 (3.8) | 17 (3.1) | 27 (2.3) | 88 (3.0) |
| Rash on breast and skin peeling | 30 (2.6) | 8 (1.4) | 20 (1.7) | 58 (2.0) |
| Change in nipples | 21 (1.8) | 10 (1.8) | 17 (1.4) | 48 (1.6) |
| Swelling in any other parts of the body | 11 (0.9) | 8 (1.4) | 7 (0.6) | 26 (0.9) |
| General symptoms[1] | 28 (2.4) | 33 (6.0) | 26 (2.2) | 87 (3.0) |
| Don't know | 130 (11.1) | 93 (16.8) | 168 (14.1) | 391 (13.4) |

[1]General symptoms include fatigue, chest pain, loss of weight, change in eye color, liver trouble, no appetite, sweating, cough, chills, and fever.

In both surveys, the number of respondents was limited only by the capacity of trained interviewers to administer the BCAM, since almost all potential participants approached were willing to participate, but the interviewers had to limit their workdays to catch transportation back to Eldoret. In the health center sample, attendees were given the option to be interviewed after completing an informed consent document. If attendees wished to skip the interview and proceed directly to clinical breast examination, they were given this choice. A total of 1511 volunteers (1238 women and 273 men) presented themselves to the health centers for CBE screening and about 48% (594) of this number were interviewed. In the community surveys, research assistants were dispatched in groups of 2 or 3 and walked or were driven to specific locations within the administrative units of the

**Table 3 Demographics of participants in Breast Cancer Awareness Measure survey in Western Kenya**

| Participant attribute | | Kapsokwony (*n* = 481) *n* (%) | Mosoriot (*n* = 277) *n* (%) | Turbo (*n* = 577) *n* (%) | Total (*n* = 1335) *n* (%) |
|---|---|---|---|---|---|
| Age (yr) | ⩽ 30 | 187 (38.9) | 114 (41.2) | 223 (38.9) | 524 (39.3) |
| | 31-60 | 265 (55.1) | 142 (51.3) | 320 (55.5) | 727 (54.5) |
| | 61-90 | 28 (5.8) | 17 (6.1) | 31 (5.4) | 76 (5.7) |
| | 91+ | 0 | 2 (0.7) | 1 (0.2) | 3 (0.2) |
| | Missing data | 1 (0.2) | 2 (0.7) | 2 (0.4) | 5 (0.4) |
| Sex | Female | 414 (86.1) | 198 (71.5) | 449 (77.8) | 1061 (79.5) |
| | Male | 67 (13.9) | 79 (28.5) | 126 (21.8) | 272 (20.4) |
| | Missing data | 0 | 0 | 2 (0.4) | 2 (0.2) |
| Marital status | Married | 383 (79.6) | 202 (72.9) | 423 (73.3) | 1008 (75.5) |
| | Single | 60 (12.5) | 59 (21.3) | 121 (20.9) | 240 (18.0) |
| | Divorced | 3 (0.6) | 0 | 3 (0.5) | 6 (0.5) |
| | Separated | 10 (2.1) | 8 (2.9) | 14 (2.4) | 32 (2.4) |
| | Widowed | 25 (5.2) | 8 (2.9) | 14 (2.4) | 47 (3.5) |
| | Missing data | 0 | 0 | 2 (0.4) | 2 (0.2) |
| Education | None | 31 (6.4) | 14 (5.1) | 37 (6.4) | 82 (6.1) |
| | Primary | 193 (40.1) | 130 (46.9) | 253 (43.9) | 576 (43.2) |
| | Secondary | 160 (33.3) | 94 (33.9) | 202 (35.0) | 456 (34.2) |
| | Certificate/diploma | 87 (18.1) | 35 (12.6) | 67 (11.6) | 189 (14.2) |
| | University | 10 (2.1) | 4 (1.4) | 16 (2.8) | 30 (2.3) |
| | Missing data | 0 | 0 | 2 (0.4) | 2 (0.2) |
| Occupation | Business | 84 (17.5) | 33 (11.9) | 136 (23.6) | 253 (19.0) |
| | Casual laborer | 9 (1.9) | 15 (5.4) | 19 (3.3) | 43 (3.2) |
| | Employed | 100 (20.8) | 58 (20.9) | 93 (16.1) | 251 (18.8) |
| | Farming | 157 (32.6) | 63 (22.7) | 121 (21.0) | 341 (25.5) |
| | Self employed | 22 (4.6) | 17 (6.1) | 30 (5.2) | 69 (5.2) |
| | Unemployed | 105 (21.8) | 91 (32.9) | 176 (30.5) | 372 (27.9) |
| | Missing data | 4 (0.8) | 0 | 2 (0.4) | 6 (0.5) |
| Transportation | Boda boda | 138 (28.7) | 53 (19.1) | 79 (13.7) | 270 (20.2) |
| | Car | 2 (0.4) | 9 (3.3) | 8 (1.4) | 19 (1.4) |
| | Matatu | 25 (5.2) | 88 (3.2) | 227 (39.3) | 340 (25.5) |
| | Walking | 314 (65.3) | 122 (44.0) | 259 (44.9) | 695 (52.1) |
| | Other | 1 (0.2) | 4 (1.4) | 0 | 5 (0.4) |
| | Missing data | 1 (0.2) | 1 (0.4) | 4 (0.7) | 6 (0.5) |

district served by the health center. From these drop-off points the interviewers chose the first household at random, after which they would proceed to every third household until they reached the target sample size for the day (or the transport back was ready to depart). The community resident survey used the same BCAM and was conducted the day following the screening special event. A total of 741 respondents participated in community surveys.

As shown in Table 3, most respondents were female and married. The mean age was 36.9 (SD = 13.7) years and very few (19.3%, 10% and 21% in Kapsokwony, Mosoriot and Turbo respectively had post-secondary education. A small proportion reported no formal education at all, with Turbo showing the highest proportion of the uneducated (*n* = 37, 3%). Three-hundred and seventy-two respondents (28%) reported unemployment. Not less than 8%, 7% and 13% were unemployed in Kapsokwony, Mosoriot and Turbo respectively. The most common occupations were farming (*n* = 341, 26%), business (*n* = 253, 19%), and employed positions (*n* = 251, 19%). The most common means of transport to health care included walking (52%), use of public small-van transportation (matatu) (25%) and motorcycle taxis (bodaboda) (20%).

**Causes of breast cancer:** In Table 1, we present data on perceptions of the causes of breast cancer. In general, perceptions are similar across sites. A majority of respondents could offer no opinions about probable causes of this condition. Altogether, 822 or more than half of those surveyed (52.5%) had no specific knowledge of the factors that may cause breast cancer. Among those with opinions about causality, the following (in order of higher to lower prevalence) were cited as potential causes of the condition: genetic factors or heredity (*n* = 193, 12.3%); types of food consumed (*n* = 187, 11.9%); witchcraft and curses (*n* = 108, 6.9%); some family planning methods (*n* = 56, 3.6%); and use of alcohol and tobacco (*n* = 46, 2.9%). Compared to other sites, Mosoriot respondents less often cited the possible role of substance abuse and family planning methods: only two participants attributed breast cancer to substance abuse while another eight implicated family planning.

Other causes reported by a few respondents included fertility, pregnancy and breastfeeding practices; environmental factors (toxins, radiation, vibrations); diverse sexual behaviors (few/high encounters, early debut); wearing of tight-fitting clothing, poor mental health; dirty bodies; presence of other diseases and

unfitting use of medicines; lack of exercise; male circumcision; and having large breasts.

**Severity of breast cancer:** As shown in Table 2, when asked what they thought of breast cancer's severity, the most popular response was "it is a killer disease" (*n* = 266, 19.7%) a lethal condition about which little or nothing can be done. A smaller number of respondents believed that it can be cured if found early (18, 1.3%) and it is a disease like any other (*n* = 14, 1%). Surprisingly, a few said breast cancer doesn't exist (*n* = 4, 0.3%). No less than 25 participants in Kapsokwony (*n* = 10), Mosoriot (*n* = 3) and Turbo (*n* = 12) discussed removal of breasts as they considered the severity of breast cancer.

**Symptoms and signs of breast cancer:** The most common symptoms/signs of breast cancer cited across all three communities were typical of late-stage disease (Table 2): changes in breast size (*n* = 582, 20%); pain, tingling or tenderness of the breast (*n* = 526, 18.1%); growth of a lump in the breast (*n* = 366, 12.6%); presence of a discharge of pus or blood from the breast (*n* = 352, 12.1%); development of a wound on the breast - including occurrence of a bad smell and maggots (*n* = 221, 7.6%); and itching (*n* = 165, 5.7%). Other less-often cited symptoms included: change in breast skin color (*n* = 88, 3%); development of a rash on the breast and peeling of the skin (*n* = 48, 1.6%); and changes in nipples including size and direction (*n* = 48, 1.6%). About 22.8% (*n* = 304) of the total study sample did not cite any presenting breast cancer symptom or signs. This represents 20%, 26% and 24% of participants from Kapsokwony, Mosoriot and Turbo sites respectively.

**Management of breast cancer:** In other BCAM structured question responses, lay opinions on breast cancer management showed 14% (*n* = 185) of all respondents (1335) were completely ignorant of available treatment (17%, 14% and 11% of Kapsokwony, Mosoriot and Turbo sites respectively). Some (*n* = 95, 7.1%) believed complementary alternative medicine provides relief to breast cancer patients. A few (*n* = 18, 1.4%) thought it is potentially curable, however, 7 (0.5%) said breast cancer treatment is expensive. Other rare opinions suggested mastectomy causes death (*n* = 7, 0.5%), biopsy spreads cancer in the body (*n* = 5, 0.4%), and the disease attracts social stigma (*n* = 7, 0.5%).

## DISCUSSION

This study illustrates the productivity of using open-ended, free-text inquiry as an element in surveys intended to explore the prevalence of perceptions and beliefs about a condition like breast cancer. We believe that strategic educational campaigns to inform the general public and secure their participation in primary and secondary prevention should be founded upon an appreciation of the state of lay public knowledge and beliefs, accurate or not. Because we intend to design and deliver educational messages to clinical and non-clinical populations in the AOI catchment area in Kenya, using written and spoken content at health centers and local radio stations for reaching the public, having a rich vein of information such as that summarized in Tables 1 and 2 of this paper will be an asset.

Biomedical and epidemiological evidence supports a multitude of risk factors and causes for breast cancer, including genetic endowment, obstetrical and breast feeding history, use of tobacco, low fruit and vegetable dietary intake, lack of exercise and obesity, alcohol intake, and exposure to physical and chemical carcinogens among others[18-20]. By contrast, women in the general Kapsokwony, Mosoriot and Turbo populations have very little knowledge of risk factors for breast cancer and espouse some misconceptions. As others have found, this lack of sound information may adversely affect preventive and curative behaviors[21]. To compound this problematic situation, the women we surveyed in Western Kenya - irrespective of site - perceived breast cancer to be a lethal disease about which little could be done, characterized by symptoms and signs that would be typical only of late-stage cancer. Biomedical treatments, especially surgery, were thought not to be helpful, perhaps dangerous (promoting spread of the cancer) and certainly unaffordable. This kind of mistaken information needs to be remedied to engage the public in our AOI prevention efforts.

This background of popular knowledge is not unique to Kenya. In a Zambian study[22] 82% of rural and 58% of urban women had no knowledge of breast cancer. There is a need for health care workers to deliberately design and promote educational programs to create awareness on the dangers of breast cancer. Notwithstanding the burden of breast cancer in developing countries, these countries have low public awareness of the condition; myths and misconceptions are rampant; and the affected delay initiation of treatment[23-26]. Past research shows that it is common for women in developing countries to be aware of lumps for a long time and not seek care until complications such as pain, ulcer, foul-smelling discharge or symptoms of metastatic disease occur[27-30]. Additionally, the health care work force does not seem to be an active source of breast cancer information. For example, Oluwatosin's[31] (2006) Nigerian study found that the leading source of information about breast cancer was "elders, neighbors and friends" while only 4.4% of the respondents acknowledged health workers as sources of information[31]. It is troubling to find that primary health care workers - who are expected to promote breast cancer awareness - are not the leading source of cancer information.

The important role of mainstream health care providers in patient and public education cannot be overemphasized. There is evidence that some primary health care workers have inadequate knowledge and poor client teaching on early detection of breast cancer.

For instance, only 20% of nurses in a Nigerian teaching hospital considered a painless lump an early sign of breast cancer. Further, 41% considered pain an early sign[32]. The role of health care workers as sources of information and instruction about breast cancer is imperative, for without them, the general population will continue muddling through lay explanatory models instead of gaining factual knowledge about breast cancer causes, risks, symptoms, and management.

Patients must also know more about breast cancer care and what is available. According to KEMRI, about 80% of reported cases of cancer are diagnosed at advanced stages due to the low awareness of cancer signs and symptoms, inadequate screening services, inadequate diagnostic facilities and poorly structured referral facilities[15]. Indeed, research from Kenya shows many with breast cancer symptoms do not seek medical attention until their cancer is very advanced, and knowledge of breast cancer and early detection differentiates with women's social and economic backgrounds[33].

Whatever the context of prevailing popular knowledge, as we seek to promote widespread breast cancer education in our communities, we must remember the role of culture and lay beliefs for they often reflect the framework within which local populations interpret known and emerging diseases. Accordingly, indigenous knowledge should be considered a key element in the development of culturally sensitive breast cancer control and curative programs. Simon[34] (2006) offers four practice principles that can be especially useful when appreciating the role of culture in health behavior: (1) Inclusion and use of indigenous support; (2) Cross-application of approaches for diverse populations; (3) Honor and incorporation of culture; and (4) Paying attention to language, literacy, and cultural information. By so doing, we stand to spur timely diagnosis and associated care uptake in all social contexts[34]. Whatever the accuracy or inaccuracy of common community knowledge about breast cancer, we probably need to use opinions such as those uncovered in this survey as "points of departure" and "information anchors" when seeking to change opinions and advance alternative knowledge.

This study has strengths as well as limitations. It was undertaken in three different regions of Western Kenya and recruited participants from health facilities as well as at the household level in the communities they serve. The participants were thus interviewed at their usual place of residence or familiar environments. The use of a semi-structured tool allowed participants to express their personal perceptions and opinions on the subject matter without restrictions. The utility of open-ended survey questions in such surveys was demonstrated. In general, study participants had very low breast cancer knowledge and wanted to be informed about all types of cancer. Among study limitations, we should first emphasize that Kenya is a melting pot of diverse ethnic cultures and indigenous knowledge and beliefs. This study provides valuable information on lay explanations of breast cancer but it is not robustly generalizable, even within Kenya. Second, breast cancer rates are on the increase in Kenya, and the role of health workers in breast cancer awareness and care remains only partially explored. The state of breast cancer in the country calls for involvement of all stakeholders, but our study included only lay people and no clinicians, community health workers or health policymakers.

This project reports on lay beliefs about causes, severity, symptoms and treatment of breast cancer in Western Kenya. Lay explanatory models for breast cancer are common and risk factors are not well known in this population. This lack of knowledge has been partly blamed for delay in breast cancer care uptake in Kenya[2]. Development of strategies to spur early detection and enrolment in treatment is critical and should involve health care workers, policy makers and community members at all levels[6]. Organizations such as the Kenya Breast Health Program should be used to educate the public on causal factors, symptoms, and management of breast cancer. Kenyan programs must also build capacity for treating new patients presenting with early-stage disease even as they continue to treat those reporting late with advanced conditions.

The National Cancer Control Strategy - which is based on the World Health Organization's global cancer control strategy - is the first cancer control strategy document to be developed in Kenya. It consolidates aspects in cancer prevention, screening, diagnosis, treatment and care for cancer patients as well as investment needed to deliver these services[15]. This effort is overdue and laudable, but it fails to emphasize the importance of public education in engaging the participation of at-risk populations. With combined efforts that involve international, government, and private partners, a strategy should be pursued that creates breast cancer awareness, in the overall effort to reduce mortality associated with cancer and ensure quality of life for those affected[15].

The role of health care workers in breast cancer education and symptom identification requires attention. Training and research in breast cancer remains a critical need in developing countries whereby available training programs have low levels of funding, suboptimal infrastructure, and continually experience brain drain[6]. In Kenya, although cancer continues to burden many, cancer research is diminutive due to inadequate funding and limited training facilities[15]. Promising areas for future breast cancer work in developing countries include development of training models that can be translated into several languages and applied to diverse cultural settings, and establishment of centers of excellence[6].

## COMMENTS

### *Background*

Globally, breast cancer has increasingly become a significant cause of morbidity and mortality in adult populations. This trend has been noted in developing countries like Kenya, where screening, prevention, curative and relevant data systems remain underdeveloped. It is believed in Kenya that levels of

awareness may be low and lay explanatory models for breast cancer persist. The objective of this project was to explore lay perception of causes, severity, presenting symptoms and treatment of breast cancer.

### Research frontiers

Approaches are needed to rapidly assess the state of public knowledge to order to tailor health education that might remediate ignorance and misperceptions, especially mis-information that could interfere with timely participation in programs of screening and care. This case report illustrates the use of open-ended questions to assess knowledge of relevance to the early detection of breast cancer.

### Innovations and breakthroughs

This case study has unearthed more sheer lack of information and lay misperceptions of how breast cancer presents itself that have been shown to prevail in other settings. The use of open-ended questions permitted a so-called "rapid-ethnographic" approach to characterizing knowledge, one that with the potential to uncover richer information than forced-choice questions.

### Applications

Since Kenya has proposed implementing a breast cancer control campaign that lacks a public health education component, the data suggest that such a component may be necessary and could be tailored to remediate apparent deficiencies. Other ministries of health may wish to contemplate the use of open-ended questions to characterize population-based knowledge of chronic diseases that are emerging as major causes of morbidity and mortality.

### Terminology

"Open-ended" questions are ones that pose a question but do not force the respondent to choose among limited response options. Open-ended questions require interviewers to record verbatim responses that are subjected to text-based qualitative analysis once compiled. Open-ended questions avoid one disadvantage of forced-choice (multiple choice or single-best answer) questions, which require good knowledge of what the respondent pool will be likely to say about a question *a priori*.

### Peer-review

The manuscript by Naanyu *et al* presents an important study exploring breast cancer awareness, knowledge and practices in Western Kenya. According to the results of a survey of people's knowledge and beliefs concerning breast cancer, the authors found significant ignorance and misperceptions. The major limitation of such research is whether its results apply elsewhere. The major strength is illustration of a methodologic approach others may wish to emulate.

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ORIGINAL ARTICLE

Prospective Study

# Effects of selenomethionine on acute toxicities from concurrent chemoradiation for inoperable stage Ⅲ non-small cell lung cancer

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## Abstract

**AIM:** To prospectively determine the safety and tolerability of oral L-selenomethionine (SLM) with concurrent chemoradiation (CCRT) for Stage Ⅲ non-small cell lung cancer (NSCLC) and estimate if the incidence and/or severity of adverse events could be reduced by its use.

**METHODS:** Sixteen patients with stage Ⅲ NSCLC were accrued to this single arm, phase Ⅱ study. CCRT consisted of radiation given at 2 Gy per fraction for 30-33 fractions, 5 d per week with concurrent weekly Ⅳ paclitaxel 50 mg/m$^2$ followed by carboplatin dosed at an area under the time-concentration curve of 2. SLM was dosed in a loading phase at 4800 μg twice daily for one week prior to CCRT followed by once daily dosing during treatment.

**RESULTS:** No selenium-related toxicity was observed. Analysis revealed grade 3 or higher esophagitis in 3 of 16 patients (19%), pneumonitis in 0, leukopenia in 2 (12.5%), and anemia in 1 (6%); the latter two were significantly reduced when compared to the protocol-stated expected rate of 35% ($P$ = 0.045 for leukopenia, and $P$ < 0.01 for anemia). Median overall survival was 14.9 mo and median failure-free survival was 9 mo (95%CI: 3.3-21.5).

**CONCLUSION:** There may be some protective benefit of selenium in the setting of CCRT for inoperable NSCLC. The data suggests decreased rates of myelosuppression when compared to similarly-treated historical and contemporary controls. Further evaluation of selenium in this setting may be warranted.


**Key words:** Selenium; Chemoprotective; Radioprotector; Toxicity; Radiotherapy



**Core tip:** This was a prospective international phase Ⅱ trial with 16 patients seeking to evaluate the effect of selenomethionine on acute toxicity in the setting of concurrent chemoradiaiton for locally advanced, inoperable non-small cell lung cancer. Selenium proved to be well tolerated and led to significantly reduced rates of myelosuppression.

Mix M, Ramnath N, Gomez J, de Groot C, Rajan S, Dibaj S, Tan W, Rustum Y, Jameson MB, Singh AK. Effects of selenomethionine on acute toxicities from concurrent chemoradiation for inoperable stage Ⅲ non-small cell lung cancer. *World J Clin Oncol* 2015; 6(5): 156-165 Available from: URL: http://www.wjgnet.com/2218-4333/full/v6/i5/156.htm DOI: http://dx.doi.org/10.5306/wjco.v6.i5.156

## INTRODUCTION

Concurrent chemoradiation (CCRT) is the standard of care for inoperable, locally-advanced non-small cell lung cancer (NSCLC)[1]. Even though there have been improvements in radiation delivery and less utilization of elective nodal irradiation (ENI), a significant proportion of patients continue to experience severe acute toxicities including esophagitis, myelosuppression and pneumonitis. Grade 3-4 esophagitis rates as high as 28% were reported in one study utilizing weekly carboplatin and paclitaxel in CCRT for inoperable NSCLC[2]. A meta-analysis reports that the addition of chemotherapy to radiation in this setting increases severe esophagitis rates from 4% to 18%[3]. Significant rates of high grade leukopenia and neutropenia have also been seen in the literature, with upper limits approximating 50%[4,5].

Given their short- and long-term effects on quality of life and the potential to interrupt therapy, it is important to reduce the incidence and severity of acute toxicities caused by CCRT. Several pharmacological agents that may protect against normal tissue toxicity have been studied, including organic thiophosphates such as amifostine. Although some protection by this agent during CCRT in NSCLC was suggested in Radiation Therapy Oncology Group (RTOG) study 9801, amifostine was not able to significantly reduce esophagitis rates[6,7]. In addition, side effects including marked hypotension and the requisite Ⅳ route of delivery have precluded its widespread adoption in this setting.

Preclinical data from our institution and others suggest that the organic selenium (Se) compound L-selenomethionine (SLM) has properties that confer protection on normal tissues from toxicities of chemotherapy and radiation, while enhancing their anti-tumor effects[8-17]. The dual properties of SLM to reduce normal tissue toxicity while increasing antitumor efficacy led to consideration[18] and implementation of early human studies combining chemotherapy with Se in a variety of tumors[19,20]. On the basis of this early clinical work, we hypothesized that SLM might reduce the major toxic effects of CCRT in NSCLC patients including esophagitis, pneumonitis, and myelosuppression. This might, in turn, reduce treatment interruptions and lead to increased local tumor control and survival. We therefore conducted a phase Ⅱ multi-institutional study to determine the effects of SLM on acute toxicities as well as efficacy of concurrently-administered carboplatin, paclitaxel, and radiation in patients with unresectable stage Ⅲ NSCLC.

## MATERIALS AND METHODS

### *Patient selection*

Patients with Stage Ⅲ NSCLC from Roswell Park Cancer Institute (RPCI) and Waikato Hospital were eligible for recruitment. The study was approved by the RPCI institutional review board and the Northern Y Regional Ethics Committee in New Zealand. Patients were screened for eligibility during clinic visits. Eligible

patients were given information describing the study in readily understandable language and detailing the investigational nature of the study. Patients were subsequently required to provide their written consent in order to participate in the study. ClinicalTrials.gov identifier: NCT00526890.

### Patient eligibility

Patients were eligible if: they had histologically- or cytologically-confirmed stage ⅢA-ⅢB squamous cell carcinoma, adenocarcinoma, large cell carcinoma, or NSCLC not otherwise specified; age ≥ 18; ECOG PS 0-1; weight loss ≤ 5% in the 3 mo before study entry; no invasive malignancy in the prior 3 years; no prior radiotherapy to the thorax/neck or chemotherapy; no pleural effusion; serum creatinine ≤ 1.5 mg/dL; serum bilirubin and glutamic-oxaloacetic transaminase ≤ 1.5 times the upper limit of normal; hemoglobin ≥ 8.0 g/dL; absolute granulocyte count ≥ 2000/mm$^3$; and platelet count ≥ 100000/mm$^3$. Patients were ineligible if they: were pregnant or of childbearing potential and refusing appropriate contraception; had a prior myocardial infarct within the preceding 6 mo or had symptomatic heart disease (angina, congestive heart failure, uncontrolled arrhythmia); had a serious concomitant infection including post-obstructive pneumonia; or had undergone major surgery other than biopsy in the previous 2 wk.

### Patient evaluation and follow-up

The pre-treatment evaluation included a complete medical history and physical examination with determination of the Eastern Cooperative Oncology Group (ECOG) performance status (PS) and questions about recent weight loss and concurrent non-malignant diseases. A complete blood count with differential and platelet count was also required, along with a biochemical survey, measurement of electrolytes, magnesium and serum transaminase levels, all of which had to be performed within 14 d of enrolment. Imaging studies included computed tomography (CT) scans of the chest and upper abdomen and CT or magnetic resonance imaging of the brain. At least weekly, an interval history and physical examination was performed by a member of the study team to prospectively assess and collect data regarding PS, weight loss, and symptoms of esophagitis and other toxicities. The complete blood count with differential, absolute granulocyte count, platelet count and serum creatinine levels were determined weekly. Particular attention was paid to patients' pain levels and the medications required for control of symptomatic esophagitis. Toxicity was scored using National Cancer Institute Common Toxicity Criteria (CTC), version 3.0. Patients were evaluated with the same assessments 1 and 3 mo after treatment completion, at 3-mo intervals for 2 years then every 6 mo. CT scanning of the thorax was performed 3 mo after treatment and at each follow-up visit thereafter. Blood selenium levels were drawn at baseline, then weekly for the duration of therapy in order to monitor response of serum levels to supplementation.

### Study design

An exact two-stage design was used to evaluate excess toxicity early on, and cease treatment if appropriate. The goal was for 10 patients in stage 1, with plan to stop accrual if ≥ 4 patients experienced excessive toxicity. Stage 2 was planned to accrue an additional 20 patients, with the bar set at ≥ 7 patients with excessive toxicity for stopping early. Total accrual was therefore set at 30 patients, and was expected to take a maximum of 6 years. Excessive toxicity was defined as: Grades 3-4 esophagitis, pneumonitis, or myelosuppression which caused delay of CCRT > 2 wk despite corrective measures. The study closed due to poor accrual in 2010 after the recruiting 16 patients. Changing practice patterns including desires to use alternative systemic agents, and a shift away from ENI (see below) were the primary reasons for unacceptable accrual. The decision to terminate the trial was made by the investigators for the aforementioned reasons. As the accrual goal exceeded 50%, we elected to retrospectively evaluate the collected data according to protocol specifications.

### Radiation therapy

CT simulation was performed for all patients. Intravenous contrast was recommended but not required for improved delineation of targets. Dose inhomogeneity corrections were not used. The radiation therapy (RT) delivered was determined according to optimal dose distribution. Dose was 2 Gy per fraction, 30-33 fractions, 5 d per week for 6-6½ wk. Patients received megavoltage portal imaging for verification prior to treatment initiation, and at least weekly thereafter. Patients were treated with megavoltage equipment with at least 6 MeV photons using 3D conformal radiotherapy techniques. The planning target volume included a minimum margin of 1.5 cm around the gross tumor volume (GTV). A clinical tumor volume (CTV) was treated to an intermediate dose ranging from 40-46 Gy. The CTV included the elective nodal volumes, consisting of ipsilateral hilar, upper and lower paratracheal (levels 2, 4), and subcarinal lymph nodes. Aortic nodes (levels 5-6 were also included for left sided tumors. Ipsilateral supraclavicular lymph nodes were included if the primary tumor was located in the upper lobe or mainstem bronchus. Electron beams were permitted for elective treatment of supraclavicular lymph nodes. Individual custom blocking was used to spare normal tissues. Each field was treated each day. Protocol-specified dose constraints were as follows; total lung V20 < 32%, esophagus V55 < 66%, mean esophageal dose < 45 Gy, and maximal spinal cord dose < 45 Gy.

### Chemotherapy and SLM

Patients did not receive induction chemotherapy. Concurrent chemotherapy consisted of paclitaxel (50 mg/m$^2$) infused over 1 h, followed by carboplatin dosed at an area under the plasma concentration-time curve of 2 mg/mL per minute, infused over 30 min. These were given intravenously once weekly, 30 min before thoracic

**Table 1 Patient characteristics ($n$ = 16)**

| Characteristic | $n$ (%) | Characteristic | $n$ (%) |
|---|---|---|---|
| Sex | | Performance status | |
| Male | 5 (31) | 0 | 7 (44) |
| Female | 11 (69) | 1 | 9 (57) |
| Age | | Stage | |
| Mean | 63.25 | ⅢA | 7 (44) |
| Median | 61 | ⅢB | 7 (44) |
| Range | 49-78 | Ⅲ NOS | 2 (13) |
| Race | | Smoking status | |
| White | 11 (69) | Current | 3 (19) |
| Black | 2 (13) | Former | 13 (81) |
| Other | 3 (19) | | |
| Histology | | | |
| Adenocarcinoma | 8 | | |
| Squamous Cell | 6 | | |
| NSCLC-NOS | 2 | | |

NSCLC: Non-small cell lung cancer; NOS: Not otherwise specified.

RT, for 6 wk, beginning on day 1 of RT. Patients received pre-medications and antiemetics as per institutional standards. The use of erythropoietin was permitted. The use of granulocyte colony-stimulating factors was discouraged, and was not allowed as prophylaxis, or with intent to prevent delay of protocol-specified therapy. SLM 800 μg capsules (Sabinsa Corp., NJ) were dosed as follows for a total of 7 wk: patients received loading doses of SLM 4800 μg orally twice daily for one week prior to beginning CCRT followed by a maintenance dose of 4800 μg daily for six weeks, or until the completion of therapy. This loading dosing schedule was based on pharmacokinetic modeling aiming to achieve a serum level prior to commencing CCRT that approximated the steady-state concentration expected with prolonged daily dosing of 4800 μg[19].

### *Treatment outcome*

Treatment response was determined as follows: Complete response (CR) required disappearance of all measurable disease, signs, symptoms, and biochemical changes related to the tumor. Partial response (PR) required a reduction of ⩾ 50% of the sum of the products of the perpendicular diameters of all measurable lesions. Stable disease (SD) required < 50% reduction and ⩽ 25% increase in the sum. An increase > 25% was registered as progressive disease (PD).

### *Statistical analysis*

The primary endpoint examined was toxicity resulting from SLM/CCRT (in particular, the anticipated esophagitis, pneumonitis and myelosuppression). Secondary endpoints included effects of SLM on efficacy and survival. A protocol-dictated 35% rate of CTC grade ⩾ 3 esophagitis, pneumonitis, and myelosuppression was utilized for comparative statistics. The lower bound of the statistical power for correctly concluding acceptable toxicity of SLM/CCRT is 0.81 if the true toxicity rate is reduced by 20% compared to historical controls. A 0.05 level was set for Type 1 error, and 95%CI were calculated using the Jennison and Turnbull method[21]. One-sided $P$-values were calculated. Median, overall, and failure-free survival rates were calculated using the Kaplan-Meier method, with 95%CI.

**Table 2 Adverse events**

| $n$ = 16 | Grade 1-2 | Grade 3 | Grade 4 | Grade 3-4 (%) |
|---|---|---|---|---|
| Esophagitis | 6 | 3 | 0 | 19 |
| Pneumonitis | 4 | 0 | 0 | 0 |
| Anemia | 7 | 1 | 0 | 6 |
| Leukopenia | 8 | 2 | 0 | 13 |
| Neutropenia | 4 | 0 | 0 | 0 |
| Hypokalemia | 3 | 0 | 1 | 6 |
| Fatigue | 7 | 1 | 0 | 6 |
| Weight loss | 2 | 0 | 0 | 0 |

## RESULTS

After the first 10 patients were enrolled, no excess toxicity was noted and the cohort was expanded. Patients were enrolled between January 2007 and December 2009. After enrollment of 16 patients, there was still no selenium-related excess toxicity but the study was closed due to poor accrual. Pre-treatment characteristics are shown in Table 1.

Treatment was completed as planned in 14/16 (87.5%) patients. Treatment was discontinued indefinitely in one patient due to severe esophagitis. In a second patient, the patient was given a treatment break, and was subsequently re-planned using an IMRT technique, thus was no longer receiving protocol-specified treatment. These discontinuances did not meet stopping rules per protocol, as they were not deemed to be selenium-related. From available dosimetric data (13/16), median radiation dose to the GTV and CTV was 66 Gy and 46 Gy respectively. Regarding mean esophageal dose in treated patients, mean and median values were 19 Gy and 21 Gy respectively. The median follow-up time was 14.9 mo (3.3-62). Adverse events are summarized in Table 2. Grade 3 esophagitis was seen in 3 patients, none of whom were current smokers [18.75% (95%CI 4.05-45.7)]. There were no instances of grade 3-4 pneumonitis, and rates of grade 3-4 anemia, leukopenia, and neutropenia were 6% (95%CI: 0.16%-30.2%), 12.5% (95%CI: 1.55-38.4), and 0% respectively. When compared to the protocol-specified expected toxicity rate of 35%, anemia was significantly reduced ($P$ < 0.01) when compared to the protocol-specified expected toxicity rate of 35%, leukopenia was significantly reduced ($P$ = 0.045). There were no adverse effects attributed to SLM alone.

Median overall survival (OS) and failure-free survival (FFS) were 14.9 mo (95%CI: 7.5-43.8) and 9.1 mo (95%CI: 3.3-21.5) respectively. Eight patients (50%) had a PR, 4 patients (25%) had SD, and 3 patients (19%) exhibited PD as their best response. The overall

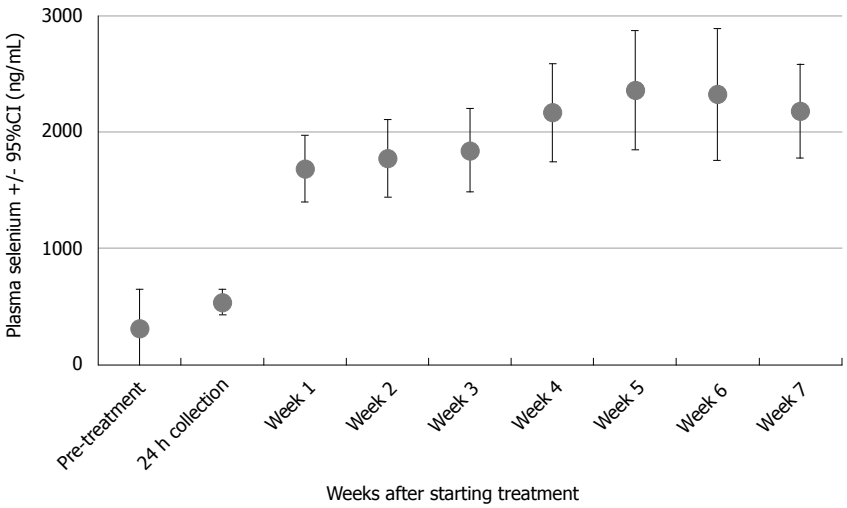


**Figure 1 Serum selenium levels before and during concurrent chemoradiation.**

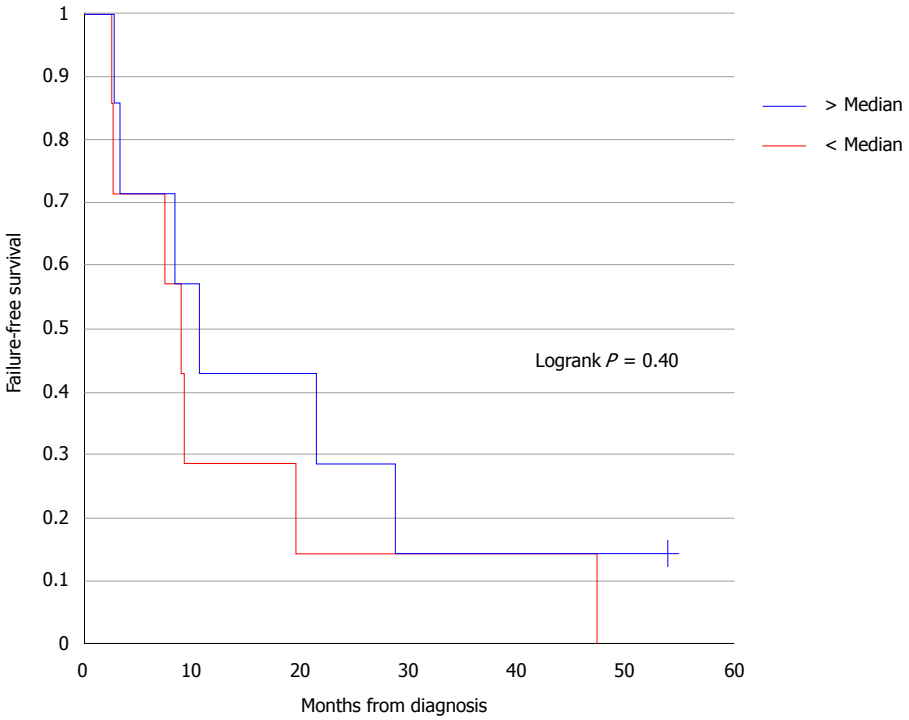


**Figure 2 Failure-free survival stratified by baseline selenium level.**

response rate was 50% (95%CI: 24.7-75.4). One patient was not evaluable for response.

### *Selenium levels*

Baseline serum Se levels were available for 14 of 16 patients: the mean (standard deviation) value was 304 (604) ng/mL and the median value was 98 ng/mL. Trough Se levels rose for all patients during supplementation, shown in Figure 1. Levels were available for 14 of 16 patients at week 6, when mean and median values were 2324 and 2179 ng/mL respectively.

Baseline Se values and their relationship to FFS were analyzed. Baseline levels were dichotomized into two groups relative to the median value. No significant correlation was detected between baseline Se and FFS ($P$ = 0.4016) (Figure 2). Similarly, baseline values were compared to severe esophagitis and/or myelosuppresion rates using Fisher's exact test and there was no significant association with either toxicity ($P$ = 1.00). Due to a paucity of data, an association between toxicity outcomes and week 7 serum Se levels could not be analyzed.

## DISCUSSION

The addition of SLM 4800 μg daily to CCRT in inoperable

**Table 3 Esophagitis and pneumonitis rates in prospective trials evaluating concurrent chemoradiation in inoperable stage Ⅲ non-small cell lung cancer**

| Ref. | Year | Design | No. of patients | Nodes | RT dose (Gy) | Chemo | Grade 3-4 esophagitis | Grade 3-4 pneumonitis |
|---|---|---|---|---|---|---|---|---|
| Furuse *et al*[27] | 1999 | Ind → RT | 314 | ENI | 56 | Cis/Vnd/Mit | 3% | - |
| | | CCRT | | | 56[1] | | 2% | 1% |
| Zatloukal *et al*[28] | 2004 | Ind → RT | 102 | ENI | 60 | Cis/Vno | 4% | |
| | | CCRT | | | | | 18% | 4% |
| Fournel *et al*[26] | 2005 | Ind → RT | 205 | ENI | 66 | Cis/Vno | 2% | |
| | | CCRT → Cons | | | | Cis/Eto → Cis/Vno | 32% | 5% |
| Belani *et al*[4] | 2005 | Ind → RT | 257 | IFRT | 63 | Cbp/Pac | - | - |
| | | Ind → CCRT | | | | | 19% | 4% |
| | | CCRT → Cons | | | | | 28% | 16% |
| Vokes *et al*[2] | 2007 | CCRT | 366 | ENI | 66 | Cbp/Pac | 28% | 4% |
| | | Ind → CCRT | | | | | 30% | 10% |
| Belderbos *et al*[25] | 2007 | Ind → RT | 158 | ENI | 66[2] | Cis/Gem | 5% | |
| | | CRT | | | | Cis | 14% | 18% |
| Socinski *et al*[41] | 2008 | Ind → CCRT | 69 | "ENI discouraged | 74 | Cbp/Pac | 16% | 16% |
| | | Ind → CRT | | but allowed" | | Cbp/Gem | 39% | 37% |
| Blumenschein *et al*[23] | 2011 | CCRT | 87 | "Selective nodal | 63 | Cbp/Pac/ | 8% | 22% |
| | | | | irradiation" | | Cet | | |
| Curran *et al*[42] | 2011 | Ind → RT | 407 | ENI | 63 | Cis/Vnb | 4% | - |
| | | CCRT | | | 63 | Cis/Vnb | 22% | 13% |
| | | CCRT | | | 69.6[3] | Cis/Eto | 45% | 15% |
| Hoang *et al*[5] | 2012 | CCRT | 546 | IFRT | 60 | Cbp/Pac | < 1% | 1% |
| | | CCRT + Thl | | | | Cbp/Pac/Thl | < 1% | 1% |

[1]Split course; [2]2.75 Gy/d; [3]BID (twice daily). Cis: Cisplatin; Vnd: Vindesine; Mit: Mitomycin; Vno: Vinorelbine; Eto: Etoposide; Cbp: Carboplatin; Pac: Paclitaxel; Gem: Gemcitabine; Cet: Cetuximab; Vnb: Vinblastine; Thl: Thalidomide; Doc: Docetaxel; Ind: Induction chemotherapy; RT: Radiation therapy; CCRT: Concurrent chemoradiotherpy; Cons: Consolidation; ENI: Elective nodal irradiation; IFRT: Involved field radiation therapy; NSCLC: Non-small cell lung cancer.

stage Ⅲ NSCLC was safe and well-tolerated. To our knowledge, this is the first study evaluating the use of SLM in this population. Leukopenia, anemia, neutropenia, and esophagitis rates appear to be improved compared to the protocol-specified incidence of 35%, however this figure was likely set too high in the context of more recent publications with regard to esophagitis. A more reasonable estimate for high grade esophagitis would be 18%[3]. Regarding the myelosuppresive endpoints, estimates based on similarly treated patients for leukopenia, anemia, and neutropenia, are 23%-51%, 3%-10%, and 15%-51%, respectively[2,4,5]. Given these estimates, the addition of selenium may have improved myelosuppresion.

### *Expected toxicity rates with chemoradiation in stage Ⅲ NSCLC*

At the time of this protocol's inception, treatment of uninvolved regional nodal basins was standard of care, thus trials which utilized ENI are the best comparators for these data. Regarding esophagitis, our rate of 19% esophagitis compared favorably to the CCRT arm using both ENI and the same chemotherapeutic regimen in a phase Ⅲ trial by Vokes *et al*[2] at 28%. Based on the observation that ENI doesn't significantly reduce regional recurrence[22] while increasing toxicity, current paradigms have shifted towards involved field radiotherapy (IFRT) with consequent decreases in normal tissue irradiation and therefore toxicity. As expected, our results exceed esophagitis rates seen in similar patients treated using an IFRT technique, reported as low as 1%-8%[5,23,24]. One such trial, however, revealed numerically-increased rates of esophagitis compared to ours, with grade 3-4 toxicity of 28%[4]. Table 3 summarizes esophagitis rates for several studies evaluating CCRT in Stage Ⅲ NSCLC, using a variety of CTV parameters and concurrent chemotherapeutic regimens.

There were no instances of grade ≥ 3 pneumonitis in our study, which compares favorably with studies using a comparable CCRT regimen as well as other chemoradiation regimens (Table 3).

Regarding myelosuppression, we report rates of anemia, leukopenia, and neutropenia of 6%, 13%, and 0% respectively. The leukopenia rate is significantly decreased from the 35% benchmark dictated in protocol. The rates of both leukopenia and neutropenia are numerically decreased when compared to patients receiving CCRT with identical chemotherapeutic regimens (Table 4). The avoidance of severe neutropenia by adding SLM, if confirmed, would be clinically significant.

### *Expected response rates and survival with CCRT in stage Ⅲ NSCLC*

The current trial reports 50% PR as best response (95%CI: 24.7-75.4), and 19% PD. This figure is somewhat less than expected from historical controls. Vokes *et al*[2] reported 67% CR/PR and 9% PD, while Blumenschein *et al*[23] report 62% and 11%. Our results should be interpreted with caution given small patient numbers and wide confidence intervals, remembering

**Table 4 Myelosuppression rates from prospective trials evaluating concurrent chemoradiation in inoperable non-small cell lung cancer**

| Ref. | Year | Design | No. of patients | Chemo | Grade 3-4 | | |
|---|---|---|---|---|---|---|---|
| | | | | | Anemia | Leukopenia | Neutropenia |
| Belani *et al*[4] | 2005 | CCRT → Cons | 92 | Cbp/Pac | 10% | 51% | 26% |
| Vokes *et al*[2] | 2007 | CCRT | 184 | Cbp/Pac | 5% | 36% | 15% |
| Hoang *et al*[5] | 2012 | CCRT | 275 | Cbp/Pac | 3% | 23% | 51% |
| Blumenschein *et al*[23] | 2011 | CCRT | 87 | Cbp/Pac/Cet | "Blood/bone marrow": 48% | | |

Cbp: Carbo; Pac: Paclitaxel; Cet: Cetuximab; CCRT: Concurrent chemoradiotherpy; Cons: Consolidation.

that preclinical work with SLM strongly suggests a benefit in terms of tumor response with RT. However, it is important to be critically aware of the slightly lower response rate seen in this study when compared to similarly treated historical cohorts. It is critically important to be vigilant of tumor response rates when investigating agents purported to protect normal tissues.

The median OS in the current study is 14.9 mo. Similar survival rates were seen in larger groups of similarly-treated patients, ranging from 12-16.6 mo[2,4,25-28]. It should be noted that more recently-published series, using more contemporary radiation methods (*i.e.*, IFRT as opposed to ENI) have demonstrated improved survival. For example, RTOG 0117 treated similar patients with similar chemotherapy, but used higher doses of radiation, and did not electively treat nodal volumes. This phase Ⅱ study reported median survival of 25.9 mo[29]. It is not clear if the data presented here are directly comparable to this more modern cohort. Nevertheless, this represents a more current estimation of median survival in this patient population.

### *Prior studies combining chemotherapy and selenium*

Broadly supportive of our findings, prior studies have found that Se compounds may limit chemotherapy toxicity. Jahangard-Rafsanjani *et al*[30] found that selenium significantly reduced oral mucositis in the setting of busulfan and cyclophosphamide-based high-dose chemotherapy followed by allogeneic stem cell transplantation for leukemia. In this 77-patient double-blind, randomized, placebo-controlled study, those receiving SLM (200 μg BID) experienced significantly less grades 3-4 oral mucositis (10.8% *vs* 35.1%, $P < 0.05$). The duration of grades 2-4 oral mucositis was also significantly shorter in the selenium group (3.6 ± 1.84 *vs* 5.3 ± 2.2 d, $P = 0.014$). Another trial evaluating Se in the form of selenokappacarrageenan given prior to cisplatin-based chemotherapy led to higher white blood cell counts on day 14 than in its absence; no comment on antitumor effect was made[31].

In a double-blind trial involving 62 women receiving cisplatin and cyclophosphamide for ovarian cancer, patients were randomized to antioxidant capsules with or without Se as selenized yeast[32]. Those receiving Se were found to have fewer toxicities including nausea, vomiting, stomatitis, alopecia, abdominal pain, weakness, and loss of appetite (all with $P < 0.05$). A formal assessment of antitumor activity wasn't performed, however CA-125 levels were numerically lower in the Se group. Another trial randomized 50 patients receiving cisplatin-based chemotherapy to concurrent supplementation with sodium selenite, vitamin C and vitamin E *vs* placebo. There was no observed difference in toxicity, although 64% of patients within the experimental arm were noncompliant with therapy due to GI side effects and serum Se levels did not differ between the two groups, suggesting that Se intake was not significant[33]. A series of small randomized controlled trials has been reported from one group using sodium selenite 200 μg/kg per day in conjunction with chemotherapy for patients with non-Hodgkin lymphoma[34,35]. While outcomes varied, the Se groups tended to have less toxicity. In the 2007 report, an increased response rate was seen, and a small but statistically significant survival advantage was seen in those achieving CR[35]. Finally, a phase Ⅰ study from our group has shown that SLM did not significantly impact irinotecan toxicity[19].

### *Combining radiotherapy and selenium*

Other studies have examined the potential of Se to mitigate radiation-induced toxicity. Muecke *et al*[36], in a multi-center open-label randomized phase Ⅲ study with the primary endpoint of improving baseline Se levels, found in 81 post-operative patients with cervical or endometrial cancer a significant reduction in grade ⩾ 2 diarrhea (20.5% *vs* 44.5%, $P = 0.04$) in the group given selenite 500 μg/d with RT and 300 μg/d on non-RT days compared to controls. Büntzel *et al*[37] performed a randomized phase Ⅱ study of 39 patients with advanced stage squamous cell carcinoma of the head and neck (HNSCC) and found less obvious benefit using the same Se regimen as Muecke. There was no statistically significant incidence of severe toxicity overall; however the weekly patient analysis showed a significant reduction of dysphagia in the experimental group during the final week of irradiation ($P = 0.05$) and overall trends towards prevention of taste loss.

Our study group conducted a phase Ⅱ, randomized, placebo-controlled study in 18 HNSCC patients undergoing CCRT with cisplatin, in which SLM supplementation at 3600 μg/m$^2$ per day was well-tolerated. While no statistically significant differences were noted in acute CCRT toxicities, nor in patient-reported quality of life measures, a trend was seen for decreased rates of severe mucositis[38].

### *Plasma selenium levels*

Trough Se levels rose in all patients for whom baseline plasma Se values were available. No association was seen between baseline Se levels and toxicity in this cohort. A recent review of Se supplementation highlighted the tendency of serum Se levels to fall during the course of radiotherapy[39]. This fact suggests that there may be a correlation between toxicity and Se levels. A report from Eroglu *et al*[40], however, found no correlation between Se levels and radiation toxicity. This cohort was found to have plasma Se levels between 56-58 ng/mL, which is below the reported levels seen in those undergoing supplementation[19]. The association of plasma Se levels and incidence of radiation of chemotherapy induced toxicity remains unclear.

### *Limitations*

Our study is limited by a number of factors that require attention. First, the early closure due to poor accrual resulted in a smaller than intended cohort. This calls into question the observed decreased rate of myelosuppression (albeit a significant one), given small patient numbers. These results may be due to other factors, and their influence can't be assessed without a placebo group. Second, the 35% benchmark set for grade $\geqslant$ 3 esophageal toxicity in this patient population may need to be reconsidered in light of newer radiation techniques, including the shift towards IFRT as opposed to ENI. The true rate of severe esophagitis in this setting should perhaps be closer to 20%. Nevertheless, we did see a decrease relative to the most closely-matched cohort.

In conclusion, SLM 4800 μg/d was safe and well tolerated when combined with CCRT in patients with inoperable stage Ⅲ NSCLC in this multicenter, international, phase Ⅱ trial. The data suggests the feasibility of investigating SLM to reduce rates of myelosuppresion. Response rates were slightly less than expected when compared to the aforementioned controls. Survival rates are comparable when considering those treated with similar radiation techniques. Treatment-induced toxicity continues to be a significant issue, thus there may be some role for future investigation of Se as a protector from chemotherapy related toxicity, and possibly from radiotherapy-related toxicity in NSCLC.

## COMMENTS

### *Background*

Concurrent chemoradiation (CCRT) is the standard of care for advanced stage, inoperable non-small cell lung cancer (NSCLC). The use of CCRT has been shown to improve survival, but can lead to significant treatment-related toxicity. Selenium compounds have shown promise in their ability to confer protection on normal tissues during treatment with radiotherapy and/or chemotherapy. The current trial was designed to evaluate the tolerability of selenomethionine (SLM) and its potential to reduce the incidence/severity of treatment-related toxicity during CCRT.

### *Research frontiers*

Outcomes of patients treated with CCRT are improving, and there is increasing focus on ways to minimize toxicity during cancer treatment. In this study, there is suggestion that SLM may reduce rates of myelosuppression compared to similarly-treated historical controls.

### *Innovations and breakthroughs*

The literature suggests a benefit for selenium in protection from radiation and chemotherapy induced toxicity. The current trial adds to that literature, with the suggestion of decreased rates of myelosuppression with the addition of SLM to CCRT in locally advanced NSCLC.

### *Applications*

This study serves as additional evidence supporting the investigation of selenium's potential role in mitigating chemotherapy and radiotherapy toxicity.

### *Terminology*

SLM: A naturally occurring amino acid containing selenium, found in certain nuts, beans, and legumes. Myelosuppression: The decrease in production of blood cells that compose the immune system (leukocytes), delivering oxygen to tissues (erythrocytes), and/or those responsible for blood clotting (thrombocytes).

### *Peer-review*

The authors have performed a good study, the manuscript is interesting.

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ORIGINAL ARTICLE

Randomized Controlled Trial

# Randomized phase Ⅱ trial of selenomethionine as a modulator of efficacy and toxicity of chemoradiation in squamous cell carcinoma of the head and neck

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Author contributions: Singh AK participated in study design, data collection, data analysis, and drafting of the manuscript; Mix M participated in data analysis, and drafting of the manuscript; Tills M participated in study design, data collection, and data analysis; Dibaj S participated in study design and performed statistical analysis; Groman A participated in study design and performed statistical analysis; Jaggernauth W participated in study design, as well as data collection and analysis; Rustum Y participated in study design and data analysis, including selenium analyes; Jameson MB participated in designing the study, data analysis, selenium analyses, and drafting the manuscript; all authors read and approved the final manuscript.

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## Abstract

**AIM:** To investigate whether selenomethionine (SLM) reduces mucositis incidence in patients with head and neck squamous cell cancer (HNSCC) undergoing concurrent chemoradiation (CRT).

**METHODS:** In this multi-institutional, randomized, double-blind phase Ⅱ trial, patients with Stage Ⅲ or Ⅳ HNSCC received SLM 3600 μg/m$^2$ or placebo twice daily

for 7 d prior to CRT, once daily during CRT, and daily for 3 wk following CRT. CRT consisted of 70 Gy at 2 Gy per fraction with cisplatin 100 mg/m$^2$ Ⅳ on days 1, 22, and 43.

**RESULTS:** Eighteen patients were randomized, 10 received SLM, and there were no differences in baseline factors. There was no difference in mucositis or patient-reported side effects between groups. There was no difference in overall or relapse-free survival at 12 mo.

**CONCLUSION:** Addition of SLM to CRT for HNSCC was well-tolerated but did not lower the incidence of severe mucositis or improve quality of life or survival outcomes.

**Key words:** Selenium; Chemotherapy; Radiation therapy; Squamous cell cancer; Radioprotector; Chemoprotective



**Core tip:** This is an international, randomized, double-blind, placebo-controlled phase Ⅱ trial evaluating the addition of selenomethionine (SLM) to concurrent chemoradiation for locally advanced squamous cell carcinoma of the head and neck. The addition of SLM was well tolerated, but did not lead to a difference in the rates of mucositis, or quality of life outcomes *vs* placebo.

Mix M, Singh AK, Tills M, Dibaj S, Groman A, Jaggernauth W, Rustum Y, Jameson MB. Randomized phase Ⅱ trial of selenomethionine as a modulator of efficacy and toxicity of chemoradiation in squamous cell carcinoma of the head and neck. *World J Clin Oncol* 2015; 6(5): 166-173 Available from: URL: http://www.wjgnet.com/2218-4333/full/v6/i5/166.htm DOI: http://dx.doi.org/10.5306/wjco.v6.i5.166

## INTRODUCTION

Head and neck squamous cell cancers (HNSCC) are occurring with increasing incidence[1]. Worldwide, approximately 350000 diagnoses are expected annually[2]. HNSCC is often related to tobacco and alcohol exposure[3], human papilloma virus exposure[4], or some combination of these factors.

Over the past 2 decades, concurrent chemoradiation therapy (CRT) without surgery has demonstrated the ability to cure many HNSCC patients and preserve important functions such as speech and swallowing. Nevertheless, even with the improvements of modern therapy, 5 year overall survival (OS) can be as low at 30%-40%[5,6]. Moreover, both the acute and late side effects with concurrent CRT (*e.g.*, mucositis, xerostomia, *etc.*) can be severe. Acute effects can be sufficiently severe to necessitate a treatment "break" during therapy. Each day of treatment prolongation can reduce local control and survival by 2%-5%[7-9].

Pre-clinical literature suggested that organic selenium (Se) compounds including L-selenomethionine (SLM) might have both anti-tumor[10-15] and anti-toxicity[12,14,16-19] effects when combined with CRT, potentially widening the very narrow therapeutic window in HNSCC. This promising dual anti-tumor and anti-toxicity effect lead to human studies combining chemotherapy and Se supplementation[20-22].

This double blind, randomized, multi-institutional trial was performed to assess whether SLM supplementation can reduce the incidence of grades 3 or 4 mucositis in HNSCC patients treated with concurrent CRT over 7 wk.

## MATERIALS AND METHODS

### *Eligibility*

Patients with stage Ⅲ-Ⅳ HNSCC who were planned for definitive treatment with 7 wk of concurrent cisplatin and radiation were offered the opportunity to participate on this phase Ⅱ trial. All patients had biopsy-proven locally-advanced HNSCC of oral cavity, oropharynx, hypopharynx, larynx, nasopharynx or paranasal sinuses, and had an eastern cooperative oncology group (ECOG) performance status of 0-2. Excluded were those who underwent definitive surgery (anything beyond excisional biopsy) or those with Stage Ⅳc disease (non-regional metastatic disease), as well as those with malignancy within the previous five years. Prior radiotherapy was not permitted. HIV or hepatitis C positivity, platinum hypersensitivity, inability to tolerate oral medications (in absence of feeding tube), symptomatic peripheral neuropathy, planned use of amifostine, and significant comorbidity were all excluding factors.

### *Trial design*

This double blind, placebo-controlled, randomized, multi-institutional trial was designed to assess whether SLM supplementation can reduce the incidence of grades 3 or 4 mucositis in HNSCC patients treated with concurrent CRT over 7 wk. The trial was planned to recruit 80 patients but, due to funding constraints, recruitment was suspended after 18 patients and an interim analysis was performed to see if a sufficiently promising effect could be discerned to warrant further funding.

The primary objective of this trial was to assess whether SLM reduces the incidence of grades 3 or 4 mucositis in HNSCC patients treated with concurrent CRT over 7 wk. Secondary objectives included assessment of the effect of SLM on tumor complete response (CR) rate, progression-free survival (PFS), OS and quality of life (QOL). In addition, an assessment of whether SLM reduces incidence and severity of other treatment-related toxicity including xerostomia, renal impairment, hearing loss, and myelosuppression was performed. In New Zealand patients only, an exploratory objective was to assess the impact of SLM on plasma free cisplatin and

plasma Se pharmacokinetics and on pharmacodynamics markers of biological activity of Se.

Written informed consent was obtained from all patients. Following registration and fulfillment of all eligibility criteria, patients were allocated to either the control or treatment arm in a 1:1 fashion using a permuted block randomization scheme based on blocks of size 4, stratified by site. The randomization list was generated by the study biostatistician. The trial was approved by the Roswell Park Cancer Institute Institutional Review Board and the Northern Y Regional Ethics Committee in New Zealand. The ClinicalTrials.gov identifier is NCT01682031.

### Radiation therapy

Radiation therapy structures and doses were consistent with the radiation therapy oncology group 0522 trial that was current at the time of this protocol. Briefly, the primary tumor, gross adenopathy and margin were treated to 70 Gy at 2 Gy per fraction in 35 daily treatments, 5 d a week over 7 wk. The at-risk but clinically-negative nodal regions were treated to 56 Gy in 35 daily treatments, 5 d a week over 7 wk.

Simulation was performed with appropriate immobilization in the treatment position. CT-based planning was required, and dose was specified at the ICRU-50 reference point. Volumes were created according to the 1993 ICRU Report #50[23]. 3D conformal planning was used, and IMRT was acceptable where feasible. Heterogeneity corrections were not utilized. The planning target volume was encompassed by the 90% isodose line. Beam energies of ⩾ 6 MeV were utilized.

### Cisplatin chemotherapy

Cisplatin was dosed at 100 mg/m$^2$ intravenously over 3 h in 1000 mL of normal saline on days 1, 22, and 43 of radiation therapy. Institution-specific standard premedication protocols for hydration and anti-emetics were used.

### SLM/placebo dosing

SLM was supplied as 800 μg capsules or matching placebo capsules (Sabinsa Corp., NJ). The number of capsules taken was the closest equivalent to a dose of 3600 μg/m$^2$. This dose was taken twice daily orally for 7 d prior to initiation of CRT, based on pharmacokinetic modeling aiming to achieve a serum level prior to commencing CRT that approximated the steady-state concentration expected with prolonged once-daily dosing of 3600 μg/m$^2$. Once CRT commenced, SLM/placebo dosing was once daily and continued until 3 wk after completion of CRT. Only for patients who were unable to tolerate capsules was dosing allowed division to 2-3 doses/d. Patients who were unable to swallow capsules or required tube feeding during or after CRT were asked to open the capsules and add the contents to their liquid feed. All patients were provided a diary to record capsule usage.

### QOL measures

QOL assessments were carried out with the EORTC quality of life questionnaire (QLQ) C-30 version 3, and the EORTC QLQ - H and N35 module. Patients completed QOL assessments at baseline visit, weeks 4 and 7 during treatment, 6-8 wk post-treatment, and at 3 mo intervals following completion.

### Follow-up

After completion of therapy, patients were seen in follow-up every 3 mo for 2 years, then every 6 mo to 5 years. This included physical examination and speech/swallow evaluation, assessment for adverse events and QOL, as well as documentation of weight, ECOG performance status, and adverse events. Relapse was defined as local, regional, or distant. Disease was measured where appropriate using the RECIST 1.0 Criteria[24]. Completion surgery to sites of remaining disease after CRT was performed if clinically appropriate.

### Statistics

Sample size calculations were based on a ⩾ grade 3 mucositis rate of 50% in published randomized studies of similar schedules of concurrent cisplatin and radiation for HNSCC. This study used the Phase Ⅱb 3-region design concept allowing decisions of: (1) clearly improved proportion with endpoint of interest; (2) promising benefits in the proportion with endpoint of interest; or (3) not worth pursuing[25]. With this design the chance of concluding there is an improvement in the proportion with ⩾ grade 3 mucositis remains the same as the standard 0.025 (one-sided) cut-off for evidence of benefit. The lower cut-off fixes a 12.5% chance of concluding SLM is not worth pursuing if the true benefit is a reduction from 50% to 30% in rates of ⩾ grade 3 mucositis.

The primary analysis was by intention-to-treat. Grade 3-4 mucositis, overall grades 3 and 4 toxicity, and tumor response were to be compared as difference in proportions with 95%CIs. Kaplan-Meier PFS curves and the proportion with an event at 1 year for PFS were to be compared simultaneously to obtain more global sensitivity to differences in time-to-event. The means between study groups and the proportion of patients completing CRT as initially planned were to be compared between groups using the student's *t* test. Comparisons will be adjusted for baseline differences in prognostic factors using logistic, Cox or linear regression as appropriate. Distributions of time to event variables will be estimated using the Kaplan-Meier method. Log-rank tests were used for the comparison of survival distributions among study groups. Continuous endpoints will be summarized using means, standard deviations and percentiles. Statistical analysis was done using SAS, version 9.1, statistical software (SAS Institute Inc., Cary, NC).

Three interim analyses were planned: the first after

**Table 1 Baseline characteristics**

| Characteristic | | Placebo ($n$ = 8) | Selenium ($n$ = 10) | $P$ value |
|---|---|---|---|---|
| Median age | | 55.5 | 59.5 | 0.700 |
| Male sex | | 7 | 10 | 0.165 |
| Race | White | 4 | 8 | 0.180 |
| | Other | 4 | 2 | |
| Best response | CR | 7 | 6 | 0.196 |
| | Not evaluable | 1 | 4 | |
| T stage | 1 | 3 | 0 | 0.063 |
| | 2 | 2 | 5 | |
| | 3 | 0 | 3 | |
| | 4 | 3 | 1 | |
| | X | 0 | 1 | |
| N stage | 1 | 0 | 1 | 0.103 |
| | 2 | 6 | 8 | |
| | 3 | 1 | 1 | |
| | X | 1 | 0 | |
| M stage | 0 | 5 | 10 | 0.105 |
| | X | 3 | 0 | |
| Stage group | ⅣA | 7 | 8 | 0.108 |
| | ⅣB | 1 | 1 | |
| | Unkn | 0 | 1 | |

Unkn: Unknown; CR: Complete response.

20 patients have completed CRT to ensure toxicity in the SLM arm was not unacceptably high and the second and third after one third and two thirds of the patients had been followed for at least 18 mo.

## RESULTS

Ten patients received SLM and 8 received placebo capsules. Median age was 57, 17 patients were male. There was no significant difference in race between the two groups. Stage was evenly matched, all patients having either stage ⅣA or ⅣB disease. See Table 1 for patient and disease characteristics.

### *Treatment compliance*

One patient randomized to SLM took one dose, complained of a "bad taste" and withdrew from the trial. All patients except one received the protocol-prescribed dose of radiation. This patient experienced a cerebrovascular event due to tumor involvement of the carotid artery, leading to abandonment of treatment. Eight patients received all three cycles of cisplatin as planned, 6 patients received two cycles, two received one cycle, and one patient had chemotherapy held altogether.

### *Adverse events*

There was no grade 4 mucosal toxicity. Grade 3 mucositis was seen in 3 of 8 patients in the placebo group, and 2 of 10 patients in the SLM group. These results are summarized in Table 2. Hearing dysfunction was reported in 1 patient from each group. Elevated creatinine was noted in 1 patient in the placebo group, and was not seen within the SLM group. Regarding myelosuppression

**Table 2 Mucositis scores**

| Mucositis grade | Placebo ($n$ = 8) | SLM ($n$ = 10) |
|---|---|---|
| 0 | 1 | 2 |
| 1 | 1 | 3 |
| 2 | 3 | 3 |
| 3 | 3 | 2 |

SLM: Selenomethionine.

**Table 3 Other adverse events**

| Toxicity ⩾ grade 2 | Placebo | SLM |
|---|---|---|
| Dermatitis | 0 | 2 |
| Dry mouth | 2 | 0 |
| Dysgeusia | 1 | 2 |
| Anemia | 1 | 0 |
| Leukopenia | 2 | 3 |
| Thrombocytopenia | 0 | 0 |
| Odyno-/dysphagia | 2 | 1 |
| Oral/throat pain | 2 | 0 |
| Phlegm | 1 | 3 |
| Elevated creatinine | 1 | 0 |
| Hearing dysfunction | 1 | 1 |

SLM: Selenomethionine.

of placebo and SLM groups; anemia occurred in 1 and 0, leukopenia in 2 and 3, respectively. Non-mucosal adverse events are summarized by treatment group in Table 3.

### *Response and survival*

Only one patient (in the SLM group) failed to achieve a CR and died of locally persistent and widely metastatic disease. There was no discernible difference in OS or PFS. Kaplan-Meier survival curves are shown in Figure 1.

EORTC QOL questionnaire scores at baseline, weeks 4 and 7 of CRT, and during the 1 year follow-up period showed no significant differences between treatment groups (data not shown).

### *Plasma Se*

Blood draws to evaluate changes in plasma Se concentrations were undertaken in 8 patients from the NZ site. Baseline mean Se was similar in the SLM and placebo groups: 80.2 ng/mL and 105.1 ng/mL, respectively. Plasma concentrations tended to fall in the placebo group during and after CRT (Figure 2). In contrast, after taking SLM twice daily for 1 wk mean plasma Se rose to 890.4 ng/mL (range 475.0-1104.7) and similar levels were maintained with SLM once daily thereafter. About 1-2 wk after finishing SLM, plasma Se remained similar to on-treatment levels.

## DISCUSSION

This small trial underwent an interim analysis after 18 of a planned 80 patients were accrued, to see if there was a sufficiently strong indication of efficacy to warrant

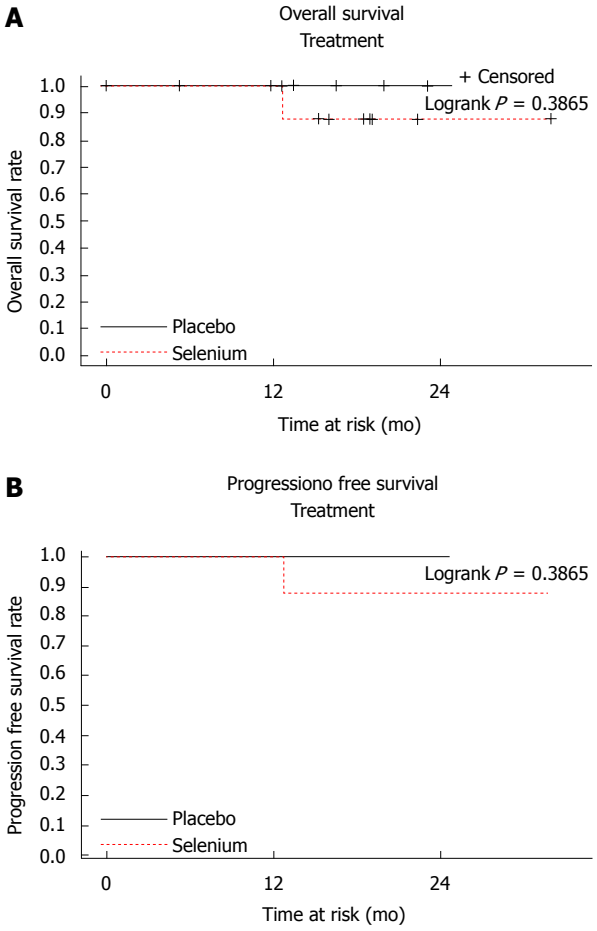


**Figure 1 Overall and progression-free survival.**

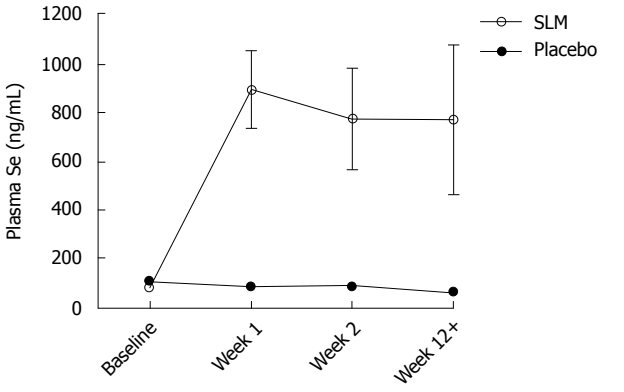


**Figure 2 Mean (± SE) trough selenium concentrations in plasma prior to selenomethionine, after 1 and 2 wk of selenomethionine intake, and 1-2 wk after the end of treatment.** SLM: Selenomethionine; Se: Selenium.

further funding. No such signal of efficacy in either reduction of toxicity or improved therapeutic benefit was found, though given the single failure to achieve CR, no conclusion regarding the effect of SLM on CRT efficacy can be drawn from this trial. The reduction in incidence of grades 3-4 mucositis from 37.5% to 20% in the experimental group was consistent with the projected effect size of 20%, however patient numbers were too small for this difference to be significant.

### *Adding Se in treatment of HNSCC*

Our findings agree with 2 other small studies of Se in HNSCC patients. Eroglu *et al*[26], in an observational study (without Se supplementation) of 47 consecutive patients receiving radiotherapy for HNSCC, found no correlation between serum Se levels and radiation toxicity[26]. Büntzel *et al*[27] performed a randomized phase Ⅱ trial of 39 patients with advanced head and neck cancer. Patients either received no Se substitution or 500 μg sodium selenite orally on the days of radiotherapy and 300 μg on days without radiotherapy. There was no statistically significant difference in the incidence of severe toxicity overall; however the weekly patient analysis showed a significant reduction of dysphagia in the Se group at the last week of irradiation[27].

### *Studies of Se in other patient populations*

Our trial results stand in contrast to the findings of 3 other studies in patients with cancers other than HNSCC, which did show benefit to the addition of Se. Muecke *et al*[28], in a multi-center phase Ⅲ trial with the primary endpoint of improving baseline serum Se levels in Se-deficient patients, found in post-operative patients with cervical cancer ($n$ = 11) and uterine cancer ($n$ = 70) a significant reduction in grade 2 or worse diarrhea (20.5% compared with 44.5%; $P$ = 0.04) in the group supplemented with sodium selenite using the schedule by Buntzel above[28].

Jahangard-Rafsanjani *et al*[29] found that oral Se 200 μg twice daily significantly reduced oral mucositis in the setting of allogeneic stem cell transplantation for leukemia. In this 77 patient double-blind, randomized, placebo-controlled trial, the incidence of severe oral mucositis (grades 3-4) was significantly lower in the Se group (10.8% *vs* 35.1%, $P$ < 0.05). Also, the duration of grades 2-4 mucositis was significantly shorter in the Se group (3.6 ± 1.84 d *vs* 5.3 ± 2.2 d, $P$ = 0.014)[29]. A series of randomized trials reported by Asfour *et al*[30,31] using sodium selenite in conjunction with chemotherapy for patients with non-Hodgkin lymphoma revealed a small but significant survival advantage in those who achieved a CR to therapy.

Our own trial in stage Ⅲ non-small cell lung cancer patients showed that SLM 4800 μg daily was well-tolerated in patients undergoing concurrent chemoradiation. The addition of SLM significantly reduced the incidence of myelosuppression and displayed a trend towards decreased rates of esophagitis and pneumonitis[32].

In contrast, a prior phase Ⅰ trial from our group has shown that SLM did not limit irinotecan toxicity[21]. Furthermore, a phase 2, randomized, placebo-controlled trial of 140 localized prostate cancer patients undergoing active surveillance showed no difference in prostate specific antigen (PSA) velocity with 200 μg/d or 800 μg/d Se supplementation (as selenized yeast). In

fact, in patients in the highest quartile of baseline Se, supplementation with high dose Se showed statistically significantly higher PSA velocity as compared with placebo ($P = 0.018$)[33].

There are a multitude of studies that have used Se supplementation to try to prevent the development of cancer in healthy patients, with mixed results[34-37]. While these studies are not directly relevant for comparison to our trial, some have argued that perhaps the discrepant results of prevention studies stem from the particular Se compound and dose selected for supplementation[38]. Similarly, it is possible that the discrepant results on toxicity and efficacy trials as described may stem from the use of different Se compounds and doses, in the setting of different tumor types.

### The optimum form and dosing of Se

With a mixed picture in human trials, the optimum form and dosing of Se is not yet known. The pre-clinical literature on the dual anti-tumor[10,11,14,15] and anti-toxicity[14,16-19] effects of organic Se compounds' ability to widen narrow therapeutic windows in patients remains compelling. The organic Se compounds, such as Se-methyl-L-selenocysteine and selenite, are currently being evaluated for safety, pharmacokinetics and dose-dependency of pharmacodynamic mechanisms in phase I trials at our institutions.

### Conclusion

Though the addition of SLM to concurrent chemoradiation for HNSCC was well-tolerated in this small trial, it did not significantly lower the incidence of severe mucositis or improve QOL outcomes. This is consistent with reports from 2 other studies of Se in HNSCC patients. Given that only a single failure to achieve CR was seen in this trial, no conclusion regarding effect of Se on treatment efficacy can be drawn from this trial.

## COMMENTS

### Background

Squamous cell carcinoma of the head and neck represents a significant worldwide health burden, and composes a substantial proportion of all cancer diagnoses. Concurrent radiotherapy and chemotherapy (CRT) has demonstrated the ability to cure a substantial number of patients, while maintaining important functions such as speech and swallowing. CRT, however, has significant acute side effects. Mucositis is one CRT side effect which can lead to interruptions of treatment. These interruptions are known to be associated with inferior outcomes. Because selenium (Se) -containing compounds have been suggested to effective protectors from radiation toxicity, the current trial was designed to evaluate the potential benefit of selenomethionine (SLM) in reducing rates and severity of mucositis during CRT. Patients received either SLM 3600 $\mu g/m^2$ twice daily for one week prior to CRT, and once daily during CRT, or placebo, through a multicenter, randomized clinical trial.

### Research frontiers

As outcomes in cancers treated with radiotherapy continue to improve, there is increasing emphasis on the importance of toxicity mitigation. In this study, SLM failed to reduce the incidence and severity of mucositis during treatment with CRT.

### Innovations and breakthroughs

The literature suggests a benefit for Se in protection from radiotherapy and chemotherapy induced toxicity. The current trial, however, failed to show benefit from the addition of Se to CRT treatment for head and neck cancer.

### Applications

This study serves as additional evidence contributing to the current knowledge regarding Se as a potential radioprotcetor.

### Terminology

SLM: A naturally occurring amino acid containing Se, found in certain nuts, beans, and legumes. Mucositis: Painful inflammation of mucous membranes. This is a common side effect of cytotoxic therapies, such as chemotherapy and radiotherapy.

### Peer-review

This is a good study to evaluate Se supplementation in CRT.

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CASE REPORT

# Liposarcoma of the breast arising in a malignant phyllodes tumor: A case report and review of the literature

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Author contributions: Banys-Paluchowski M and Paluchowski P designed the report; Burandt E, Quaas A, Wilczak W and Sauter G performed the histopathological analyses; Geist S and Krawczyk N collected the patient's clinical data; Banys-Paluchowski M, Burandt E, Pietzner K and Paluchowski P analyzed the data and wrote the paper.

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## Abstract

Liposarcoma of the breast is a very rare malignant tumor. It can clinically manifest as a palpable breast mass and mimic primary breast cancer. We report an unusual case of a 51-year-old female who presented with an asymptomatic right breast mass, which was histologically diagnosed as well differentiated liposarcoma arisen within malignant phyllodes tumor. The patient underwent breast conserving surgery, received no adjuvant treatment and is disease-free after 2 years. Radiological and histopathological features are presented and described in detail. Data from the literature are presented and therapy recommendations discussed.

**Key words:** Liposarcoma; Soft tissue sarcoma; Breast cancer; Phyllodes tumor; Rare malignancies



**Core tip:** Liposarcoma is a very rare malignant tumor

of the breast and may mimic invasive breast cancer on imaging studies. The definite pathological diagnosis may be challenging.

Banys-Paluchowski M, Burandt E, Quaas A, Wilczak W, Geist S, Sauter G, Krawczyk N, Pietzner K, Paluchowski P. Liposarcoma of the breast arising in a malignant phyllodes tumor: A case report and review of the literature. *World J Clin Oncol* 2015; 6(5): 174-178 Available from: URL: http://www.wjgnet.com/2218-4333/full/v6/i5/174.htm DOI: http://dx.doi.org/10.5306/wjco.v6.i5.174

## INTRODUCTION

Breast cancer is the most common female malignancy worldwide[1]. In rare cases, the histopathological work-up of a suspicious breast mass shows not epithelial (carcinoma) but sarcomatous differentiation. One of such rare malignant tumors is a liposarcoma, which may present as a pure liposarcoma or arise within a phyllodes tumor (PT). Upon imaging studies, liposarcoma often resembles primary invasive breast carcinoma. Given the rarity of the disease, there are no randomized trials specifically addressing treatment modalities in breast sarcoma and therapy guidelines are based on data from non-breast soft tissue sarcoma trials. In the following, we report an unusual case of a 51-year-old female with a well differentiated liposarcoma arisen within malignant PT and present current data and evidence-based therapy recommendations for breast liposarcoma.

## CASE REPORT

A 51-year-old Caucasian postmenopausal female presented at the certified Breast Cancer Center, Klinikum Pinneberg, Germany, with a newly diagnosed palpable, asymptomatic mass located in the lower inner quadrant of her right breast. Clinical examination showed a nodular movable mass of 2 cm diameter; the overlying skin was unremarkable. She had no concomitant diseases at time of presentation beside obesity (BMI 31 kg/m$^2$); her previous surgeries included cholecystectomy and she was a nonsmoker. She denied any first- or second degree family medical history of cancer of any type and she never received radiotherapy. At mammography, the lesion was scored BI-RADS 5. Breast ultrasound and mammograms are presented in Figures 1 and 2, respectively. Axillary lymph nodes were unremarkable on sonography. Ultrasound guided minimal-invasive 14-gauge core biopsy revealed a biphasic tumor of the phyllodes type with suspicious stroma. We conducted a lumpectomy; histopathological workup described a malignant PT of 21 mm diameter with a specific heterologous component identified as well differentiated liposarcoma; mitotic rate was 21/10 high-power field (Figure 3). The case was discussed in the interdisciplinary tumor board. Because of close margins (min. 1 mm) a wide excision was recommended, which was conducted 4 wk after the lumpectomy and showed no further malignant lesion (resection margins after wide excision > 10 mm). The case was discussed again in the tumor board, which recommended further follow-up care including clinical examinations, mammography and breast sonography at regular intervals. Neither chemotherapy nor radiotherapy was recommended. The patient had an uneventful recovery, received no further therapy and is free of disease since surgery (two years).

## DISCUSSION

Soft tissue sarcomas (STS) amount to less than 1% of all malignant tumors with an incidence estimated at 2-5 cases per 100000 yearly[2]. The exact diagnosis may pose a significant challenge since there are over fifty subtypes of STS, which determine their prognostic and therapeutic features[3]. Eight percent to 14% of all newly diagnosed STS have liposarcomatous differentiation making primary liposarcoma a common subtype. Lucas *et al*[4] reported on 58 consecutive cases of well differentiated liposarcoma treated at the Mayo Clinic; of these, the majority involved the extremities (32 cases) and the retroperitoneum (20), followed by the scrotum (4), the abdominal wall (1) and the cheek (1). Liposarcoma localized in the breast has been reported in the literature before but remains a very rare neoplasm.

Primary sarcomas of the breast account for 0.1% of all malignant breast tumors. A thorough review on breast sarcomas, along with a series of 25 cases, was published by Adem *et al*[5]. The incidence of liposarcoma among breast sarcomas vary in the literature from 2% to 10%[6-10]. Its definite pathological diagnosis is challenging and may require a cooperation with a reference center. Liposarcomas of the breast may occur either as pure primary liposarcoma or arise in cystosarcomas phyllodes. The patient in our case report presented with a suspicious breast mass that was revealed as malignant PT with heterologous liposarcomatous differentiation. Liposarcomatous differentiation is rarely diagnosed in PTs; the malignant stroma transformation of PT usually shows fibrosarcomatous differentiation and rarely heterologous sarcomatous elements[11]. Other uncommon sarcomatous stromal elements may include leiomyosarcoma, osteosarcoma, angiosarcoma, chondrosarcoma and rhabdomyosarcoma. PT with liposarcomatous differentiation may resemble breast cancer on imaging studies. The prognosis is strongly influenced by histologic subtype: dedifferentiated liposarcomas are aggressive tumors with high metastatic potential while well differentiated and myxoid types generally have a more favorable outcome[12]. Further features associated with favorable survival include complete surgical excision of tumor with tumor-free margins[13].

### *Therapy of breast sarcomas*

Given the rarity and heterogeneity of the disease, there are no prospective randomized trials on the surgical

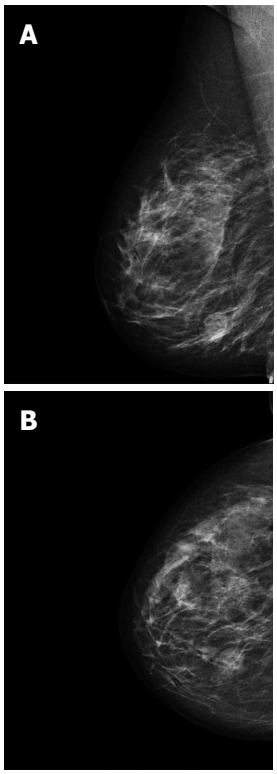


**Figure 1 Mammography of the right breast shows a round lesion with smooth margins measuring 2.6 cm in the lower inner quadrant (A and B).**

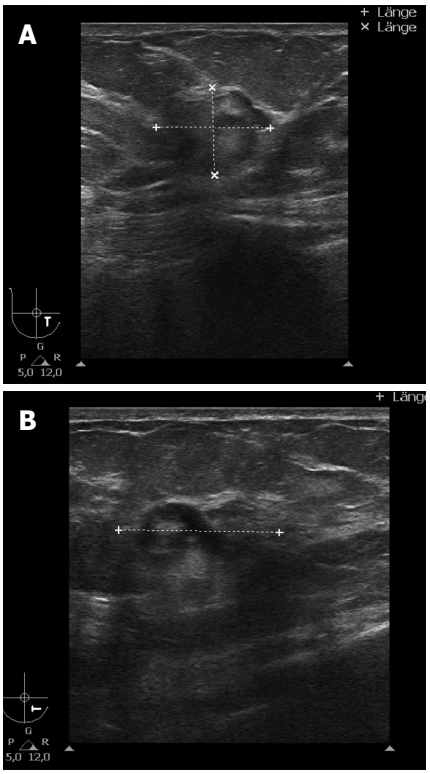


**Figure 2 Breast ultrasound shows an irregular structure of complex echogenicity measuring 2.4 cm × 2.0 cm × 1.6 cm (BI-RADS 5) (A and B).**

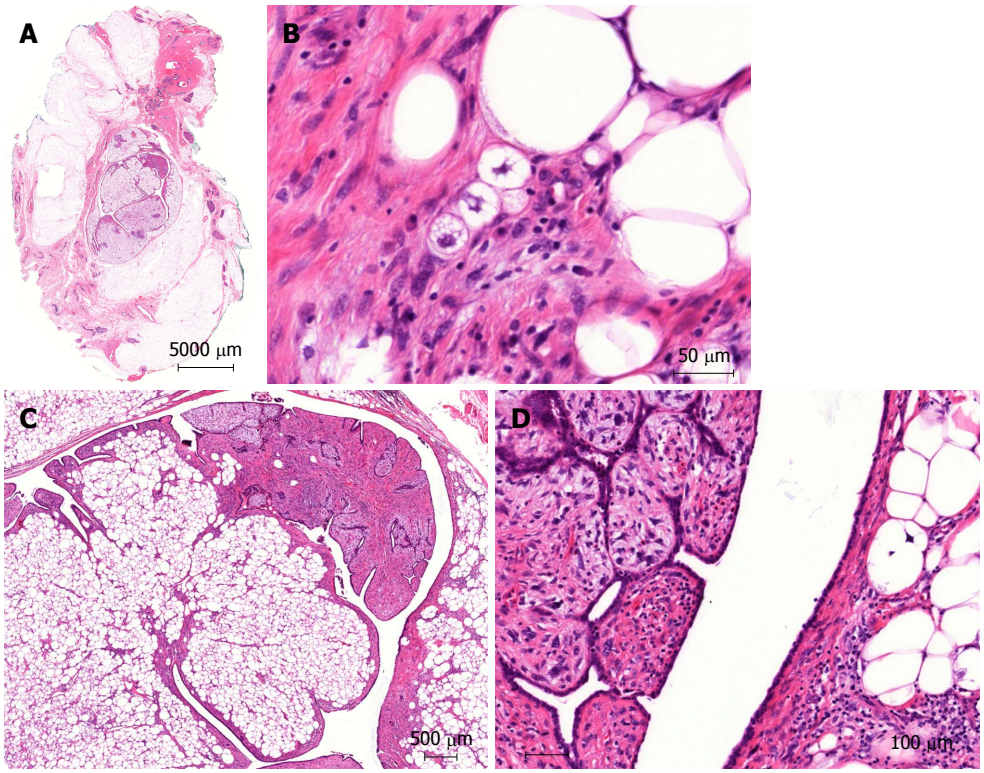


**Figure 3 Lumpectomy; histopathological workup described a malignant phyllodes tumor of 21 mm diameter with a specific heterologous component identified as well differentiated liposarcoma.** A: Breast excision with centrally located phyllodes tumor (zoom × 3); B: Atypical stroma component of the phyllodes tumor including lipoblasts with multiple vacuoles (× 400); C: Intraductal phyllodes tumor with typical architecture harboring the liposarcomatous component (× 27); D: Hypercellular stroma of the phyllodes tumor showing striking atypia (left) and multivacuolated atypical lipoid cells (right) (× 200).

and systemic treatment of breast sarcomas and the optimal therapy remains yet to be defined. Current recommendation of the European Society for Medical Oncology (ESMO) and the European Sarcoma Network Working Group[14] is to treat non-radiation induced breast sarcomas as other STS by performing breast conserving surgery (*e.g.*, wide excision as in our case), with the exception of angiosarcoma because of its high local recurrence rates[15,16]. Since an adequate resection margin is the most important prognostic factor, negative margins are crucial for long-term survival[8,17]. Given clear margins of resection, survival rates after mastectomy and breast conserving surgery are similar[18]. Sarcomas tend to spread by direct local invasion or hematogenously. Since lymphatic dissemination is rare, neither axillary lymph node dissection nor sentinel node biopsy are recommended in the absence of clinical evidence of lymph node involvement[14,19,20]. In the retrospective analysis, Shabahang *et al*[21] found no positive nodes in ten patients treated with axillary lymph node dissection for primary breast sarcoma. The role of adjuvant radiotherapy for breast sarcoma remains unclear due to the rarity of the disease and lack of randomized trials. Data from single institution studies are contradictory: some observational studies suggest improved local control[9,19] while others reported no benefit of radiotherapy[18,20,22,23]. The subgroup that might particularly benefit from adjuvant radiotherapy consists of patients with large tumors (> 5 cm), high-grade sarcoma and positive margins. The patient presented in the case report had a small tumor (2.1 cm) removed with clear margins of > 1 cm; based on the available data, the interdisciplinary tumor board did not recommend adjuvant radiation. As far as chemotherapy is concerned, since there are no trials specifically addressing breast sarcoma, current recommendations are based on randomized trials conducted in patients with non-breast STS. In the current ESMO guidelines, adjuvant chemotherapy is not standard treatment in adult-type STS[14]. The benefit of chemotherapy must be discussed on an individual basis, taking into account the tumor size, histologic subtype and grade. Patients with whom a chemotherapy should be discussed are those with high-risk primary sarcomas (tumor size > 5 cm, high-grade or lymph node positive). Due to their particularly poor prognosis, chemotherapy may be offered to angiosarcoma patients presenting with smaller tumor size as well (*e.g.*, 3-5 cm). In the present case report, tumor board decided against adjuvant chemotherapy for well differentiated small (< 3 cm) liposarcoma. Another systemic option typically used in breast cancer, the endocrine therapy, is not recommended in breast sarcoma due to the lack of efficacy since these tumors tend to be hormone receptor negative. Regarding adjuvant options, one should keep in mind that neither radiotherapy nor chemotherapy can compensate for inadequate surgery, and re-excision to obtain clear margins should be pursued whenever possible. Surgical treatment of breast sarcoma should be carried out in centers specialized in oncological breast surgery[14].

Liposarcoma of the breast arising within a malignant PT is a rare neoplasm and may mimic breast cancer on clinical and radiological examination. Malignant stroma may be present in only part of the tumor, so thorough sampling is essential. Surgery is a potentially curative modality; the role of adjuvant chemo- and radiotherapy remains yet to be clarified.

## COMMENTS

### *Case characteristics*

An 51-year-old female presented with an asymptomatic breast mass.

### *Clinical diagnosis*

Nodular movable mass of 2 cm diameter in the lower inner quadrant of the right brass, the overlying skin unremarkable.

### *Differential diagnosis*

Invasive breast carcinoma.

### *Imaging diagnosis*

Mammography: suspicious round lesion with smooth margins measuring 2.6 cm in the lower inner quadrant (BI-RADS 5). Breast ultrasound: irregular structure of complex echogenicity measuring 2.4 cm × 2.0 cm × 1.6 cm (BI-RADS 5), axillary lymph nodes unremarkable.

### *Pathological diagnosis*

Core biopsy revealed a biphasic tumor of the phyllodes type with suspicious stroma. Lumpectomy showed a malignant phyllodes tumor (PT) with a specific heterologous component identified as well differentiated liposarcoma.

### *Treatment*

The patient was treated by a lumpectomy and subsequent wide excision.

### *Experiences and lessons*

This case report describes a rare malignant tumor and emphasizes the importance of thorough histopathological workup in case of PT with suspicious heterologous component.

### *Peer-review*

This is a well-written manuscript. It defines a rare case of liposarcoma arising from PT of the breast.

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CASE REPORT

# Malignant peripheral nerve sheath tumor of proximal third tibia

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Author contributions: Rao A and Rajurkar P designed the study; Goyal V and Dokrimare N prepared the first draft of the manuscript; Ingle SB carried outthe pathological diagnosis, and critically revised the intellectual content of the manuscript and gave itfinal approval.

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Informed consent statement: As we are not disclosing theidentity of the patient, it wasnot needed.

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## Abstract

A 16-year-old man had aswelling over the anterior aspect of the proximal third of the tibia for 1 year, which was peanut size initially and progressively increased to its present size of 10 cm × 8 cm. He underwent fine needle aspiration cytology (FNAC) twice during this period and reported aspindle cell sarcoma. Malignant peripheral nerve sheath tumor (MPNST) is a malignancy of the connective tissue surrounding the nerves. Previously, MPNST was also known as neurofibrosarcoma, malignant schwannoma, andneurogenic sarcoma. We are reporting this case for its rarity and peculiar mode of presentation. FNAC/core biopsy can be used as an effective tool to achievethe correct pathological diagnosis.

**Key words:** Tibial malignant peripheral nerve sheath tumor; Fine needle aspiration cytology; Histopathology



**Core tip:** In cases of malignant peripheral nerve sheath tumor of the tibia, fine needle aspiration cytology/core biopsy can be used as an effective tool to achieve the correct pathological diagnosis. In such cases, *en bloc* resection is the treatment of choice. Adjuvant radiotherapy/chemotherapy plays a vital role in achieving a good outcome.

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peripheral nerve sheath tumor of proximal third tibia. *World J Clin Oncol* 2015; 6(5): 179-183 Available from: URL: http://www.wjgnet.com/2218-4333/full/v6/i5/179.htm DOI: http://dx.doi.org/10.5306/wjco.v6.i5.179

## INTRODUCTION

Malignant peripheral nerve sheath tumors (MPNSTs) are sarcomas originating from cells associated with the nerve sheath. The lifetime risk of MPNST is 0.001% in the general population. As MPNSTs arise from different types of cells associated with nerve sheaths, for example, Schwann cells and fibroblasts, the clinical presentation and histopathological features varies from case to case. So, it is a real challenge to diagnose and classify this rare entity. Generally, a sarcoma originating from a peripheral nerve or a neurofibroma is assumed clinically as MPNST[1,2].

## CASE REPORT

A 16-year-old man was admitted to YCR Hospital Latur, with apeanut-size swelling when it was first noticed,which progressively increased to its present size of 10 cm × 8 cm. Pain was intermittent in the right proximal tibia, with tingling sensationin theright leg for the previous year.

Physical examination revealed a swelling over the anterior aspect of the proximal end of the tibia (10 cm × 8 cm; Figure 1), shiny skin, a scab in the center of swelling, dilated veins over the swelling, and local temperature increase with tenderness. The swelling was mobile and not attached to underlying structures. The range of movements of the right knee joint was full and free, with intact neurovascular status. There was no history of exposure to radiation and no evidence of signs and symptoms of neurofibromatosis (NF) (Figure 2).

### *Management*

Anteroposterior and lateral radiography of the right knee and tibia showed an expansile soft-tissue mass destroying the adjacent cortex on lateral view, but it did not extendinto the medullary cavity. Congruency of the knee joint was well maintained (Figure 3).

Magnetic resonance imaging showed alobulated mass lesion (7.5 cm × 3.9 cm × 1.6 cm) along the anterior surface of the tibial shaft, which caused periosteal elevation. There was no extension of the lesion within the medullary space of the tibia and no significant bone marrow edema in the adjacent tibia (Figure 4).

Considering the nature of the growth and high clinical propensity for malignancy, it was treated by *en bloc* resection and immobilization for 2 wk.

In this procedure, through an antero-medial approach, around 20 cm an elliptical incision of around 20 cm was made and radical *en bloc* resection of the tumor was performed.

Care was taken to preserve the neurovascular bundle during resection of the tumor from the surrounding soft tissue. The wound was washed thoroughly with $H_2O_2$ and the excised mass was sent for histopathological examination. On gross examination, the cut surface was gray-white (Figure 5) and on histopathological examination, the mass was diagnosed as malignant spindle cell sarcoma, *i.e.*, low-grade MPNST (Figure 6). The tumor cells were immunopositive for S-100, thus confirming the final diagnosis of MPNST (Figure 7).

The limb was immobilized in a long/medium knee brace for 2 wk and followed by active knee mobilization. The patient was discharged and advised to attend monthly review. He was also advised to consult an oncologist for chemotherapy/radiotherapy.

## DISCUSSION

MPNSTs constitute 5%-10% of all soft-tissue malignancies. They are associated with NF-1, or may occur independently in a spontaneous manner.

The cause is not known, but they are strongly associated with history of exposure to radiation[3,4]. Fifty percent of the cases occur in patients with NF-1[5-7], and they usually occur in apre-existing neurofibroma.

The genesis ofMPNSTs is associated with genetic mutations in *p53* and *p16* genes[8-10]. *NF*-1 gene activity acts as a predisposing factor.

MPNSTs are commonly seen in adults, aged 20-50 years. In the first two decades of life, the incidence is 10%-20%[6], with exceptional casesseen in infants[11].

The plan of treatment for MPNSTs is surgical excision with wide margins. Adjuvant chemotherapy or radiotherapy does not achieve a better outcome[12,13].

It has been clearly stated that these tumors have a tendency to spread for considerable distances along nerves. In such a scenario, frozen sections are advised to ensure clear margins[14].

In a 10-year institutional review, chemotherapy did not seem to reduce mortality, so its effectiveness is questionable. With recent approaches inthe molecular biology of MPNSTs, new therapies and prognostic factors are being examined[15].

## COMMENTS

### *Case characteristics*

A 16-year-old manpresented with apeanut-size swelling, when first noticed, which progressively increased to its present size of 10 cm × 8 cm, and intermittent pain in theright proximal tibia and atingling sensation in theright leg for the past year.

### *Clinical diagnosis*

The case was diagnosed as soft tissue sarcoma.

### *Differential diagnosis*

Soft tissue sarcomas, that is, fibrosarcoma, malignant fibrous histiocytoma, and

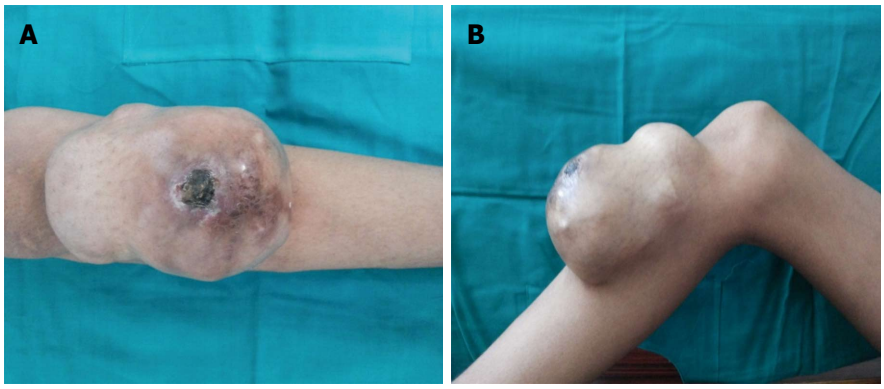


**Figure 1 Preoperative clinical photographs (A and B).**

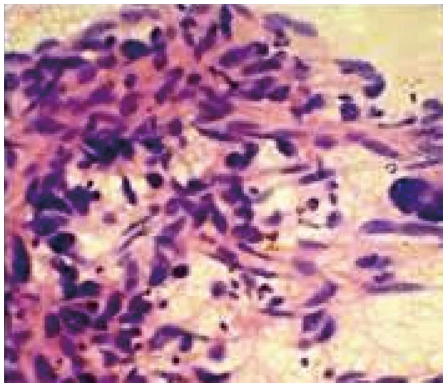

**Figure 2 Fine needle aspiration cytology showing loosely scattered malignant spindle cells.**

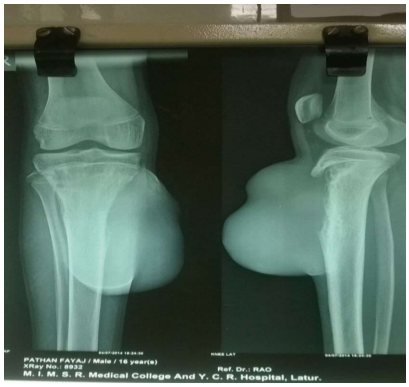


**Figure 3 Preoperative X-ray.**

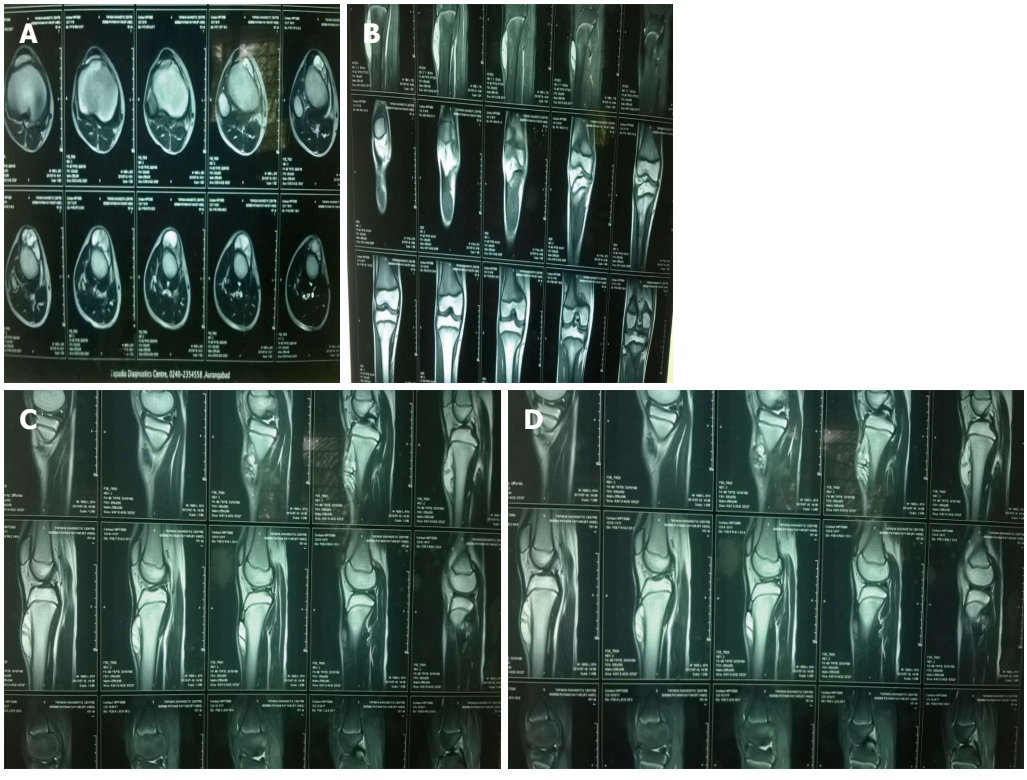


**Figure 4 Magnetic resonance imaging transverse (A), coronal (B) and saggital (C, D) section.**

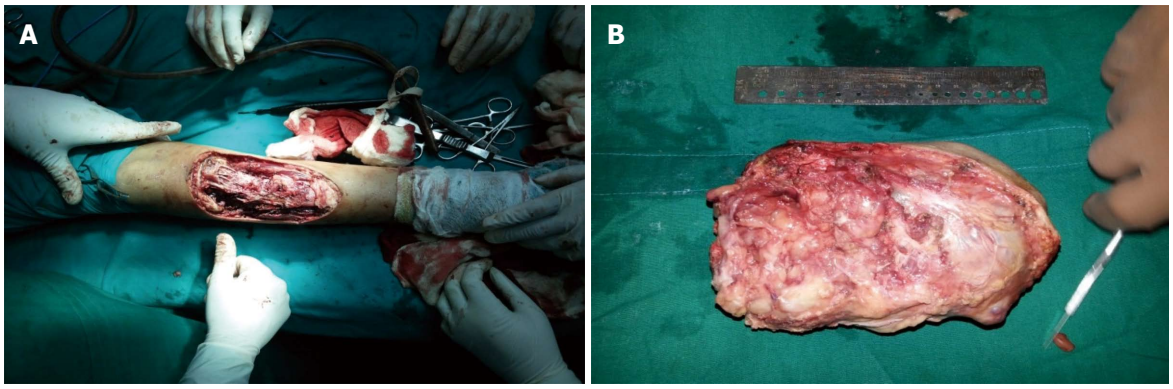


**Figure 5 Intraoperative photograph showing excised mass (15 cm × 8 cm × 4 cm) (A and B).**

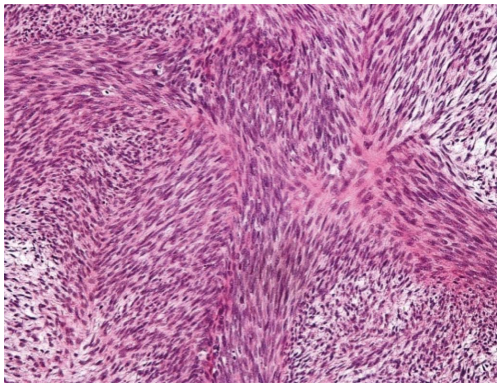

**Figure 6 Malignant peripheral nerve sheath tumor on microscopy (LP 10 ×).**

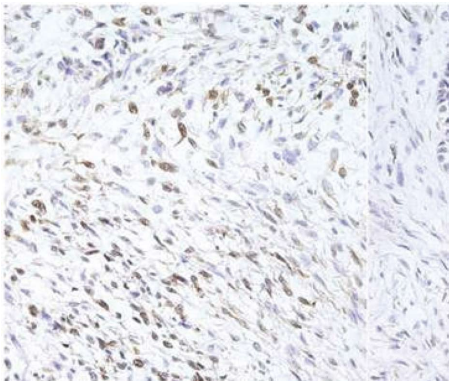

**Figure 7 S-100 immunopositive tumor cells.**

malignant peripheral nerve sheath tumor (MPNST).

### *Laboratory diagnosis*

On fine needle aspiration cytology (FNAC), the case was diagnosed as spindle cell sarcoma, which was confirmed by histopathology and immunostaining.

### *Imaging diagnosis*

X-ray: Anteroposterior and lateral radiography of the right knee and tibia showed an expansile, soft tissue mass destroying adjacent cortex on lateral view, but it didnot extendinto the medullary cavity; congruency of the knee joint waswell maintained. Magnetic resonance imaging showed a lobulated mass lesion (7.5 cm × 3.9 cm × 1.6 cm) along the anterior surface of the tibial shaft, causing periosteal elevation. There was no extension of the lesion within the medullary space of the tibia and no significant bone marrow edema in the adjacent tibia.

### *Pathological diagnosis*

MPNST was confirmed by immunohistochemistry.

### *Treatment*

*En bloc* resection followed by chemotherapy/radiotherapy.

### *Experiences and lessons*

FNAC/core biopsy can be used as an effective diagnostic tool to achieve early diagnosis.

### *Peer-review*

It is a well written paper describing an interesting case report of MPNST of proximal third tibia treated by *en bloc* resection.

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