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## Axillary lymph node management in breast cancer with positive sentinel lymph node biopsy

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### Abstract

The surgical treatment of localized breast cancer has become progressively less aggressive over the years. The management of the axillary lymph nodes has been modified by the introduction of sentinel lymph node biopsy. Axillary dissection can be avoided in patients with sentinel lymph node negative biopsies. Based on randomized trials data, it has been proposed that no lymph node dissection should be carried out even

in certain patients with sentinel lymph node positive biopsies. This commentary discusses the basis of such recommendations and cautions against a general omission of lymph node dissection in breast cancer patients with positive sentinel lymph node biopsies. Instead, an individualized approach based on axillary tumor burden and biology of the cancer should be considered.

**Key words:** Tumor sub-types; Micro-metastatic; Node positive; Breast cancer; Axillary lymph node dissection; Macro-metastatic; Axillary recurrence

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**Core tip:** Management of the axilla in breast cancer has been modified by the introduction of sentinel lymph node biopsy. Axillary dissection can be avoided in sentinel lymph node negative patients. More recently, it has been proposed that lymph node dissection could be avoided even in patients with sentinel lymph node positive biopsies. The basis of such proposals is discussed here and caution is advised against a universal omission of lymph node dissection in breast cancer patients with positive sentinel lymph node biopsies. Instead, an individualized approach based on axillary tumor burden and biology of the cancer should be considered.

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### INTRODUCTION

Over the years the surgical management of the primary tumor in localized breast cancer has become

less extensive and less mutilating. It has progressively been reduced in extent from radical mastectomy to total mastectomy and later to lumpectomy with corresponding decrease in morbidity<sup>[1]</sup>. Reduction of surgical extent has become possible because of increasing awareness and screening that have contributed to earlier diagnosis and stage migration and because of the introduction of other effective treatments such as radiation and systemic therapies. Progress in biologic understanding of the disease has allowed the integration of targeted therapies in the adjuvant treatment of breast cancer. Complete resection of the primary tumor with negative margins remains a cornerstone of treatment for localized breast cancer. More recently, the introduction of the sentinel lymph node biopsy has changed the management of the axilla in patients with localized breast cancer allowing avoidance of lymph node dissection in patients with pathologically negative sentinel nodes<sup>[2]</sup>. This development has led to a decrease of the percentage of patients with local adverse effects of dissection such as lymphedema and paresthesia which may significantly decrease quality of life and functionality and sometimes cause severe effects such as wound infections, cellulitis and systemic infections<sup>[3,4]</sup>.

The standard management of the axilla in patients with positive sentinel lymph nodes remains a complete lymph node dissection but recent data have challenged this posit and produced controversy<sup>[5]</sup>. The American Society of Clinical Oncology (ASCO) recently published a revised guideline on sentinel lymph node biopsy for patients with early stage breast cancer<sup>[6]</sup>. The guideline advises against completion axillary lymph node dissection (ALND) for patients who meet criteria that include T1 or T2 primary lesions, one or two positive axillary sentinel lymph nodes (SLN) without extra-capsular infiltration and a plan to undergo breast conserving surgery followed by conventionally fractionated whole-breast radiotherapy. Patients with larger tumors, more than two positive SLN, inflammatory breast cancer, undergoing mastectomy or planned to receive unconventional radiation treatments are excluded from this recommendation. The recommendation is based on data from one randomized trial, the American College of Surgeons Oncology Group (ACOSOG) Z0011 trial<sup>[7]</sup>. Some additional trials provide related, although circumferential evidence, on the issue and will be included in this discussion (Table 1). Another randomized trial, for example, the International Breast Cancer Study Group (IBCSG) 23-01 trial addresses a similar clinical question in a different patient population with only micrometastatic lymph node disease<sup>[8]</sup> and thus its relevance for the decision of omission of ALND in patients with macrometastatic disease is questionable. IBCSG 23-01 showed the non-inferiority of avoiding ALND vs performing an ALND regarding disease-free survival

(DFS) and overall survival (OS) in 931 patients with mainly (90%) positive Estrogen Receptor (ER) status and T1 or T2 primary tumors (70% T1 and more than 90% less than 3 cm in major diameter)<sup>[8]</sup>. DFS was 84% and 88% ( $P = 0.16$ ) and OS 98% in both arms ( $P = 0.73$ ). Omission of lymph node dissection in patients with isolated tumor cells (less than 0.2 mm in diameter) or micrometastatic (0.2-2 mm in diameter) only disease in the axilla could be advocated with less controversy given the results of IBCSG 23-01 and the predicted lower incidence of additional positive nodes and lower risk of recurrence in patients with micrometastatic only disease in the axilla compared with counterparts with macrometastases<sup>[9]</sup>. In addition, in a large retrospective analysis, patients with micrometastatic disease in the axilla, in contrast to patients with macrometastases in whom there was a trend towards inferior outcomes, had equivalent survival if no complete dissection was performed<sup>[10]</sup>. Nevertheless, even in these patients, about 20% can be expected to have additional axillary involvement<sup>[9]</sup>. It is interesting to note that patients with micrometastatic disease have a lower disease-free survival compared with lymph node negative patients and benefit from adjuvant therapy<sup>[11]</sup>.

The Z0011 trial randomized 891 patients with T1 or T2 breast cancers and one or two positive axillary SLN (both patients with macrometastases and micrometastases were included) to further ALND or no further surgical treatment<sup>[7]</sup>. The initial trial plan was for randomization of 1900 patients but had to be modified due to slow accrual and lower than expected mortality rate. The study was able to demonstrate the non-inferiority of no further surgical treatment for the end-points of OS and DFS. Being the only randomized trial attempting to answer a very important clinical question, Z0011 has been scrutinized and shortcomings previously discussed<sup>[12]</sup>. The two groups were well-balanced but ALND group had slightly more patients with T2 disease (32.1% vs 29.4%, and even some T3 tumors, as the upper range of size in this group was 7 cm), lymphovascular invasion (40.6% vs 35.2% in the SLN only group) and grade II/III tumors (78% vs 74.4% in the SLN only group). In addition, the ALND group had less patients with the good prognostic features of ER and PR positivity (66.8% vs 68.9%) and no positive SLN (1.2% vs 7%, these patients should have been excluded but were included for the intention-to-treat analysis). Micrometastatic lymph node disease was present in a statistically significant higher percentage of patients in the SLN group (44.8% vs 37.5%). Despite these inequalities all favoring the SLN group over the ALND group, OS and DFS were similar in the two groups (92.5% vs 91.8% and 83.9% vs 82.2% respectively). The study had a high rate of loss to follow-up (166 of 891 patients, 18.6%), a source of potential bias. Her2/Neu testing was not standard at the time of the study and no data were

**Table 1** Trials discussed in this paper informing directly or indirectly on the question of completion of axillary lymph node dissection in patients with sentinel lymph node biopsy-positive breast cancer patients

Trial [Ref.]	Comment
ACOSOG Z0011 <sup>[7]</sup>	The main randomized trial informing the clinical question
IBCSG 23-01 <sup>[8]</sup>	Randomized trial in patients with micrometastatic only axillary disease
Yegiyants <i>et al</i> <sup>[14]</sup>	Small study of patients followed after micrometastases found in SLN biopsy
Martelli <i>et al</i> <sup>[15]</sup>	Randomized trial comparing ALND <i>vs</i> no surgery in older patients
IBCSG Trial 10-93 <sup>[16]</sup>	Similar to reference 15
Avril <i>et al</i> <sup>[17]</sup>	Similar to references 15 and 16 but in a younger patient population
Louis-Sylvestre <i>et al</i> <sup>[19]</sup>	Randomized trial comparing ALND with axillary radiation treatment
Wang <i>et al</i> <sup>[21]</sup>	Retrospective SEER-based study in patients with lobular carcinomas

SLN: Sentinel lymph node; ALND: Axillary lymph node dissection; SEER: Surveillance, Epidemiology and End Results.

reported. ER and PR negative patients represented a minority with only about 16% of patients in each arm. After exclusion of the 166 patients lost to follow-up and 301 patients with only micrometastatic lymph node disease, there remain 424 patients (about two thirds ER and PR positive) who can inform us on the question of macrometastatic disease.

The LN tumor burden in the patient population of the Z0011 study appears to be low with only 21% of patients in the ALND group having additional positive nodes and 13.7% having a total of four or more positive nodes<sup>[7]</sup>. One can predict that a similar (or even lower based on somewhat better prognostic characteristics) percentage of patients in the SLN group would have additional disease. The low tumor burden is also depicted in the higher OS rates in the study compared with the rate of 80% anticipated. Available models predict that patients with T2 disease and two positive sentinel nodes (that is still meeting the eligibility criteria of Z0011) may have much higher probabilities of additional positive nodes. For example the Memorial Sloan Kettering Cancer Center (MSKCC) model predicts that a patient with a grade III tumor of 5 cm, and 2 positive SLN may have a probability of over 90% for additional positive nodes<sup>[13]</sup>. The question remains if patients with higher axillary LN burdens will have similar outcome without ALND as seen in Z0011. It is important to note that the predicted risk of additional positive lymph nodes may not be the only determinant of risk of recurrence. For example in a small study of 47 patients with positive SLN (33 with micrometastases only) and a low risk of 11.5% of additional LN positivity by the MSKCC model two patients (4.2%) had an axillary recurrence<sup>[14]</sup>. On the other hand, as studies performed before the routine introduction of SLN biopsy that compare ALND with no dissection in older patients (mean age over 70 years old) with small primaries (mostly T1a and T1b), ER positive and clinically negative axilla have shown, most patients with a predicted low burden axillary disease will not have a clinically apparent axillary recurrence even if left untreated<sup>[15,16]</sup>. These patients are spared the adverse effects of ALND without adverse

oncologic outcome in the short term. Nevertheless even these studies suggest that axillary recurrences are much more frequent (up to three times) if no axillary intervention is undertaken, although low in absolute numbers<sup>[15,16]</sup>. In addition another study with the same design that included younger patients (mean age 62 years old) showed a statistically significant worse overall and disease-free survival in patients with no axillary intervention as compared with the group that underwent ALND<sup>[17]</sup>.

An additional question is the generalizability of results to sub-types of breast cancer not well-represented or not studied in Z0011 (ER negative and Her2 positive). Trying to address this short-coming, the authors of the study performed an exploratory analysis of the positive and negative ER/PR groups that showed no statistically significant differences. Nevertheless, this represents weak evidence for the negative sub-group and optimally clinical decisions based on exploratory analyses should be avoided. Overall the less common sub-types of breast cancer such as triple negative and Her2/Neu positive benefit from very little high quality evidence to support omitting ALND. This is particularly worrisome in triple negative patients for whom there is currently no proven targeted therapy to control residual disease. Moreover, for Her2/Neu positive patients that have efficacious targeted therapies available to them, these therapies can produce only small percentages of complete responses in the metastatic setting and thus their ability to control significant residual disease burden in the axilla remains unproven<sup>[18]</sup>. The same is true for radiation therapy which is an efficacious treatment for localized or oligometastatic disease but is not possible for more widespread disease. In addition in a randomized study of ALND *vs* radiation therapy in breast cancer patients with clinically negative axillary nodes the axillary recurrence was almost 4 times higher in the radiation treatment arm (2.2% *vs* 0.6% at 5 years)<sup>[19]</sup>. In this study patients were mostly ER positive and had a low incidence of 21% of positive nodes in the ALND and similar axillary disease positivity could be expected to have been present in the radiation arm.



**Table 2** Considerations for omission of completion axillary lymph node dissection in patients with positive sentinel lymph node

Axillary lymph node dissection may be omitted
T1 or T2 primary
One or two positive SLN without extra-capsular extension
Lumpectomy and conventional radiation therapy planned
ER and PR positive, Her2/Neu negative (equivalent to Luminal A) biology
Patient older than 65 yr old
Ductal histology
Axillary lymph node dissection should be the standard but omission could be discussed in an individualized basis
Patient younger than 65 yr old
Biology other than Luminal A
Lobular histology
Axillary lymph node dissection should be performed
T3, T4 or inflammatory primary
More than two positive SLN and/or extra-capsular extension
Mastectomy or unconventional radiation therapy planned

SLN: Sentinel lymph node.

Another sub-type of breast cancer not well-represented in the Z0011 study is lobular carcinoma. Only 7.7% of patients had this sub-type which has a different biologic behavior from ductal carcinoma, is more often multifocal and tends to metastasize to unusual locations such as the gastrointestinal tract and peritoneal surfaces<sup>[20]</sup>. A retrospective study of the Surveillance, Epidemiology and End Results database suggested that there are no differences in overall or disease-specific survival between patients with lobular carcinoma who fulfilled the Z0011 criteria and did or did not undergo completion ALND<sup>[21]</sup>. Most of the patients had T1 tumors (median size of the two groups 1.7 and 1.8 cm), 70% had one positive LN and over 90% had ER positive tumors.

A final issue that should be considered in evaluating the strength of the available data as a basis for clinical decisions is the length of follow-up. As the authors of Z0011 note, it would have taken more than 20 years to observe the pre-specified 500 deaths in their study population and thus the trial was closed prematurely with less than the target accrual number. Although the fact that no differences were observed in OS and local recurrences at 6 years is reassuring, at least for the shorter term, concerns remain regarding longer term applicability. The population studied with mainly ER positive disease (more than 80% of whom would be expected to be Her2 negative) has, often, even in the metastatic setting, a more indolent course (not unusually with bone only disease) and thus OS at 6 years may be too short for definite conclusions when studying localized disease. Moreover, and at closer scrutiny, local recurrence rates display some discernible differences between the two groups in Z0011<sup>[22]</sup>. The ALND group had numerically more (3.6% vs 1.9% in the SLN group) in-breast recurrences. These recurrences should not have been influenced by the treatment assigned and probably reflect the aforementioned base-line differences in the two groups. In contrast, axillary

recurrences were more prevalent in the SLN group (0.9% vs 0.5% in the ALND group). Although this difference was not statistically significant and small in absolute numbers (reflecting the low disease burden in the axilla), it is still an 80% difference and may imply that the two treatments are not equal in controlling axillary disease. Increased axillary tumor burden may accentuate such differences in local control and eventually global outcomes.

Fisher's model proposes that breast cancer is a systemic disease from an early point in time and has been used to justify less aggressive surgical interventions<sup>[23]</sup>. In fact this model, in conjunction with the current models of the plasticity of tumor initiating cells and their genetic instability, predict that the lower the residual tumor burden is the lower becomes the probability of resistant clones to emerge spontaneously or as a result of treatment pressure<sup>[24,25]</sup>. These cannot currently be satisfactorily treated with systemic therapies.

In conclusion, the randomized data available are not sufficient to recommend omitting completion ALND in all patients with T1/T2 disease and up to two positive SLN that will undergo lumpectomy and whole breast irradiation. A more prudent policy would be to consider ALND omission only in older post-menopausal patients with ductal carcinomas, clinically negative axilla, no extra-nodal extension and ER/PR positive disease. In other patients with one or more deviations from the studied population of Z0011 trial the standard should remain a completion ALND (Table 2) and an individualized decision should be reached, optimally with involvement of the patient, awaiting more confirmatory data, especially in sub-types other than luminal A (immunohistochemically determined as ER/PR positive and Her2/Neu negative). Longer follow-up is required to ensure that ALND omission is safe and oncologic outcomes are equivalent at long term in patients with luminal A sub-type breast cancers and extent of disease within the acceptable

for consideration of ALND omission rate but higher expected tumor burdens such as patients with T2 tumors and 2 positive SLN.

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## Optimal management of the elderly patient with head and neck cancer: Issues regarding surgery, irradiation and chemotherapy

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### Abstract

Head and neck cancer (HNC) represents the sixth most common malignancy and accounts for approximately 6% of new cancer cases annually worldwide. As life expectancy constantly increases, the onset of HNC in patients older than 65 years of age at diagnosis is not rare and up to one fourth of cases occurs in patients older than 70 years at age. Because elderly cancer patients are severely under-represented in clinical trials, there is a clear need to address the particular aspects

of this specific patient group, especially in the context of novel multidisciplinary therapeutic approaches. The frailty of elderly patients with HNC is attributed to the high incidence of smoking and alcohol abuse in this malignancy and the presence of substantial cardiovascular, respiratory or metabolic comorbidities. In the current work, I provide an overview of current and emerging treatment approaches, in elderly patients with HNC. In particular, I discuss modern surgical approaches that improve radical excision rates while preserving functionality, the incorporation of modern radiotherapeutic techniques and the introduction of novel chemotherapeutic combinations and molecular targeted agents in an effort to reduce toxicity without compromising efficacy. Finally, there is an urgent need to increase accrual and active participation of elderly patients with HNC in clinical trials, including biomarker evaluation in biopsy specimens towards an individualized therapeutic approach.

**Key words:** Elderly patients; Head and neck cancer; Radiotherapy; Surgery; Chemotherapy; Molecular targeted agents

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**Core tip:** Elderly patients with head and neck cancer represent an increasing but frail patient group that require the implementation of multidisciplinary therapeutic approaches. Organ-sparing modern surgical techniques, sophisticated radiotherapy and novel chemotherapeutic and molecular targeted drug combinations enable delivery of optimal treatment in an effort to minimize toxicity without compromising efficacy.

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## INTRODUCTION

Cancer-related incidence and mortality increase progressively with age. It is estimated that by year 2030, 20% of the Europe population will be  $\geq 65$  years of age<sup>[1]</sup> and despite a decrease in overall cancer death rates, the expansion of the elderly and their inherent cancer propensity are expected to ultimately increase cancer prevalence<sup>[2]</sup>. About 60% of all tumors arise in patients older than 65 years and 70% of all deaths due to cancer occur in this elderly patient population<sup>[3-5]</sup>.

Tumors of the head and neck (HNC) represent the sixth most common malignancy and account for 6% of all cancer cases. Although the majority of HNC occur between the fifth and sixth decade of life, their onset in patients older than 60 years is not rare<sup>[3]</sup>, since up to 24% of HNC cases are diagnosed in patients older than 70 years<sup>[4,5]</sup>. In European case series, patients aged between 70 and 75 years represent a proportion of up to 6%-32% of all patients with HNC<sup>[3]</sup>.

The aforementioned evidence underscore the necessity to focus clinical and research efforts on elderly patient population, which is often neglected in current treatment guidelines. The aim of this Review is to draw attention to this frail and under-represented, yet so important group of patients that require a multidisciplinary therapeutic approach.

## METHODOLOGY

For the purposes of this Review, a comprehensive search in the literature was performed using the databases "MEDLINE", "SCOPUS", "EMBASE" and "GOOGLE SCHOLAR" using the key words "Head and neck cancer", "Elderly" and either "Surgery", "Chemotherapy", "Targeted treatment" or "Irradiation/radiotherapy" from January 1973 to December 2012. Initial search with the two terms "Head and neck cancer" and "elderly" yielded 182191 references in the whole literature. After computerized filtering for exclusion of papers not written in English or French, duplications, overlaps, reviews or letters to the editor and irrelevant manuscript titles, the research yielded 7182 original papers. Manual quick review of these publications excluded another 4883 references irrelevant to the topic, ending up to 2299 original articles that were abstract-reviewed. This final process yielded 886 articles that were used as source for the present work (Figure 1).

## DEFINING THE ELDERLY PATIENT WITH HNC

As a general rule, elderly patients are excluded from randomized clinical trials resulting in paucity of evidence-based data regarding efficacy and safety of available treatment modalities. In the literature, few studies have focused on therapeutic strategies in patients aged  $\geq 75$  years<sup>[6-10]</sup>. Finally socioeconomic issues such as access to medical centers and availability of caregivers may influence therapeutic strategy, as well as clinical outcomes.

Several studies indicate that older patients with HNC are less likely to receive curative treatment as compared to the younger age population<sup>[6-9]</sup>. In particular, a strikingly lower prevalence of radical treatments, including surgery and combined modalities such as surgery plus irradiation or chemotherapy plus irradiation, was evident among elderly patients as compared to their younger counterparts. The same was true for overall survival too, with an incremental rate at 5 years of 17%-31% vs 30%-44%, in the same patient cohorts<sup>[7-10]</sup>. These results were challenged, however, by other studies showing that radical surgical or radiotherapy treatment can be performed safely in elderly patients without an increase in overall complication rates, as long as the patients do not have severe comorbidities<sup>[6-8]</sup>, creating thus an ongoing debate.

The definition of the elderly patient with cancer remains controversial and the thresholds used often tend to differ among various malignancies. The "traditional" age limit of 65 years used by the European Organization for Research and Treatment of Cancer (EORTC) has been challenged in the recent years by the increasing life expectancy, as well as the improvements in global cancer care and survival rates. Moreover, a sub-categorization of "younger old" (65-70 years), and "older old" (more than 80 years) has been introduced to allow allocation of elderly patients with cancer to homogenous patient groups.

## SPECIAL FEATURES OF HNC IN THE ELDERLY

### *Risk factors and biology*

HNC is a predominantly male disease with the usual male to female ratio ranging from 8:1 to 15:1<sup>[7]</sup>. Nevertheless, Ang *et al*<sup>[7]</sup> reported an increased incidence of females among the elderly patient population compared to the younger one (15.8% vs 4.4%,  $P < 0.001$ ). The major risk factors for HNC include tobacco use (85%) and alcohol consumption both reported in up to 70% of HNC patients<sup>[7-9]</sup>. However, elderly patients have been reported to



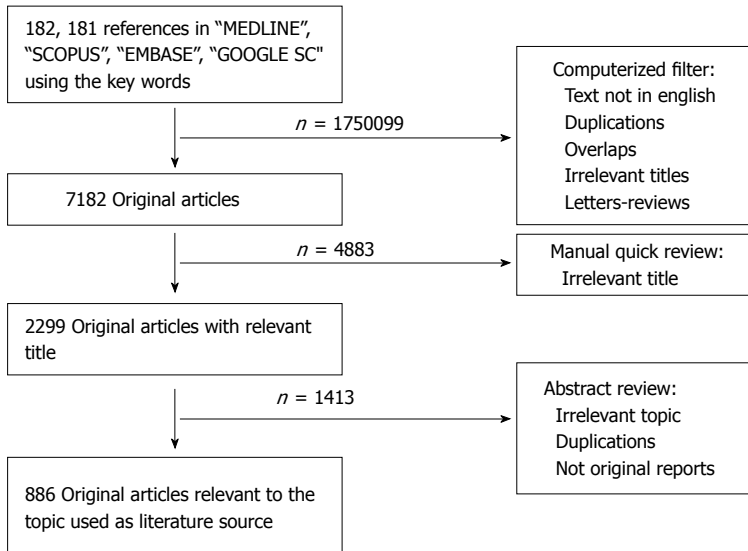


Figure 1 Graphic representation of the research methodology.

have a significantly lower prevalence of alcohol and tobacco exposure, as compared to their younger counterparts<sup>[3]</sup>. This finding follows the rational that malignant tumors occur earlier under the influence of risk factors, but are also likely to occur without them as time passes by<sup>[6]</sup>. As a proof of this concept, in a French cohort of 270 consecutive patients aged  $\geq 80$  years with cancer of the oral cavity, tobacco or alcohol intoxication was the main risk factor among male individuals<sup>[10]</sup>. HPV-positive tumors are also more likely to occur in younger patients, although it has been suggested that they can contribute in HNC carcinogenesis in older patients as well<sup>[7]</sup>.

HNC in the elderly seem to share a specific epidemiological profile thus implying that the molecular profile of these tumors could also be specific. According to the results from studies assessing carcinogenesis in the elderly patient population, a series of spontaneous mutations rather than an increased exposure to carcinogenic substances is responsible for tumour cell transformation<sup>[8]</sup>. Because of those mutations, as well as the ageing process, hypomethylation of the DNA was recognized as the underlying mechanism leading to malignant transformation<sup>[9,10]</sup>. Moreover, recently published Genome-Wide Association studies implicate a number of important cellular processes in NHC neoplastic transformation, including deregulation of tumour-suppressor-genes, inactivating mutations in the molecular pathway of squamous differentiation (Notch/TP63 pathway), loss-of-function mutations in proliferative cell-signaling and impairment of epigenetic integrity<sup>[11]</sup>. These studies discovered mutations in genes involved in the differentiation program of squamous epithelium and Notch/p63 axis (such as NOTCH1, TP63 and FBXW7), and validated findings derived from previous genetic studies (such as mutations in TP53, CDKN2A, PIK3CA, CCND1 and HRAS) as driver genetic events in SCCHN neoplastic transformation.

### Tumor distribution and histology

HNC is a heterogeneous disease entity comprising cancers originating from the paranasal sinuses, nasal cavity, oral cavity, pharynx and larynx<sup>[12]</sup>. In western countries, the three latter locations are the ones most usually affected in the elderly patients. In a recent study evaluating 316 patients with HNC of 80 years of age or more, Italiano *et al*<sup>[13]</sup> reported that 46% of the tumors were located in the oral cavity, 23% in the laryngeal, 19% in oropharyngeal, and 4% in hypopharyngeal sites whereas in 7% of the patients, another site was also involved. Finally, Jun *et al*<sup>[14]</sup> reported a series of 159 patients aged  $> 80$  years in whom 53% of tumours were located in the oral cavity, 10.9% in the larynx, and only 5.8% in the pharyngo-laryngeal area.

The most common histological type of HNC is squamous cell carcinoma (95%), followed by less common types including salivary gland tumors, lymphomas and sarcomas<sup>[15]</sup>. Nevertheless, a rare histological type of well differentiated squamous cell carcinoma, called verrucous type of SCCHN, is more prevalent in elderly patients<sup>[16]</sup>. In the following paragraphs, the term HNC, as well as the presented data will refer to squamous-cell carcinoma, which is the pre-dominant histological subtype.

### Stage and clinical features

In general, nearly two-thirds of HNC patients present with locally advanced disease, whereas metastatic disease at diagnosis is documented in about 10% of patients<sup>[3]</sup>. Elderly patients have been reported to present more often with locally advanced disease (T3 or T4, in TNM staging) but with a lower incidence of regional lymph node metastasis at diagnosis<sup>[3,8]</sup>. Cancer stage at diagnosis was equally distributed between the older patients and the younger ones-31.1% vs 29.8% for those with stages I and II, 37.9% vs 37% for those with stage III, and 31% vs 33.2% for those with stage IV, respectively<sup>[8]</sup>.

Metastatic disease usually occurs in distal lymph-nodes and, in late stages of the disease, in lung parenchyma or the liver *via* hematogenous spread.

In elderly patients the length of their symptoms history is of potential clinical importance, since a median duration of complaints dating up to 15.5 wk until the patients sought medical advice has been reported<sup>[7]</sup>. Older patients tend to perceive several-otherwise alerting-symptoms as normal in the ageing process, or to attribute them to common colds or upper aero-digestive tract infections. Additional medical and social problems of the elderly generation such as social isolation due to the loss of partner or friends, the distance to children or other relatives, limited mobility, hearing loss, visual loss, other physical handicaps, or already existing diseases occupy more space in the awareness of the patients than cancerous diseases which they are not used to discuss openly<sup>[6]</sup>. Thus an eventual newly developed malignant disease is likely to be neglected, as long as the symptoms do not influence daily routine<sup>[6,17]</sup>. Importantly, elderly patients should be educated to report their symptoms to the family physician or a specialist, who should have a high level of suspicion for diagnosis of this particular disease and should alert the patient in case of heralding symptoms and signs.

## THERAPEUTIC APPROACH OF HNC IN THE ELDERLY

### General principles

Because of their frail nature, treatment of geriatric patients with HNC sometimes necessitates compromises and the use of suboptimal regimens which are better tolerated than standard treatment. Surgery was reported to be less often used for older patients: 13.9% vs 27.4% for the primary site and 15.4% vs 35.6% for neck lesions<sup>[18]</sup>. Combined modalities including surgery and irradiation or chemoradiotherapy were also less frequently administered in the elderly patient population than in the younger patients: 22.3% vs 9.7% and 14.1% vs 0.2%, respectively<sup>[19]</sup>. Systemic chemotherapy as exclusive treatment was less frequently used for elderly: 5.5% vs 17.6% and, even in these cases, it was mainly used as palliative treatment.

For a long time undertreatment was attributed to assumed poor tolerance and compliance to treatment in older patients. The available data currently suggest that curative therapy should be offered in elderly HNC patients, not only because of the reversible nature of therapy-associated toxicities but also because of the relatively good prognosis<sup>[6]</sup>. Chronological age by itself is an unreliable parameter for decision making. The treatment of choice should be based on a medical assessment and the preferences of the patient, not on chronological age alone<sup>[18]</sup>.

### Surgery

**Feasibility:** Surgery should be offered as the preferred treatment when the primary tumor can be removed with clear margins without causing major functional compromise; Such an aggressive approach with a curative intent can also be considered for the elderly HNC patients<sup>[19]</sup>. The choice of radical local therapy must be based on a number of aspects, including the potential functional outcome of treatment, the comprehensive geriatric assessment, the life expectancy and, importantly, the patient's wishes.

As has been previously suggested<sup>[3,19,20]</sup>, chronological age alone should not be a contraindication to aggressive surgical approach, which should be attempted whenever risk-assessment ration is favorable. In a large retrospective series of 810 patients > 65 years who had undergone major head and neck resections, Morgan *et al*<sup>[21]</sup> reported an acceptable overall mortality rate of 3.5%. Aggressive surgical approach should include attempting a radical surgical excision that removes thoroughly the tumor without compromising functional outcomes.

**Preoperative risk assessment:** According to the series from Serletti *et al*<sup>[22]</sup> prolonged surgical time longer than 10 h serves as a predictive factor for the development of postoperative surgical complications. In another study<sup>[22]</sup> assessing 121 patients treated for HNC, after stratification according to their age, a number of surgery-related complications, occurred in 53% of the elderly patient group. Notably, tumor-specific 5-year survival rates were absolutely comparable 85.2% for the younger patients and 84.5% for the aged (> 65 years)<sup>[22]</sup>. In a third large study<sup>[23]</sup> among 242 patients aged 70 years or more who underwent surgery with curative potential for HNC, co-morbidities were present in 87.6% of the patients and 56.6% had some type of postoperative complication.

### Radiotherapy

**Dose intensity:** As a general rule, the primary tumor and gross lymphadenopathy require a total dose of 70 Gy at a dose fractionation of 2 Gy/d, while radiation to suspected unresected microscopic disease in nodal levels requires a total of 50 Gy or more at 2 Gy/d<sup>[6]</sup>. An important issue is whether the reduction of the clinical target volume (CTV)-in an effort to minimize adverse events, is acceptable in this frail group of patients. In this context, Ortholan *et al*<sup>[23]</sup> reported that the omission of regional lymph-node irradiation for T1-T2 N0 oral cavity cancer in patients more than 80 years of age is associated with a high risk of node recurrence.

**Toxicity and efficacy:** Several studies have shown that RT is effective and well tolerated in the elderly patient population and advanced age alone should

not be considered a contraindicator for radiation therapy<sup>[24-28]</sup>. Nevertheless, it has been reported more than a decade ago that elderly patients with HNC recruited in clinical trials had a better performance status compared to those who were not included in clinical trials; therefore, the results from clinical trials might be biased and not generalizable to the general aged HNC population<sup>[29]</sup>. Besides those limitations a meta-analysis of HNC patients enrolled in the European Organization for Research and Treatment of Cancer (EORTC) trials using standard treatment approaches demonstrated that survival and late toxicity were similar for each age group<sup>[20]</sup>. However, a subsequent meta-analysis found that the benefit of intensified radiotherapy (RT) regimens was diminished in the elderly patients enrolled in chemoradiotherapy or accelerated RT<sup>[30]</sup> trials, probably owing to competing risks from co-morbidities. In another study<sup>[31]</sup>, among 1487 patients who received definitive radiotherapy, no differences were found between the elderly and younger patients in terms of treatment interruption, completion and treatment-related death. Within the subset of 760 patients who received intensified treatment (concurrent chemoradiotherapy or hyperfractionated accelerated RT), no difference was seen between the elderly and younger patients with respect to the outcome. Of note, after a median follow-up of 2.5 years, the two-year cause-specific survival rate after definitive RT for the elderly and for the younger patients was 72% and 86%, respectively<sup>[31]</sup>. Despite their retrospective nature, these results suggest that the outcome in elderly patients is comparable to that of younger patients<sup>[32]</sup>.

**Palliative radiotherapy:** It has been argued that the use of palliative RT in HNC elderly patients is potentially hazardous due to the presumed significant toxicity resulting from the dose used in order to achieve a clinical benefit<sup>[33]</sup>. However, regarding the tolerance of radiotherapy, in a series of 1589 patients included in the EORTC trials between 1980 and 1995 with 20% of patients aged > 65 years and 2% aged > 75 years, the aforementioned meta-analysis<sup>[20]</sup> concluded that adverse mucosal rates increased with age of the patients, with 8% of severe toxicity in patients aged less than 50 years and 31% in those more than 70 years of age. Nevertheless, there was no statistically significant difference in survival or severe mucosal reactions and in weight loss rates more than 10% between the two age groups. However, older patients experienced more frequently severe functional toxicity, as compared to their younger counterparts.

#### **Fractionation and transportation issues**

An aspect of particular clinical importance is the number of daily transportations required for HNC radiotherapy, which in standard fractionation,

necessitates approximately 35 daily transportations for more than 7 wk, a process that has been associated with increased fatigue in the elderly patients over time<sup>[34]</sup>. Inevitably, treatment interruption due to fatigue or to socioeconomic reasons is frequent in elderly patients<sup>[6,35]</sup>. Many hypofractionated palliative schedules for HN cancers have been proposed including 20 Gy in five fractions<sup>[35]</sup>, 30 Gy in five fractions<sup>[36]</sup>, 14 Gy in four fractions<sup>[37]</sup>, and 50 Gy in 16 fractions<sup>[38]</sup>. Nonetheless, increased late toxicity rates were still reported for those patients treated using a hypofractionated schedule<sup>[39]</sup>. In every case, practical issues regarding transportation and number of hospital visits should be always taken into account in the multidisciplinary care of elderly patients with HNC because they can profoundly affect the quality of life of the patient.

#### **Chemotherapy**

**Feasibility and tolerability:** Chemotherapy in HNC can be administered with different potentials: (1) in combination with locoregional therapy (surgery or radiotherapy) in patients with locally advanced disease; (2) as neoadjuvant/induction when it is delivered before surgery or radical radiotherapy; (3) as adjuvant when it is delivered following radiotherapy or surgery; and (4) as the only treatment in recurrent or metastatic disease. The standard chemotherapy regimen for advanced HNC is a combination of cisplatin and infusional 5 fluorouracil (5FU) and usually achieves response rates of up to 40%-50% in the recurrent or metastatic setting, whereas in the induction setting attempted for organ preservation, this combination may yield response rates of up to 70%-80%<sup>[40]</sup>. The incorporation of chemotherapy to locoregional treatment (surgery or irradiation) for patients with locally advanced HNC has been consistently reported to improve survival<sup>[41]</sup>. The incorporation of cisplatin in post-operative irradiation has been reported to be beneficial in cases of either positive surgical margins or extracapsular node involvement<sup>[42]</sup>. A synchronous study suggested for the first time that the size of benefit with concurrent chemoradiotherapy is age dependent, with the largest benefit in patients younger than 60 years of age and at the expense of increased acute early and late toxicity<sup>[42]</sup>. Docetaxel has been the only chemotherapeutic regimen that offered an absolute survival benefit when added to the cisplatin-fluorouracil combination, including the elderly<sup>[43]</sup>. In a confirmatory study with 10% of the patients aged between 65 and 71 years old, induction chemotherapy with the addition of docetaxel to the standard regimen of cisplatin and fluorouracil significantly improved progression free and overall survival in patients with initially inoperable-advanced HNC<sup>[44]</sup>.

Elderly patients have been considered subjects at high risk for toxicity from cytotoxic agents<sup>[3,6,45]</sup>,

since age has been associated with pharmacokinetic and pharmacodynamic changes and with increased susceptibility of normal tissues to toxic complications or reduced capacity of healthy tissues to recuperate. Besides the “classical” toxicities observed with cytotoxic agents (anemia, neutropenia, thrombocytopenia, fatigue, anorexia and gastrointestinal abnormalities), chemotherapy may affect cognition, continence, vision, balance and even mood<sup>[6]</sup>. In elderly patients with other tumor types, no major age-related differences in drug clearance were demonstrated for docetaxel and paclitaxel<sup>[46,47]</sup>. The hematopoietic reserve is also reduced in the elderly, which renders them more susceptible to chemotherapy-induced myelotoxicity<sup>[48]</sup>.

**“To chemo or not to chemo?” The ongoing debate:** A number of retrospective studies in various solid tumors have reported that toxicity in general is not increased in the elderly<sup>[49-51]</sup>, although these results have been challenged by other studies<sup>[52-54]</sup>. In elderly patients with advanced HNC in particular, combined data from two phase III randomized trials<sup>[43]</sup>, conducted by the Eastern Cooperative Oncology Group attempted to clarify the obscure landscape: The trial E1393 compared cisplatin plus paclitaxel at two dose levels and the trial E1395 compared cisplatin plus fluorouracil to cisplatin plus paclitaxel; Both trials evaluated clinical outcomes and toxicity in patients 70 years or older as compared to their younger counterparts<sup>[55]</sup>. As commented by the authors, “fit” elderly patients sustained increased toxicities with platinum-based combinations, but had comparable survival outcomes to younger patients. It should be noted, however, that the number of elderly patients was strikingly low (13% were 70 years or older and 30% 65 years or older), highlighting thus the problem of participation of elderly patients with HNC in clinical trials, even when the crucial question of the clinical trial is the age effect.

#### **Targeted and combined therapy**

Cetuximab is a chimeric IgG1 monoclonal antibody against the ligand-binding domain of EGFR that has been proven to possess synergistic cytotoxic effect with radiation against HNC<sup>[55-58]</sup>. The largest to date randomized controlled trial involved 424 patients and demonstrated that in locally advanced disease, concurrent administration of cetuximab, with radical external beam radiotherapy resulted in an 11% reduction in progression and a 10% improvement in overall survival<sup>[55]</sup>. There are no clinical studies assessing the efficacy or tolerability of cetuximab particularly in the elderly patient population: In the aforementioned study only 11% of patients were 70 years of age or older at study entry; however, the demonstrated activity of cetuximab with concurrent radiotherapy, along with the reported good

tolerance of the combination and low toxicity, suggest that it could represent a valid alternative to the combination of platinum compounds with radiotherapy in elderly patients unfit for cisplatin administration due to nephrotoxicity, ototoxicity or sensory neuropathy. Other targeted agents are currently under early clinical evaluation in HNC including angiogenesis inhibitors, tyrosine kinase inhibitors and immunotherapy.

#### **Clinical endpoints and their assessment in elderly patients with HNC**

Survival is not easy to assess in a population that has, by definition, a rather short life expectancy even when a malignant disease has not been diagnosed. An 80-year-old person, for example, had a life expectancy of a further 8 years and a 70-year-old person had a life expectancy of a further 15 years in the ninth decade of the twentieth century in western countries. Patients older than 80 years have a 1.68-fold increased risk of death, even when adjusted for variables such as the severity of comorbidities, clinical stage and functional status. Thus, as expected overall survival was significantly lower in elderly patients, with an actuarial rate at 5 years of 17%-31% vs 30%-44% in younger patients in the same case series of HNC patients<sup>[56-58]</sup>. However, these differences tend to deteriorate or even disappear, in the case series where cancer-specific survival is analyzed and/or the groups of patients are homogeneous in terms of radicality of treatment. When considering cancer-specific overall survival, the difference between the two groups was at borderline statistical level, being at 5 years 55% vs 59%, respectively<sup>[59,60]</sup>. Cancer-specific overall survival was similar between the two groups for oral cavity and oropharyngeal cancer, whereas elderly patients with laryngeal and hypopharyngeal cancer had a significantly worse 5-year cancer-specific overall survival compared to their younger counterparts (71% vs 78%,  $P = 0.02$  and 30% vs 42%,  $P < 0.01$ )<sup>[61]</sup>. In the case-control study by the surveillance, epidemiology and result data base of Baltimore, on 2508 cases of HNC in patients older than 50 years, cancer-specific survival of patients older than 70 years has been shown to be comparable to that of patients of 50-69 years, with the exception of stage I and IV glottic carcinoma and stage III tonsil carcinoma, where cancer-specific prognosis has been demonstrated to be worse and better in elderly patients, respectively<sup>[3]</sup>.

## **CONCLUSION**

Elderly patients can cope, tolerate and adapt remarkably well and several studies have shown that the quality of life of elderly patients undergoing curative treatment for cancer of the head and neck



is comparable to that of the younger population<sup>[61-63]</sup>. Despite the aforementioned data, elderly patients with cancer of the head and neck are less likely to receive standard treatment including radical surgery or postoperative chemo-radiotherapy, which probably contributes to poor outcomes reported in those patient populations<sup>[6]</sup>. There is thus an absolute necessity to improve the framework of cancer care for this frail subgroup of patients and provide them with adequate physical and emotional support that they need, especially at this period of their life.

Today, it is well recognized that elderly patients with HNC tend to receive suboptimal treatment, mainly due to fears of poor adherence and/or tolerance, excessive toxicity or lack of support from their environment. Nevertheless, it becomes increasingly apparent that medical intervention in the elderly should be guided by the benefit/risk ratio, as estimated by the co-evaluation of the expected treatment outcome, the life expectancy of the patient, the possible therapy-related toxicities and the patient tolerability. There is now international consensus, that elderly patients affected by HNC should be treated on the basis of a curative intent, as long as comprehensive preoperative evaluation of existing comorbidities is performed and optimal management of concomitant morbidities is completed. Age itself should never guide therapeutic decision, but a holistic, multidisciplinary approach addressing the real needs of the patient, as well as her/his wishes, should be implemented and maintained throughout the whole therapeutic process.

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## Sentinel lymph node mapping of a breast cancer of the vulva: Case report and literature review

James Cripe, Ramez Eskander, Krishnansu Tewari

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### Abstract

Ectopic breast tissue is rare and typically presents as an axillary mass. Previous reports have identified ectopic breast tissue in the vulva, but malignancy is exceedingly uncommon. We present a 62 years old with

locally advanced breast carcinoma arising in the vulva demonstrates the utilization of sentinel lymph node mapping to identify metastatic lymph nodes previously unable to be identified *via* traditional surgical exploration. Our case supports the principles of adjuvant therapy for breast cancer to be applied to ectopic breast cancer arising in the vulva. A literature review highlights common key points in similar cases to guide management.

**Key words:** Vulvar cancer; Ectopic breast; Sentinel lymph node; Breast cancer; Vulvar breast cancer

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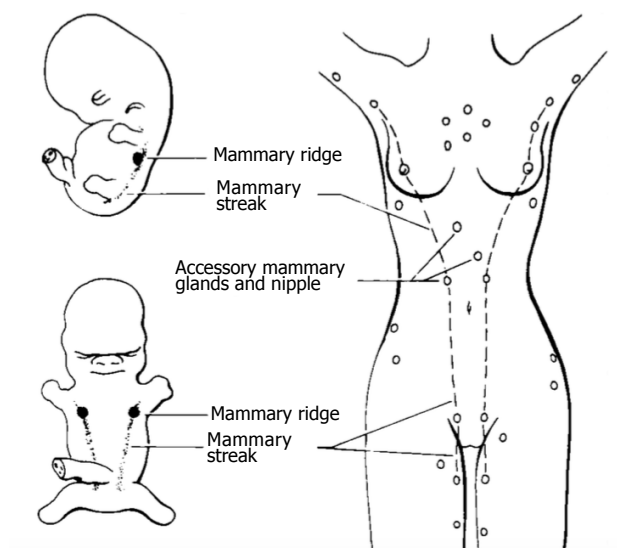
**Core tip:** Our findings describe the presentation of ectopic breast cancer in the vulva. We demonstrate use of sentinel lymph node technology with identification of the sentinel node, only possible after the use of this technology. We conclude with a review of the literature outlining treatment of this enigmatic disease.

Cripe J, Eskander R, Tewari K. Sentinel lymph node mapping of a breast cancer of the vulva: Case report and literature review. *World J Clin Oncol* 2015; 6(2): 16-21 Available from: URL: <http://www.wjgnet.com/2218-4333/full/v6/i2/16.htm> DOI: <http://dx.doi.org/10.5306/wjco.v6.i2.16>

### INTRODUCTION

Ectopic breast tissue has been previously reported in various locations along the primitive milk line, from the axilla to the vulva (Figure 1). Axillary ectopic breast tissue is the most frequent location and the vulva being the least common site<sup>[1]</sup>. Malignant ectopic breast tissue is rare, typically presenting as an axillary mass, with vulvar breast malignancy being exceedingly rare<sup>[1]</sup>. In 1935, Green *et al*<sup>[2]</sup> published the first case report





**Figure 1** Ectopic breast tissue has been previously reported in various locations along the primitive milk line, from the axilla to the vulva.

of adenocarcinoma arising from breast tissue in the vulva. Although 22 cases of malignant vulvar breast tissue have been reported since then, there are no clear guidelines regarding surgical or adjuvant treatment. We present a case that outlines the diagnosis and management of primary breast cancer of the vulva, highlighting diagnostic dilemmas, the utility of sentinel node mapping and reinforcing the importance of a multidisciplinary approach in the management of this rare clinical entity.

## CASE REPORT

A 62 years old Hispanic multiparous women noted a new 1.3 cm left labial mass for approximately 1 year and presented to her primary gynecologist for evaluation. She underwent a wide local excision that was noteworthy for an invasive ductal carcinoma arising in ectopic breast tissue. Final pathology was confirmed by independent review at two separate institutions. Immunohistochemical staining showed the lesion to be 95% estrogen receptor (ER) positive, 10% progesterone receptor (PR) positive, and human epidermal growth factor 2 (HER2) negative (Figure 2).

The patient underwent a magnetic resonance imaging of the breast that was negative for a breast primary malignancy. Approximately 1 mo after initial presentation in September of 2012, the patient was referred to gynecologic oncology and underwent a partial radical vulvectomy at the prior vulvar scar site. Final pathology was negative for residual disease and the patient, given absence of metastatic disease declined adjuvant therapy. The patient initiated close surveillance and had a Fluoro-deoxyglucose (FDG) Positron emission tomography (PET) scan in January 2013 with findings of suspicious left inguinal-femoral lymphadenopathy, with standard uptake value (SUV) of 8.1.

The patient was counseled to undergo left inguinal-femoral lymphadenectomy (LND). The dissection was completed superficial to the cribiform fascia and final pathology identified 14 lymph nodes ranging from 1.2-2.5 cm that were all negative for tumor. On follow up examination in April 2013, the patient was found to have a 1-2 mm firm, non-tender nodule under her healing scar. In office biopsy confirmed recurrent invasive ductal carcinoma, with identical histology to the previous primary lesion. A repeat wide local excision was performed in June 2013. Pathology from that surgical resection was negative for tumor.

A PET-CT in August 2013 was repeated and was significant for suspicious left inguinal lymph node measuring 1.1 cm × 1.6 cm with SUV of 8.2 (Figure 3). The patient returned to the operating room with preoperative technetium 99 lymphoscintigraphy and lymphazurin blue (injected into the previous left surgical site) lymph node localization (Figure 4). An inguinal incision was created and the Geiger counter was used to identify "hot" areas. Dissection continued until area of maximum radioactivity was encountered. A hot, blue, slightly firm, 1.2 cm left sentinel was identified superficial to the cribiform fascia and excised. Intraoperative frozen section was positive for metastasis and comprehensive LND was performed. Two additional left sentinels (both hot and blue) were positive for ductal carcinoma. A right-sided sentinel node was not identified, but given contralateral positive nodes a comprehensive right LND was performed. Final pathology (Figure 2) confirmed three positive sentinels and 14 negative left and right inguinofemoral nodes.

Metastatic workup was negative and the patient underwent intensity modulated radiation therapy (4500 cGy) with 5900 cGy boosts to the left groin. Chemotherapy included weekly taxol followed by adriamycin and cyclophosphamide. Following adjuvant therapy she started maintenance therapy with an aromatase inhibitor.

She is currently without evidence of disease recurrence 13 mo after sentinel lymph node detection.

## DISCUSSION

Ectopic breast tissue is rare and accounts for 0.2%-0.6% of all breast cancers. Only 4% of these ectopic breast cancers are located in the vulva, making vulvar breast cancer exceedingly rare<sup>[3]</sup>. Ectopic breast tissue originates in the fetus at the ectodermal mammary streak extending from the axilla to the groin as demonstrated in Figure 1. Most of this structure disappears with small portions persisting in the thorax. This primordial ectoderm penetrates the underlying mesenchyme and gives rise to small solid out buddings that canalize and form the lactiferous ducts and alveoli of the mammary gland<sup>[4,5]</sup>.

There have been 22 reported cases since Greene's index case report in 1935. The majority of these patients

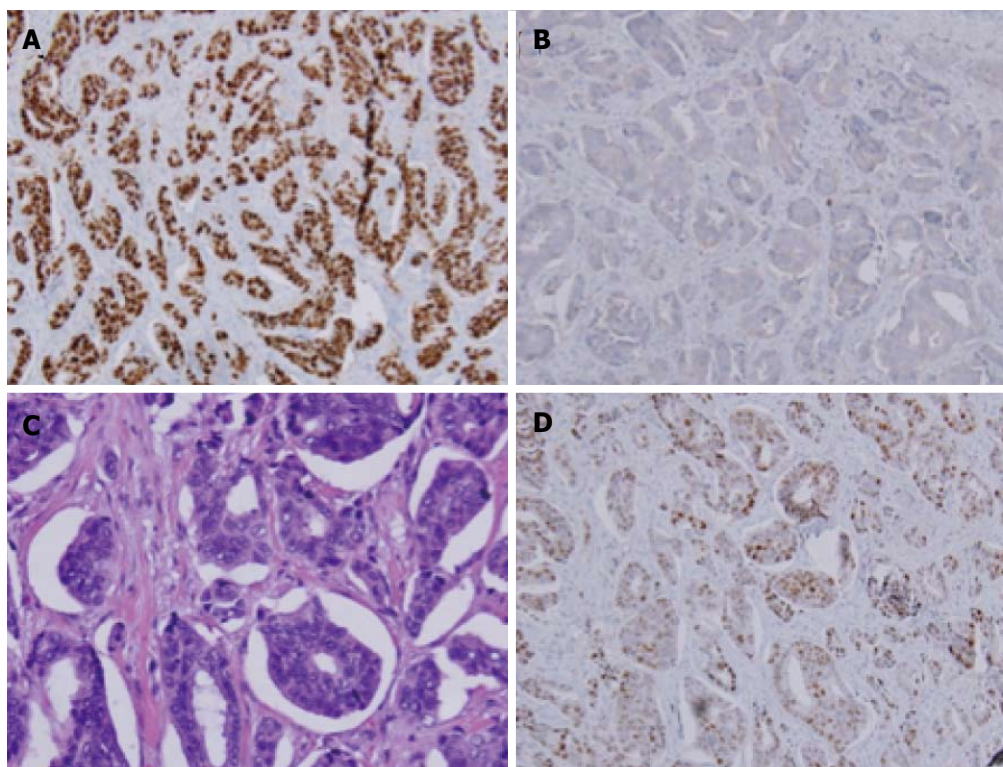


Figure 2 Estrogen receptor staining of primary tumor (A), Her2neu staining of primary tumor (B), metastasis to the lymph node (C), progesterone receptor staining of primary tumor (D).

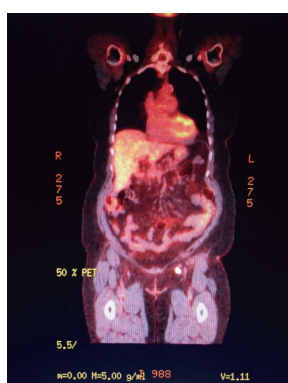


Figure 3 Patient returned to the operating room with preoperative technetium 99 lymphoscintigraphy and lymphazurin blue (injected into the previous left surgical site) lymph node localization.

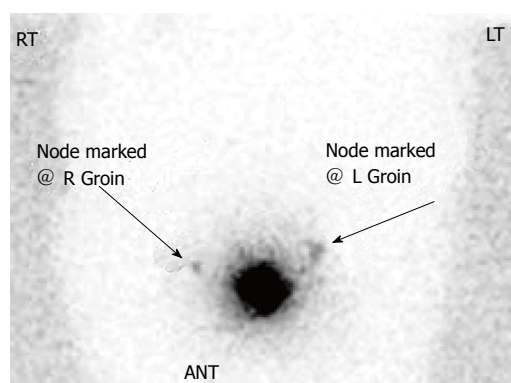


Figure 4 Ectopic breast tissue originates in the fetus at the ectodermal mammary streak extending from the axilla to the groin. RT: Radiation therapy.

presented with an innocuous solitary lesion of the vulva (Table 1); upon surgical excision, adenocarcinoma or ductal carcinoma arising in normal appearing breast tissue was identified. Extensive preoperative imaging is traditionally used to exclude metastasis of a primary breast malignancy. Two of these reported cases were indeed metastatic from a primary breast lesion<sup>[6,7]</sup>. Adjuvant chemotherapy and radiation treatment protocols are heterogeneous (Table 2) given the rare frequency of these lesions, and absence of standardized treatment paradigms. Anti-hormonal therapy has been used in 14 (13 Tamoxifen and 1 Aromatase) patients with ER/PR positive specimens with various outcomes. The use of trastuzumab in

HER2 positive cases has not been previously reported.

The presence of metastatic tumor in regional lymph nodes remains the most significant prognostic factor for several malignancies, including breast cancer. Sixteen patients underwent inguinal LND with all 16 patients having lymph node involvement. Survival and adjuvant therapy data are outlined in Table 2. Sentinel lymph node mapping is a technique that minimizes morbidity while maintaining diagnostic accuracy by isolating the first or "sentinel" node to drain the affected area burdened with tumor. This is traditionally performed with injection of the tumor with isosulfan blue and a radiolabeled colloid, most often technetium 99. This technique was pioneered by Morton in the treatment

Table 1 Clinical presentations of vulvar-breast cancer

Ref.	Year	Age (yr)	Location	Size (cm)	Symptoms and duration	Duration (mo)
Greene <sup>[2]</sup>	1935	59	Right labia majora	20 × 15	Mass	12
Hendrix <i>et al</i> <sup>[14]</sup>	1956	58	Right Labia minora	3	Ulcerated mass	84
Guerry <i>et al</i> <sup>[16]</sup>	1976	62	Right labia minora	1.5	Mass	4
Guercio <i>et al</i> <sup>[15]</sup>	1984	49	Left labia majora	2	Mass	Unk
Cho <i>et al</i> <sup>[16]</sup>	1985	70	Right labia majora	3 × 4	Mass	30
Simon <i>et al</i> <sup>[17]</sup>	1988	60	Right labia majora	2 × 2	Ulcerated mass	36
Rose <i>et al</i> <sup>[18]</sup>	1990	68	Right labia majora	3.5 × 3.5	Mass	36
Di Bonito <i>et al</i> <sup>[19]</sup>	1992	46	Right labia majora	1.5	Ulcerated mass	24
Bailey <i>et al</i> <sup>[20]</sup>	1993	65	Right labia majora	3 × 2	Ulcerated mass	36
Levin <i>et al</i> <sup>[21]</sup>	1994	62	Left clitoris	2.5	Mass	Unk
Kennedy <i>et al</i> <sup>[7]</sup>	1997	71	Left labia majora	5	Ulceration and dysuria	1
Irvin <i>et al</i> <sup>[4]</sup>	1998	64	Left lateral mons	3	Indurated mass	48
Gorisek <i>et al</i> <sup>[22]</sup>	2000	81	Left labia majora	2 × 3	Ulcerated mass	Unk
Piura <i>et al</i> <sup>[23]</sup>	2002	69	Left labia majora	3	Ulcerated mas	Unk
Chung-Park <i>et al</i> <sup>[24]</sup>	2002	47	Right labia minora	2	Ulcerated mass	12
Yin <i>et al</i> <sup>[25]</sup>	2003	84	Mons	5	Swelling	24
Lopes <i>et al</i> <sup>[26]</sup>	2006	44	Left vulva	2	Mass	48
Fracchioli <i>et al</i> <sup>[27]</sup>	2006	57	Left vulva	1	Mass	Unk
North <i>et al</i> <sup>[28]</sup>	2006	49	Right labia minora and clitoris	1.5	Pain and pressure	Unk
			Left groin	2		
Naseer <i>et al</i> <sup>[29]</sup>	2011	57	Right labia majora	1.5	Non painful lesion	Unk
McMaster <sup>[30]</sup>	2013	60	Left labia majora	3	Pedunculated ulcerated mass	6
Bogani <i>et al</i> <sup>[13]</sup>	2013	71	Left labia Majora	4	Painful ulcerated mass	Unk
			Left groin	3		

Unk: Unknown.

of melanoma in the early 1990's<sup>[8]</sup>. The assessment of regional lymph nodes in breast cancer paralleled the work in melanoma, in an effort to limit the morbidity of axillary lymph node dissection<sup>[9]</sup>. Numerous clinical trials have detailed the effectiveness and reduced morbidity associated with sentinel lymph node dissection in breast cancer patients in both the primary surgical setting and following neoadjuvant therapy. Current American Society of Clinical Oncology (ASCO) guidelines recommends sentinel lymph node mapping as standard of care in breast cancer<sup>[10]</sup>.

Sentinel node mapping in vulvar cancer is a more contemporary topic with evolving literature, and has paralleled some advances in penile carcinoma lymphatic mapping. GROINS-V, an observational study, followed 403 patients with primary vulvar tumors less than 4 cm treated with sentinel node mapping. Eight patients had groin recurrence with a false negative rate of 5.9% and a false negative predictive value of 2.9%<sup>[11]</sup>. Similar results were replicated in GOG protocol 173<sup>[12]</sup>, a phase 3 multi-institutional study of intraoperative lymphatic mapping in patients with invasive squamous cell carcinoma of the vulva. Inclusion criteria included depth of invasion > 1 mm and primary tumor size 2-6 cm. Four hundred and fifty-two patients underwent sentinel node mapping with 418 patients having a sentinel node identified. Eleven (8.3%) patients with negative sentinel lymph nodes had groin recurrence. The false negative rate in primary lesions < 4 cm was 2%, but with lesions > 4 cm the false negative rate was 4%<sup>[11]</sup>. It is important to note that all patients in GOG protocol 173 underwent comprehensive LND after sentinel LND

regardless of its status. GROINS-V also identified a statistically significant decrease in wound breakdown, cellulitis, and lymphedema in patients undergoing sentinel lymph node mapping. Both studies provide evidence to support the incorporation of sentinel LND in the management of vulvar malignancies.

Our case utilized sentinel lymph node mapping at time of recurrence and precisely aided in the identification of the metastatic lymph nodes. This technology was not utilized in the primary surgical management due to uncertainties on how it would perform when the primary lesion was comprised of ectopic breast tissue. However, after failing to identify the PET positive nodes with standard LND, SLD was employed to identify and excise the lymph nodes.

Primary breast cancer originating in the vulva is rare and management strategies stem from individual case reports or case series. Current literature supports the use of sentinel lymph node mapping in vulvar cancer and we anticipate that future cases will utilize this practice. Bogani *et al*<sup>[13]</sup> as well as our case have been the only published literature to utilize sentinel lymph node mapping after a previous LND. Both cases identified positive sentinel lymph nodes that were previously unable to have been resected. These findings may support the up-front use of sentinel lymph node localization.

From our review of the literature there are several key concepts in managing this rare malignancy. First, exclusion of a primary breast malignancy needs to be confirmed by pretreatment imaging and physical examination. Next, occurrence of positive nodes is

**Table 2** Previously published reports

Ref.	Treatment	Histology	ER	PR	Her2-neu	LN	Status	Follow up (mo)
Greene <sup>[2]</sup>	None	AC + S	NA	NA	NA	NA	DOD	1
Hendrix <i>et al</i> <sup>[14]</sup>	Surgery	AC	NA	NA	NA	NA	DOD	4
Guerry <i>et al</i> <sup>[6]</sup>	Surgery	DC	NA	NA	NA	NA	DOD	24
Guercio <i>et al</i> <sup>[15]</sup>	Surgery + RT	LO	NA	NA	NA	11/24	NED	36
Cho <i>et al</i> <sup>[16]</sup>	Surgery + tamoxifen	AC	+	+	NA	2/9	NED	24
Simon <i>et al</i> <sup>[17]</sup>	Surgery + tamoxifen + cyclophosphamide, adriamycin, 5FU Recurrence cisplatin/etoposide then carboplatin/etoposide	AC	+	+	NA	3/11	DOD	27
Rose <i>et al</i> <sup>[18]</sup>	Surgery + RT + tamoxifen	DC	+	-	NA	1/15	Unk	Unk
Di Bonito <i>et al</i> <sup>[19]</sup>	Surgery	Unk			NA	11/13	NED	4
Bailey <i>et al</i> <sup>[20]</sup>	Surgery + tamoxifen	DC	+	+	NA	2/20	NED	12
Levin <i>et al</i> <sup>[21]</sup>	Surgery + tamoxifen Recurrence restarted tamoxifen/RT	AC	+	-	+	4/11	NED	24
Kennedy <i>et al</i> <sup>[7]</sup>	Surgery + adriamycin/cyclophosphamide	Unk	-	-	NA	9/9	NED	15
Irvin <i>et al</i> <sup>[4]</sup>	Surgery + cytoxan/Mtx/5FU + RT + tamoxifen	AC	+	+	NA	1/14	NED	4
Gorisek <i>et al</i> <sup>[22]</sup>	Surgery + tamoxifen	AC	+	+	NA	NA	NED	19
Piura <i>et al</i> <sup>[23]</sup>	Surgery + adriamycin/cyclophosphamide/paclitaxel + RT + tamoxifen	AC	+	+	NA	7/15	NED	14
Chung-Park <i>et al</i> <sup>[24]</sup>	Surgery	MU	+	+	-	NA	NED	36
Yin <i>et al</i> <sup>[25]</sup>	Surgery	MU	+	+	-	1/11	NED	9
Lopes <i>et al</i> <sup>[26]</sup>	Surgery + docetaxel/doxorubicin/cyclophosphamide + tamoxifen	MU	+	NA	-	2/13	Unk	Unk
Fracchioli <i>et al</i> <sup>[27]</sup>	Surgery + 5FU/adriamycin/cyclophosphamide + tamoxifen Recurrence-paclitaxel	AC	-	NA	NA	7/7	Rec Unk	36 Unk
North <i>et al</i> <sup>[28]</sup>	Surgery + cyclophosphamide/epirubicin/5FU + weekly docetaxel + tamoxifen	DC	+	+	-	5/7	Unk	Unk
Naseer <i>et al</i> <sup>[29]</sup>	Surgery + 5FU/epirubicin/cyclophosphamide + weekly docetaxel + RT + aromatase	DC	+	+	-	3/13	Unk	Unk
McMaster <i>et al</i> <sup>[30]</sup>	Surgery + RT	DC	+	NA	NA	NA	Unk	Unk
Bogani <i>et al</i> <sup>[13]</sup>	Surgery + epirubicin/cyclophosphamide + tamoxifen	DC	+	+	NA	1/8	NED	24

AC: Adenocarcinoma; DC: Ductal carcinoma; DOD: Dead of disease; LO: Lobular; MU: Mucinous; Mtx: Methotrexate; NA: Not applicable; NED: No evidence of disease; Rec: Recurrence; RT: Radiation therapy; S: Squamous; Unk: Unknown; 5FU: 5-fluorouracil.

high and can be difficult to locate; primary surgical excision (radical vulvectomy) with sentinel lymph node dissection to help identify the sentinel lymph node should be considered.

Systemic chemotherapy based on adjuvant therapy platforms for breast cancer with docetaxel or paclitaxel, plus doxorubicin, and cyclophosphamide should be considered. Given the biologic parallels between primary breast and vulvar breast cancer, treatment paradigms mimicking primary breast cancer are a rational approach and are advisable. This is supported by the median survival of patients treated with adjuvant therapy for breast cancer had a mean survival of 30 mo in comparison to 12 mo for those receiving a vulvar treatment (surgery followed by radiation). Finally, patients with estrogen and progesterone receptor positive specimens may be maintained on tamoxifen or aromatase inhibitors.

## COMMENTS

### Case characteristics

A 62 years old Hispanic women presented with a 1.3 cm left labial mass.

### Clinical diagnosis

Suspected neoplasm originating from the left labia.

### Differential diagnosis

Gartner's duct cyst, labial myoma, vulvar squamous cell carcinoma, and vulvar

melanoma.

### Pathological diagnosis

Histologic examination identified the ectopic breast tissue and carcinoma present in excised specimen.

### Treatment

Excisional procedure.

### Related reports

Previous reports of vulvar breast cancer present similarly with an isolated labial mass, treated with surgical excision, however many do not receive chemotherapy that parallels breast cancer treatments.

### Experiences and lessons

When vulvar breast cancer is encountered, the physician should exclude a primary breast malignancy, perform an excision procedure, and utilize sentinel lymph node mapping as recommended in breast cancer.

### Peer-review

Well written case report.

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## Robotic technology: Optimizing the outcomes in rectal cancer?

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### Abstract

Minimally invasive rectal resection remains a challenging procedure, even in experienced hands. Technical limitations explain at least in part the reasons of a relatively poor adoption of laparoscopy for rectal resection, in particular for low tumors in a deep and narrow pelvis. Robotics is intended to overcome these limitations. Potentially better short-term outcomes have been

published: reduced conversion rates, better functional outcomes, shorter learning curve, reduction of positive margins, better specimen... However, robotic surgery has not yet taken over as the gold standard approach for low anterior resection. Several drawbacks might indeed discourage the most fervent surgeon: the size of the robot, the lack of tactile feedback, the risk and difficulties during multiquadrant surgery, and, of course, costs. Whilst new systems might overcome most of these drawbacks, it seems obvious that the development of robotic surgery is underway. Robotics is not just another interesting technical tool, but more a new concept, which should play a role in the future.

**Key words:** Robot; Laparoscopy; Total mesorectal excision; Transanal total mesorectal excision; Transanal endoscopic microsurgery; Outcomes; Rectal cancer

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**Core tip:** The current evidences of robotic rectal resection are presented, as its potential limitations. While several better short-term outcomes have been reported (notably reduced conversion rates, better functional outcomes, shorter learning curve, reduction of positive margins, and better specimen), robotics has not yet taken over as the gold standard for low anterior resection. The reasons for this are analyzed, as the future developments in the robotic rectal field.

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### TEXT

Rectal resection for cancer remains a difficult operation

especially when using a minimally invasive approach. This explains at least in part the reasons for the limited diffusion of laparoscopy in the colorectal field. The technical explanations for this relatively low adoption are well known: unstable instrumentations, two-dimensional vision, narrow space, and poor ergonomics. These limitations are particularly relevant during low rectal dissection in the confines of the pelvis.

On the other hand, the recently published Colorectal cancer Laparoscopic or Open Resection (COLOR) II study has confirmed that in selected patients with rectal cancer treated by skilled surgeons, laparoscopic surgery resulted in similar safety, resection margins, and completeness of resection to that of open surgery, while recovery was improved after laparoscopic surgery. However, even in highly experienced hands, the authors still reported a conversion rate of 17%<sup>[1]</sup>.

The use of robotic technology is intended to overcome these limitations. The initial reports were encouraging with promising outcomes, although a clear advantage has not yet been demonstrated. More than 10 years after the initial experience, robotic surgery has not (yet?) taken over as the gold standard approach for low anterior resection (LAR), and the main question is why?

Focusing on the published evidences, there are yet potentially better short-term outcomes, as shown in several systematic reviews<sup>[2-6]</sup>, notably better functional outcomes<sup>[7]</sup> and a shorter learning curve<sup>[8]</sup>. This is particularly true when applied in selected patients such as obese and/or male patients, especially those with preoperative radiotherapy, and tumors in the lower two thirds of the rectum<sup>[3]</sup>. Indeed, robotics may overcome the challenges associated with difficult pelvic anatomy and might reduce the risk of conversion (ranging from 1% to 7.3% for robotics vs 3% to 34% for laparoscopy)<sup>[3,9]</sup>. An open conversion in these difficult cases can be still technically challenging, leading to potentially worse short-term or oncological outcomes<sup>[9,10]</sup>. On the other hand, it is not clear why robotics might prevent conversion. There are some hypothetical explanations: (1) better vision that could allow better dissection; (2) a more stable platform; (3) a self-controllable camera; (4) instruments with more degrees of freedom and without tremor; (5) improved opportunity to control unexpected bleeding; and (6) better ergonomics.

According to the CLASICC trial (up to 34% of conversion!), the main reasons for conversion from laparoscopy were: tumor fixity or uncertainty of tumor clearance, obesity, anatomic problems, and tumor inaccessibility<sup>[9]</sup>. All these parameters are crucial from an oncological point of view when performing a LAR or an ultra-LAR. The risk of positive margins for low rectal tumor is indeed still high (9% with a laparoscopic approach, but up to 22% with an open approach)<sup>[1]</sup>. The corollary of these relatively poor outcomes has been the introduction and the development of different technical options to reduce the risk of positive margins.

Firstly, robotics might reduce the rate of positive circumferential resection margins (CRM)<sup>[5]</sup>. In addition,

it might improve the quality of the specimen, with more complete total mesorectal excision (TME)<sup>[11]</sup>, which might reduce the risk of local recurrence<sup>[12]</sup>. However, this advantage of the robotic approach remains hypothetical, and so far oncological outcomes seem to be comparable between robotic and laparoscopic approaches<sup>[13]</sup>.

Secondly, transanal TME has been developed, based on the concept to start first the distal dissection from the anus (so called "bottom-up technique"), allowing to define precisely the distal margin. The early data are encouraging, with a reduced positive margins rate in comparison to standard approach<sup>[14]</sup>. However, this technique, still in its infancy, remains technically challenging, and again the robot could be applied to overcome the difficulties associated with this new technique<sup>[15]</sup>. Interestingly, the same advantages and drawbacks were seen when using robotics for transanal endoscopic microsurgery<sup>[16]</sup>.

Looking at the published experience, it would seem obvious that robotic surgery is a valid option for low rectal cancer. However, the enthusiasm has been dampened by several drawbacks, which could discourage the most fervent surgeon: the size of the robot, the lack of tactile feedback, the risk and difficulties during multiquadrant surgery, and, of course, costs. While part of these disadvantages might be overcome with the new Xi system (Intuitive Surgical Inc., Sunnyvale, CA), the global economic impact of robotic surgery remains unclear and the increase in overall costs is probably the most limiting factor for a wide diffusion of robotic technology. The real benefits for the institution remain to be scrutinized (marketing impact, increased referral, reduced global costs), and beyond this local economic problem, the risk that this technology will be restricted to rich countries is real.

So far, the best indications for this technology are not yet clear. However, it seems obvious that the development of robotic surgery is underway. The number of series to date is significant and the safety and feasibility of the robotic approach have been proven, along with its oncological outcomes (at least the short-term outcomes). However, comparison between robotics and laparoscopy did not give the expected results in favor of robotics. While still in its youth, it should be noted that the perioperative outcomes associated with robotic LAR are at least as good as laparoscopy, and could be achieved with a shorter learning curve and better functional results, in particular in difficult patients. Regarding the learning curve, it is not clear if open colorectal surgeons (who probably did not embark on laparoscopy) would be interested by robotics (as were the urologists in those days). The learning curve might be then slightly different for an open surgeon starting robotic surgery than an already experienced laparoscopic colorectal surgeon embarking on robotics. The evidences concerning the learning curve are indeed mainly based on skilled minimally invasive surgeons.

So far, the main difference remains the reduction in conversion rate after a robotic LAR. The clinical

corollary of this fact is still hypothetical, but might give some benefits to robotic patients. From an oncological point of view, similar outcomes have been reported. However, better TME and a reduction in positive CRM were reported in selected robotic series, especially when applied for low tumors.

To conclude, the main question is not whether robotic surgery will take over from laparoscopy, but when and how. However, technical challenges and barriers (such as costs, size of the robot, and lack of tactile feedback) still need to be overcome. Looking at the history of surgery, it seems obvious that robotics is not just another interesting technical tool, but more a new concept, creating a computer interface between the patient and the surgeon. The possibilities appear really interesting, notably in terms of planning, teaching, automation, and telemedicine. However, this technology has a cost, and it is not yet clear whether the surgical community, or even the overall community, is ready to pay for this.

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## Present status of endoscopic mastectomy for breast cancer

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incision can be created. A retractor with an endoscope, CO<sub>2</sub>, and an abrasion device with the endoscope are used for operation space security. It is extremely rare that an endoscope is used for lymph node dissection. For breast reconstruction, it may be used for latissimus muscle flap making, but an endoscope is rarely used for other reconstructions. Endoscopic mastectomy is limited to certain institutions and practiced hands, and it has not been significantly developed in breast cancer surgery. On the other hand, endoscopic surgery may be used widely in breast reconstruction. With respect to the spread of robotic surgery, many factors remain uncertain.

**Key words:** Endoscopy; Video-assisted; Breast cancer; Surgery; Mastectomy

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**Core tip:** Endoscopic mastectomy is limited to certain institutions and practiced hands, and has not yet been significantly developed in breast cancer surgery. However, endoscopic surgery may be used widely in breast reconstruction. Many factors remain uncertain with respect to the spread of robotic surgery.

Owaki T, Kijima Y, Yoshinaka H, Hirata M, Okumura H, Ishigami S, Nerome Y, Takezaki T, Natsugoe S. Present status of endoscopic mastectomy for breast cancer. *World J Clin Oncol* 2015; 6(3): 25-29 Available from: URL: <http://www.wjgnet.com/2218-4333/full/v6/i3/25.htm> DOI: <http://dx.doi.org/10.5306/wjco.v6.i3.25>

### Abstract

Endoscopy is now being used for breast cancer surgery. Though it is used for mastectomy, lymph node dissection, and breast reconstruction, its prime use is for mastectomy. Because an incision can be placed inconspicuously in the axillary site, a relatively large

### INTRODUCTION

Surgery using an endoscope began with intra-abdominal surgery and progressed to intra-articular surgery and thoracic surgery. Surgery using an endoscope is said to

be minimally invasive surgery, but its low invasiveness is actually difficult to prove. However, it is definitely useful for shortening the length of hospital stay and alleviation of postoperative pain. A major advantage of endoscopic surgery over normal surgery is that the operative incision can be small. A small wound is a major factor related to shortening of the length of hospital stay and alleviation of postoperative pain. In this way, endoscopic surgical techniques have been applied to surgical procedures in a variety of organs. And this technique is used to minimize the skin incision and improve breast reconstruction outcomes in breast surgery in 2002<sup>[1]</sup>. Prior to it in 1996, endoscopic axillary lymph node dissection was reported<sup>[2]</sup>. Furthermore, prior to it, the use of endoscopes to assist in latissimus muscle harvest has been effectively since 1994<sup>[3]</sup>. In breast cancer surgery, an endoscope is used most particularly for partial or total mastectomy, as well as for lymph node dissection and breast reconstruction.

## MASTECTOMY

In most breast cancer surgery, an endoscope is used in order to have a small wound; the purpose of using an endoscope in breast cancer surgery is not to reduce the invasiveness of surgery. Depending on the site of the tumor, the operative method of mastectomy, lymph node dissection, and mammary reconstruction, the moving window method from the small incised part of the skin is used under direct vision<sup>[4,5]</sup>.

However, most reports show a method to exfoliate breast from the skin through a small incision using an abrasion device with an endoscope, the retractor with the endoscope, and the appliance that exfoliates with a balloon under endoscopic observation. For an endoscope with an abrasion device, a vein abrasion retractor with a 30° endoscope<sup>[6-9]</sup> or optical tracker<sup>[10]</sup> is used, and for a retractor with the endoscope, an Ultra Retractor (Johnson and Johnson Company, New Brunswick, NJ)<sup>[11,12]</sup> or Optical Retractor (Karl Storz GmbH and Co. KG, Tuttlingen, Germany)<sup>[11]</sup> with a 30° endoscope is used. Under endoscopic observation, a round balloon dissector (for example, PDB balloon: autosuture or preperitoneal distention balloon: United States Surgical) is used as an appliance for exfoliating with a balloon<sup>[10,13,14]</sup>. Carbon dioxide and an appliance for pulling skin are used to secure the virtual cavity of the operation. Nakajima *et al.*<sup>[15,16]</sup> introduced an exclusive device, called the HIROTECK retractor, for pulling the breast in the ventral aspect. The authors also introduced a device to pull skin using a Kirschner wire (two wire retractors)<sup>[17]</sup>. Serra-Renom *et al.*<sup>[18]</sup> reported an appliance for skin lifting and tractioning the muscle upward, which they designed originally as the Serra-Renom endoscopic retractor.

A 2.5-5 cm incision is placed in the axillary region in many cases<sup>[6,10,16,19-21]</sup>. The semi-arc incision is placed in the areolar edge, and an abrasion device is used through this wound<sup>[14]</sup>. Some articles show that both axillary and periareolar incisions are used as windows

for manipulating instruments<sup>[1,7-9,12,13,17]</sup>. Most of these reports are from Japan and Korea. It is thought that the small volumes of the breasts of Asian women and the small extent of resection are reasons for using endoscopy to treat breast cancer.

## LYMPH NODE DISSECTION

Axillary lymph node dissection is performed through an axillary finesse incision with direct observation in many cases. A major reason for its use is that there are few cosmetic problems and the wound does not attract attention, even if the axillary wound area is slightly larger. Dissection of only sentinel lymph nodes or dissection of level 1 or 2 lymph nodes can be performed in the above-mentioned manner.

A method of endoscopic lymph node dissection has also been reported. Salvat *et al.*<sup>[2]</sup>, Suzanne *et al.*<sup>[22]</sup>, Brun *et al.*<sup>[23]</sup>, and Cangioti *et al.*<sup>[24]</sup> performed axillary lymph node dissection by securing the surgical field with carbon dioxide after liposuction with an axilloscope (a normal rigid endoscope device). Kamprath *et al.*<sup>[25]</sup> and Lim *et al.*<sup>[26]</sup> reported axillary lymph node dissection using an endoscope without a liposuction device. Moreover, Tagaya *et al.*<sup>[27]</sup> reported axillary lymph node dissection using an endoscope without a liposuction device with an insufflated space using carbon dioxide. Saimura *et al.*<sup>[9]</sup> and Nakajima *et al.*<sup>[16]</sup> reported axillary lymph node dissection using an endoscope with a vein retractor without using carbon dioxide. Conrado-Abrão *et al.*<sup>[28]</sup> and Long *et al.*<sup>[29]</sup> reported a method of parasternal lymph node dissection using thoracoscopic technique. Long *et al.*<sup>[29]</sup> performed internal mammary node dissection simultaneously with mastectomy, and Conrado-Abrão *et al.*<sup>[28]</sup> performed this dissection 18 mo after radical mastectomy.

After reports such as that of Owaki *et al.*<sup>[17]</sup> in 2005, in the case of endoscopic mastectomy, not only axillary lymph node dissection but also sentinel lymph node dissection has been performed. Sentinel lymph node dissection was performed under direct vision in all reports. For the sentinel lymph node biopsy, the operation area is limited, and it is not necessary to use an endoscope, because the dissection field is just beneath the axillary incision.

## BREAST RECONSTRUCTION

Mobilizing the remnant breast gland and fatty tissue or an autologous lateral tissue flap using the latissimus muscle (for reconstruction after total extirpation of the breast and in reconstruction after partial extirpation) and the insertion of an implant after total breast extirpation are used for breast reconstruction.

Owaki *et al.*<sup>[17]</sup> reported reconstruction of the defect using the remaining mammary gland tissue with endoscopic assistance after quadrantectomy by endoscopic technique.

To make a latissimus muscle flap as a caulescent

flap, it is isolated from the trunk part using an abrasion appliance with an endoscope through a small axillary incision<sup>[6,16,18,30,31]</sup>. Yang *et al.*<sup>[31]</sup> used Pediatric Omni-tract retractors to maintain the surgical view. Alternatively, Pomel *et al.*<sup>[32]</sup>, Missana *et al.*<sup>[33]</sup>, and Selber *et al.*<sup>[34]</sup> reported a method using carbon dioxide to secure the surgical field when they prepare a latissimus muscle flap. In particular, Selber *et al.*<sup>[34]</sup> reported an operative method to make a latissimus muscle flap using the da Vinci system under insufflation with carbon dioxide.

Cothier-Savey *et al.*<sup>[35]</sup> and Zaha *et al.*<sup>[36]</sup> used the greater omentum, which was isolated as a caulescent flap using laparoscopic technique, for breast reconstruction. Yenumula *et al.*<sup>[37]</sup> performed breast reconstruction using a transverse rectus abdominis musculocutaneous flap, which was isolated by the extraperitoneal approach using a laparoscopic dissector and balloon dissector.

Implant insertion is performed after having secured space for its insertion by exfoliation of the pectoralis major muscle from the chest wall using an abrasion appliance with an endoscope<sup>[20,21]</sup>. In many cases, implant instruments are inserted under direct visualization after mastectomy using endoscopic technique<sup>[1,9,10,13]</sup>.

Methods of breast reconstruction using remnant mammary gland under direct visualization after mastectomy using endoscopic technique have also been reported<sup>[7,8,11,15]</sup>.

## PROGNOSIS AFTER RESECTION

There are few reports of follow-up, recurrence rates, and survival rates after endoscopic mastectomy. Many authors may think that endoscopic breast surgery does not greatly affect the survival rate compared with open breast surgery. Regarding the rates of local recurrence, Kitamura *et al.*<sup>[20]</sup> demonstrated that there was no significant difference between endoscopic mastectomy and open mastectomy in a retrospective study. Furthermore, Kitamura *et al.*<sup>[20]</sup> showed that overall survival following endoscopic and open mastectomy for early stage breast cancer was comparable. In 2011, Leff *et al.*<sup>[38]</sup> summarized many previous reports of mastectomy using the endoscope. In their review, they reported that it is possible to achieve disease control with high rates of overall survival and a low rate of local relapse recurrence and/or distant metastasis.

## DISCUSSION

Recently, in cases of breast cancer, the approach has been to reduce the surgical field and prevent recurrence by postoperative irradiation. In addition, for lymph node dissection, sentinel lymph node dissection has come to be widely accepted, and wide resection of axillary lymph nodes is not commonly performed. Particularly in the case of sentinel lymph node dissection, lymph node dissection under direct vision may be adequate, and the necessity of using an endoscope through a small, non-conspicuous, axillary incision is low. If normal axillary

dissection is required following sentinel node dissection, the wound can simply be enlarged, and more lymph nodes can be dissected without an endoscope. Even with a larger axillary wound, the wound is covered under the armpits and remains inconspicuous, thus obviating the need for using an endoscope. Thus, the need to use an endoscope may not be very great, even for normal axillary lymph node dissection.

Given this situation, the method of using an endoscope for breast cancer surgery has not shown significant development, and endoscopic mastectomy has not been performed widely. Alternatively, robotic surgery with the da Vinci system has been used for breast cancer resection<sup>[34]</sup>. The advantages of robotic surgery include a smaller wound and the potential for moving the incision from the anterior chest to the axillary region. However, robotic surgery is expensive and appears unlikely to become commonly used, because the expense outweighs the small advantages it offers.

However, for breast reconstruction, we think that an endoscopic abrasion device is useful for latissimus muscle isolation through an incision only for the discreet axillary part. Using an abrasion device with the endoscope is important, because an expander implant can be inserted through a small incision in the process of preparing the expander implant insertion space. By the development of materials and the shape of the implant, we resect the whole breast and reconstruct neatly. On this occasion, skin-sparing approach is achieved to resect breast using the endoscope *via* an axillary and/or periareolar operation wound<sup>[39]</sup>. The endoscope enables the mastectomy *via* small incision at the site which is not conspicuous, and provides cosmetic advantage.

## CONCLUSION

Endoscopic mastectomy is limited to some institutions and practiced hands, and it has not been significantly developed in breast cancer surgery. On the other hand, in breast reconstruction, endoscopic surgery may be used widely. With respect to the spread of robotic surgery, many factors remain uncertain.

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## Oligometastatic disease, the curative challenge in radiation oncology

Amalia Palacios-Eito, Sonia García-Cabezas

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### Abstract

The concept of oligometastatic disease was first described by Hellman and Weichselbaum in 1995. The mere insight of this concept led to the hypothesis that this disease may be cured using local ablative weapons. Surgery has already demonstrated this hypothesis. Surgery limitations, either technical or due to refusal

or associated comorbidity, have led to implement alternative ablative options such as stereotactic body radiation therapy (SBRT). SBRT evolved from (stereotactic radiosurgery) because of the need to irradiate extracranial lesions and has been shown to be safe and effective. SBRT achieves local control rates ranging from 70%-90%, but highly variable survival rates depending on the group analyzed. Series with heterogeneous metastatic sites and tumor origin have reported 20% survival rates at 2-3 years, similar to those achieved with surgery. Despite its excellent results, SBRT still faces significant clinical challenges. Its optimal integration with systemic treatment is unknown, and response assessment is very difficult. However, the greatest challenge lies in selection of patients most likely to remain oligometastatic, those who will most benefit from the technique. Biomarkers, molecular signatures, that accurately predict the biological behavior of malignancy are needed. The expression profile of specific miRNAs has been shown to have a potential in this regard.

**Key words:** Oligometastases; Radiotherapy; Stereotactic body radiotherapy; Stereotactic body radiation therapy; Stereotactic ablative body radiotherapy; Curative intent

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**Core tip:** Surgery has been shown to be able to cure a proportion of oligometastatic patients. Surgery limitations, either technical or due to refusal, advanced age, or associated comorbidity, have led to progressive implementation of stereotactic body radiation therapy (SBRT) as an alternative local ablative weapon. SBRT has been shown to be safe and effective and to achieve local control rates around 80%, with a variable impact on survival depending on other associated prognostic factors. Despite its good results, SBRT still faces significant clinical challenges, including identification of optimal patients to be treated.

Palacios-Eito A, García-Cabezas S. Oligometastatic disease, the curative challenge in radiation oncology. *World J Clin Oncol* 2015; 6(4): 30-34 Available from: URL: <http://www.wjgnet.com/2218-4333/full/v6/i4/30.htm> DOI: <http://dx.doi.org/10.5306/wjco.v6.i4.30>

## EDITORIAL

The current approach to staging of cancer patients is based on identification of two large groups, patients with local/locoregional tumors and those with distant metastases. This approach stems from the possibility to perform local eradication treatment with curative intent in localized tumors, while metastatic disease is usually treated with palliative intent using systemic drugs. Tumor burden of metastatic disease varies widely for one patient to another, and most systemic treatment schemes do not make differences depending on extent of disease.

The term "oligometastases" was introduced in 1995 by Hellman and Weichselbaum<sup>[1]</sup>. Based on their clinical experience, these authors reported an intermediate state of metastatic dissemination between localized disease and multiple dissemination, and considered this as a different clinical entity characterized by a lower capacity of metastatic dissemination. This concept was revised by Niiibe *et al.*<sup>[2]</sup> in 2006 as oligo-recurrence. The biggest difference between oligometastases and oligo-recurrences lies in the uncontrolled or controlled primary lesion. They postulated the hypothesis that if this oligometastatic disease is eradicated using local ablative procedures, patient may be cured, as occurs in locoregional tumors. This hypothesis has been supported by surgery by finding that a group of oligometastatic and oligo-recurrence patients may be cured when their metastases are resected. Thus, in a series of over 1000 patients, resection of liver metastases from colorectal cancer achieved 20% survival rates at 10 years<sup>[3]</sup>. Similarly, 10- and 15-year survival rates of 26% and 22% have been reported in an analysis of more than 5000 patients in The International Registry of Lung Metastases<sup>[4]</sup>. It may therefore be stated that the oligometastatic status exists, and that a proportion of patients with disseminated disease may be cured. Therapeutic options for local ablation alternative to surgery are currently needed for this purpose. Surgery is associated with significant morbidity and mortality, the group of candidates who are elderly patients with associated comorbidity is increasing, and other patients have already undergone one or more procedures for resection of metastases. Moreover, the cost of this surgery is high<sup>[5]</sup>.

Radiation therapy is, together with radiofrequency, one of the main local ablative options alternative to surgery. Technological development in the past decade, mainly associated to computer systems and advances in radiographic imaging, has allowed for clinical use

of a radiotherapeutic technique with high precision and ablative capacity now known as stereotactic body radiotherapy (SBRT)<sup>[6]</sup> or stereotactic ablative body radiotherapy (SABR), a high-precision external radiotherapy technique that administers ablative doses (> 100 Gy) in a minimal number of sessions (1-8) with high doses per fraction. SBRT requires specific systems to immobilize patients, as well as guided images to ensure its precision. Sophisticated calculation systems allowing for a high gradient between the doses administered to the tumor tissue and to the surrounding healthy tissue are needed. The name of the procedure (SBRT/SABR) is confusing, because administration under stereotactic conditions is no longer required thanks to the availability of image-guided systems. It is a natural evolution of the field of knowledge of cranial stereotactic radiosurgery, already established because of the need to treat extracranial sites. The non-optimal results of conventional radiotherapy in early non-small cell lung cancer (NSCLC) in patients not amenable to surgery caused this to be the first extracranial indication investigated both in Europe and North America. Both the Nordic Group and the Radiation Therapy Oncology Group showed that in this clinical condition, a dose of 45-54 Gy, administered in three fractions, achieved local control rates of approximately 90% and 3-year survival in 60% of cases, which more than doubled the historical rates achieved with conventional fractionation<sup>[7,8]</sup>.

Radiation therapy with curative intent had never been considered in patients with extracranial oligometastases. Current technological advances allow for aspiring to that ambitious goal, but multiple unresolved challenges still exist<sup>[9]</sup>.

### Results of SBRT in oligometastatic disease

Limited toxicity, good clinical results, and the experience gained using SBRT in stage I NSCLC have driven use of SBRT for oligometastatic disease. Phase I/II prospective studies have shown SBRT to be a safe and effective treatment for metastases in oligometastatic patients<sup>[10-14]</sup>. Multiple institutions have reported excellent control rates of irradiated metastases, either pulmonary<sup>[15-20]</sup>, hepatic<sup>[11,12]</sup>, adrenal<sup>[21]</sup>, vertebral<sup>[13,14]</sup>, lymph node<sup>[22]</sup> or mixed<sup>[23-27]</sup> (Table 1). The highly diverse prognosis of the population tested makes comparison of survival results impossible. Since Niiibe *et al.*<sup>[28]</sup> showed that the most important prognostic factor was the status of the primary lesion, the status of this and whether all tumoral disease is treated with ablative dose should be clarified.

Results of SBRT may be summarized referring to the last systematic review published<sup>[29]</sup>. This review includes the phase I and II trials available to date and the main case series. Series are highly heterogeneous: They include patients with up to five metastases, distributed in no more than three organs and of diverse histology. Pulmonary and hepatic metastases were most often treated, followed by adrenal gland metastases. Single bone metastases in the spine or nodal metastases were

**Table 1** Summary of selected series of stereotactic body radiation therapy

Ref.	No. of patients (number of lesions)	Primary site	Treated site (s)	Total dose (Gy)	Local control	Toxicity
SBRT for mixed oligometastatic sites						
Milano <i>et al</i> <sup>[26]</sup> (2012)	121 (293)	All (mostly breast and colorectal)	Mostly liver lung, lymph nodes	Median 50 Gy in 10 fr	74% (2 yr) 65% (6 yr)	G3 in 1 patient
Greco <i>et al</i> <sup>[27]</sup> (2011)	103 (126)	All (mostly prostate, renal, colorectal)	Majority bone, lymph node, soft tissue	18-24 Gy in 1 fr	64% (82% if > 22 Gy) (2 yr)	G3 late < 4%
SBRT for lung oligometastases						
Norihisa <i>et al</i> <sup>[17]</sup> (2008)	34 (43)	All (mostly lung)	Lung	48 Gy/4 fr-60 Gy/5 fr	90% (2 yr)	G2, 12%/G3, 3%
Navarria <i>et al</i> <sup>[16]</sup> (2014)	76 (118)	All (mostly colorectal)	Lung	60 Gy/3 fr (peripheral) < 2 cm 48 Gy/4 fr (peripheral) 60 Gy/8 fr (central)	89% (3 yr)	G1, 80%
SBRT for liver oligometastases						
Rusthoven <i>et al</i> <sup>[11]</sup> (2009)	47 (63)	All (mostly colorectal and lung)	Liver	36-60 Gy /3 fr	92% (2 yr)	≥ G3 in 1 patient
Lee <i>et al</i> <sup>[12]</sup> (2009)	68	All (mostly colorectal and breast)	Liver	Median 41.8 Gy (range 27.7-60 Gy/6 fr/2w)	71% (1 yr)	G3 in 8 patients G4 in 1 patient
SBRT for spinal metastases						
Wang <i>et al</i> <sup>[13]</sup> (2012)	149 (166)	Mixed (mostly renal, breast and NSCL)	Spine	27-30 Gy in 3 fr	72.4% (2 yr)	G3 in 6 patient
Schipani <i>et al</i> <sup>[14]</sup> (2012)	124 (165)	Mixed (mostly lung and prostate)	Spine	18 Gy in 1 fr	92% (1 yr)	G2-G4, 0%
SBRT for adrenal oligometastases						
Casamassima <i>et al</i> <sup>[21]</sup> (2012)	48	Mixed (mostly NSCLC and colon)	Adrenal	36 Gy in 3 fr (most common dose)	90% (2 yr)	G2 in 1 patient (adrenal insufficiency)
SBRT for lymph-node oligometastases						
Jerezek-Fossa <i>et al</i> <sup>[22]</sup> (2014)	69 (94)	Mixed (mostly urological, gastrointestinal and gynecologic)	Single abdominal lymph node recurrence	Median 24 Gy in 3 fr	64.3% (3 yr)	G3 acute in 2 patients G4 late in 1 patient

SBRT: Stereotactic body radiation therapy; NSCLC: Non-small cell lung cancer.

treated in some cases. Local control rates ranging from 70%-90% were reported, with an excellent toxicity profile (> G3 < 5%). Mean survival rate at 2-5 years was 20% (11%-44%), with great variability and closely related to patient selection in each series. Reports show that several metastatic sites may be safely irradiated at the same time, provided the dose limits for healthy organs are respected, and that 25%-30% of patients benefit from a second course of SBRT<sup>[23]</sup>.

No randomized trials are available quantifying the efficacy of SBRT as compared to other local ablative options or its contribution to survival when it is part of a systemic therapy for disseminated disease. Ethical issues and the lack of alternative treatments in most cases make randomization of these patients difficult, and first level evidence may probably never become available. However, SBRT is already part of standard treatment in this group of patients, although its implementation varies widely depending on the hospital because of the technical infrastructure required<sup>[6]</sup>.

### Oligometastatic signature

Today, selection of patients for SBRT is based on clinical criteria only<sup>[30]</sup>. Despite the excellent local control achieved, the main progression pattern in these patients is systemic, and a group of them progress rapidly (< 4 mo) to polymetastatic patients. Some clinical factors

have been shown to be associated to poorer survival, including brain metastases, "non-adenocarcinoma" histology, and synchronous vs metachronous metastatic disease<sup>[31]</sup>.

Despite these clinical selection criteria, survival rates of approximately 25% show that most patients selected for local aggressive therapy are not cured<sup>[29]</sup>. A method is needed to objectively categorize patients as oligometastatic or with a trend to progress to polymetastatic patients in short time periods. This would avoid expensive treatments of little clinical benefit and with potential associated toxicity. Alternatively, a group of oligometastatic patients could be initially offered a curative treatment.

Biomarkers that objectively and unequivocally identify oligometastatic patients are needed. miRNAs have provided promising results for this purpose. miRNAs are small single-stranded, non-coding RNA molecules 18 to 22 nucleotides in length that regulate transduction of messenger RNA. miRNAs may therefore be considered as the conductors of the gene expression orchestra. More than 1500 miRNAs have been identified to date in humans. They are involved in regulation of multiple metabolic and cell pathways, particularly those that control the changes occurring during development, embryogenesis, stem cell preservation, differentiation of hemopoietic cells, and brain development. Altered



miRNA expression is likely to contribute to human disease and, among other processes, has been related to tumor progression, which includes tumor growth, differentiation, adhesion, apoptosis, invasion, and metastasis formation. miRNA expression profile appears to classify tumors and to reflect their origin and differentiation state<sup>[32]</sup>. Since altered miRNA expression is related to cancer development and metastasis formation, miRNAs have a great potential to serve as biomarkers. Moreover, it is widely known that tumor tissues release miRNAs to biological fluids (blood, urine and/or saliva) inside exosomes, making them ideal molecular biomarkers for performing non-invasive biopsies, known as liquid biopsies<sup>[33]</sup>. Several clinical trials are currently analyzing circulating miRNAs in patients with different cancers subject to different therapies (NCT01722851 Circulating miRNAs. ICORG 10-11, V2; NCT01541800 Circulating microRNAs as Disease Markers in Pediatric Cancers; NCT01598285 A Combined GWAS and miRNA for the Identification of Bevacizumab Response Predictors in Metastatic Breast Cancer) to use them as patterns to stratify cancer patients and monitor the efficacy of treatment with a non-invasive method<sup>[34]</sup>.

To date, only Lussier *et al.*<sup>[35]</sup> in Chicago has tested miRNA profiles in tissues from oligometastatic and polymetastatic patients. In 2011 they identified a list of miRNAs that reflects the metastatic progression rate in oligometastatic patients treated with SBRT. One year later, these same authors validated in two case series their prioritized list of miRNAs and were able to predict metastatic behavior in a homogeneous study where only pulmonary metastases treated with surgical resection were included<sup>[36]</sup>. After a recent combined analysis of both databases<sup>[37]</sup>, this group concluded that oligometastases and polymetastases are different biological conditions and have therefore different molecular profiles which are partly regulated by miRNAs. In this study, they were able to successfully stratify patients treated with surgery or SBRT with oligometastases and polymetastases based on their different miRNA expression. Authors recognize that the study is limited by the small sample analyzed. These are the only databases of miRNAs associated to oligometastases available to date<sup>[37]</sup>.

In conclusion, use of SBRT in oligometastatic disease still faces many challenges: The standard dose scheme and fractionation has not been established, assessment of the local response achieved is very difficult, and the optimal form of integration with systemic treatment is unknown. However, the key factor is probably the identification of the group of patients in whom this local treatment may potentially be curative or provide long survival, delaying or avoiding systemic treatment. Objective parameters for adequate identification of candidate patients are needed.

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## Concurrent chemoradiation for high-risk prostate cancer

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### Abstract

There are estimated to be 220800 cases of prostate cancer diagnosed in 2015, making up 26% of all cancer diagnoses. Fortunately, adenocarcinoma of the prostate is often a highly treatable malignancy. Even though the majority of prostate cancer patients present with localized disease, prostate cancer still accounts for over 27000 deaths a year. There is a subset of patients that

are likely to recur after locoregional treatment that is thought of as a "high-risk" population. This more aggressive subset includes patients with clinical stage greater than T2b, Gleason score greater than 7, and prostate specific antigen greater than 20 ng/dL. The rate of biochemical relapse in this high risk group is 32%-70% within five years of definitive focal therapy. Given these discouraging outcomes, attempts have been made to improve cure rates by radiation dose escalation, addition of androgen deprivation therapy, and addition of chemotherapy either sequentially or concurrently with radiation. One method that has been shown to improve clinical outcomes is the addition of chemotherapy to radiotherapy for definitive treatment. Concurrent chemoradiation with 5-fluorouracil, estramustine phosphate, vincristine, docetaxel, and paclitaxel has been studied in the phase I and/or II setting. These trials have identified the maximum tolerated dose of chemotherapy and radiation that can be safely delivered concurrently and established the safety and feasibility of this technique. This review will focus on the addition of concurrent chemotherapy to radiotherapy in the definitive management of high-risk prostate cancer.

**Key words:** Prostate cancer; Chemoradiation; High-risk prostate cancer; Concurrent chemotherapy; Chemotherapy; Intensity modulated radiation therapy

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**Core tip:** Over half of patients with high-risk prostate cancer will have a biochemical relapse within 5 years when treated primarily with radiotherapy as shown in multiple studies. One method that has been shown to improve local control, and in some disease sites overall survival, is the addition of chemotherapy to radiotherapy for definitive treatment. We review the safety and efficacy data of combined chemoradiation in patients with high risk adenocarcinoma of the prostate.

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## INTRODUCTION

Prostate cancer is the leading non-cutaneous oncologic diagnosis in males with an estimated 220800 cases to be diagnosed in 2015, making up 26% of all cancer diagnoses and leading to over 27000 deaths<sup>[1]</sup>. While adenocarcinoma of the prostate is often thought of as a relatively indolent malignancy, disease presentation and clinical outcome are often quite heterogeneous. Even though the majority of prostate cancer patients are diagnosed with localized disease, there is a subset of patients that are likely to recur after locoregional treatment that is thought of as a "high-risk" population. Disease characteristics that portend a more aggressive phenotype and place patients in a high-risk category are clinical stage greater than T2b, Gleason score greater than 7, and prostate specific antigen (PSA) greater than 20 ng/dL<sup>[2]</sup>. Patients in this high risk group have a biochemical relapse rate of 32%-70% five years following definitive focal therapy<sup>[2-8]</sup>.

Given these discouraging results, efforts have been made to improve outcomes by radiation dose escalation. Early results in the setting of advanced disease were only available from centers with proton beam radiation therapy (RT), such as Harvard University, due to gastrointestinal (GI) and genitourinary (GU) toxicity associated with higher radiation doses delivered with less focused radiation techniques<sup>[9,10]</sup>. Patients with T3-4, Nx-2, M0 prostate cancer were randomly assigned to radiation to the whole pelvis *via* a 4-field box technique to 50.4 Gy, followed by a photon (16.8 Gy) or proton (25.2 CGE) boost. There was an improvement in local control only in patients with high Gleason score with dose escalation and a 8-year failure rate of 23% in the entire cohort<sup>[11]</sup>. The advent of computed tomography based planning allowed for radiation doses above 70 Gy to be delivered safely with three dimensional conformal radiation therapy (3D-CRT)<sup>[12]</sup>. A prospective, 3D-CRT dose escalation study mandating sextant prostate biopsy after treatment demonstrated a 7% positive biopsy rate after doses of 81 Gy vs 57% positivity in patients receiving 64.8 Gy. Despite this low biopsy rate, patients with 2 or more high risk features (T-stage > 2, pretreatment PSA > 10.0 ng/dL and Gleason score > 6) had a 65% chance of PSA failure<sup>[13]</sup>.

Another strategy to augment cure rates is the addition of androgen deprivation therapy (ADT) to radiation therapy. EORTC 22863, a multi-intuitional randomized trial of 415 patients testing RT alone vs RT + ADT (concurrently and adjuvantly for 3 years) demonstrated a 16% overall survival benefit supporting the strategy of combined ADT and RT<sup>[14]</sup>. However, RTOG 86-10 testing neoadjuvant and concurrent ADT

(4 mo total) vs RT alone did not demonstrate an overall survival benefit in 471 patients<sup>[15]</sup>. Furthermore, the biochemical failure rate at 10 years was 65% in the combined treatment arm of RTOG 86-10.

These uninspiring results demonstrate the need for better treatment options in patients with high-risk prostate cancer. One method that has been shown to improve local control, and in some disease sites overall survival, is the addition of chemotherapy to RT for definitive therapy<sup>[16-25]</sup>. This review will focus on the addition of concurrent chemotherapy to radiation therapy in the definitive management of high-risk prostate cancer.

## EARLY EXPERIENCE WITH CONCURRENT 5-FLUOROURACIL

Drawing on over 30 years of experience from the treatment of adenocarcinoma of the gastrointestinal tract<sup>[26]</sup> and *in vitro* evidence that 5-fluorouracil (5-FU) is a radiosensitizer in DU-145 human prostate cell lines<sup>[27,28]</sup>, the Southwest Oncology Group (SWOG 9024) initiated a phase II trial testing chemoradiation with 5-FU in locally advanced prostate cancer<sup>[29]</sup>. Patients were included if they were cT3 or greater and node negative/metastasis free. Patients were treated with a 4-field approach to 45 Gy followed by a CT-defined boost to the prostate and seminal vesicles to a total dose of 70.2 Gy in 39 fractions. Continuous infusion 5-FU at a dose of 200 mg/m<sup>2</sup> daily was administered from day 1 until the completion of radiotherapy. Thirty eligible patients were accrued from 1991 to 1993 with 13 patients achieving a PSA < 1.0 ng/dL with 6 of these 13 patients also having a negative post-treatment biopsy. Seven patients had grade 3 toxicity and 2 had grade 4 toxicity, but no toxicity necessitated a treatment break. The most common toxicity was diarrhea with 2 patients having grade 3 and 1 patient having grade 4 acute toxicity. While the results of this trial were not overly encouraging, the demonstration that chemotherapy could be combined with radiotherapy to 70.2 Gy with acceptable toxicity paved the way for future trials combining chemotherapy and radiation.

## CONCURRENT AND NEOADJUVANT PLUS CONCURRENT ESTRAMUSTINE PHOSPHATE

Estramustine Phosphate (EP) is a cytotoxic agent that binds to microtubule associated proteins and inhibits spindle formation<sup>[30]</sup>. This results in G<sub>2</sub> phase arrest and accumulation of cells in the radiosensitive G<sub>2</sub>/M phase of the cell cycle<sup>[31]</sup>. For this reason EP was tested as a radiosensitizer both *in vivo* and clinically and found to have an enhancement ratio of 1.3-1.6<sup>[32,33]</sup>. Vinblastine, another microtubule inhibiting agent<sup>[34]</sup>, when combined with EP has led to tumor regression in patients with



hormone-refractory, metastatic prostate cancer<sup>[35]</sup> and was therefore a logical doublet (EV) to test in the concurrent setting. EV and concurrent RT was first tested by Khil *et al.*<sup>[36]</sup> in 65 patients between 1991 and 1996 with either: cT2b-c and Gleason Score 9-10, cT3, or cTxN1M0 prostate cancer. Patients were treated with EP at 450 mg/m<sup>2</sup> by mouth daily with a weekly infusion of vinblastine (3 mg/m<sup>2</sup>) and concurrent whole pelvis conventional radiation to 45 Gy followed by a prostate boost of 20-25 Gy. Patient Gleason score ranged from 4-10 and pretreatment PSA was defined in cohorts of less than 20 (32%), 20 to 50 (35%) and greater than 50 (32%). Six weeks following the completion of chemoradiation all patients had a complete response on rectal exam. With a median follow-up of 43 mo, 86% of patients had an undetectable PSA at nadir and 48% remained in biochemical remission. Clinical control was achieved in 81% of the patients. Biochemical relapse free survival was 49%, 38% and 17% for patients with stage T2, T3 and T4 disease, respectively. Furthermore, biochemical relapse free survival was 60% or greater in patients with a PSA  $\leq$  50 ng/dL compared to 0% in the patients with a PSA greater than 50 ng/dL, highlighting the importance of disease burden at diagnosis on response<sup>[36]</sup>.

This study on EV combined with radiation therapy lent evidence for initiating a phase II study at Memorial Sloan-Kettering Cancer Center published initially in 2000<sup>[37]</sup>. The impetus for this study was to combine EV with high-dose 3DCRT (75.6 Gy). Patients with the following 5 clinical scenarios were included: (1) Gleason score  $\geq$  8 and PSA > 10 ng/dL; (2) Gleason score of 7 and PSA > 20 ng/dL; (3) cT3 and PSA > 20; (4) cT4; or (5) cTxN1M0. Estramustine was given by mouth daily at 10 mg/kg in three divided doses with two neoadjuvant 8-wk cycles of intravenous vinblastine (weekly as 4 mg/m<sup>2</sup>) followed by 8 wk of concomitant EV and high-dose 3DCRT. Twenty seven patients were enrolled from 1996 to 1998 and 2 patients could not tolerate the entire treatment course due to liver dysfunction likely secondary to EP. Acute grade 3 GI and GU toxicity was observed in 35% and 48% of patients, respectively. Late toxicity was uncommon with no grade 3 or greater GI toxicity, and only 12% grade 3 GU toxicity. The efficacy was reported in a follow-up manuscript in 2004 with a 34% 5-year biochemical free survival in patients who tolerated the entire treatment regimen at a median follow-up of 5 years<sup>[38]</sup>. The median time to PSA failure was 1 year with 22% of patients developing metastases. There were no severe long-term toxicities and 48% of patients received no further therapy, supporting neoadjuvant and concurrent EV as a viable alternative to ADT.

There was a similar, contemporaneous pilot study accruing from 1996 to 1999 which was reported by Ben-Josef *et al.*<sup>[39]</sup> in 2001. Patients were eligible if they had cT3-4 or cT1c-2c prostate cancer with a Gleason score > 7 and a serum PSA > 15 ng/dL. Fourteen of the 16 patients accrued completed the treatment

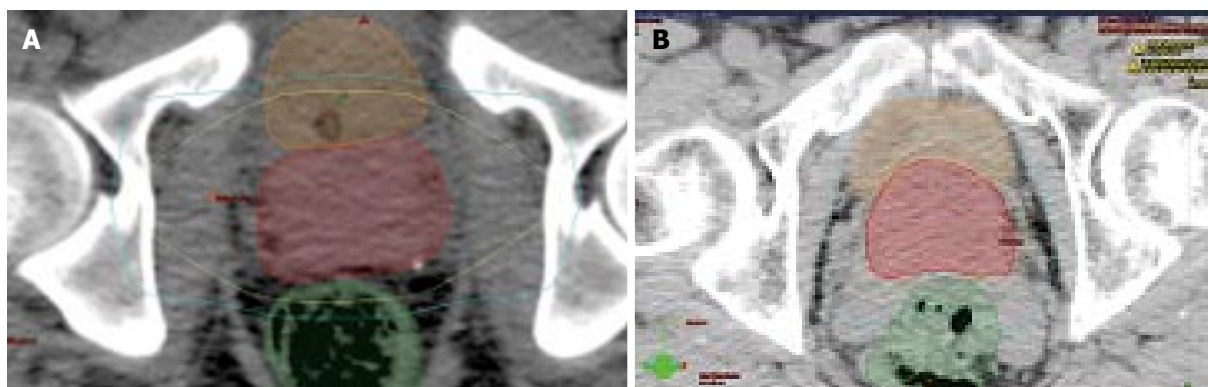
regimen consisting of 2 neoadjuvant 21-d cycles of oral EP (10 mg/kg per day in three divided doses) and oral etoposide (50 mg/m<sup>2</sup> per day, in two divided doses), followed by concurrent EP (10 mg/kg per day, PO) and 3DCRT (70.2 Gy). Etoposide resulted in temporary epilation in all patients. With a median follow-up of only 20 mo, 5 of 7 assessable patients demonstrated biopsy negative disease. Actuarial overall survival and disease-free survival at three years were 88% and 73%, respectively. Grade 3 toxicity occurred in 3 patients total (19%; hematologic in 2, venous thrombosis in 1). One patient experienced grade 4 cardiac toxicity. Overall, while the follow-up is too short to draw definitive efficacy conclusions, the regimen was relatively well tolerated and warrants further exploration.

## TAXANES AS RADIOSENSITIZERS

Paclitaxel (Taxol), discovered to have anti-tumor activity in the late 1970s, is a diterpenoid isolated from the bark of the Pacific yew, *Taxus brevifolia* and functions as a potent inhibitor of cell replication due to microtubule stabilization<sup>[40]</sup>. While early clinical studies of the drug were promising<sup>[41-43]</sup>, the slow growth rate of the Pacific yew tree and resulting tree death upon Taxol extraction made extraction in quantities sufficient for large scale clinical trials difficult. This clinical need lead researchers at multiple institutes in France to prepare the semisynthetic Taxol derivative docetaxel (Taxotere/Docedad)<sup>[44,45]</sup>. Because of the ability of docetaxel to stabilize cells in the G<sub>2</sub>/M-phase of the cell cycle, docetaxel was tested as, and found to be a radiosensitizer by a factor of 2.5-3.0<sup>[46-48]</sup>. In the setting of metastatic hormone refractory prostate cancer, docetaxel was demonstrated to be safe and effective in multiple phase I and II trials<sup>[49-51]</sup>. The combined systemic efficacy and radiosensitization led researchers to investigate the combination of docetaxel and radiation therapy in patients with high-risk prostate cancer.

The first trial to test the combination of docetaxel and radiation therapy was the phase I study by Kumar *et al.*<sup>[52]</sup> conducted from 2000-2002. This docetaxel dose escalation trial tested node negative prostate cancer patients with any of the following advanced features: cT3-4, cT1b-2 and Gleason Score  $\geq$  8, or cT1c-2 with Gleason Score 5 to 7 and PSA  $\geq$  10 ng/dL. Patients were treated with 3DCRT to a dose of 70.2 Gy in 5 cohorts of docetaxel dosing, ranging from 5-20 mg/m<sup>2</sup>. The maximum tolerated dose (MTD) of docetaxel delivered concurrently with radiation was determined to be 20 mg/m<sup>2</sup> with a dose limiting toxicity of diarrhea. One patient required intermittent urinary catheterization for 10 mo after the completion of therapy, which resolved without any surgical intervention. The overall incidence of grade 2 diarrhea and dysuria was 36% and 23%, respectively leading the authors to conclude that this treatment was well tolerated and this regimen should move on to phase II testing.





**Figure 1** Comparison of three dimensional conformal radiation therapy (A) and intensity modulated radiation therapy (B) treatment plans. The yellow line denotes the area that is receiving the prescription dose. The outer blue line denotes 90% of the prescription dose. Notice in (B) the yellow line conforms to the prostate (red) and does not enter into the bladder or rectum as it does in (A), demonstrating the increased dose given to the bladder and rectum with 3DCRT (A). 3DCRT: Three dimensional conformal radiation therapy.

With the MTC and safety established, a multicenter, phase II trial of 50 men with high-risk, locally advanced, or node-positive prostate cancer was conducted between 2003 and 2005<sup>[53]</sup>. Patients were treated concurrently with 3DCRT to 70 Gy with weekly docetaxel (20 mg/m<sup>2</sup>) and a luteinizing hormone-releasing hormone agonist. This was followed by a 3-wk treatment break and three consecutive 21 d cycles of docetaxel (60 mg/m<sup>2</sup>). Forty-six of the 50 patients completed full-dose chemoradiation. Treatment was well tolerated with 15 and 5 patients experiencing grade 2 and 3 toxicity, respectively. There were no late grade 3 or greater toxicities. Two-thirds of patients were clinically disease free with a median follow-up of 54 mo with a 5-year survival of 92%. These results are promising and a phase III trial is warranted.

With improvements in plant cell fermentation and biosynthesis, paclitaxel production and availability are no longer reliant on the Pacific yew tree and the drug is now widely accessible for clinical use<sup>[54]</sup>. Paclitaxel, for reasons similar to docetaxel, was found to be a potent radiosensitizer as well<sup>[55-59]</sup>. Clinical trials of the delivery of chemoradiation using paclitaxel have been efficacious and well tolerated in other malignancies<sup>[60-63]</sup> leading Sanfilippo *et al.*<sup>[64]</sup> to conduct a phase I/II trial investigating the use of biweekly paclitaxel in combination with escalating doses of 3DCRT in high-risk prostate cancer patients receiving ADT. Between 2000-2006, 22 patients with cT2-T4 and Gleason score  $\geq 8$ , PSA > 10 ng/dL, or node-positive disease were treated with biweekly paclitaxel (30 mg/m<sup>2</sup>) and escalating doses of 3DCRT (cohorts of 3; 63 Gy, 66.6 Gy, 70.2 Gy, and 73.8 Gy) to determine the MTD of radiation delivered with biweekly paclitaxel. The radiation was initially to be given to the whole pelvis to a dose of 39.6 Gy *via* a 4-field technique followed by a 3DCRT cone-down to the prostate but this was later amended to treat the whole pelvis after the 3DCRT prostate boost after all patients receiving 66.6 Gy had grade 3 diarrhea, in an attempt to limit toxicity. There were subsequently no grade 3 toxicities in the 70.2 Gy

cohort and 1 grade 3 toxicity in the 73.8 Gy cohort out of the 6 total patients treated at this dose level. Six of the twenty-two patients experienced a PSA relapse at a median follow-up of 38 mo. The authors concluded that combined chemoradiation with paclitaxel is safe and effective and they suggested a MTD of 73.8 Gy when using 3DCRT.

## CONCURRENT CHEMORADIATION WITH INTENSITY MODULATED RADIATION THERAPY

While the increased therapeutic ratio achieved from the more accurate target delineation and beam shaping ability of 3DCRT was profound, there was still an excess of normal tissue being treated to relatively high doses (Figure 1A). A new method of treatment planning recommend by Brahme<sup>[65]</sup>, and soon adopted by other investigators<sup>[66-68]</sup>, approached radiation dose delivery by modulating the intensity of individual radiation beams to conform more closely to the target, thus avoiding treating excess normal tissue (Figure 1B). This new form of radiation, termed intensity modulated radiation therapy (IMRT), was shown to cause less toxicity when compared to 3DCRT in an early trial of prostate cancer patients<sup>[69]</sup>. The combination of taxanes combined with IMRT was first explored by Perrotti *et al.*<sup>[70]</sup> in a phase I/II trial of weekly docetaxel (20 mg/m<sup>2</sup>) and concurrent IMRT (72 Gy). Seventeen of twenty men with cT3, Gleason score  $\geq 8$ , or Gleason score 7 with PSA > 10 ng/dL prostate cancer completed the treatment course without interruption. No significant hematologic toxicities (grades 2-4) were encountered among the 20 patients. Three patients had grade 3 toxicity (2 with dehydration, 1 with dyspnea) and no patients experienced grade 4 or 5 acute toxicity. At a short median follow-up of 11.7 mo, 15% of the treated patients experienced relapsed disease with no patient deaths.

The advent and widespread utilization of IMRT for

**Table 1 Comparison of trials investigating chemoradiation for high-risk prostate cancer**

Institution/group	n	Radiation technique	Comp Rx	GI toxicity	GU toxicity	Other toxicity
Continuous infusion 5-fluorouracil SWOG <sup>[29]</sup>	30	Whole pelvis if not surgically negative to 45 Gy + 70.2 Gy 3DCRT to prostate	97%	Gr 3: 7% Gr 4: 3%	Gr 3: 3% Gr 4: 3%	Multiple Gr 3: 13% (cumulative)
Daily estramustine phosphate + weekly vincristine Henry Ford Hospital <sup>[36]</sup>	65	4-field pelvis to 45 Gy + 65-70 Gy 3DCRT to prostate	71%	Gr 3: 0% Gr 4: 2%	Gr 3: 0% Gr 4: 0%	Leukopenia Gr 3: 2%
MSKCC <sup>[37]</sup>	27	3DCRT to prostate and SV	85%	Gr 3: 35% Gr 4: 0%	Gr 3: 48% Gr 4: 11%	Hematologic Gr 3: 8% Liver Gr 3: 7%
Daily estramustine phosphate Wayne State <sup>[39]</sup>	18	Prostate and SV to 50.4-70.2 Gy <i>via</i> 4-field + 3DCRT to prostate 70.2 Gy	78%	Not reported	Not reported	Leukopenia Gr 3: 12% Venous thrombosis Gr: 6% MI Gr 4: 6%
Weekly docetaxel UMDNJ <sup>[52]</sup>	22	3DCRT to prostate to 70.2 Gy	100%	Gr 3: 9% Gr 4: 0%	Gr 3: 0% Gr 4: 0%	No Gr 3 or 4
Europe <sup>[53]</sup>	50	4-field pelvis to 46 Gy + 70 Gy to prostate and proximal SV <i>via</i> 3DCRT or IMRT	92%	Gr 3: 6% Gr 4: 2%	Gr 3: 4% Gr 4: 0%	MI Gr 4: 2%
St. Peter's <sup>[70]</sup>	20	72 Gy delivered <i>via</i> IMRT (no further details)	85%	Gr 3: 0% Gr 4: 0%	Gr 3: 0% Gr 4: 0%	Dyspnea Gr 3: 5% Dehydration Gr 3: 10%
UNC <sup>[72]</sup>	18	Prostate and proximal SV 78 Gy with IMRT	89%	Gr 3: 11% Gr 4: 0%	Gr 3: 0% Gr 4: 0%	Leukopenia Gr 3: 28% Liver Gr 3: 6%
Medical University of South Carolina <sup>[73]</sup>	19	Prostate and proximal SV 45 Gy + 77.4 Gy prostate with IMRT	89%	Gr 3: 11% Gr 4: 0%	Gr 3: 0% Gr 4: 0%	Fatigue Gr 3: 11%
Biweekly Paclitaxel NYU <sup>[64]</sup>	22	4-field pelvis to 39.6 Gy + 63-73.8 Gy to prostate and proximal SV <i>via</i> 3DCRT	100%	Gr 3: 18% Gr 4: 0%	Gr 3: 0% Gr 4: 0%	No Gr 3 or 4

Comp Rx: Percentage of patients completing all protocol treatment; GI: Gastrointestinal; GU: Genitourinary; Gr: Grade; SV: Seminal vesicles; MI: Myocardial infarction; SWOG: Southwest Oncology Group; MSKCC: Memorial Sloan Kettering Cancer Center; UMDNJ: University of Medicine and Dentistry of New Jersey; UNC: University of North Carolina; NYU: New York University; 3DCRT: Three dimensional conformal radiation therapy; IMRT: Intensity modulated radiation therapy.

prostate cancer has led to radiation oncologists routinely treating patients to doses of 78 Gy or greater<sup>[71]</sup>. Combining high-dose IMRT with chemotherapy for prostate cancer was first published by Chen *et al*<sup>[72]</sup> in 2012 in a phase I docetaxel dose escalation feasibility study. Eighteen patients with node-negative prostate cancer and cT3-4, Gleason score  $\geq 8$ , or PSA  $\geq 20$  ng/dL disease characteristics were treated with 24 mo of leuprolide started 3 mo before the chemoradiotherapy consisting of 78 Gy delivered *via* IMRT and escalating dose levels of weekly docetaxel (10, 15, and 20 mg/m<sup>2</sup>). Grade 3 diarrhea occurred at each of the first two docetaxel dose levels but upon cohort expansion no further grade 3 toxicity was seen. There were no grade 4 or 5 toxicities reported leading the authors to conclude that docetaxel given weekly at 20 mg/m<sup>2</sup> appears safe. At a median follow-up of 26 mo biochemical progression-free survival was 94%.

From 2006-2010, another phase I docetaxel dose escalation study was performed with docetaxel doses higher (up to 30 mg/m<sup>2</sup>) than those investigated in the aforementioned studies<sup>[73]</sup>. Nineteen patients with node-

negative prostate cancer and cT2c-4, pretreatment PSA level  $\geq 20$ , or Gleason score  $\geq 8$  disease characteristics were treated with combined androgen blockade for 4 mo starting 2 mo before the start of chemoradiation as well as treatment with a gonadotropin-releasing hormone (GnRH) analog alone for 24 mo after the completion of chemoradiation. Patients were treated with IMRT to 77.4 Gy with escalating weekly docetaxel in planned cohorts of 3 patients (10-30 mg/m<sup>2</sup>). No grade 3 toxicities were seen in any of the patients treated up to a docetaxel dose of 25 mg/m<sup>2</sup>. One patient of the three that were treated with docetaxel at 30 mg/m<sup>2</sup> experienced grade 3 dose-limiting diarrhea and this was determined to be the MTD of weekly docetaxel. At a median follow-up of 41 mo all patients achieved a PSA nadir of  $< 1$  ng/dL, including 13 patients who had an undetectable PSA level with a biochemical progression-free survival of 78.9% in the entire cohort.

## CONCLUSION

The studies discussed and summarized in Table 1

provide evidence supporting the safety and preliminary efficacy of a combined chemoradiation approach in men with high-risk prostate cancer; a disease with a historically poor cure rate. Current technology has allowed for radiation dose escalation and higher doses of chemotherapy to be given with even less toxicity. These results, while promising, are only useful if randomized, phase III trials are undertaken to prove the utility of chemoradiation over androgen deprivation and radiation alone. Chemoradiation could even be investigated in an intermediate-risk population in men who wish to avoid ADT. For this to be proven safe, randomized trials examining efficacy and carefully measured patient reported outcomes need to be conducted.

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## Concise review on the safety of exercise on symptoms of lymphedema

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### Abstract

Lymphedema is an atypical accumulation of high-protein

fluid located just beneath the skin, which often occurs in the arm or leg. Exercising with lymphedema was traditionally considered to be unsafe. However, recent research indicates that exercise may be beneficial to individuals with lymphedema. Studies indicate that exercise can improve the range of motion and strength of the afflicted limb(s), as well as overall fitness and functional quality of life, and can be performed without exacerbating symptoms of lymphedema.

**Key words:** Quality of life; Lymphedema; Exercise; Breast cancer

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**Core tip:** Recent research lends credibility to the safety and efficacy of strength training in women with breast cancer-related lymphedema. Appropriately prescribed upper body resistance exercise, carried out under the supervision of a certified cancer exercise trainer is not likely to cause an increased risk of lymphedema or symptom exacerbation.

Morris C, Wonders KY. Concise review on the safety of exercise on symptoms of lymphedema. *World J Clin Oncol* 2015; 6(4): 43-44. Available from: URL: <http://www.wjgnet.com/2218-4333/full/v6/i4/43.htm> DOI: <http://dx.doi.org/10.5306/wjco.v6.i4.43>

### INTRODUCTION

Lymphedema is an atypical accumulation of high-protein fluid located just beneath the skin, which often occurs in the arm or leg<sup>[1]</sup>. The fluid, or lymph, is a part of the lymphatic system. It is a colorless fluid containing white blood cells, which make it very important to the immune system. Its primary purpose is to remove toxins from the body by draining through the lymphatic system into the bloodstream. As cancer attacks the

tissue, white blood cells flood the area in support of healing. The lymphedema experienced by individuals battling cancer is known as secondary lymphedema and is primarily seen following surgery or radiation. The most common cancers with this side effect are melanoma, breast cancer, testicular and prostate cancer, bladder and colon cancer, or any surgery that requires the removal of the lymph nodes<sup>[1]</sup>. Those afflicted with these forms of cancer often have multiple lymph nodes removed during treatment.

### Signs and symptoms of lymphedema

Patients experiencing secondary lymphedema often describe it as a heavy feeling in the affected limb(s), tightness of the skin or tissue, decreased flexibility in the limb, or tightness and/or difficulty fitting into clothing in<sup>[1]</sup>. Lymphedema presents further risks such as cellulitis and lymphangitis, which are swelling of the connective tissues and lymphatic vessels. Signs and symptoms of lymphedema should not be ignored and should be treated by a medical professional or a certified lymphedema therapist.

### Treatment of lymphedema

While there is no cure for lymphedema, there are tactics that can be used to treat the symptoms, manage ongoing edema, and prevent injury due to swelling. The two most popular methods of control are pressure garments and compression devices. Pressure garments are often made specifically for the afflicted patient, and are worn at all times, whereas compression devices are used intermittently. Compression devices are pumps that are attached to a sleeve that is wrapped around the area<sup>[2]</sup>. Both work by keeping constant pressure on the area, keeping lymph from building up by helping the fluid move. During exercise, one should wear a pressure garment while exercising the affected limb to further prevent swelling.

## EXERCISE AND LYMPHEDEMA

Exercising with lymphedema was traditionally considered to be unsafe. However, recent research indicate that exercise may be beneficial to individuals with lymphedema<sup>[1,3]</sup>. A recent 8-wk home-based exercise study on postmastectomy patients experiencing lymphedema revealed an improvement in the affected limb regarding both volume and circumference, as well as an improved quality of life<sup>[4]</sup>. The weight loss that often accompanies exercise can help reduce the effects of lymphedema by improving overall circulation, which

helps remove the lymph out of the affected area and can decrease swelling<sup>[3]</sup>.

A second study involving heavy resistance exercise for the upper body revealed that exercise was effective in improving muscular strength, endurance, and quality of life. In addition, no differences were noted with regards to arm swelling and symptom severity. Therefore, the researchers concluded resistance training was safe in patients with lymphedema.

A systemic review of existing literature concluded that resistance exercise was did not exacerbate breast cancer-related lymphedema. Provided the exercise trainer had the proper training, researchers concluded that it was safe for breast cancer survivors to perform both aerobic and strength training exercise during and after cancer treatment<sup>[5]</sup>.

## CONCLUSION

In conclusion, research indicates that resistance exercise is safe and effective in women with lymphedema<sup>[6]</sup>. Women with breast cancer-related lymphedema who perform appropriately prescribed upper body resistance exercise under the supervision of a certified cancer exercise trainer can do so without fear of an increased risk of lymphedema or symptom exacerbation<sup>[7]</sup>. Exercise can improve the range of motion and strength of the afflicted limb(s), as well as overall fitness and functional quality of life.

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## Is there a role for epidermal growth factor receptor tyrosine kinase inhibitors in epidermal growth factor receptor wild-type non-small cell lung cancer?

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**Author contributions:** The research was designed by Arriola E; Taus A and Casadevall D conducted this work; Arriola E, Taus A and Casadevall D analysed the data; Arriola E, Taus A and Casadevall D wrote the paper.

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### Abstract

Non-small cell lung cancer (NSCLC) is the most common type of lung cancer with a world-wide annual incidence of around 1.3 million. The majority of patients are

diagnosed with advanced disease and survival remains poor. However, relevant advances have occurred in recent years through the identification of biomarkers that predict for benefit of therapeutic agents. This is exemplified by the efficacy of epidermal growth factor receptor (EGFR) tyrosine kinase inhibitors for the treatment of EGFR mutant patients. These drugs have also shown efficacy in unselected populations but this point remains controversial. Here we have reviewed the clinical data that demonstrate a small but consistent subgroup of EGFR wild-type patients with NSCLC that obtain a clinical benefit from these drugs. Moreover, we review the biological rationale that may explain this benefit observed in the clinical setting.

**Key words:** Non-small cell lung cancer; Tyrosine kinase inhibitors; Epidermal growth factor receptors

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**Core tip:** Epidermal growth factor receptor (EGFR) tyrosine kinase inhibitors are well established as the treatment of choice in EGFR-mutant non-small cell lung cancer. However, they are approved and have shown efficacy in patients with wild-type disease. Here, we review the clinical data showing this consistent benefit in a subgroup of patients and the potential biological mechanisms of this clinical effect.

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## CLINICAL ACTIVITY OF ERLOTINIB IN STUDIES WITH EGFR WILD-TYPE NSCLC PATIENTS

The activity of epidermal growth factor receptor (EGFR) tyrosine kinase inhibitors (TKIs) in non-small cell lung cancer (NSCLC) patients harbouring EGFR mutations has changed the way we diagnose and treat patients. Since the role of oncogenic driver mutations was first recognised, several other genes have been identified as predictors of dramatic and sustained response to other targeted therapies in lung cancer.

Despite this tight link between driver and benefit with specific drugs, we have targeted agents, such as erlotinib or gefitinib, approved for the treatment of molecularly unselected NSCLC patients. Since the design of the trials<sup>[1,2]</sup> that led to the approval of erlotinib in Western countries or of gefitinib in Asia did not include obligatory assessment of molecular status, some have argued that the benefit observed with TKI vs placebo could derive from the undetected EGFR-mutant population in these trials. Other studies have demonstrated activity of EGFR-TKIs in wild-type (wt) EGFR patients with advanced NSCLC (Table 1). This happens in studies treating unselected populations of NSCLC patients, but does it hold true when we select for EGFR-wt tumours? There are three studies<sup>[3-5]</sup> that have put this into question. The TAILOR trial<sup>[3]</sup> demonstrates superiority, in terms of progression-free survival (PFS), of docetaxel vs erlotinib in second-line treatment in EGFR-wt NSCLC patients. The DELTA trial<sup>[4]</sup> found that, in a pre-specified subgroup analysis, the EGFR-wt population did better in terms of PFS with docetaxel vs erlotinib<sup>[4]</sup>. The third study<sup>[5]</sup> compares gefitinib to pemetrexed in an Asian population and demonstrates superiority of pemetrexed in the second-line setting in terms of response rates (RR) and PFS.

Although for the general population of wt patients the benefit of erlotinib might be inferior to chemotherapy, there are still patients who respond and achieve disease control with EGFR-TKIs in those trials. Here, we review the clinical data supporting this potential benefit and the scientific evidence that may underlie the efficacy of EGFR-TKIs in selected EGFR-wt patients.

## CLINICAL EVIDENCE FOR ACTIVITY OF EGFR-TKIS IN EGFR-WT NSCLC PATIENTS

Platinum-based doublets are the first-line treatment for unselected advanced NSCLC patients and three drugs are approved for second-line treatment: docetaxel, pemetrexed and erlotinib. Docetaxel has demonstrated effectiveness in prolonging PFS and OS in second-line treatment of NSCLC when compared to single agent chemotherapy<sup>[6]</sup>. Pemetrexed has shown similar efficacy to docetaxel in the same setting<sup>[7]</sup>.

The BR.21 trial<sup>[1]</sup> showed that erlotinib improved PFS, OS and quality of life compared with placebo in molecularly unselected patients with advanced NSCLC not suitable for second- and third-line chemotherapy. These results led to the approval of erlotinib in second- and third-line treatment in patients with wt or unknown EGFR mutations. Although EGFR-TKIs are clearly superior to chemotherapy in patients with EGFR-mutant NSCLC<sup>[8,9]</sup>, their role in wt patients is still controversial. Several trials have compared EGFR-TKIs with chemotherapy in unselected patients with NSCLC, but the majority were not properly designed to investigate the treatment benefit according to EGFR mutations, and retrospective analysis according to EGFR genotype was restricted by the high percentage of patients with unknown EGFR status<sup>[10]</sup>.

### First-line trials

**Combination with chemotherapy:** The combination of EGFR-TKIs with platinum-based chemotherapy doublets in the first-line setting was evaluated in phase III trials (Table 1); both gefitinib and erlotinib were studied in combination with cisplatin and gemcitabine (INTACT 1 and TALENT)<sup>[11,12]</sup> and with carboplatin and paclitaxel (INTACT 2 and TRIBUTE)<sup>[13,14]</sup>. The addition of gefitinib or erlotinib to standard first-line chemotherapy did not result in a survival benefit in the general population, but in the TRIBUTE study<sup>[14]</sup>, never-smoker patients treated with erlotinib and chemotherapy experienced an improvement in survival. The proportion of patients with a non-adenocarcinoma histology and thus likely to be EGFR-wt ranged from 39.3%-61.6%. No difference in efficacy according to histology was found in the subgroup analysis of these studies. Clinical trial results are summarised in Table 1. It seems that the combination of EGFR-TKIs with chemotherapy in EGFR-wt patients does not provide additional benefit.

**Monotherapy:** Certain clinical characteristics (adenocarcinoma histology, Asian race, female gender and never-smoking status) were related with an increased probability of response to EGFR-TKIs. The IPASS trial<sup>[8]</sup> included only patients with these characteristics, comparing the efficacy of first-line gefitinib monotherapy to the combination of carboplatin and paclitaxel. This trial demonstrated the inefficacy of clinical selection in predicting mutational status, as up to 40% of these clinically selected patients were EGFR-wt. Gefitinib was non-inferior to chemotherapy in the general population. The subgroup analysis clearly showed superiority of gefitinib over chemotherapy in patients harbouring EGFR mutations, but also showed that gefitinib was inferior to chemotherapy in EGFR-wt cases. Of note, however, was that the disease control rate with gefitinib in the EGFR-wt population was 39.6%, with one patient achieving a partial response. The results of these trials are summarised in Table 1. Taken together, these trials show a subset of EGFR-wt patients with some benefit from EGFR-TKIs, in general in the form of stabilisation

**Table 1 First-line and maintenance phase III trials**

Trial	Comparison	Population characteristics	Efficacy in all patients	Efficacy in subgroup enriched for EGFR wt	Mutational analysis	Efficacy by mutational status
INTACT 1 <sup>[11]</sup>	C + Gem + G (n = 730) vs C + Gem + P (n = 363)	First line; nonADC, 53.9%; non-Asian, 94.7%	PFS for C + Gem + G, 5.5 mo; PFS for C + Gem + P, 6 mo; $P = 0.763$ ; OS for C + Gem + G, 9.9 mo; OS for C + Gem + P, 10.9 mo; $P = 0.45$	NR	NR	NR
INTACT 2 <sup>[13]</sup>	Cb + T + G (n = 692) vs Cb + T + P (n = 345)	First line; nonADC, 44.9%; non-Asian, 95.8%	PFS for Cb + T + G, 5.3 mo; PFS for Cb + T + P, 5 mo; $P = 0.056$ ; OS for Cb + T + G, 9.8 mo; OS for Cb + T + P, 9.9 mo; $P = 0.638$	NR	NR	NR
TALENT <sup>[12]</sup>	C + Gem + E (n = 580) vs C + Gem + P (n = 579)	First line; nonADC, 61.6%; non-Asian, 93.6%	PFS for C + Gem + E, 5.9 mo; PFS for C + Gem + P, 6.1 mo; HR = 0.98; $P = 0.74$ ; OS for C + Gem + E, 10.7 mo; OS for C + Gem + P, 11 mo; HR = 1.06; $P = 0.486$	NR	NR	NR
TRIBUTE <sup>[14]</sup>	Cb + T + E (n = 539) vs Cb + T + P (n = 540)	First line; nonADC, 39.3%; non-Asian, 96.9%	PFS for Cb + T + E, 5.1 mo; PFS for Cb + T + P, 4.9	NR	n = 228 (21.1%); activating mutation, 29	NR
IPASS <sup>[8]</sup>	G (n = 609) vs Cb + T (n = 608)	First line; only Asians with ADC and never or light former smokers	PFS for G, 5.7 mo; PFS for Cb + T, 5.8 mo; HR = 0.74; $P < 0.001$		n = 437 (35.9%); activating mutation, 261	EGFR mutated: PFS HR, 0.83; EGFR wt: PFS HR, 2.85; interaction $P < 0.001$
First-SIGNAL <sup>[15]</sup>	G (n = 159) vs C + Gem (n = 154)	First line; only Asians with ADC and never smokers	PFS for G, 5.8 mo; PFS for C + Gem, 6.4 mo; HR = 1.198, $P = 0.138$ ; OS for G, 22.3 mo; OS for C + Gem, 22.9 mo; HR = 0.932; $P = 0.604$		n = 96 (31%); activating mutation, 42	EGFR mutated: PFS HR, 0.54; EGFR wt: PFS HR, 1.41
SATURN <sup>[16]</sup>	E (n = 438) vs P (n = 451)	Maintenance; no progression after prior platinum-doublet; nonADC, 55%; non-Asian, 85%	PFS for E, 3 mo; PFS for P, 2.77 mo; HR = 0.71; $P < 0.001$ ; OS for E, 12 mo; OS for P, 11 mo; HR = 0.81; $P = 0.0088$	Squamous PFS HR, 0.76; non-Asian PFS HR, 0.75; squamous OS HR, 0.86; non-Asian OS HR, 0.86+	n = 446 (50.1%); EGFR activating mutation, 49	EGFR mutated: PFS HR, 0.10; EGFR wt: PFS HR, 0.78; interaction $P < 0.001$ ; EGFR mutated: OS HR, NR; EGFR wt: OS HR, 0.77

ADC: Adenocarcinoma; C: Cisplatin; Cb: Carboplatin; D: Docetaxel; E: Erlotinib; EGFR: Epidermal growth factor receptor; G: Gefitinib; Gem: Gemcitabine; HR: Hazard ratio; NR: Not reported; OS: Median overall survival; P: Placebo; Pem: Pemetrexed; PFS: Median progression free survival; T: Paclitaxel; wt: Wild type.

of disease.

### Maintenance therapy trials

The sequential Tarceva in unresectable NSCLC (SATURN) trial<sup>[15]</sup> was a phase III study that randomised patients without progression after 4 cycles of platinum-doublet

chemotherapy to erlotinib or placebo as maintenance treatment. Maintenance therapy with erlotinib produced a modest benefit in terms of PFS (HR = 0.71;  $P < 0.01$ ) and OS (HR = 0.77;  $P < 0.008$ ) in the overall population. The subgroup analysis revealed that the benefit was greater in EGFR-mutant patients. However,



this benefit still persisted in EGFR-wt cases, both for PFS (HR = 0.78;  $P = 0.018$ ) and OS (HR = 0.77;  $P = 0.243$ ). One of the main caveats of this study is that maintenance treatment with pemetrexed is currently indicated in non-squamous tumours<sup>[16]</sup>, so the benefit observed in wt patients could be inferior to that offered by pemetrexed. Another phase III study<sup>[17]</sup> (ATLAS) evaluated the addition of erlotinib to maintenance treatment with bevacizumab after first-line chemotherapy in unselected patients. The addition of erlotinib to bevacizumab improved PFS (HR = 0.71;  $P < 0.001$ ) but not OS (HR = 0.92;  $P = 0.534$ ). It should be noted that the study was not powered to detect differences in OS, it was unblinded after the interim analysis, and further survival follow-up was not pursued based on the low likelihood of observing significant differences between arms. Lastly, a phase III trial<sup>[18]</sup> evaluating maintenance therapy with gefitinib showed similar results, with an improvement in PFS (HR 0.61;  $P = 0.001$ ) but not in OS (HR = 0.83;  $P = 0.2$ ). Results of the above trials are summarised in Table 1.

### Second- and third-line trials

The BR.21<sup>[1]</sup> and ISEL<sup>[19]</sup> trials compared erlotinib and gefitinib respectively with placebo and best supportive care in second- and third-line settings in unselected populations.

Despite an RR of only 8%, in the BR.21 trial, erlotinib showed an improvement in OS (6.7 mo with erlotinib vs 4.7 mo with placebo; HR = 0.70;  $P < 0.001$ ). This benefit was also observed in patients with squamous histology, a subgroup more likely to be EGFR-wt. In retrospective analysis the results for EGFR-wt patients were similar to the overall population, with an RR of 7% in EGFR-wt patients treated with erlotinib<sup>[20,21]</sup>. In a retrospective analysis 21 of the 15% of cases with available tissue from the ISEL study, the RR to gefitinib in EGFR-wt patients was 2.6%.

In the INTEREST trial<sup>[22]</sup>, a non-inferiority trial comparing second-line treatment with gefitinib and docetaxel in an unselected population, gefitinib was non-inferior to docetaxel. This non-inferiority was maintained in the non-adenocarcinoma and non-Asian subgroups. The EGFR-mutated cases had better PFS than those with EGFR-wt tumours, but no differences were shown in terms of OS. The RR of EGFR-wt patients treated with gefitinib was 6.6%<sup>[23]</sup>.

The TITAN study<sup>[24]</sup> included patients who progressed on first-line platinum-doublet chemotherapy in the run-in period shared with the SATURN trial. Second-line erlotinib was compared with docetaxel or pemetrexed. Erlotinib showed a similar efficacy to docetaxel or pemetrexed, but the trial was not powered to detect non-inferiority because it was prematurely halted due to poor accrual. EGFR mutational status was determined in 40% of patients. No differences between treatment arms were shown in the EGFR-wt population. The HORG trial<sup>[25]</sup> showed no differences in

efficacy between erlotinib and pemetrexed in second- or third-line settings in unselected patients. The limited efficacy of pemetrexed in squamous histology may have decreased the performance of the pemetrexed arm. Focusing on EGFR-wt patients, the RR with erlotinib was 7.3%, with a disease control rate of 21.8%.

Recently the TAILOR phase III study<sup>[3]</sup> compared second-line treatment with erlotinib or docetaxel in EGFR-wt tumours. Docetaxel was superior to erlotinib in terms of PFS (2.9 mo with docetaxel vs 2.4 mo with erlotinib; HR = 0.71;  $P = 0.02$ ), and showed a trend towards superiority over erlotinib in OS (OS 8.2 mo with docetaxel vs 5.4 mo with erlotinib; HR = 0.73;  $P = 0.05$ ). Despite this, 3% of patients in the erlotinib arm achieved a partial response, and 23% disease stabilisation. In the CTONG 0806 study<sup>[26]</sup> conducted in China, comparing pemetrexed with gefitinib in EGFR-wt patients, overall results favoured pemetrexed, with PFS of 5.6 vs 1.7 mo. However, some benefit was still observed in the gefitinib arm in the form of ORR and disease stabilisation of 2.4% and 12.2%, respectively. The results of second- and third-line phase III trials are summarised in Table 2.

In conclusion, the efficacy of second- and third-line treatment in non-mutant patients with advanced NSCLC is limited. Moreover, the toxicity of chemotherapy, in particular docetaxel, can deteriorate the quality of life of patients at this stage. The main advantages of EGFR-TKIs in this setting are basically the convenience of oral administration and mild and manageable toxicity. Although the studies presented above show limited efficacy of erlotinib or gefitinib for EGFR-wt patients, a response rate of approximately 8% has consistently been observed, with stabilisation in 25% of patients. This small, but significant population may have relative dependence on the EGFR pathway independent of mutational status that may explain these clinical observations.

In the next part of the article, we review potential biological explanations for this clinical effect.

## BIOLOGICAL EVIDENCE OFR EGFR INHIBITION IN EGFR WILD-TYPE NSCLC

### EGFR pathway

The EGF receptor (EGFR/HER1) belongs to a family of receptors with a common architecture (HER2, 3 and 4). These receptors have an extracellular ligand-binding portion, a single transmembrane helix and an intracellular tyrosine kinase domain and C-terminal tail that serve as a scaffold for adaptor molecules. A variety of EGF receptor ligands, mainly amphiregulin, TGF- $\alpha$  and EGF for EGFR, upon binding, drive the formation of homo- or heterodimers that activate the receptors and amplify their signal. In cancer cells, the phosphorylation of the tyrosine kinase domain eventually results in the recruitment of intracellular substrates and binding of adaptor molecules that

**Table 2** Relevant second- and third-line phase III trials

Trial	Comparison	Population characteristics	Efficacy in all patients	Efficacy in subgroup enriched for EGFR wt	Mutational analysis	Efficacy by mutational status
BR.21 <sup>[1,21]</sup>	E (n = 488) vs P (n = 243)	Second line (51%) or third line (49%); nonADC, 50%; non-Asian, 87%	OS for E, 6.7 mo; OS for P, 4.7 mo; HR 0.70; P < 0.001	NonADC HR, 0.8; non-Asian HR, 0.8	n = 204 (27.9%); EGFR activating mutation, 34	EGFR mutated: OS HR, 0.55; EGFR wt OS HR, 0.74; interaction P = 0.47
ISEL <sup>[20,23]</sup>	G (n = 959) vs P (n = 480)	Second line (49%) or third line (51%); nonADC 52%; non-Asian, 90%	OS for G, 5.6 mo; OS for P, 5.1 mo; HR, 0.89; P = 0.089	NonADC HR < 1.0 <sup>1</sup> ; non-Asian HR = 0.92	n = 215 (14.9%); activating EGFR mutation, 26	NR
INTEREST <sup>[24,25]</sup>	G (n = 723) vs D (n = 710)	Second line (84%) or third line (16%); nonADC, 44%; non-Asian, 78%	OS for G, 7.6 mo; OS for D, 8 mo; HR, 1.02 (met non inferiority criteria)	NonADC HR < 1 <sup>1</sup> ; non-Asian HR = 1 <sup>1</sup>	n = 297 (20.7%); activating EGFR mutation, 44	EGFR mutated: OS HR, 0.83; EGFR wt: OS HR, 1.02; interaction P = 0.59
TITAN <sup>[26]</sup>	E (n = 203) vs D/Pem (n = 221)	Second line; non-Asian, 86%; nonADC, 55%	OS for E, 5.3 mo; OS for D/Pem, 5.5 mo; HR = 0.96; P = 0.73	Squamous OS HR = 0.86; non-Asian OS HR = 0.94	n = 167 (39.3%); activating EGFR mutation, 18	EGFR mutated: OS HR = 1.19; EGFR wt: OS HR = 0.85
HORG <sup>[27]</sup>	E (n = 166) vs Pem (n = 166)	Second line (57%) or third line (43%); nonADC, 77.5%; non-Asian, 100%	PFS for E, 3.6 mo; PFS for Pem 2.9 mo; P = 0.136; OS for E, 8.2 mo; OS for Pem, 10.1 mo; P = 0.986	Squamous OS HR = 1.97	n = 123 (37%); activating EGFR mutations, 11	NR

<sup>1</sup>HR estimated from forest plot in publication. ADC: Adenocarcinoma; C: Cisplatin; Cb: Carboplatin; D: Docetaxel; E: Erlotinib; EGFR: Epidermal growth factor receptor; G: Gefitinib; Gem: Gemcitabine; HR: Hazard ratio; NR: Not reported; OS: Median overall survival; P: Placebo; Pem: Pemetrexed; PFS: Median progression free survival; T: Paclitaxel; wt: Wild type.

activate downstream signalling pathways. One of the major signalling pathways downstream of EGFR is the Ras-Raf-MAP kinase pathway. Another important target of EGFR signalling is the PI3K-Akt pathway. Lastly, EGFR activation also recruits PKC and Jak/Stat. The activation of these pathways induces transcriptional programmes that result in increased proliferation, survival, motility, and invasion<sup>[27]</sup>.

Different mechanisms for activation of the EGFR pathway have been postulated.

### Overexpression of EGFR ligands

Eleven ligands have been reported to bind to the ErbB receptor family, including epidermal growth factor (EGF), transforming growth factor alpha (TGF $\alpha$ ), amphiregulin, betacellulin, heparin-binding EGF and epiregulin. These are synthesised as membrane-anchored precursor forms that are then cleaved to generate soluble ligands. In some cases, these membrane-anchored isoforms can also act as biologically active ligands. Moreover, stromal cells have been described as releasing amphiregulin and TGF $\alpha$ . Thus, activation of EGFR by its ligands can happen through paracrine, autocrine and juxtacrine mechanisms<sup>[28]</sup>. Upon binding, they induce a conformational change in the receptor and activate

several signalling pathways (see above).

Preclinical studies have been performed to evaluate the role of these ligands in the response to the treatment with EGFR-TKIs, showing conflicting results. A study by Yonesaka *et al.*<sup>[29]</sup> demonstrated that high levels of amphiregulin (Areg) produced by EGFR-wt NSCLC cells through an autocrine mechanism predicted sensitivity to gefitinib in the form of cell cycle arrest. This happened preferentially by inhibition of signal-regulated kinase 1/2, but not the Akt pathway. In contrast, some studies<sup>[30,31]</sup> showed that autocrine Areg confers resistance to gefitinib in NSCLC cells through inhibition of apoptosis. In this case, inhibition of Areg secretion by siRNA was able to restore sensitivity to gefitinib in EGFR-wt cell-line models (H358) *in vitro* and *in vivo*. Moreover, addition of recombinant Areg to previously sensitive NSCLC cell lines (H322) conferred resistance to these cells<sup>[30]</sup>. Intriguingly, Areg reduced acetylation of ku70, preventing the release of the proapoptotic form of BAX and, consequently, inhibiting the gefitinib toxicity in NSCLC cells<sup>[31]</sup>, suggesting a role for the combination of EGFR-TKIs with HDAC inhibitors (see below-EMT). These discrepancies in preclinical data may be due to differential downstream effects produced by the ligands in each particular cell-line model.

Regarding clinical data on EGFR ligands and response to EGFR inhibitors, most studies have focused on the role of amphiregulin and TGF $\alpha$ . A retrospective study by Chang *et al.*<sup>[32]</sup> evaluates amphiregulin expression by immunohistochemistry in NSCLC specimens. This work showed an association between amphiregulin expression (H-score >100) and better OS in patients treated with erlotinib or gefitinib.

Several publications<sup>[33,34]</sup> have reported on the role of serum levels of circulating amphiregulin (cAreg) and TGF $\alpha$  (cTGF $\alpha$ ) in NSCLC patients treated with EGFR-TKIs.

Detection of these circulating markers may allow measurement of the total expression of these markers in different compartments and be a surrogate marker of the EGFR signalling intensity. A Japanese study showed that high levels of cAreg in serum were associated with lack of benefit from gefitinib<sup>[33]</sup>. In contrast, a study performed in the Netherlands concluded that patients presenting high levels of cAreg benefited from treatment with EGFR-TKIs<sup>[34]</sup>. The authors speculate that these differences may be based on ethnic differences.

More consistent data have been reported for cTGF $\alpha$ . It seems that high baseline TGF $\alpha$  predicts lack of benefit from erlotinib or gefitinib<sup>[33,35]</sup> or, accordingly, low levels before treatment predict benefit from EGFR-TKIs<sup>[34]</sup>.

The convenience of measuring circulating levels of a protein to select patients for a treatment underlines the importance of validating these results in prospective trials. Validated cut-offs and techniques for these measurements are essential to ensure the applicability of these findings to day-to-day clinical practice.

### Other members of the ERBB family

Upon ligand stimulation, EGFR forms homodimers or heterodimers with the other HER family members. Several studies<sup>[36,37]</sup> demonstrated the importance of the status of other EGFR family members in response to EGFR-TKIs. HER2 is overexpressed in various cancers through gene amplification that constitutively activates the protein. In lung cancer, HER2 amplification has been identified in a low percentage of patients and has been associated with poor prognosis<sup>[36]</sup>. HER2 mutations have also been identified in NSCLC in about 2% of patients<sup>[37]</sup>. The impact of these genetic abnormalities or overexpression of HER2 in the response to EGFR-TKI treatment in NSCLC has been evaluated.

For instance, HER2 mutations seem to predict for resistance to EGFR-TKIs<sup>[38]</sup> in NSCLC cells, while these remain sensitive to anti-HER2 treatments. In this study, knockdown of mutant HER2 induced cell death and sensitised these cells to EGFR-TKIs. In contrast, amplification/overexpression of the *HER2* gene has been associated with moderate sensitivity to gefitinib and erlotinib<sup>[39]</sup>. Cell-line studies in NSCLC models show that overexpression of HER2 in EGFR-wt cells enhances sensitivity to gefitinib that acts specifically through the inhibition of the PI3K/Akt pathway<sup>[40,41]</sup>. In these models, a relevant role of HER3 in the observed

response could not be ruled out; either through specific abrogation by gefitinib of HER2/3 heterodimers<sup>[40]</sup> or by the presence of coexpression of this receptor<sup>[41]</sup>.

In the clinical setting, there is retrospective evidence showing that patients with EGFR-positive tumours (by immunohistochemistry) which harbour high HER2 copy numbers have better response and disease control rates when treated with gefitinib<sup>[42]</sup>. The clinical data on patients, whose tumours harbour HER2 mutation support the use of drugs such as trastuzumab or afatinib for these patients<sup>[37]</sup>.

As discussed above, HER3 expression has also been associated with sensitivity to EGFR-TKIs. ErbB3 is unique among the ErbB family members because it lacks significant tyrosine kinase activity. However, it heterodimerizes with other members of the family and couples to the PI3K/Akt pathway, initiating intracellular signalling pathways. In preclinical models, it has been shown that EGFR-wt NSCLC cell lines are growth-inhibited by gefitinib when downregulation of the PI3K/Akt pathway is observed through ErbB3<sup>[41]</sup>, and this has also been suggested in pancreatic cancer cells<sup>[43]</sup>.

Results from a clinical study<sup>[44]</sup> suggest that HER3 expression is a predictor of response to EGFR-TKIs independent of EGFR mutational status, although more data are needed. HER4 mutations have also been identified in lung cancer<sup>[45]</sup>. The role of this receptor in lung cancer seems to be associated with chemoresistance<sup>[46]</sup> and there is one study that shows that a HER4 mutant cell line was resistant to gefitinib<sup>[39]</sup>.

Overall, it seems that HER-family receptor status has an impact on the response of wild-type EGFR lung cancer to EGFR-TKIs and that combining EGFR-TKIs with other receptor inhibitors or the use of pan-HER inhibitors could be a promising strategy for the treatment of patients with activation of the HER family members.

### Epithelial to mesenchymal transition

Epithelial-to-mesenchymal transition (EMT) is a cellular process that occurs both during critical phases of embryonic development and in carcinogenesis<sup>[47]</sup>. This transition is characterised by the loss of epithelial markers and acquisition of a mesenchymal phenotype, which enables cancer cells to invade surrounding tissues and generate distant metastases<sup>[48]</sup>. Loss of E-cadherin expression, a key protein in adhesive junctions between epithelial cells, is central to EMT. Therefore, E-cadherin-negative cells show a more invasive phenotype<sup>[47,48]</sup>. This process is initiated by the transcriptional factor Snail1. Although Snail1 is induced at the early phases of EMT its expression is not maintained in most mesenchymal cells; instead, E-cadherin silencing is dependent on other transcriptional repressors induced by Snail1, such as Zeb1 and 2<sup>[49]</sup>. Other markers of a mesenchymal phenotype are expression of vimentin, fibronectin or N-cadherin<sup>[47]</sup>.

EMT has been associated with poor prognosis and chemoresistance in different tumour models<sup>[50-52]</sup>.

Many studies<sup>[53-55]</sup> have demonstrated a correlation between sensitivity to EGFR-TKIs and EMT in lung cancer. A gene expression analysis<sup>[53]</sup> in NSCLC cell lines showed a correlation between expression of epithelial- or mesenchymal-related genes and growth inhibitory effect of erlotinib. Cell lines with an epithelial phenotype showed a lower IC<sub>50</sub> compared to cells with a mesenchymal phenotype<sup>[54]</sup>. A similar study<sup>[55]</sup> reported differences in expression of vimentin and fibronectin between erlotinib-sensitive and erlotinib-insensitive cell lines. Cell lines overexpressing fibronectin and/or vimentin were insensitive to growth inhibition by erlotinib *in vitro* and *in vivo*, and no or little expression of these proteins was found in erlotinib-sensitive cells. Conversely, expression of E-cadherin and ErbB3 was found in erlotinib-sensitive cell lines and was absent in insensitive cell lines<sup>[20]</sup>. Comparable results have been observed using gefitinib in NSCLC, head and neck Squamous Cell Carcinoma (HNSCC) and hepatoma cell lines<sup>[56,57]</sup>, which supports the hypothesis that EMT status is predictive of EGFR-TKI sensitivity. Moreover, Frederick *et al.*<sup>[56]</sup> found gefitinib sensitivity to be more strongly related with epithelial or mesenchymal phenotype than with tumour origin, and, in the gene expression analysis, gefitinib-sensitive NSCLC clustered together with sensitive HNSCC cells, as did gefitinib-resistant cell lines of both histological origins. However, within the two sensitivity groups, HNSCC cells formed a cluster of distinct NSCLC cells.

To explore the clinical relevance of these observations, Yauch *et al.*<sup>[53]</sup> evaluated E-cadherin membranous and cytoplasmatic staining in tumour samples from a subset of patients who had participated in the TRIBUTE trial. E-cadherin staining intensity was determined on a scale of 0-3, and patients divided into two groups: E-cadherin positive (2-3+); and E-cadherin negative (0-1+). No statistically significant differences were found between groups in terms of RR and OS. However, within the E-cadherin-positive staining subgroup, there was a statistical significant difference in time to progression favouring those receiving CHT + erlotinib vs those receiving CHT alone (34 vs 19.3 wk respectively;  $P = 0.003$ ). Comparable results were observed analysing tumour samples of a subset of patients with chemorefractory NSCLC who had participated in the BATTLE trial<sup>[58]</sup>. Of 20 KRAS-wt/EGFR-wt tumours that received erlotinib, 8-wk disease control was superior in those tumours with an epithelial phenotype, although this was of borderline significance.

Several pathways have been explored as a mechanistic link between EMT and the EGFR pathway. In cell-line cultures, the biological activity of the EGFR pathway has been related to erlotinib sensitivity. In EGF-stimulated cells, erlotinib inhibited phosphorylation of Akt and Erk independent of EMT status. However, under baseline conditions, this effect could only be observed in epithelial-like cells<sup>[53]</sup>.

The findings presented above point to a common capacity for mesenchymal-like cancer cells for by-

passing the EGFR pathway and/or having alternative mechanisms to resist apoptosis and maintain their proliferative potential. Increased Akt and STAT3 activation through elevated expression of Integrin-linked kinase (ILK) was found in gefitinib-resistant hepatoma cell lines with a mesenchymal-like phenotype<sup>[57]</sup>. ILK is a serine/threonine protein kinase that is localised to focal adhesions and stimulated by engagement of integrins to the extracellular matrix<sup>[59]</sup>. ILK regulates E-cadherin levels through interaction with transcription factors such as Zeb1 and Snail<sup>[60]</sup>, and up-regulation of ILK has been detected in mesenchymal-like cell lines. Fuchs *et al.*<sup>[57]</sup> found that inhibition of ILK in two EGFR-TKI resistant hepatoma cell lines and mouse xenografts caused a decrease in p-Akt levels and restored cell sensitivity to gefitinib, partly through an EMT. Furthermore, ILK expression has been related to shorter survival and risk of recurrence in Japanese patients with Stage Ia-IIIa resected NSCLC<sup>[61]</sup>.

Acquisition of platelet-derived growth factor receptor (PDGFR) and fibroblast growth factor receptor (FGFR) is another way for mesenchymal-like NSCLC cells to maintain survival independent of EGFR activity. In a study<sup>[57]</sup> with NSCLC cell lines, both epithelial and mesenchymal-like cells showed expression of PDGF ligands. However, expression of PDGFR alpha and beta was only detected in mesenchymal-like cells. In this study, EGFR blockade by erlotinib showed increased PDGFR autophosphorylation and downstream activation in mesenchymal-like cells. Similar findings were detected in regard to FGFR and FGF-ligand expression and activity. Interestingly, in a cell line that underwent an epithelial-to-mesenchymal-like transition induced by TGF-beta stimulation, increased levels of PDGFR, PDGF-ligands, FGFR, FGF-ligands and transcription factors (Snail, Zeb1 and Zeb2) were detected, along with a significant decrease in erlotinib-sensitivity. Treatment of this cell line with a TGF-beta receptor inhibitor reversed this process and re-sensitised the cells to erlotinib.

Moreover, transfection of E-cadherin in NSCLC cell lines resistant to gefitinib resulted in a decrease in cellular growth that was further enhanced in the presence of gefitinib. The apoptotic effect of gefitinib was increased in transfected cell lines compared to the parental cell controls. The activity of transcription factors such as Snail, Zeb1 and Sip1 ultimately leads to recruiting of histone deacetylases (HDAC) and, consequently, to chromatin condensation and gene silencing. HDAC inhibitors are currently being studied as anticancer treatment<sup>[62]</sup>. Inhibiting HDAC induces E-cadherin expression<sup>[63]</sup>.

Witta *et al.*<sup>[64]</sup> demonstrated a synergic effect of HDAC inhibitor MS-275 (entinostat) and gefitinib in 4 NSCLC cell lines resistant to EGFR-TKIs. Growth inhibitory and apoptotic effects of gefitinib increased after pre-treatment with 24 h of MS-275, and was similar to the effect of gefitinib alone in a cell line harbouring the L858R mutation. A phase II randomised study<sup>[64]</sup> in non-selected, previously treated patients



with advanced NSCLC failed to show a benefit in PFS with the combination of erlotinib-entinstat vs erlotinib-placebo. However, subset analysis showed increased OS in patients with high E-cadherin levels in their tumour samples (9.5 mo vs 5.4 mo;  $P = 0.03$ )<sup>[64]</sup>. Thus, it appears that patients with a more epithelial-like tumour were the ones who benefited from the combination, while patients whose tumours had mainly lost E-cadherin expression did not. Therefore, reversion of EMT through HDAC inhibition would only be possible in an initial state of transformation, being more effective in preventing EGFR resistance than in restoring it.

### Gene signatures

As the status of the EGFR or other family members does not completely explain the potential benefit from EGFR-TKIs, efforts have been made to evaluate gene signatures that may better predict for response to these drugs.

Several studies<sup>[65,66]</sup> have identified gene expression profiles that discriminate patients who benefit from EGFR-TKIs from those who do not. Kakiuchi *et al.*<sup>[65]</sup> described a 12-gene signature obtained from human lung carcinoma samples with differential expression between responders and non-responders to gefitinib. Interestingly, some of these genes, such as *Areg*, *TGF $\alpha$*  and *ADAM9*, are directly related to the EGFR pathway. They also obtained serum samples from an independent cohort of patients and concluded that those with higher levels of circulating TGF $\alpha$  were classified as non-responders. They finally validated the *Areg* results with *in vitro* models, suggesting that *Areg* expression was associated with lack of response to gefitinib. Tan *et al.*<sup>[66]</sup> described that the gene signature is not a strong predictor of benefit from erlotinib.

Another strategy has been to obtain the gene expression signature from lung cancer cell lines and then validate it in independent cell line or tumour samples. In this regard, Balko *et al.*<sup>[67]</sup> and Coldren *et al.*<sup>[68]</sup> generated > 100-gene signatures that exhibited enrichment in signal transduction functions between EGFR-inhibition sensitive and EGFR-inhibition resistant and were more robust than prediction based on mutational status alone.

The clear advantage of this approach is that study of the complexity of the tumour can be addressed by simultaneously evaluating multiple genes that may be involved in the behaviour of a particular tumour. However, in the attempt to limit the number of genes to be used in a platform, we are probably leaving out genes that are more relevant than the ones we include. Further validation in human samples from patients treated with these drugs is warranted.

### MicroRNAs

MicroRNAs are regulatory RNAs that are responsible for post-transcriptional gene silencing by degrading the mRNA or preventing its translation. One study<sup>[69]</sup> using NSCLC-cell-line expression data identified a 13-gene

miRNA signature that predicted sensitivity to erlotinib. These miRNAs were involved in the control of the expression of proteins involved in EMT.

There are also studies that identify single miRNAs as predictors of response to EGFR-TKIs. Chen *et al.*<sup>[70]</sup> identify miR-146a as overexpressed in cell-line models with activated EGFR. This microRNA was also a predictor of inhibitory response to erlotinib, gefitinib and afatinib. An additional study<sup>[71]</sup>, based on head and neck cancer cell lines, identifies miR-7 as a tumour-suppressor gene that regulated EGFR expression and downstream signalling and enhanced sensitivity to erlotinib. An unpublished study by Li *et al.*<sup>[72]</sup>, performed in NSCLC cell lines and then validated in patients' samples, demonstrates an association between expression of miR-200c, epithelial phenotype and response to EGFR-TKIs in EGFR-wt patients.

This area is currently being actively investigated and will probably provide interesting data on other regulatory mechanisms of EGFR that may affect the response to EGFR-TKIs.

### Proteomics

Lastly, there are some publications reporting evidence of serum- or plasma-based assays as predictors of response to EGFR-TKIs. VeriStrat<sup>®</sup> is the test with the most solid data that we will review here.

VeriStrat<sup>®</sup> is a commercially available serum- or plasma-based test which uses matrix- assisted laser desorption ionisation (MALDI) mass spectrometry methods. It was developed through a training set of serum samples obtained before treatment from patients who experienced long-term stable disease or early progression on gefitinib therapy<sup>[73]</sup>. Mass spectra (MS) from these patients' serum samples were used to define eight MS features, differentiating these two outcome groups.

The commercial test uses a fixed set of parameters established during the development phase and assigns each spectrum a binary classification of Good or Poor. Two independent cohorts of patients<sup>[73]</sup> who were treated with gefitinib or erlotinib confirmed that patients classified as Good had better outcomes than patients classified as Poor (HR for death 0.47,  $P = 0.009$  and HR for death 0.33,  $P = 0.0007$ ). VeriStrat<sup>®</sup> was not predictive of benefit in patients receiving other treatments<sup>[73]</sup>. A more recent study<sup>[74]</sup> further validated the role of VeriStrat<sup>®</sup> as a predictor of benefit from EGFR-TKIs. Good VeriStrat<sup>®</sup> classification was associated with better outcome in patients in the placebo arm. Regarding prediction of response, Good patients had a higher response rate than poor patients (11.5% vs 1.1%,  $P = 0.002$ ), with a Good classification remaining independently correlated with response after adjustment for potential confounding factors. However, for both OS and PFS, VeriStrat<sup>®</sup> was prognostic but not predictive of differential benefit from erlotinib, leading to doubts about the clinical utility of this test for decision making. A prospective study<sup>[75]</sup> to test this hypothesis



was finally set up and preliminary data show that patients classified as VeriStrat Poor performed worse when treated with erlotinib compared to chemotherapy.

## CONCLUSION

Similar data have been consistently reported about the limited but significant benefit of EGFR-TKIs in a subset of patients with EGFR-wt NSCLC. Many potential biological mechanisms could underlie these observations. However, lack of prospective and validated data preclude drawing robust conclusions. Moreover, combination therapies blocking the EGFR pathway with other “escape” pathways provide an additional potentially beneficial approach to the treatment of patients with somewhat EGFR-dependent tumours. Additional studies specifically designed for the validation of these hypotheses are warranted in order to be able to translate these findings to the clinic.

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## Retrospective Study

# Isolated limb infusion chemotherapy with or without hemofiltration for recurrent limb melanoma

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## Abstract

**AIM:** To better define the efficacy and the safety of intra-arterial infusion performed with or without hemofiltration for recurrent limb melanoma.

**METHODS:** Patients with the following characteristics were included in the study: recurrent limb melanoma not indicated for surgical resection, measurable disease in the extremity, > 18 years, performances status (Eastern Cooperative Oncology Group ) was 0-1 and life expectancy of at least 6 mo. Twenty nine consecutive patients were enrolled in the study. Patients underwent fluoroscopic placement of angiographic arterial and venous catheters to infuse the drug in the artery [isolated limb infusion (ILI)], and to stop the out flow (venous). Melphalan was rapidly infused into the isolated limb *via* the arterial catheter after the inflation of venous balloon catheter. Then the circulation of the limb was completely blocked with a pneumatic cuff at the root of the limb. Haemofiltration (HF) was available only in the main center, and was performed with an extracorporeal perfusion system, in order to reduce high systemic toxic peaks of drug.



**RESULTS:** Thirty seven ILI were done in 29 cases (31 ILI-HF and 6 ILI) between 2001 and 2014 at Ancona and Pesaro Hospitals, Italy. Clinical outcomes were monitored 30 d after treatment. Eleven patients (38%) received infusion of melphalan alone, 7 (24%) melphalan associated to mitomycin C and 7 (24%) melphalan associated to cisplatin, the remaining 4 were treated with cisplatin, melphalan and epirubicin or cisplatin and mitomycin C. The overall response rate was 66%, in particular, 3 patients (10%) were complete responders and 16 (56%) were partial responders; whereas 7 patients (24%) had stable disease, and 3 (10%) showed progressive disease. Limb toxicity was assessed adopting Wieberdink scale, with evidence of 90% of low grade (I and II) toxicity.

**CONCLUSION:** ILI-HF and ILI are effective and safe treatments for recurrent non-resectable limb melanoma. They present evidence of favorable clinical benefit and is effective in delaying progression.

**Key words:** Metastatic melanoma; Melphalan; Intra-arterial infusion; Hemofiltration

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**Core tip:** Isolated limb infusion (ILI) is a regional treatment of limb melanoma, allowing selective delivery of toxic agents to the arm or leg with the tumor, with limited leakage. Hemofiltration can reduce the high toxic peaks of drug in the blood, hence, limiting post-procedural side effects. In this paper we report results of an Italian Registry applying the ILI technique with or without Hemofiltration to recurrent non-resectable limb melanoma. The overall response rate was 66%. Low grade toxicity was observed in 90% of patients. ILI with/without hemofiltration is an efficacious and safe treatment for recurrent non-resectable limb melanoma.

Cecchini S, Sarti D, Ricci S, Vergini LD, Sallei M, Serresi S, Ricotti G, Mulazzani L, Lattanzio F, Fiorentini G. Isolated limb infusion chemotherapy with or without hemofiltration for recurrent limb melanoma. *World J Clin Oncol* 2015; 6(4): 57-63 Available from: URL: <http://www.wjgnet.com/2218-4333/full/v6/i4/57.htm> DOI: <http://dx.doi.org/10.5306/wjco.v6.i4.57>

## INTRODUCTION

The treatments for recurrent limb melanoma are several, including, repeated surgical excision, local injection of drugs, chemotherapy and immuotherapy. Systemic chemotherapy, radiotherapy or regional chemotherapy are the most used techniques when the metastases are several and large<sup>[1-3]</sup>. Doses of general chemotherapy are restrictive because of adverse events; for this reason isolated limb perfusion (ILP, surgical method) and isolated limb infusion (ILI)

(radiological method) are the preferred approaches<sup>[4-6]</sup>.

Melphalan is used as referring drug for melanoma and sarcoma therapy (primary tumor and recurrences at the limbs)<sup>[7]</sup>. In-transit metastases are observed in 3%-8% of melanoma cases<sup>[8]</sup>. In this circumstance, treatment of these metastases with ILP or ILI can be very indicated, since they allow using high doses of toxic agents directly in the affected limb with limited outflow<sup>[9,10]</sup>.

ILI is a less invasive procedure than ILP, moreover, it is safe and can be repeatable also in the old and frail patients<sup>[11,12]</sup>. ILI avoids the use of complex surgical procedures, is shorter in execution, generate lower temperatures, but more hypoxic and acidotic environment, improving in this way the clinical outcome<sup>[11-16]</sup>.

ILI has, moreover, comparable tumor response to ILP, for this reason it may be the future first choice treatment of isolated limb melanoma, alone or in association with systemic targeted therapies<sup>[17]</sup>.

ILI can also be associated to hemofiltration (ILI-HF), for the reduction of unbound drug that can be present in the plasma after ILI procedure, which can cause side effects. This method can be particularly useful for elderly and frail patients<sup>[18,19]</sup>. The interruption of limb blood circulation can be obtained placing external tourniquets or vascular catheters with terminal inflating balloon, in the main blood vessel of the interested arm or leg<sup>[19,20]</sup>.

The selective exposition of the tumor area allows the administration of higher drug doses (also 10 ×) with an acceptable general toxicity<sup>[13,16]</sup>.

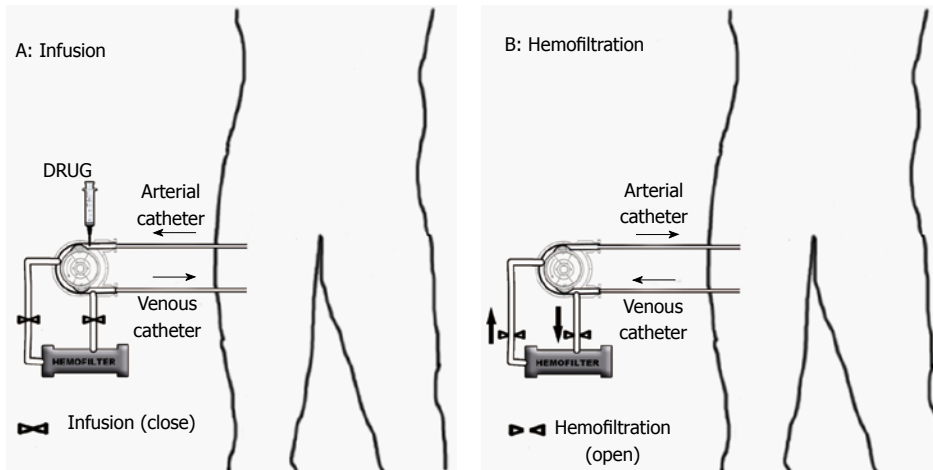
Our angiographic method permits an almost complete vascular isolation of the tumor-affected limb, and is combined with an extra-corporeal circuit that maintains the limb perfusion for 30 min. In this way, the perfusion is performed under hypoxic conditions and with normal thermal conditions.

We have been performing ILI for our patients for several years, and decided to collect data on our procedures, in order to verify if this procedure can be comparable to ILP, which normally is performed by surgeons, and is a more complex and expensive procedure.

The results of our study confirm that the new research avenue ILI and ILI-HF, which are non invasive loco-regional treatments, can be as effective as more aggressive treatments, showing similar responses to ILP.

ILI is a simple method allowing the treatment even of patients with distant metastases which would be excluded from ILP. ILI permits also a good palliation of painful lesions and control of disease. Literature reports in this field are still limited and require further confirmations.

Our purpose is to monitor effectiveness and tolerability of ILI and ILI-HF in patients with limb relapsed melanoma, data are collected from a regional registry of



**Figure 1** Limb infusion/hemofiltration scheme. A: Infusion: the blood is drawn from the venous line and, once enriched with the drug, is reintroduced in the arterial line. The hemofilter is excluded from the circuit. The occlusion of the blood inflow and outflow was achieved by catheters with terminal balloon inflated; B: Hemofiltration: lines are reversed: the blood is drawn from the arterial side and reintroduces into the venous line. The hemofilter is connected to the circuit.

Marche Region (situated in the central part of Italy with 1.4 million of inhabitants).

## MATERIALS AND METHODS

### Patient selection

This was a retrospective study; data were collected from 29 consecutive patients that were treated with ILI or ILI-HF from January 2001 to September 2014 at INRCA Hospital of Ancona, and at Department of Oncology-Hematology of Pesaro General Hospital. Patients were included in the study if they were older than 18 years, and had histological proven recurrent, arm or leg metastatic melanoma that was not suitable for surgical resection, performances status was 0-1 (ECOG scale) and life expectancy of at least 6 mo.

### ILI procedure

Upon admittance to the radiology room, hydration, antibiotic and analgesic therapy were administered as previously reported by Thompson *et al.*<sup>[6]</sup>. It included the use tropisetron before the beginning of procedure; and 1 vial of 10 mg morphine hydrochloride during melphalan infusion. Intra-arterial premedication was performed with verapamil 5 mg, and lidocaine 2%, 20 mg.

The catheters were introduced into the vessels by percutaneous vascular puncture according to Seldinger's technique, after loco-regional anesthesia and systemic heparin treatment (100 UI/kg). The catheters with terminal balloon (Boston Scientific Occlusion Balloon Catheters 6F) were placed in the main vessels of the affected limb under fluoroscopic guidance. The balloons were then cuffed, in order to occlude the vascular lumen and to start drug administration. The circulation of the limb was completely interrupted applying a pneumatic tourniquet at the root of the affected limb. The simultaneous blockage of both blood inflow and outflow allowed the complete vascular isolation and the following infusion of the affected limb. Vascular

isolation created a local pH decrease and hypoxia, which enhanced the cytotoxic activity of melphalan, doxorubicin and mitomycin. The arterial and the venous catheters were connected with an extra-corporeal circuit sustained by a roller pump that allowed the perfusion of the affected limb at a flow rate range 100-150 mL/min (Figure 1A). Melphalan 1 mg/kg was administered rapidly for the first 4 min and then was recirculated for 30 min. Longer drugs exposure could create severe tissue acidosis and compromise the pre-cellular drug concentration. Haemofilter (two capillary filter system, model FH88, Gambro Dialysontaen, KG, Hechingen, FRG) was included in the extracorporeal perfusion system, in order to eliminate the excess of drug at the end of the procedure. Limb blockade was performed for 30 min, during the last five minutes the hemofiltration was started, and it was prolonged after the opening of limb circulation, by clearing the blood for 45 min or till the production of at least five liters of ultrafiltrate (Figure 1B). Our ILI procedure respected the standard Thomson protocol<sup>[6,9,16]</sup>, with the only exception that we did not use hyperthermia, because our hospital did not have the required equipment. We believed that hyperthermia could increase the therapeutic results, but unfortunately we were not able to perform it due to technical issues.

Sequential arteriograms were obtained before intra-arterial infusion of chemotherapeutic agents, in order to detect and localize the lesions (Figure 2). The rationale for the use of this angiograms was that in responders we observed the disappearance and/or reduction of tumor blush in the lesions.

Patients with little or no response after 4 wk from first ILI were indicated to receive up to 2 additional treatments (after 30 and 90 d). Patients were hospitalized for 24-48 h, keeping the limb elevated.

ILI was performed with the most used drugs for loco-regional treatments, such as Melphalan, the standard drug, which was associated to Mitomycin C (bio-reductive agent), because of its property of being more



**Figure 2 Angiogram of melanoma.** In a patient with extremity melanoma AJCC stage IIIC, angiogram shows in the middle third of the leg rounded nodule with a homogeneous, hypervascular stain, nourished by muscular branches of the peroneal artery. Additional deep-seated lesions, which were not palpable clinically, were detected by angiography distally in the leg, nourished by branches of the anterior tibial artery.

active under hypoxic conditions<sup>[10,21-23]</sup>.

### Toxicity

Complete blood counts were obtained daily for the first week following therapy. The Wieberdink Toxicity Grading scale was used for the assessment of limb toxicity (I-V)<sup>[24]</sup>.

### Tumor response evaluation

Tumor response was assessed using clinical evaluation, according to the Recist 1.1 version; CT scans were performed only in case of profound lesions. First assessments were made at 30 d after the procedure, because in our experience the response was promptly observed, and was maintained for several months, as it could be noticed from the progression free survival, and time to progression (TTP).

### Assessment of quality of life

We used the validated Italian version of the Edmonton Symptom Assessment Scale (ESAS), which monitored nine of the most frequent cancer symptoms<sup>[25]</sup>. Patients were asked to assess the severity of each item giving a score from 0 (not at all serious) to 10 (the worst possible severity) on a visual numeric scale (VNS).

Final scores were summed to obtain the total score (overall distress). Overall distress > 5 indicated a poor quality of life (QoL). QoL was monitored at 30 d after treatment. Time to walking recover was another monitored parameter as index of quality of life. Fifteen to twenty days were normally needed for walking recovery after surgical ILP<sup>[4,8]</sup>. ILI was less invasive procedure and allowed to recover the walking ability in a shorter time range (1-3 d).

### Statistical analysis

Data of the whole sample ( $n = 29$ ) were analyzed, and quantitative variables were reported as the mean and  $\pm$  SD or median. Proportions were expressed in

**Table 1 Sample description**

$n = 29$		
Average age (yr)	74,17 (std 12,90)	
Range (yr)	34-93	
Sex	$n$	%
Females	13	45
Males	16	55
Tumor localization		
Upper limb	5	17
Lower limb	24	83
Previous therapy		
SURG + INF	10	34
SURG	9	31
SURG + CHT + INF	7	24
SURG + CHT	2	7
SURG + PERF + CHT + INF	1	3
Treatment type		
ILI + HF	30	76
ILI	4	14
ILI + (ILI + HF)	3	10
Drug type		
Melphalan	11	38
Melphalan + cisplatin	7	24
Melphalan + mitomicin C	7	24
Cisplatin	2	7
Melphalan + epirubicin	1	3
Cisplatin + mitomicin C	1	3

SURG: Surgery; CHT: Chemotherapy; PERF: Hypertermic isolated limb perfusion; ILI: Isolated limb infusion; HF: Hemofiltration; INF: Interferon.

percentage. There was no statistical test except to demonstrate the variability and trends of the response to treatment. The statistical review of the study was performed by a biomedical statistician. IBM SPSS Statistic was used for all calculations.

## RESULTS

### The sample

Thirty-seven ILI procedures were performed in 29 patients between 2001 and 2014 in Ancona and Pesaro Hospitals. Main patients' characteristics were reported in Table 1. They all had distant metastases, the suggested reason for which ILI was done. All patients had been excluded from ILP by surgeon opinion. Palpable nodes were observed in 50% of patients, however no lymph-node dissection was performed. Presenting symptoms were painful nodes, ulcerated lesions, and functional impairment.

The majority of patients, 23 (79%), received only one infusion, whereas 4 (14%) and 2 (7%) patients received two and three infusions respectively. Twenty-three patients received ILI + HF and six patients ILI, of these three underwent HF following progression after the first ILI.

The median follow-up duration after the first procedure was 14.4 mo (range 3-65 mo). The melanoma nodules were monitored with clinical observation and computed tomography scans if in case of deep lesions.

The median infusion liquid was 1.5 L in the treated leg, whereas it was of 1 L for the upper limb infusion.

**Table 2 Efficacy: Tumor response one month after isolated limb infusion and hemofiltration**

<i>n</i> = 29		
Response	<i>n</i>	%
PR	16	56
SD	7	22
CR	3	11
PD	3	11

Eleven patients (38%) received infusion of melphalan alone, 7 (24%) melphalan associated to mitomycin C and 7 (24%) melphalan associated to cisplatin, the remaining 4 were treated with cisplatin, melphalan and epirubicin or cisplatin and mitomycin C. The average melphalan dose administered was 50.63 ( $\pm$  18.46) mg per liter of infused solution, average Mitomycin C dose was 14.4 ( $\pm$  10.05) mg per liter, average cisplatin dose was 47.69 ( $\pm$  31.90) mg per liter of tissue, and epirubicin dose was 50 mg per liter.

Therapies performed before ILI infusion were: 10 (34%) surgery followed by interferon (INF) therapy; 9 (31%) surgery alone; 2 (7%) surgery followed by chemotherapy, 7 (24%) surgery followed by chemotherapy and interferon (INF) or interleukin-2 (IL-2). There was also one patient that 4 years after surgery received a hypertermic isolated limb perfusion, with complete response for 10 years, and after progression received ILI, reporting a further complete remission lasting 24 mo. The patient was included in the study.

### Clinical responses

The overall response rate (ORR) was 66% one month after treatment (Table 2). In particular, 3 (10%) were complete responders, 16 (56%) were partial responders and 7 (24%) stable disease, whereas 3 (10%) showed progression disease.

Six (21%) patients showed an early progression (3.5 mo median), whereas median overall TTP was 14.0  $\pm$  4.01 mo. Seventeen (59%) patients were lost to follow up at 36 mo after ILI. Overall survival was 36 mo (range 3-65).

### Toxicity

The toxicity (*n* = 37) was grade I in 30 cases (82%), grade II in 3 cases (8%), and grade III in 4 cases (10%); and there weren't any grade IV and V limb toxicity. The average hospitalization was 2 d after both ILI and ILI + HF.

### QoL

The majority of sample, 26 patients (90%), had an improvement of QoL according Edmonton' scale 30 d after the procedure, and came back to their daily activity. Fifteen (52%) patients, moreover, interrupted analgesic or symptomatic therapy two weeks after the local infusion procedure, for more than one year.

Walking ability and rotary motion were resumed 24

and 48 h after treatment in the lower limbs and in the upper limbs respectively.

## DISCUSSION

Regional chemotherapy is the most indicated therapy for metastatic melanoma<sup>[2-9,18]</sup>. Isolated limb perfusion is the first extensively internationally procedure for regional treatment of metastatic melanoma, lately, ILI has become widely used because of its advantages: procedural good tolerability in patients (especially old and frail patients), and relative easy repetition of the procedure<sup>[15,21]</sup>. ILI has also the important advantage of reducing the complication rate (limb toxicity and morbidity), because it is a less invasive and non surgical procedure<sup>[12]</sup>.

Our data confirm the above findings, showing an overall low toxicity with mainly grade 1 or 2 (Wieberdink scale). The median follow-up was 14.4 (range 3-65) mo, and the ORR was 66% one month after treatment. In particular, 3 (10%) were complete responders, 16 (56%) were partial responders and 7 (24%) stable disease, whereas 3 (10%) showed progression disease. Our response rates were comparable to those of previously published ILI studies that reported overall response ranging from 64% to 84%<sup>[6-9]</sup>.

Hemofiltration resulted in a clear reduction of expected drug related systemic toxicity. Patients treated with ILI-HF, indeed, had reduced fall-down of white blood cells and platelets, than ILI without HF. The repetition of the procedure was performed only in cases of none or low response after the first ILI. The best cytotoxic agent for ILI is Melphalan in our opinion; we believe that the addition of other drug for the loco-regional treatment did not add anything in terms of response.

The measurement of HF leakage has already been performed in our previous studies that showed that there is a reduction of unbounded drugs of more than 30%<sup>[10,13,23]</sup>.

The main limitation of our study is the number of patients included in the analysis, in respect to the time of enrollment and the number of patients lost to follow up. Our data, however, showed similar results to those presented in the literature, suggesting the low risk and good efficacy of ILI for metastatic limb melanoma therapy.

ILI is a simple method, less expensive and with lower morbidity than the surgical approach. Long-term survivors are reported after ILI also in our experience. Scientific advances in understanding oncogenic signaling and the immunobiology of melanoma lead to the most recent therapies in the treatment of advanced melanoma, such as the targeted therapy with BRAf inhibitor, Ipilimumab and anti PD-1<sup>[26,27]</sup>. These new drugs could be successfully integrated with ILI in the next future.

In conclusion, our study suggested that isolated limb infusion with or without HF is an efficacious procedure for the palliation of melanoma metastases confined



to a limb, showing evidence of responses and clinical benefit. For this reason we suggest ILI as an effective treatment option for patients with advanced limb melanoma, in which surgical resection is not possible.

## COMMENTS

### Background

The treatments for recurrent limb melanoma are several, including, repeated surgical excision, local injection of drugs, chemotherapy and vaccine therapy. Doses of general chemotherapy are restrictive because of adverse events; for this reason isolated limb infusion (ILI) is the preferred method in cases of limb melanoma, allowing the selective delivery of cytotoxic agents to tumor, with limited general diffusion.

### Research frontiers

ILI is a simpler procedure than isolated limb perfusion (ILP), avoiding complex surgical procedures and shortening operating duration. It is safe and can be repeatable also in the old and frail patients.

### Innovations and breakthroughs

ILI has, moreover, comparable tumor response to ILP, for this reason it may be the future first choice treatment of isolated limb melanoma, alone or in association with systemic targeted therapies, due to lack of side effects, and could be combined with other systemic target therapies.

### Applications

The results of this study suggested that isolated limb infusion with or without HF is an efficacious procedure for the palliation of melanoma metastases confined to a limb, showing. For this reason the authors suggest ILI as an effective treatment option for patients with advanced limb melanoma, in which surgical resection is not possible.

### Terminology

ILI is a regional treatment of limb melanoma, allowing selective delivery of toxic agents to the target arm or leg, with limited leakage. Hemofiltration (HF) can reduce the high toxic peaks of drug in the blood, hence, limiting post-procedural side effects. Hemofiltration can be associated to ILI (ILI-HF) in order to further reduce drug leakage and systemic diffusion.

### Peer-review

This is a retrospective review of the experience of ILI as a regional treatment of melanoma localized to a limb. The sample is short but the article is well written.

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## Comparative analysis of the effectiveness of abiraterone before and after docetaxel in patients with metastatic castration-resistant prostate cancer

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**Data sharing statement:** Technical appendix and statistical code available from the corresponding author atshenhong.wu@stonybrook.edu.

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### Abstract

**AIM:** To study the efficacy and safety of abiraterone in patients with and without prior chemotherapy.

**METHODS:** The databases including PubMed and abstracts presented at the American Society of Clinical Oncology meetings up to April 2014 were systematically searched. Eligible studies included randomized controlled trials (RCTs) in which abiraterone plus prednisone was compared to placebo plus prednisone in metastatic castration-resistant prostate cancer (CRPC) patients. The summary incidence, relative risk, hazard ratio and 95%CI were calculated using random or fixed-effects models. Heterogeneity test was performed to test between-study differences in efficacy and toxicity.

**RESULTS:** A total of two phase III RCTs were included in our analysis, with metastatic CRPC patients before ( $n = 1088$ ) and after chemotherapy ( $n = 1195$ ). Prior chemotherapy did not significantly alter the effect of abiraterone on overall survival ( $P = 0.92$ ) and prostate-specific antigen (PSA) progression-free survival ( $P = 0.13$ ), but reduced its effect on radiographic-prog-

ression-free survival ( $P = 0.04$ ), objective response rate ( $P < 0.001$ ), and PSA response rate ( $P < 0.001$ ). Prior chemotherapy significantly increased the specific risk of fluid retention and edema ( $P < 0.001$ ) and hypokalemia ( $P < 0.001$ ), but decreased the risk of all-grade hypertension ( $P < 0.001$ ) attributable to abiraterone. There was no significant difference of cardiac disorders associated with abiraterone between the two settings ( $P = 0.58$ ).

**CONCLUSION:** Prior chemotherapy may reduce the effectiveness of abiraterone in patients with metastatic CRPC.

**Key words:** Abiraterone; Docetaxel; Metastatic castration-resistant prostate cancer; Chemotherapy-naïve; Pre-chemotherapy; Post-chemotherapy

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**Core tip:** Our meta-analysis has demonstrated that pre-chemotherapy may affect the efficacy and toxicity of abiraterone treatment in patients with metastatic castration-resistant prostate cancer. Abiraterone was associated with significantly increased radiographic-progression-free survival, objective response rate, and prostate-specific antigen response rate in the pre-chemotherapy setting when compared to the post-chemotherapy setting. In addition, abiraterone in the pre-chemotherapy setting had a significant lower risk of all-grade fluid retention and edema ( $P < 0.001$ ), and hypokalemia ( $P < 0.001$ ), but had a higher risk of all-grade hypertension ( $P < 0.001$ ) when compared to post-chemotherapy.

Shameem R, Hamid MS, Xu KY, Wu S. Comparative analysis of the effectiveness of abiraterone before and after docetaxel in patients with metastatic castration-resistant prostate cancer. *World J Clin Oncol* 2015; 6(4): 64-72 Available from: URL: <http://www.wjgnet.com/2218-4333/full/v6/i4/64.htm> DOI: <http://dx.doi.org/10.5306/wjco.v6.i4.64>

## INTRODUCTION

Metastatic castration-resistant prostate cancer (CRPC) is defined as disease progression despite the castrate levels of testosterone ( $\leq 50$  ng/dL) after medical or surgical castration<sup>[1-3]</sup>. Tumor growth in this setting may result from aberrant androgen receptor (AR) signaling, up-regulation of androgen synthesis enzymes, and persistent conversion of androgens to testosterone and dihydrotestosterone (DHT)<sup>[1,4-6]</sup>. Docetaxel as a chemotherapy taxane has shown survival benefit based on randomized controlled trials (RCTs) and has been a standard therapy in patients with metastatic CRPC since 2004<sup>[7,8]</sup>. However, the treatment of disease relapse on docetaxel had been challenging due to a former nonexistence of secondary effective

options<sup>[4,7]</sup>. Fortunately, in the recent years there has been an improved understanding of metastatic CRPC pathophysiology with the rapid introduction of new agents such as abiraterone to the metastatic CRPC armamentarium<sup>[9-11]</sup>.

Abiraterone acetate (AA) is a pro-drug pregnenolone derivative that is administered orally. Its active metabolite, abiraterone is an irreversible inhibitor of CYP-17, 20 lyase and 17- $\alpha$ -hydroxylase, essential enzymes for gonadal and extra-gonadal androgen synthesis<sup>[1,9,12,13]</sup>. In combination with prednisone, abiraterone has demonstrated favorable outcomes in metastatic CRPC patients who have progressed with docetaxel<sup>[14]</sup> and in the pre-chemotherapy setting as well<sup>[15]</sup>.

However, as of today the optimal sequence for abiraterone in relationship to chemotherapy is unknown, with a clear absence of evidence-based guidance<sup>[16]</sup>. We conducted a meta-analysis of RCTs to compare the efficacy and safety of abiraterone in the pre- and post-chemotherapy settings.

## MATERIALS AND METHODS

### Data source

The PubMed database ([www.pubmed.gov](http://www.pubmed.gov)) was independently searched from January 1<sup>st</sup> 2008 to March 31<sup>st</sup> 2014 using the key words "abiraterone" and "metastatic castrate resistant prostate cancer" or "metastatic castration resistant prostate cancer". In addition, we searched abstracts presented at the American Society of Clinical Oncology annual conferences from 2010 to 2014 using the same keywords. Selected abstracts and publications were reviewed for complete safety and efficacy information, and verified so that the most recent and up to date version was identified.

### Study selection

The goal of this study was to determine the impact of prior chemotherapy on the efficacy and toxicity of abiraterone in the treatment of metastatic CRPC. Therefore, we have included RCTs in which abiraterone plus prednisone was compared to placebo plus prednisone in patients with and without prior chemotherapy. All non-RCTs including phase 1 trials and single-arm phase 2 trials were excluded. Trials that met the following criteria were chosen for analysis: phase II or III prospective RCTs, patients with metastatic CRPC, and patients assigned to treatment with abiraterone plus prednisone or placebo plus prednisone. To assess study quality, the 7-item scale (score 0-5) Jadad Score was used for each included clinical trial<sup>[17]</sup>.

### Clinical end points

Efficacy end points included overall survival (OS), defined from the date of randomization to the date of death from all causes, prostate-specific antigen (PSA) progression-free survival (PFS), and Radiographic-PFS.

Radiographic-PFS was defined as progression on bone scanning defined by the Prostate Cancer Working Group 2 (PCWG2) criteria<sup>[18]</sup> or progressive soft-tissue lesions measured with the use of computed tomography or magnetic resonance imaging according to the modified Response Evaluation Criteria in Solid Tumors (RECIST) criteria<sup>[19]</sup>. PSA progression was defined by the PCWG2 criteria<sup>[18]</sup>. Additional clinical endpoints included PSA response rate ( $\geq 50\%$  decline in PSA level from baseline) and rate of objective response based on the RECIST criteria<sup>[18,19]</sup>.

Safety endpoints were based on the National Cancer Institute-Common Terminology Criteria for Adverse Events (NCI-CTCAE) version 3.0. Hypokalemia was categorized as grade 1:  $< 3.5$  mmol/L, grade 3:  $< 3.0$ - $2.5$  mmol/L and, grade 4:  $< 2.5$  mmol/L. Hypertension was categorized grade 1; asymptomatic, transient ( $< 24$  h) increase by  $> 20$  mmHg (diastolic) or to  $> 150/100$  if previously within normal limit with no need for intervention, grade 2; recurrent or persistent ( $\geq 24$  h) or symptomatic increase by  $> 20$  mmHg (diastolic) or to  $> 150/100$  if previously within normal limit; with monotherapy may be indicated, grade 3; requiring more than one drug or more intensive therapy than previously, and grade 4; life-threatening consequences such as hypertensive crisis. We also extracted safety data for various cardiac disorders: ischemic heart disease, myocardial infarction, supra-ventricular arrhythmia, ventricular tachyarrhythmias, cardiac failure, signs and symptoms. Edema and fluid retention was also included and was derived from edema-related adverse effects from sites such head and neck, trunk and genitals, limbs, and viscera.

### Statistical analysis

All statistical analyses were performed using Comprehensive MetaAnalysis program version 2.0 (Biostat, Englewood, New Jersey, United States). Hazard ratios (HR), the median time of OS, time to PSA-PFS, time to Radiographic-PFS of patients in months, along with the proportion of patients showing PSA response rate and rate of objective response in both arms were extracted from the included clinical trials. Safety data were collected included the number of patients with: all-grade and high-grade cardiac disorders, all-grade and high-grade hypertension, all-grade and high-grade fluid retention and edema, all-grade and high-grade hypokalemia respectively. Collected data were entered into a Microsoft Excel sheet. For each study, the proportion of patients, the relative risk (RR) was calculated and the 95%CI was derived. Because the two clinical trials were designed to have a control arm, the relative risk of safety outcome among patients assigned to abiraterone was calculated and compared to patients assigned to the control arm; in addition, the specific effect of abiraterone was calculated based on the incidence or response rate difference between abiraterone plus prednisone and placebo plus prednisone. For meta-analysis, both fixed-effects

(weighted with inverse variance) and random-effects model were considered. Prior to the meta-analysis, Cochran's Q statistic and  $I^2$  were calculated to assess the heterogeneity among the proportions of the included trials. For a  $P$  value of  $< 0.1$ , the assumption of homogeneity was considered invalid. If invalid the random-effects model was used. If the assumption of homogeneity was valid both the fixed-effects and random-effects model results were reported. A two-tailed  $P$  value of  $< 0.05$  was considered to be statistically significant.

## RESULTS

### Search results

Our literature search generated a total of 178 potentially relevant studies of abiraterone and metastatic CRPC. From these studies a total of 7 RCTs were identified, where 5 of them were excluded due to concomitant treatment with other agents [OGX-427, Sipuleucel-T, Trebanib (AMG-386), GDC-0068/GDC-0980]. Overall, two phase III RCTs were included in our final analysis<sup>[14,15]</sup> (Figure 1). Both trials included patients with metastatic CRPC; histologically or cytologically confirmed prostate cancer with disease progression based on PCWG2 criteria and ongoing androgen deprivation, with a serum testosterone level of  $\leq 50$  ng/dL<sup>[14,15]</sup>. In the COU-AA-301 trial, included patients had Eastern Cooperative Oncology Group (ECOG) performance status scores of  $\leq 2$  with established disease progression after receiving docetaxel (Table 1). COU-AA-302 included chemotherapy-naïve patients with disease progression, with good performance status (ECOG status  $\leq 1$ ) and Brief Pain Inventory-Short Form (BPI-SF) score of 0-3 ( $n = 1088$ ). In an independent review, the Jadad Score was calculated for each included trial (Table 1). The COU-AA-301 study was given the highest Jadad score of 5, while COU-AA-302 was given a score of 4, based on the 7-item scale. In the COU-AA-302 trial, radiographic progression-free survival was measured by the study investigators, rather than by a blinded review<sup>[15]</sup>.

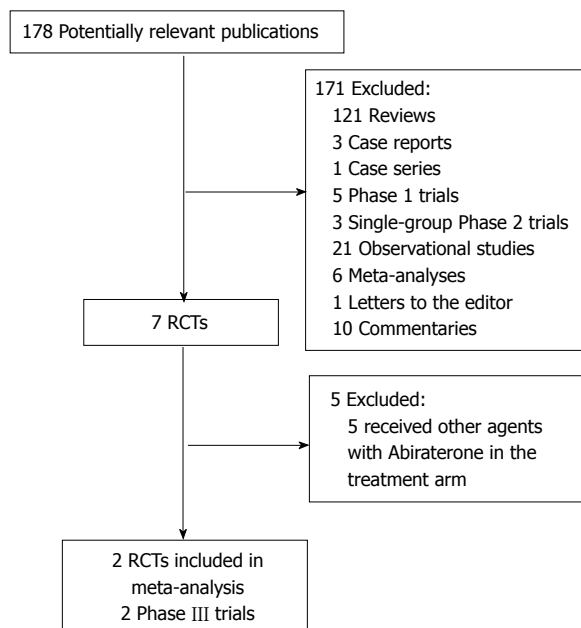
### Patients

A total of 2283 patients diagnosed with metastatic CRPC from the two RCTs were available for the analysis. A total of 1343 (58.3%) patients received the approved United States Food and Drug Administration abiraterone oral dose of 1 g daily with 5 mg of prednisone twice daily by mouth. Finally, 546 patients in the pre-chemotherapy and 797 in the post-chemotherapy arms were included in the meta-analysis (Table 2). In the post-chemotherapy trial, 70% in the abiraterone arm and 69% in the control arm received one previous cytotoxic chemotherapy regimen and approximately 30% in the abiraterone arm and 31% in the control arm received two distinct previous regimens<sup>[14]</sup>. All included patients received at least one previous cytotoxic chemotherapy regimen containing docetaxel: docetaxel

**Table 1** Characteristics of randomized controlled trials included in the meta-analysis

Clinical trial characteristics	COU-AA-302 <sup>[15]</sup>	COU-AA-301 <sup>[14]</sup>
Inclusion criteria	(1) Age $\geq$ 18 yr; (2) Confirmed metastatic disease; (3) Histologically or cytologically confirmed Prostate Adeno-CA; (4) PSA progression (PCWG2) or radiograph progression w/ or w/o PSA progression; (5) Testosterone $<$ 50 ng/dL; (6) ECOG 0 or 1; (7) BPI-SF 0-3	(1) Confirmed metastatic disease; (2) Histologically or cytologically confirmed Prostate Adeno-CA; (3) PSA progression (PCWG2) or radiograph progression w/ or w/o PSA progression; (4) Testosterone $<$ 50 ng/dL; (5) ECOG $\leq$ 2; (6) previous therapy with docetaxel
Exclusion criteria	(1) Visceral metastasis; (2) Previous use of ketoconazole lasting $>$ 7 d	(1) Elevated LFT ( $>$ 2.5 ULN) (2) Previous ketoconazole therapy; (3) Viral hepatitis; (4) Chronic liver disease; (5) Uncontrolled HTN; (6) Pituitary or adrenal dysfunction
Eligible patients	546 (abiraterone <i>vs</i> control)	797
Study arm medication and dose	Abiraterone Acetate 1 gm OD + Prednisone 5 mg BID	
Jadad Score	4	5
Median overall survival: Abiraterone <i>vs</i> Placebo (mo)	Not reached <i>vs</i> 27.2	15.8 <i>vs</i> 11.2
Median time to PSA PFS: Abiraterone <i>vs</i> Placebo (mo)	11.1 <i>vs</i> 5.6	8.5 <i>vs</i> 6.6
Median time to Radiographic PFS: Abiraterone <i>vs</i> Placebo (mo)	16.5 <i>vs</i> 8.3	5.6 <i>vs</i> 3.6
Median follow up time: Abiraterone <i>vs</i> Placebo (mo)	22.2	12.8

PCWG2: Prostate Cancer Working Group 2 Criteria; PSA: Prostate-specific antigen; BPI-SF: Brief pain inventory-short form; ECOG: Eastern Cooperative Oncology Criteria; HTN: Hypertension; ULN: Upper limit of normal range; w/o: With/without; PFS: Progression free survival; OD: Once daily; BID: Twice daily; Adeno-CA: Adenocarcinoma.



**Figure 1** Selection of randomized controlled trials included in the meta-analysis. RCTs: Randomized controlled trials.

after a treatment break, single therapy with docetaxel, or docetaxel in combination with other agents<sup>[14]</sup>.

## OS

Based on the meta-analysis of the two trials (Figure 2), the summary hazard ratio of OS associated with abiraterone acetate in comparison with the placebo yielded an increase in the OS (HR = 0.74, 95%CI: 0.66-0.84,  $P \leq 0.001$ ). An increase in OS was also seen in the pre-chemotherapy study (HR = 0.75, 95%CI:

0.61-0.93,  $P < 0.01$ ) and post-chemotherapy study (HR = 0.65, 95%CI: 0.54-0.78,  $P < 0.001$ ) respectively. However there was no significant difference in HR between the two settings (Heterogeneity test:  $Q = 0.01$ ,  $I^2 < 0.001$ ,  $P = 0.92$ ).

## PSA-PFS

Overall, abiraterone in comparison to placebo significantly reduced the risk of PSA-PFS (HR = 0.52, 95%CI: 0.45-0.59,  $P < 0.001$ ), which was also seen in the pre-chemotherapy (HR = 0.49, 95%CI: 0.42-0.57,  $P < 0.001$ ) and post-chemotherapy settings (HR = 0.63, 95%CI: 0.47-0.84,  $P = 0.002$ ). Even though HR with pre-chemotherapy was lower than post-chemotherapy (Figure 3), there was no significant difference in the risk reduction of PSA-PFS associated with abiraterone between the two settings (Heterogeneity test:  $Q = 2.255$ ,  $I^2 = 55.648$ ,  $P = 0.13$ ).

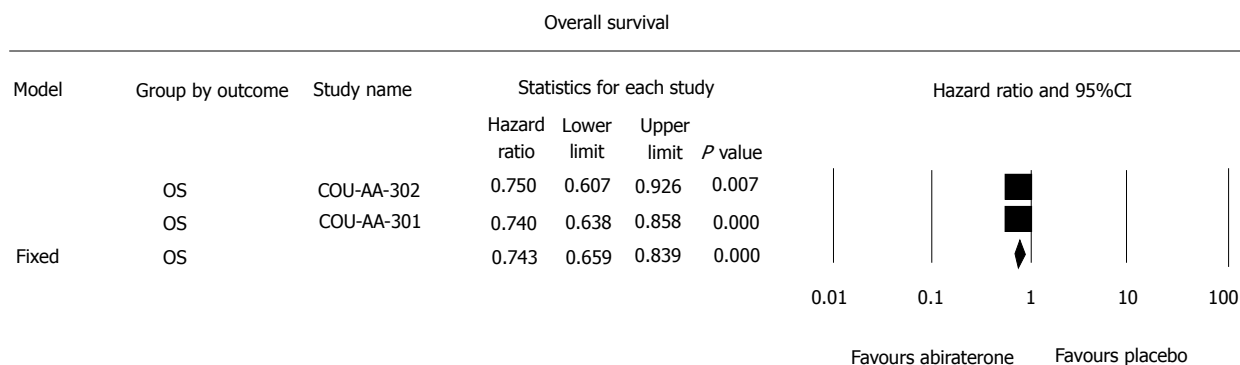
## Radiographic-PFS

As shown in Figure 4, radiographic-PFS was significantly increased with abiraterone in comparison with placebo (summary HR = 0.60, 95%CI: 0.48-0.75,  $P < 0.001$ ) in the pre-chemotherapy setting (HR = 0.53, 95%CI: 0.45-0.62,  $P < 0.001$ ) and the post-chemotherapy setting (HR = 0.67, 95%CI: 0.58-0.78,  $P < 0.001$ ). There was significant difference between the pre- and post-chemotherapy settings (Heterogeneity test:  $Q = 4.207$ ,  $I^2 = 76.203$ ,  $P = 0.035$ ).

## PSA response rate

Overall, abiraterone compared to placebo significantly increased PSA response rate (summary RR = 3.62,





Heterogeneity test:  $Q = 0.01$ ,  $I^2 < 0.001$ ,  $P = 0.92$

**Figure 2 Overall survival of abiraterone with prednisone vs placebo with prednisone.** Hazard ratio (HR) associated with abiraterone with prednisone vs placebo with prednisone for overall survival, was calculated using fixed effects model. The HR and 95%CI for each trial and the final combined results are demonstrated numerically on the left and graphically as a forest plot on the right. For individual trials: filled in square, HR; lines, 95%CI; diamond plot, overall results of the included trials. OS: Overall survival.

**Table 2 Rates of tumor response attributable to abiraterone in the pre- and post-chemotherapy settings**

	Pre-chemotherapy rate (95%CI)	Post-chemotherapy rate (95%CI)
Objective response	20.0% (16.9%-23.6%)	11.5% (9.5%-13.9%) <sup>b</sup>
PSA response	38.0% (34.0%-42.1%)	24.0% (21.2%-27.1%) <sup>b</sup>

<sup>b</sup> $P < 0.01$ , pre-chemotherapy *vs* post-chemotherapy rates. The response rate attributable to abiraterone = rate of abiraterone and prednisone minus that of placebo and prednisone.

95%CI: 1.78-7.40,  $P < 0.001$ ) in the pre-chemotherapy setting (RR = 2.58, 95%CI: 2.19-3.04,  $P < 0.001$ ) and the post-chemotherapy setting (RR = 5.36, 95%CI: 3.52-8.17,  $P < 0.001$ ). There was significant difference between the pre-chemotherapy setting and the post-chemotherapy setting (Heterogeneity test:  $Q = 4.207$ ,  $I^2 = 76.203$ ,  $P = 0.04$ ).

In addition, we determined the specific effect of abiraterone on PSA response (PSA response rate of abiraterone and prednisone minus placebo and prednisone in the same trial). As shown in Table 2, pre-chemotherapy (38.0%, 95%CI: 34.0%-42.1%) had significantly higher PSA response rate attributable to abiraterone ( $P < 0.001$ ) than post-chemotherapy (24.0%, 95%CI: 21.2%-27.1%).

#### Rate of objective response

The rate of object response with abiraterone in comparison to placebo, was significantly increased (summary RR = 3.02, 95%CI: 1.55-5.90,  $P < 0.001$ ). The difference between the post-chemotherapy setting (RR = 4.49, 95%CI: 2.57-7.830,  $P < 0.001$ ) and the pre-chemotherapy setting (RR = 2.25, 95%CI: 1.80-2.81,  $P < 0.001$ ) significantly favored the pre-chemotherapy setting (Heterogeneity test:  $Q = 4.207$ ,  $I^2 = 76.203$ ,  $P = 0.04$ ). In addition, we determined the specific effect of abiraterone on objective response (objective response rate of abiraterone and prednisone

**Table 3 Incidence of adverse events attributable to abiraterone in the pre- and post-chemotherapy settings**

Adverse events	Pre-chemotherapy incidence (95%CI)	Post-chemotherapy incidence (95%CI)
Cardiac disorders all-grade	3.0% (1.9%-4.8%)	4.0% (2.8%-5.6%)
Fluid retention and edema all-grade	4.0% (2.6%-6.0%)	9.0% (7.2%-11.2%) <sup>b</sup>
Hypertension all-grade	9.0% (6.9%-11.7%)	3.0% (2.0%-4.4%) <sup>b</sup>
Hypokalemia all-grade	9.0% (7.2%-11.2%)	4.0% (2.6%-6.0%) <sup>b</sup>
Hypokalemia high-grade	0.1% (1.0%-1.5%)	3.7% (2.6%-5.2%) <sup>b</sup>

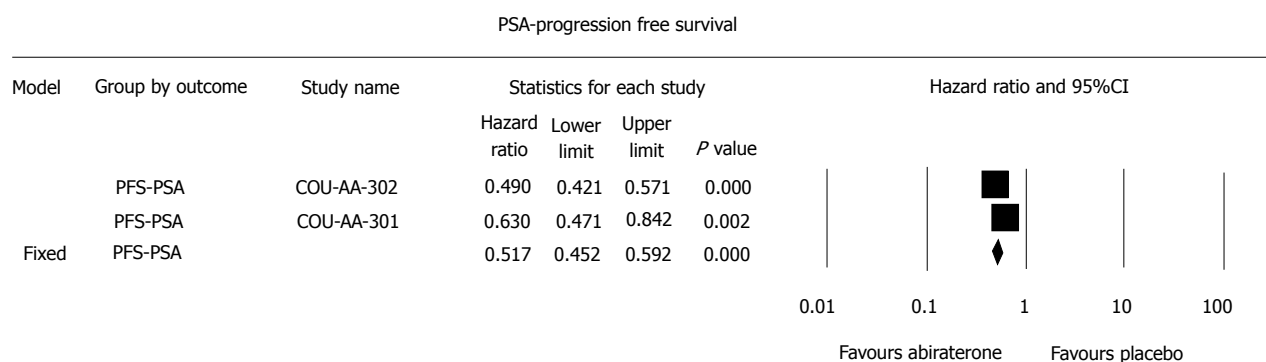
<sup>b</sup> $P < 0.01$ , pre-chemotherapy *vs* post-chemotherapy incidence. The incidence rate attributable to abiraterone = incidence rate of abiraterone and prednisone minus that of placebo and prednisone.

minus placebo and prednisone). As shown in Table 2, there was significant difference ( $P < 0.001$ ) in objective response rate attributable to abiraterone between pre-chemotherapy (20.0%, 95%CI: 16.9%-23.6%) and post-chemotherapy (11.5%, 95%CI: 9.5%-13.9%).

#### Toxicity

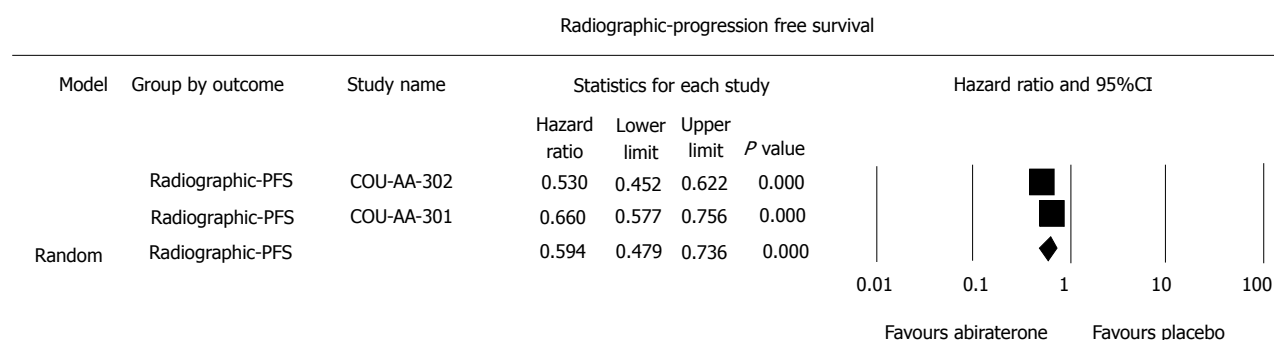
Overall, abiraterone significantly increased the risk of all-grade cardiac disorders (RR = 1.25, 95%CI: 1.02-1.52,  $P = 0.03$ ), fluid retention and edema (RR = 1.27, 95%CI: 1.08-1.49,  $P = 0.004$ ), hypertension (RR = 1.58, 95%CI: 1.27-1.98), and hypokalemia (RR = 1.60, 95%CI: 1.06-2.42,  $P = 0.027$ ). In addition, abiraterone significantly increased the risk of high-grade cardiac disorders (RR = 2.17, 95%CI: 1.37-3.45), but not high-grade fluid retention and edema, hypertension, and hypokalemia (Table 3) based on the summary results of the two trials. Subgroup analyses showed there was significant difference between pre- and post-chemotherapy in the risk of high-grade fluid retention and edema ( $P = 0.02$ ) and hypokalemia ( $P = 0.02$ ) associated with abiraterone.

We further determined the incidence rate attributable to abiraterone (incidence rate of abiraterone and prednisone minus placebo and prednisone). The



Heterogeneity test:  $Q = 2.255$ ,  $I^2 = 55.648$ ,  $P = 0.13$

**Figure 3** Prostate specific antigen progression-free survival of abiraterone with prednisone vs placebo with prednisone. Hazard ratio (HR) associated with abiraterone with prednisone vs placebo with prednisone for PSA-PFS were calculated using fixed effects model. The HR and 95%CI for each trial and the final combined results are demonstrated numerically on the left and graphically as a forest plot on the right. For individual trials: filled in square, hazard ratio; lines, 95%CI; diamond plot, overall results of the included trials. PSA: Prostate specific antigen; PFS: Progression-free survival.



Heterogeneity test:  $Q = 4.207$ ,  $I^2 = 76.203$ ,  $P = 0.04$

**Figure 4** Radiographic progression-free survival of abiraterone with prednisone vs placebo with prednisone. Hazard ratio (HR) associated with abiraterone with prednisone vs placebo with prednisone for Radiographic-PFS were calculated using random effect model. The HR and 95%CI for each trial and the final combined results are demonstrated numerically on the left and graphically as a forest plot on the right. For individual trials: filled in square, hazard ratio; lines, 95%CI; diamond plot, overall results of the included trials. PFS: Progression-free survival.

summary incidence of all-grade fluid retention and edema attributable to abiraterone was 6.1% (95%CI: 2.7%-13.2%). Post-chemotherapy was associated with significantly higher risk of all-grade fluid retention/edema attributable to abiraterone than pre-chemotherapy (9.0% vs 4.0%,  $P < 0.001$ ). Similarly, post-chemotherapy was associated with significantly higher risk of all-grade hypokalemia attributable to abiraterone than pre-chemotherapy (9.0% vs 4.0%,  $P < 0.001$ ). In contrast, pre-chemotherapy was associated with significantly higher risk of all-grade hypertension attributable to abiraterone than post-chemotherapy (3.0% vs 9.0%,  $P < 0.001$ ).

## DISCUSSION

Our meta-analysis has demonstrated that pre-chemotherapy may affect the efficacy and toxicity of abiraterone treatment in patients with metastatic CRPC. Abiraterone was associated with significantly increased radiographic-PFS, objective response rate, and PSA response rate in the pre-chemotherapy setting

when compared to the post-chemotherapy setting. However, its effects on OS ( $P = 0.92$ ) and PSA-PFS ( $P = 0.13$ ) were not significantly different between the two settings. In addition, abiraterone in the pre-chemotherapy setting had a significant lower risk of all-grade fluid retention and edema ( $P < 0.001$ ), and hypokalemia ( $P < 0.001$ ), but had a higher risk of all-grade hypertension ( $P < 0.001$ ) when compared to post-chemotherapy.

In the setting of metastatic CRPC, the optimal sequence of abiraterone in relation to chemotherapy to achieve the greatest survival benefit is a topic of emerging interest. In a previous meta-analysis of abiraterone for treatment of metastatic CRPC, abiraterone compared to placebo significantly prolonged OS and radiographic-PFS<sup>[20]</sup>. These results support the efficacy of abiraterone in metastatic CRPC: this however did not ascertain the differences in the pre-chemotherapy and post-chemotherapy settings. This highlights the significance of our results, which provides verification based on well-designed RCTs of the clinical benefits of administering abiraterone early

in the metastatic CRPC disease course. Even though, we did not appreciate a significant difference in OS, differences in validated clinical efficacy outcomes were seen. The PCWG2 criteria (used in both COU-AA-301 and COU-AA-302) for disease progression is highly reproducible and associated with OS<sup>[18,21]</sup>. Radiographic progression is defined as the presence of two or more new lesions on the bone scan, where the first post-treatment bone scan is not used to make treatment decisions to prevent treatment discontinuation due to disease flare as opposed to a true progression<sup>[18,21]</sup>. Rate of objective response and PSA response rate were also improved in the pre-chemotherapy setting. Definitions for both rate of objective response and PSA response rate were similar in both trials, thus our results are likely meaningful<sup>[14,15]</sup>. An increase in OS that did not reach statistical significance in our analysis could be explained by the early un-blinding of the COU-AA-302 trial at the time the pre-specified 43% total number of events occurred<sup>[15]</sup>. A sufficient duration for evaluation of OS likely requires a longer follow-up, highlighted by a lack of survival differences seen in the COU-AA-302 until follow-up of 12 mo<sup>[15]</sup>.

The ease of oral administration, once a day dosing, and favorable safety profile of abiraterone make it an attractive therapy in metastatic CRPC<sup>[3]</sup>. In the COU-AA-301 trial, abiraterone administered with low-dose prednisone was shown to increase radiographic-PFS with a trend toward improved OS in the post-docetaxel chemotherapy group<sup>[14]</sup>. In the COU-AA-302 trial, abiraterone administered with prednisone in the metastatic CRPC pre-chemotherapy setting significantly improved OS, and prolonged radiographic-PFS, time to PSA progression, and rate of objective response<sup>[15]</sup>. Despite favorable results, assurances on when and how to utilize abiraterone in clinical practice is imperfect. Patients with ECOG scores of  $\geq 2$  comprised only 10% of the COU-AA-301 study population, and were entirely excluded from the COU-AA-302 trial<sup>[14,15]</sup>. In a retrospective analysis, poor performance status (ECOG  $\geq 2$ ) was shown to be associated with poor survival outcomes in both pre-chemotherapy and post-docetaxel patients treated with abiraterone<sup>[12]</sup>. This highlights the benefit of early initiation of abiraterone prior to deterioration of performance status after administration of taxane chemotherapy.

Cross-resistance, where sensitivity to one compound is impaired by another compound with a similar mechanism of action is a concern with taxanes and abiraterone due to common activity on AR nuclear transport<sup>[22]</sup>. Docetaxel, a taxane, acts principally through microtubule stabilization but has a unique mechanism of action in metastatic CRPC; AR translocation inhibition in response to androgens and ligand-independent pathways, and down-expression of AR<sup>[6,10]</sup>. Pre-clinical data has suggested that the efficacy of docetaxel to perform such actions may be diminished by prior abiraterone androgen targeted therapy<sup>[10,23]</sup>. However, a retrospective analysis of a phase III RCT

did not show prior androgen synthesis inhibition with ketoconazole to negatively impact clinical outcomes with subsequent docetaxel chemotherapy<sup>[24]</sup>. Unfortunately, because the COU-AA-302 trial underwent un-blinding prior to reaching a significant difference in OS, concerns for cross-resistance have not fully been put to rest<sup>[14]</sup>. However, the significant impact of pre-chemotherapy on the efficacy of abiraterone as shown in this study supported the notion of cross-resistance between docetaxel and abiraterone.

Compared to chemotherapy, abiraterone is less toxic with a relatively low incidence of high-grade adverse effects, and can be tolerated for an extended period in metastatic CRPC patients. The importance of tolerability is especially important for the metastatic CRPC patient population given their advanced age and multiple co-morbidities. In these trials abiraterone was well tolerated with the majority of adverse events being low-grade<sup>[14,15]</sup>. Abiraterone discontinuation rates in the COU-301 and COU-302 trials were 19% and 10% respectively<sup>[14,15]</sup>.

Abiraterone inhibition of CYP-17 leads to an inhibition of both androgen and glucocorticoid synthesis, where the latter causes an elevation of adrenocorticotrophic hormone (ACTH) and excess mineralocorticoid activity<sup>[9]</sup>. Increased mineralocorticoid activity is the mechanism for many of abiraterone's adverse effects including hypokalemia, fluid retention and edema, and hypertension. Caution is advised in patients with renal failure, metabolic disturbances, and congestive heart failure<sup>[9]</sup>.

To minimize the incidence of these adverse effects, abiraterone is concomitantly administered with prednisone<sup>[9]</sup>. Early clinical data has shown that abiraterone can be administered safely without prednisone, and mineralocorticoid adverse effects can be managed with eplerenone, a non-steroidal mineralocorticoid antagonist<sup>[11]</sup>. Interestingly, our analysis has shown significantly lower incidence of fluid retention and edema and hypokalemia and a higher incidence of hypertension in the pre-chemotherapy setting compared to the post-chemotherapy setting. This is also consistent with lower discontinuation rate of abiraterone in the pre-chemotherapy setting versus post-chemotherapy (10% vs 19%).

To our knowledge, this study is the first to comment on differences in abiraterone toxicity in relation to sequencing with chemotherapy. Both docetaxel and abiraterone can cause fluid retention edema, where post-chemotherapy abiraterone treatment may lead to an additive risk for fluid retention and edema. Differences in the risk of hypokalemia and hypertension with abiraterone in relation to docetaxel that does share the same mineralocorticoid related toxicity profile requires elucidation. It is possible that chemotherapy or cancer progression may decrease food intake leading to increased risk of hypokalemia and reduced risk of hypertension.

In metastatic CRPC, the specific choice of agent and

its timing should be made on individual personalized assessment, and should incorporate multiple factors including clinical and symptomatic disease burden, tolerability, side-effect profile, performance status, and physician experience<sup>[9,25,26]</sup>. For example, patients who are given abiraterone prior to docetaxel may not be able to tolerate subsequent chemotherapy<sup>[10]</sup>. This prevents patients from receiving chemotherapy that has been proven to have a survival benefit. Abiraterone should be considered as a first-line choice in patients who cannot tolerate chemotherapy or have asymptomatic disease<sup>[25]</sup>. Opting to use docetaxel chemotherapy prior to abiraterone would be beneficial for patients who have rapid progressive disease such as with visceral metastasis, high Gleason score, rapid PSA doubling time, or with an initial poor response to androgen deprivation therapy<sup>[25,27]</sup>.

This meta-analysis has several limitations. Our findings may be limited by sample size and the accuracy of individual RCTs to assess primary and secondary outcomes that may affect our ability to discern differences in efficacy of abiraterone in the pre-chemotherapy and post-chemotherapy settings. Results regarding differences in toxicity for these two settings may also have been affected by the ability of individual RCTs to correctly grade the severity of adverse events based on NCI-CTCAE version 3.0. Sub-optimal classification could have erroneously estimated the incidence of all-grade and high-grade adverse events in this meta-analysis. Selection bias based on the exclusion of patients with poor baseline ECOG performance status as mentioned earlier may have limited the generalizability and clinical application of our results to the general population. Also mentioned previously, our results may have been affected by the early un-blinding of the COU-AA-301 trial.

In conclusion, our meta-analysis of RCTs has demonstrated that abiraterone is associated with a significantly improved radiographic-PFS, objective response rate, PSA response rate in the pre-chemotherapy setting when compared to the post-chemotherapy setting in patients with metastatic CRPC. This emphasizes that these patients may obtain the greatest clinical benefit with early treatment of androgen synthesis inhibition with abiraterone. Further studies may be necessary to determine the effectiveness of abiraterone in the pre-chemotherapy setting in comparison with other new agents including the androgen signaling pathway inhibitor enzalutamide or vaccines.

## COMMENTS

### Background

Chemotherapy with docetaxel plays an important role in the treatment of metastatic castration-resistant prostate cancer (CRPC). Abiraterone, an androgen biosynthesis inhibitor, has been shown to improve overall survival in metastatic CRPC patients who have not received previous chemotherapy. It is not clear that prior chemotherapy affects the effectiveness of abiraterone. The authors performed a meta-analysis to compare the efficacy and safety of

abiraterone in patients with and without prior chemotherapy.

### Research frontiers

In the setting of metastatic CRPC, the optimal sequence of abiraterone in relation to chemotherapy to achieve the greatest survival benefit is a topic of emerging interest. In a previous meta-analysis of abiraterone for treatment of metastatic CRPC, abiraterone compared to placebo significantly prolonged OS and radiographic-PFS. These results support the efficacy of abiraterone in metastatic CRPC.

### Innovations and breakthroughs

This meta-analysis has demonstrated that pre-chemotherapy may affect the efficacy and toxicity of abiraterone treatment in patients with metastatic CRPC. Abiraterone was associated with significantly increased radiographic-PFS, objective response rate, and prostate-specific antigen (PSA) response rate in the pre-chemotherapy setting when compared to the post-chemotherapy setting.

### Applications

Abiraterone should be considered as a first-line choice in patients who cannot tolerate chemotherapy or have asymptomatic disease. However studies with larger sample size and are required to compare abiraterone in pre and post chemotherapy setting and to compare the efficacy with other new androgen signaling blocking agents.

### Terminology

Radiographic-PFS was defined as progression on bone scanning based on the Prostate Cancer Working Group 2 (PCWG2) criteria or progressive soft-tissue lesions measured with the use of computed tomography or magnetic resonance imaging according to the modified Response Evaluation Criteria in Solid Tumors PSA progression is defined as a 25% or greater increase and an absolute increase of 2 ng/mL or more from the nadir is documented and confirmed by a second value obtained 3 or more weeks later, also based on the PCWG2 criteria.

### Peer-review

The study was clinically relevant and addressed the important topic of abiraterone sequencing in metastatic castrate resistant prostate cancer. The article provides a succinct yet relevant discussion about the impact of prior chemotherapy in the treatment of this clinical entity.

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## Second-line treatments for advanced gastric cancer: Interpreting outcomes by network meta-analysis

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**Data sharing statement:** Technical appendix, statistical code, and dataset available from the corresponding author at [andrea.messori.it@gmail.com](mailto:andrea.messori.it@gmail.com)

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treatments for advanced gastric cancer by application of Bayesian network meta-analysis.

**METHODS:** Our search covered the literature up to February 2015. The following 6 treatments were evaluated: (1) irinotecan (camptothecins); (2) paclitaxel (taxanes class); (3) docetaxel (taxanes); (4) everolimus (mammalian target of rapamycin inhibitors); (5) ramucirumab (vascular endothelial growth factor receptor 2 inhibitors); (6) ramucirumab + paclitaxel. Our methodology was based on standard models of Bayesian network meta-analysis. The reference treatment was best supportive care (BSC). The end-point was overall survival. Median survival was the outcome measure along with 95% credible intervals.

**RESULTS:** Our search identified a total of 7 randomized controlled trials. These trials included 2298 patients (in 15 treatment arms) in whom a total of 6 active treatments were evaluated as well as BSC. There were 21 head-to-head comparisons (6 direct, 15 indirect). The difference in survival between each of two active treatments (paclitaxel and ramucirumab + paclitaxel) vs BSC was statistically significant, while the other 4 showed no statistical difference. In the 6 head-to-head comparisons between active treatments, no significant survival difference was demonstrated.

**CONCLUSION:** Our results indicate that both paclitaxel monotherapy and ramucirumab + paclitaxel determine a significant prolongation in survival as compared with BSC.

**Key words:** Meta-analysis; Bayesian methods; Advanced gastric cancer; Second line therapy; Paclitaxel; Irinotecan; Docetaxel; Ramucirumab

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### Abstract

**AIM:** To study the effectiveness of second-line

**Core tip:** We carried out a Bayesian network meta-

analysis to evaluate second-line treatments for advanced gastric cancer. After scanning the literature up to February 2015, 7 randomized controlled trials were included in our meta-analysis in which the treatments for this disease condition and best supportive care (BSC) were evaluated according to overall survival (OS). Our meta-analysis investigated 21 direct or indirect comparisons. The difference in OS between paclitaxel *vs* BSC and ramucirumab + paclitaxel *vs* BSC was statistically significant, while the other comparisons showed no statistical difference. In conclusion, our results indicate that both paclitaxel and ramucirumab + paclitaxel determine a significant prolongation in survival in comparison with BSC.

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## INTRODUCTION

Gastric cancer is one of the most common malignancies and the third leading cause of cancer mortality worldwide<sup>[1-3]</sup>. This disease condition represents 3.4% of all cancers in both sexes, and ranks sixth among all cancers in incidence and fifth as mortality. The incidence varies with age and reaches its peak in the seventh decade of life.

The standard first-line chemotherapy for advanced gastric cancer (AGC) is the association of fluoropyrimidine and platinum complexes with or without anthracyclines<sup>[1-5]</sup>. However, more than half of patients with AGC do not respond to chemotherapy and even if patients show a response, its duration is only a few months. For this reason, a second-line therapy is needed in most patients.

While several pharmacological options have been proposed as second-line treatment [*e.g.*, taxanes, camptothecins, selective mammalian target of rapamycin (mTOR) inhibitors, and more recently the R2 (VEGF-R2) antagonists of endothelial growth factor VEGF such as ramucirumab], there is currently no standard of care.

In the present study, we performed an updated meta-analysis of second-line treatments for AGC including the data from the most recent randomized controlled trials (RCTs).

## MATERIALS AND METHODS

Our literature search was conducted in PubMed ([www.pubmed.org](http://www.pubmed.org)) and in Scopus ([www.scopus.com](http://www.scopus.com)) and covered the period from 1 January 1990 to present time (last query on 28 February 2015). A single search

term ("advanced gastric cancer") was employed (in combination with the filter "randomized controlled trials"). Since the number of citations retrieved through these keywords was small (less than 400 with PubMed), we analyzed all of these articles by examining the abstract or, when necessary, their full text, and we identified the RCTs that met our inclusion criteria. These criteria included: (1) metastatic or non-resectable, locally advanced gastric or gastro-esophageal junction adenocarcinoma; (2) age from 18 to 75 years; (3) adequate organ function (bone marrow function, liver function, kidney function); (4) Eastern Cooperative Oncology Group performance status (PS) of 0, 1 or 2; and (5) first-line chemotherapy with fluoropyrimidine plus platinum with or without anthracycline. The end-point of our analysis was overall survival (OS), which was handled as a continuous endpoint.

For each trial, we extracted the basic information needed for our analysis and the information on the primary end-point, *i.e.*, OS. Data on OS (median value of OS with lower and upper extremes of the 95%CI) were meant to reflect the intention-to-treat population; however, there were some occasional post-randomization exclusions in some trials, and so our clinical material in some cases reflected the so-called modified intention-to-treat population<sup>[6]</sup>. As regards the assessment of methodological quality, two reviewers (BB and DM) applied the Cochrane Collaboration's tool<sup>[7]</sup> to evaluate the risk of bias in the studies included in our analysis. This tool assesses six domains (namely: random sequence generation, concealment of allocation, blinding of participants and personnel, incomplete data, selective outcome reporting of outcomes, and other sources of bias). Studies with adequate procedures in all domains were considered to have a low risk of bias.

For our statistical analysis, we employed a Bayesian model of network meta-analysis<sup>[8]</sup>. This approach is advantageous because all treatments under comparison are incorporated into a single model; another advantage is that the Bayesian technique enables rank ordering of each treatment. This Bayesian model is available as fixed-effect model and random-effect model. For the purposes of our analysis, these two versions of the model (*i.e.*, fixed-effect and random-effect) were run separately using the same data set of primary data (median and 95%CI of OS). Thereafter, the Deviance Information Criterion (DIC) was used to choose the model that yielded the better performance. Only the results generated by the better model were presented, while those generated by the worse model were not reported.

In running our analysis, the following second-line chemotherapy treatments were evaluated: (1) irinotecan (class of camptothecin); (2) paclitaxel (class of taxanes); (3) docetaxel (class of taxanes); (4) everolimus (m-TOR inhibitor); (5) ramucirumab (VEGF-R2 inhibitor); and (6) ramucirumab + paclitaxel. Firstly, we analyzed the data of included trials to determine if the OS for each active

**Table 1** Values of overall survival reported in the 7 randomized controlled trials

Ref.	Year of publication	Patients		Control arm				Experimental arm				P value
		Age <sup>1</sup> (yr)	Race	Treatment	N	Median OS (mo)	SE <sup>2</sup> (mo)	Treatment	N	Median OS (mo)	SE <sup>2</sup> (mo)	
<sup>3</sup> Kang <i>et al</i> <sup>[4]</sup>	2012	56	Korean	BSC	69	3.8	0.36	Docetaxel	66	5.2	0.71	0.07
Hironaka <i>et al</i> <sup>[3]</sup>	2011	65	Japanese	Irinotecan	111	8.4	0.56	Paclitaxel	108	9.4	0.59	0.22
Thuss-Patience <i>et al</i> <sup>[10]</sup>	2011	56	-	BSC	19	2.4	0.82	Irinotecan	21	4	0.99	0.22
Ford <i>et al</i> <sup>[1]</sup>	2014	65 (34-84)	English	BSC	84	3.6	0.28	Docetaxel	84	5.2	0.46	0.003
Ohtsu <i>et al</i> <sup>[9]</sup>	2013	62 (22-86)	Various (white, Asian, black or other)	BSC	217	4.3	0.43	Everolimus	439	5.4	0.31	0.039
Fuchs <i>et al</i> <sup>[2]</sup>	2014	60 (51-69)	Various (white, Asian, black or other)	BSC	117	3.8	1.38	Ramucirumab	238	5.2	1.94	0.65
Wilke <i>et al</i> <sup>[11]</sup>	2014	61 (24-83)	Various (white, Asian, black or other)	Paclitaxel	335	7.4	0.54	Ramucirumab + paclitaxel	330	9.6	0.59	0.006

<sup>1</sup>Mean or median age with range in parenthesis; <sup>2</sup>Calculated from confidence intervals according to the procedure described by Altman *et al*<sup>[13]</sup>; <sup>3</sup>The trial by Kang *et al*<sup>[4]</sup> included a third arm treated with irinotecan (N = 60) in which median OS was 7.9 mo (SE = 1.02 mo). OS: Overall survival; SE: Standard error; N: Number of patients; BSC: Best supportive care.

treatment was significantly different from that of best supportive care (BSC). Next, we estimated the statistics for all pairwise comparisons (6 direct comparisons and 15 indirect comparisons) by determining the difference in OS [with 95% credible interval (CrI)]. The rank order was calculated for each treatment according to the endpoint of OS. In summary, the main output of our analysis consisted of the meta-analytic survival difference with CrIs along with ranking statistics.

Finally, to evaluate the reproducibility of our results, we changed the initial parameter estimates from which the Markov chain Monte Carlo simulation begins according to a verification that is customary employed in these Bayesian analyses. All of our analyses were conducted by using the software package WinBUGS 1.4.3 (Cambridge, United Kingdom) and by running the meta-analysis code for continuous end-points made available by the NICE Support Unit (United Kingdom)<sup>[8]</sup>. The statistical methods of our study were reviewed by AM according to his role of Lecturer in Medical Statistics at the Faculty of Pharmacy of the University of Firenze, Italy.

## RESULTS

Our literature search is summarized in Figure 1 according to the PRISMA schematic. After the initial selection of 355 articles in PubMed and 612 in Scopus, we examined the full text of 12 articles and we finally identified 7 studies that met our inclusion criteria<sup>[1-4,9-11]</sup>. The treatments evaluated in these 7 studies are shown in Table 1 along with the information on OS extracted from their respective results. In 6 out of these 7 cases, the RCTs compared a second-line treatment with BSC. Overall, these 7 RCTs enrolled 2298 patients (in 15

treatment arms). As regards the methodological quality, the 7 RCTs showed a low risk of bias. As illustrated in Figure 2, the only source of potential risk of bias was the open-label design of three randomized studies, but all the other items of the scoring method were at low risk of bias.

In running our Bayesian analysis, the value of DIC was found to be more favourable for the fixed-effect model. For this reason, only the results generated by this model are presented below.

Our results (Figure 3) revealed a statistically significant difference in the direct comparisons between two second-line active treatments vs BSC (namely, paclitaxel monotherapy and ramucirumab + paclitaxel). Furthermore, 4 indirect head-to-head comparisons reached the threshold of statistical significance (namely, the comparisons of ramucirumab + paclitaxel with irinotecan or docetaxel or paclitaxel or everolimus).

Figure 4 illustrates the ranking histograms generated by the Bayesian probabilistic analysis. Individual rankings for the 6 second-line treatments and BSC were the following (lowest rank = highest effectiveness, highest rank = lowest effectiveness; 95%CrI in parenthesis): ramucirumab+paclitaxel, 1 (1 to 2); paclitaxel, 2 (2 to 5); irinotecan, 3 (2 to 6); docetaxel, 4 (3 to 7); everolimus, 4 (3 to 7); ramucirumab, 6 (1 to 7); BSC, 7 (5 to 7).

Finally, our sensitivity analysis showed that using different initial parameter estimates did not affect the results.

## DISCUSSION

The results of our Bayesian meta-analysis provided a summary of the effectiveness data concerning the main

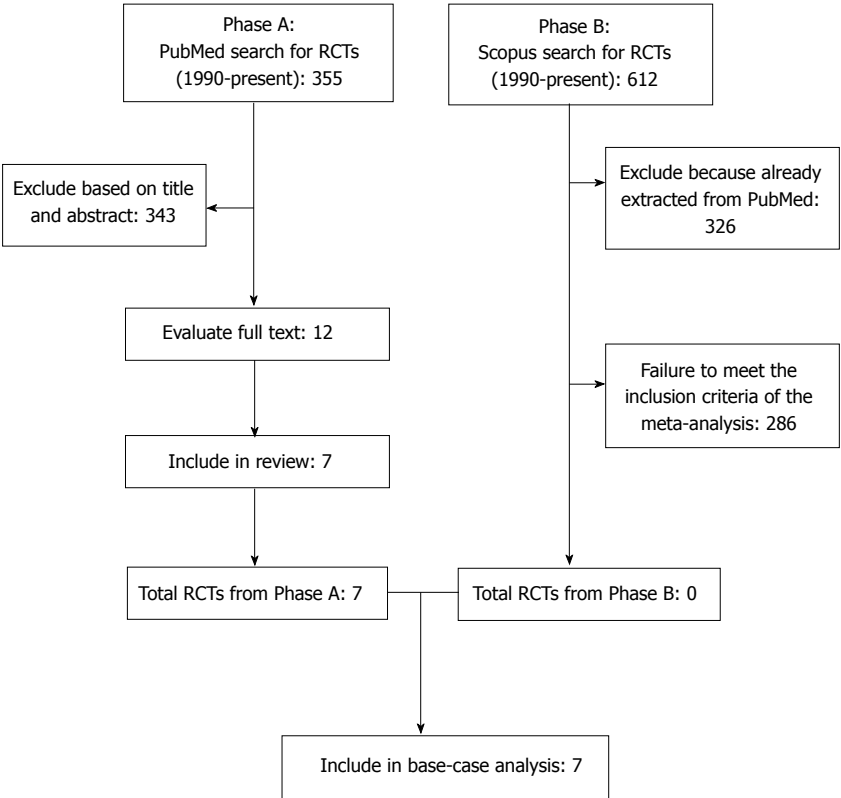


Figure 1 Phases of our literature search illustrated according to the PRISMA schematic. RCTs: Randomized controlled trials.

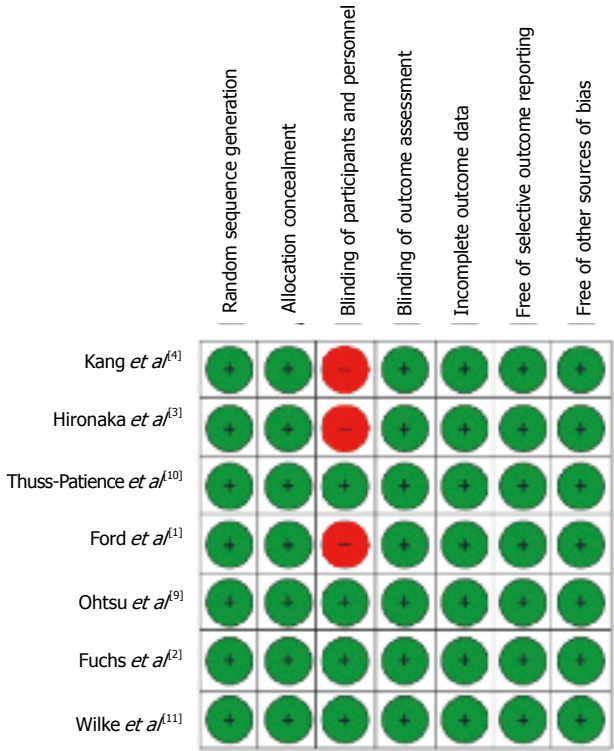


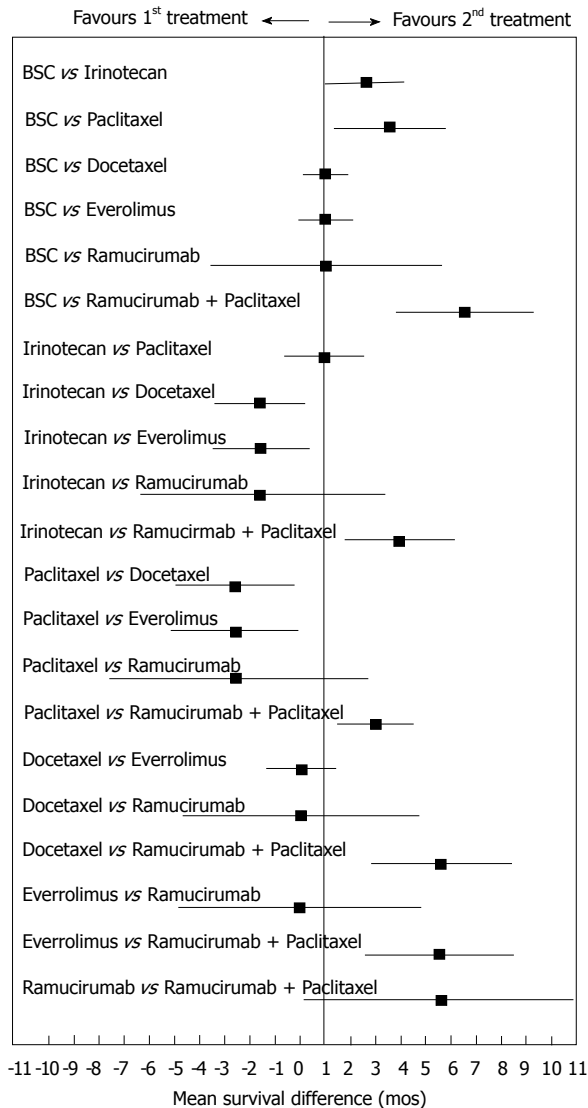
Figure 2 Application of the Cochrane Collaboration's tool for assessing risk of bias in randomised trials. The figure shows the summary of risk-of-bias assessments for the 7 randomized, controlled trials included in our analysis. Low risk of bias is represented by green circles (see Higgins *et al.*<sup>[7]</sup> for further details).

second-line treatments for AGC and were successful in evaluating the statistical significance of differences between active treatments and in defining the ranking in effectiveness for each treatment.

Overall, our results are of interest under several viewpoints. The information on rankings is, in our view, the most interesting result of our analysis. Among the 6 active treatments, ramucirumab + paclitaxel and paclitaxel monotherapy had the two best rankings, while ramucirumab monotherapy had a quite variable ranking.

As shown in Figure 3, our choice of employing an absolute outcome measure (*i.e.*, OS) was advantageous in comparison with the approaches based on relative outcome measures (*e.g.*, relative risk, odds-ratio or hazard ratio) that are commonly employed in meta-analysis<sup>[12]</sup>. In fact, absolute outcome measures allow us to better interpret the clinical relevance of the differences; for example, the differences shown in Figure 3 that proved to be statistically significant were mostly around 2 or 3 mo, but those involving ramucirumab in association were remarkably around 6 mo.

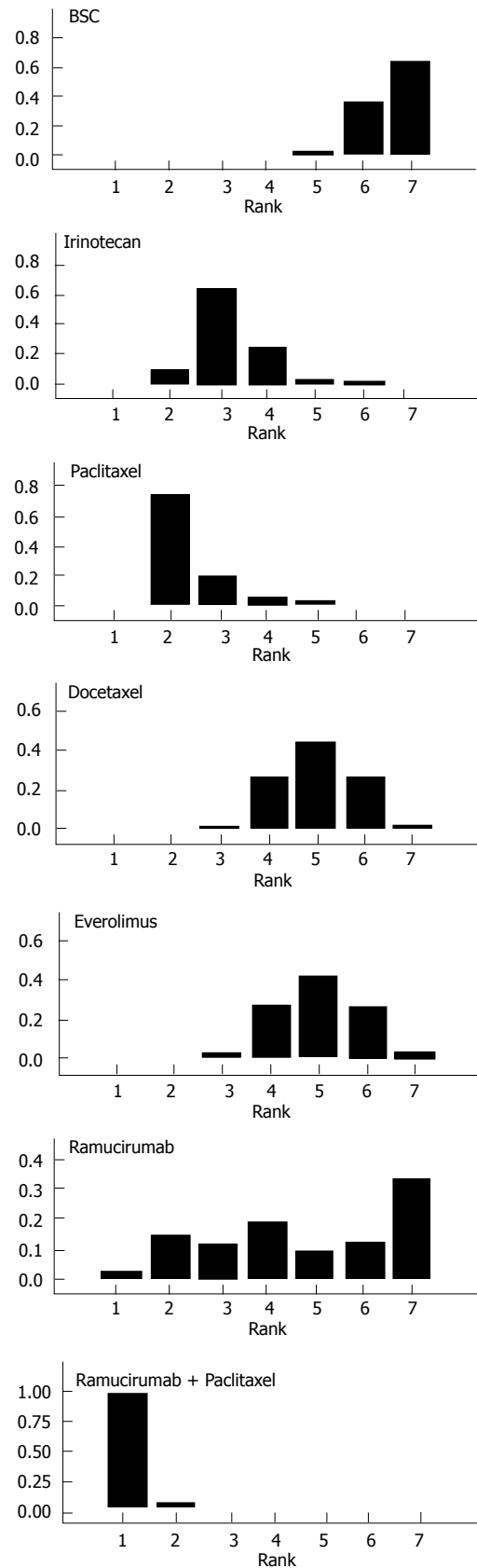
As confirmed by the present analysis, the Bayesian approach for evaluating direct and indirect comparisons according to a network of treatments shows a number of important advantages, mainly because a single programming language (*i.e.*, Winbugs) has been adopted worldwide for conducting this type of



**Figure 3** Meta-analytical values of mean survival difference estimated for 6 direct comparisons (each of the 6 active treatments vs best supportive care) and for 15 head-to-head indirect comparisons between the active treatments. Each horizontal bar indicates the two-sided 95%CrI for the mean survival difference (solid square). BSC: Best supportive care.

research. This translates into a very high degree of standardization in doing these analyses. For example, if one examines a random sample of Bayesian meta-analyses published over the past months<sup>[14-25]</sup>, it is impressive to see the extraordinary homogeneity of the models adopted by different researchers and also the important scientific impact that this type of research determines as demonstrated by the authoritativeness of the journals where these studies have been published.

The points of strength of our study included the originality of the methodological approach because this is the first "all-in-one" Bayesian network meta-analysis carried out on this topic. Another advantage is that we evaluated the main second-line active treatments currently available for advanced gastric cancer, without focusing the analysis on a single agent (like in other published papers<sup>[26]</sup>).



**Figure 4** Histogram of rankings generated by the Bayesian network meta-analysis. The graphs reflect a total of 20000 iterations and consist of as many histograms as the treatments ( $N = 6$  plus best supportive care) included in the analysis. In each panel, the histogram shows the percent distribution of the simulations across ranks 1 (most effective treatment) through 7 (least effective treatment); the y-axis shows probability on a 0 to 1 scale. BSC: Best supportive care.



In conclusion, our results convey an original information to establish the place in therapy of these 6 pharmacological second-line treatments for AGC.

## COMMENTS

### Background

In patients with advanced gastric cancer requiring second-line treatment, no meta-analysis for indirect comparisons between active treatments has been conducted. All data on effectiveness essentially refer to the comparison between an active treatment and best supportive care. In contrast, data on comparative effectiveness are needed to clarify which treatment is more effective in this disease condition.

### Research frontiers

Bayesian network meta-analysis is increasingly recognized to be the new standard for analyzing the effectiveness data from a series of randomized trials and for generate a ranking in effectiveness across the active treatments available.

### Innovations and breakthroughs

The present study is the first meta-analysis in which a Bayesian network model has been used to synthesize the data of effectiveness and to generate the ranking histograms that are a typical output of this type of statistics.

### Applications

After a standard literature search, the above Bayesian methodology was applied to a series of 7 randomized trials, that evaluated 5 active treatments in this disease condition. Two of these trials were focused on ramucirumab, a new agent proposed for this clinical indication. Ramucirumab in association with paclitaxel rank first in comparative effectiveness across the 5 active treatment.

### Terminology

While standard pair-wise meta-analysis examines a single comparison, generally between a single active agent and a single reference treatment (or no treatment), network meta-analysis evaluates all head-to-head combinations across the therapeutic options evaluated in included trials. Network meta-analysis based on Bayesian methods has a further important advantage in that a single statistical analysis (*i.e.*, an “all-in-one” statistical model) allows people to simultaneously evaluate the effectiveness of several treatment options.

### Peer-review

In this network meta-analysis study, the author investigated 21 direct or indirect comparisons of overall survival of total of 2298 advanced gastric cancer patients. The result shows that there are statistically significant differences in overall survival between paclitaxel vs best supportive care (BSC) and ramucirumab + paclitaxel vs BSC groups, indicating that both paclitaxel and ramucirumab + paclitaxel determine a significant prolongation in survival in comparison with BSC. This has significance for the second-line drugs treatment of gastric cancer. The paper is about an interesting topic.

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*WJCO* covers a variety of clinical medical topics, including etiology, epidemiology, evidence-based medicine, informatics, diagnostic imaging, endoscopy, tumor recurrence and metastasis, tumor stem cells, radiotherapy, chemotherapy, interventional radiology, palliative therapy, clinical chemotherapy, biological therapy, minimally invasive therapy, physiotherapy, psycho-oncology, comprehensive therapy, and oncology-related nursing. Priority publication will be given to articles concerning diagnosis and treatment of oncology diseases. The following aspects are covered: Clinical diagnosis, laboratory diagnosis, differential diagnosis, imaging tests, pathological diagnosis, molecular biological diagnosis, immunological diagnosis, genetic diagnosis, functional diagnostics, and physical diagnosis; and comprehensive therapy, drug therapy, surgical therapy, interventional treatment, minimally invasive therapy, and robot-assisted therapy.

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## 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

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**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<sup>[1]</sup>. In fact, nearly 30% of breast cancers will relapse years or decades after the initial treatment<sup>[2-4]</sup>. 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<sup>[2,5]</sup>. 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<sup>[6]</sup>. 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<sup>[7-15]</sup>. 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)<sup>[16]</sup>. 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<sup>[17]</sup>. 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

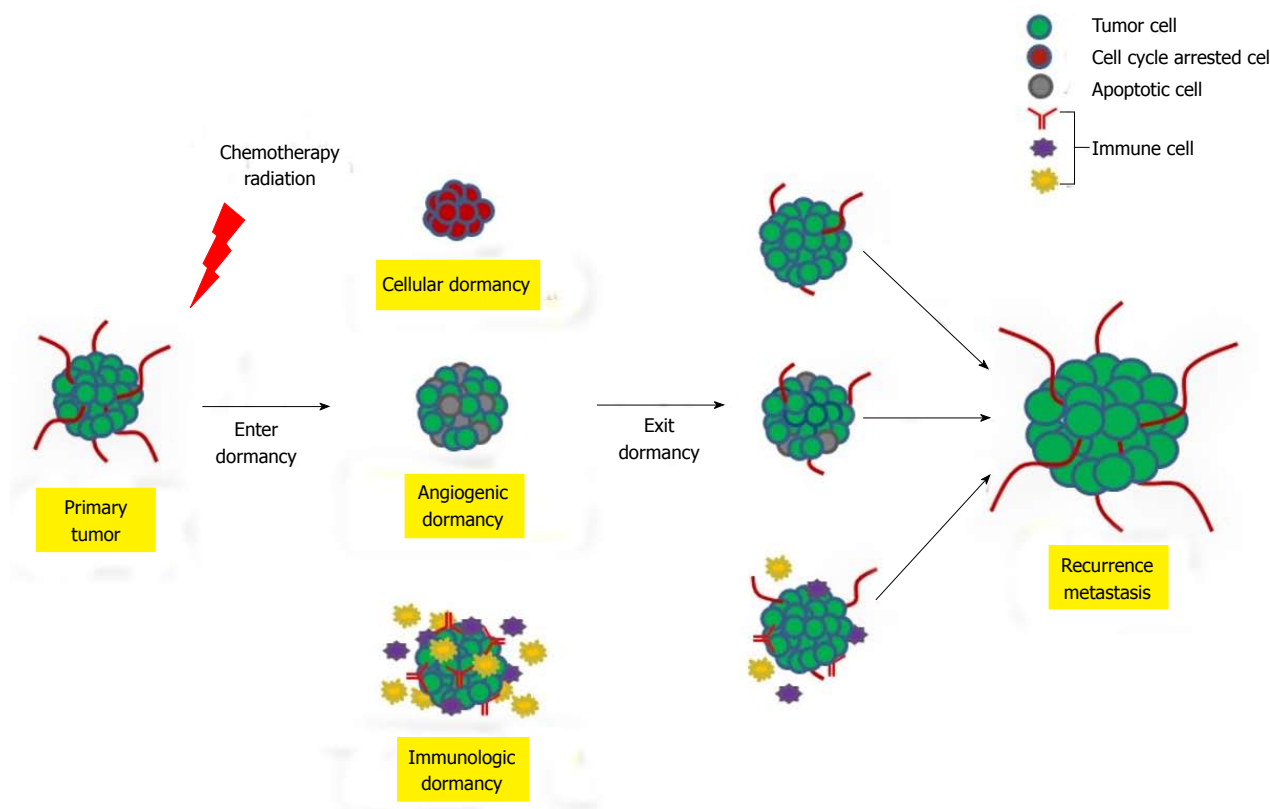
to escape treatment subsequently leading to disease recurrence<sup>[16,18-20]</sup>. Once dormant cancer cells exit G0 arrest, a second mechanism termed angiogenic dormancy can limit the tumor size by preventing angiogenesis and therefore the tumor cannot obtain the nutrients required for continual growth. These cells can maintain a balance between proliferation and apoptosis resulting in the inability to detect the tumor<sup>[6,16]</sup> (Figure 1). The immune system can also contribute to cancer cell dormancy by maintaining a balance between clearance and proliferation<sup>[16]</sup> (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<sup>[21]</sup>.

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<sup>[15,22]</sup>. 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<sup>[16,18-20]</sup> (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<sup>[23-27]</sup>. In addition, positive Ki-67 expression was correlated with breast cancer recurrence and poor prognosis<sup>[28]</sup>. 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<sup>[29]</sup>. Recently, Fitzgerald *et al*<sup>[30]</sup> (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<sup>[16]</sup> (2010) and Wang and Lin<sup>[6]</sup> (2013).

multivulval class B (MuvB) proteins, is a critical regulator of cell cycle arrest<sup>[31]</sup>. 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<sup>[32]</sup>. During dormancy, MuvB binds to all of the components of the DREAM complex and represses the transcription of all cell cycle-dependent genes<sup>[32-34]</sup>. 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<sup>[35,36]</sup>. 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 MuvB with the other core components of the DREAM complex<sup>[31]</sup>. 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<sup>[37,38]</sup>. 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<sup>[39]</sup>. 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)<sup>[40-42]</sup>. In addition, reduction in p27 (Kip1) is used as a strong prognostic marker for recurrence and poor outcomes in renal cell carcinoma patients<sup>[42]</sup>. Loss of p53, the upstream regulator of p21, was correlated with drug resistance and recurrence in colorectal cancer<sup>[43]</sup>. Overexpression of cyclin D is associated with recurrence of multiple neoplasms including breast, lymphomas, prostate, and non-small cell lung cancers<sup>[44-46]</sup>. 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<sup>[44]</sup>. Recently, Kim *et al.*<sup>[47]</sup> (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<sup>[48]</sup>. 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<sup>[49]</sup>. Blockage of the metastasis-associated u-PAR, integrins, focal adhesion kinase or epithelial growth factor receptor can result in tumor suppression and induction of tumor dormancy<sup>[49]</sup>. U-PAR can also regulate tumor dormancy by favoring p38 activation over ERK activation<sup>[50]</sup>. In addition, the activation of the PI3K/c-Myc pathway controls the level of thrombospondin (TSP), a vital factor of tumor dormancy<sup>[16]</sup>. Troyanovsky *et al.*<sup>[51]</sup> (2001) also discovered that the expression of angiostatin can control tumor dormancy by suppressing tumor growth, and one mediator of angiostatin, angiomin, was highly elevated in dormant cells.

The transition from avascular tumor to a highly vascularized tumor is termed the "angiogenic switch"<sup>[16,21]</sup>. Balancing the pro-angiogenic and anti-angiogenic factors is vital in regulating the angiogenic switch. Satchi-Fainaro *et al.*<sup>[52]</sup> (2012) discovered that dormant glioblastoma cells express high levels of anti-angiogenic factors including TSP, angiomin, 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<sup>[53,54]</sup>. Another key protein that plays a role in controlling the switch from dormancy to tumor growth is heat shock protein 27 (HSP27)<sup>[55]</sup>. 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<sup>[55]</sup>. Recently, the hypoxia inducible factor, HIF-2 $\alpha$ , was shown to promote angiogenesis in hepatocellular carcinoma<sup>[56]</sup>. HIF-2 $\alpha$  increased plasminogen activator inhibitor 1 which lowered active plasmin concentrations resulting in increased angiogenesis<sup>[56]</sup>.

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<sup>[57-59]</sup>. Overexpression of LTBP in nasopharyngeal carcinoma induced cancer cell dormancy and reduced VEGF expression thus inhibiting the migration and angiogenesis of tumor cells<sup>[57]</sup>. BMP7, a member of the TGF- $\beta$  superfamily, signaling facilitates the balance between dormant prostate cancer cells and metastasis<sup>[58]</sup>. Administration of BMP7 in mice significantly reduced tumor growth whereas inhibition of BMP7, *via* the secreted antagonist COCO, resulted in metastasis<sup>[58,59]</sup>. Leukemic dormancy occurs within bone marrow niches and is influenced by the expression of OPN<sup>[14]</sup>. Acute lymphoblastic 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<sup>[14]</sup>. This interaction drives leukemia blast into dormancy and this causes the cells to escape chemotherapy and/or radiation treatment<sup>[14]</sup>. In addition, antibody neutralization of OPN resulted in leukemia blast to exit dormancy and re-enter the cell cycle<sup>[14]</sup>. 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<sup>[60-62]</sup> and tumor metastasis (Figure 1). For example, DTC can reduce T-cell activation which weakens the cytotoxic T-lymphocyte response thus cells escape apoptosis<sup>[63]</sup>. 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<sup>[63]</sup>. 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<sup>[63]</sup>. Furthermore, loss of CD4<sup>+</sup> or CD8<sup>+</sup> T-cells can result in tumor cell dormancy escape<sup>[64]</sup>. 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<sup>[65,66]</sup>. 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<sup>+</sup> 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<sup>[67,68]</sup>. In addition, myeloid-derived suppressor cells, regulatory T-cells, and tumor-associated macrophages can also indirectly promote tumor cells to escape dormancy<sup>[63]</sup>. These cells can secrete mitogens and proangiogenic molecules which



promote cell proliferation, angiogenesis and immunosuppression causing the cells to exit dormancy<sup>[63]</sup>. 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 cancer 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 repaired 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<sup>[69]</sup>. Loss of heterozygosity of OGG1, a DNA glycosylase, is associated with papillary thyroid cancer<sup>[70]</sup>.

DSBs are considered to be the most toxic form of DNA lesions<sup>[71-73]</sup>. 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 joining (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<sup>[69]</sup>. 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<sup>[74]</sup>.

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<sup>[75]</sup>. 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<sup>[69]</sup>. DNA-PK complex acts as a molecular sensor for NHEJ repair<sup>[76,77]</sup>, and cells lacking DNA-PK function fail to show proper NHEJ<sup>[78-84]</sup>. 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<sup>[85,86]</sup>. 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<sup>[87]</sup>. 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<sup>[88]</sup>. 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<sup>[87]</sup>. Specifically, mutations in BRCA1, BRCA2, ATM, CHEK2, and RAD50 have been identified in several cancers including lung, ovarian, pancreatic, and leukemia<sup>[69]</sup>. Besides drugs that target RAD51, currently there are very little therapeutics that target other proteins involved in HR<sup>[87]</sup>. Alternatively, targeting the alternative NHEJ pathway *via* PARP1 inhibitors have been used to treat BRCA1 or BRCA2-defected cancers<sup>[69]</sup>.

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<sup>[89,90]</sup>. The dormant cells contain less DSBs after chemotherapy treatment, and Nishikawa *et al.*<sup>[15,22]</sup> (2012) demonstrated that these cells activated the NHEJ pathway to repair the DNA damage<sup>[15,22]</sup>. 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<sup>[91]</sup>. 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<sup>[92]</sup>.

## 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<sup>[93]</sup>. Recently, Kim *et al.*<sup>[94]</sup> (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<sup>[94]</sup>. 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, angiogenic 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<sup>[75]</sup>. 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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## Significant methodologic variations in calculating renal function changes following kidney tumor surgery: A quality reporting issue?

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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

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**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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### Abstract

Renal tumor surgery places patients at increased risk for chronic kidney disease (CKD). Accurate quantification

### 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	> 12 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*<sup>[1]</sup> 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, Cockcroft-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<sup>[2]</sup>. Furthermore, these factors can be affected by medications, hydration status, diet, certain disease states, and exercise<sup>[3]</sup>. 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<sup>[2]</sup>. 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 Cockcroft-Gault equation for those with a GFR less than approximately 90 mL/min per 1.73 m<sup>2</sup>. However, there are questions about its validity for persons without renal disease, persons > 70 years old, and patients with serious comorbid conditions<sup>[1,4]</sup>. 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<sup>2</sup>, thus reducing that rate of false-positive diagnoses of stage 3 CKD<sup>[5]</sup>. This was further highlighted by a study by Clark *et al*<sup>[6]</sup>, 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<sup>[6]</sup>.

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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## 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

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**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<sup>[1]</sup>, 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<sup>[2]</sup>, DNA repair<sup>[3]</sup> or microRNAs<sup>[4]</sup> 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<sup>[5]</sup>. 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<sup>[6,7]</sup>. 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<sup>[8]</sup>. 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<sup>[9]</sup>, there are many in-depth reports of pathogenic splice variants in diseases ranging from neuromuscular disorders<sup>[10]</sup> to diabetes<sup>[11]</sup> or cardiomyopathies<sup>[12]</sup>.

## 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<sup>[9,13,14]</sup>. 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<sup>[15]</sup>. 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<sup>[16]</sup>. 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<sup>[17]</sup>. 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<sup>[18,19]</sup>.

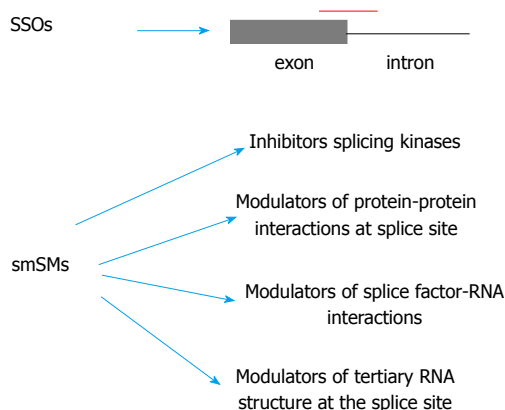
The main question that arises - especially having a therapeutic purpose in mind - are these modifications simply by-products of the oncogenic process or do they drive pathogenesis of cancer? While inevitably some splice variants are "associated noise" similar to physiology, there is compelling evidence 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<sup>[20]</sup>.

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<sub>165b</sub>, this is lost in cancers, with expression of predominantly pro-angiogenic VEGF<sub>165a</sub>, which maintains a state of high and chaotic neovascularization in tumours<sup>[21]</sup>. 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<sup>[22]</sup> 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<sup>[23]</sup>.





**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<sup>[24]</sup>.

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 potentially affect splicing of several genes involved in apoptosis and further-on to be able to decrease tumour growth in animal models<sup>[25]</sup>. 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<sup>[26]</sup> as well as melanoma xenografts growth<sup>[27]</sup> and orthotopic prostate cancer mouse models<sup>[28]</sup>.

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<sup>[10]</sup>. 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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## New findings on thymic epithelial tumors: Something is changing

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### Abstract

Thymic epithelial tumors (TETs) are uncommon neo-

plasms 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 currently 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

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**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<sup>[1,2]</sup>.

## 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<sup>[3-5]</sup>.

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*<sup>[6]</sup> 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*<sup>[7]</sup> 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*<sup>[8]</sup> 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*<sup>[9]</sup> 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<sup>[10,11]</sup>.

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

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### Abstract

Three-weekly docetaxel plus prednisone is the stan-

dard 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

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**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)<sup>[1,2]</sup>. In TAX 327 trial, which compared 3-weekly docetaxel plus prednisone vs mitoxantrone plus prednisone, 45% of patients receiving docetaxel and prednisone achieved  $\geq 50\%$  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 than 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,  $P = 0.004$ )<sup>[3]</sup>.

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<sup>[4-6]</sup>. 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<sup>[4-7]</sup>.

## 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<sup>[8-12]</sup>.

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

Cabazitaxel, a second-generation taxane, achieved a statistically significant improvement in OS when added to prednisone vs mitoxantrone plus prednisone in mCRPC patients<sup>[10]</sup>. Sipuleucel-T, an active cellular immunotherapy, prolonged OS among asymptomatic mCRPC patients<sup>[11]</sup>, and Radium-223, which has high

bone affinity, improved OS and time to first skeletal-related event<sup>[12]</sup>. Despite the availability of these new agents in mCRPC patients, their optimal sequencing remains unclear<sup>[15]</sup>.

The possibility of a docetaxel rechallenge has been largely limited by the introduction of abiraterone, enzalutamide and cabazitaxel in the treatment of CRPC patients. Nevertheless, 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<sup>[16]</sup>. Furthermore, the situation is actually complicated by recent trials which might lead to early prescription of docetaxel in combination with androgen-deprivation therapy, or for the new indications of abiraterone and enzalutamide in pre-docetaxel patients<sup>[13,14,17]</sup>. 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<sup>[18-20]</sup>.

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 careful selection of patients has to be performed. Most studies reported that  $\geq 50\%$  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 frequency of good PSA responses and symptomatic responses on docetaxel rechallenge in a large retrospective study, and encouraging 20.4 mo median OS was reported<sup>[21]</sup>. 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<sup>[22]</sup>. 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 several times, and the good responders had a median PFI of 6 mo<sup>[7]</sup>.

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<sup>[23]</sup>.

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<sup>[10]</sup>.

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<sup>[24]</sup>. 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<sup>[25]</sup>. 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<sup>[26]</sup>. 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<sup>[27-29]</sup>. Moreover, sensory neuropathy, nail disorders and fatigue might occur on docetaxel rechallenge<sup>[6,7,21]</sup>.

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<sup>[30,31]</sup>.

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. Nevertheless, a few small experiences suggested that weekly docetaxel schedule might be effective in patients not-responding to first-line 3-weekly docetaxel<sup>[7,32]</sup>. 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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## 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

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**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 55<sup>th</sup> anniversary since the first specific oncogenic alteration was described<sup>[1]</sup>. It took over 40 years since the initial observation of the Philadelphia chromosome, to bring a concrete benefit to the patients carrying such abnormality<sup>[2]</sup>. 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)<sup>[3]</sup>. 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)<sup>[4]</sup>.

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<sup>[5]</sup>. 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<sup>[5]</sup>. 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)<sup>[6]</sup> 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<sup>[7]</sup>.

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<sup>[8]</sup>. 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<sup>[4,9]</sup>. 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<sup>[10,11]</sup>. 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<sup>[12-14]</sup>. 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<sup>[14]</sup>. This may relate to the ability of Crizotinib to eradicate tumor-propagating ALCL cells<sup>[15]</sup>. 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<sup>[16,17]</sup>. In other cases, activation of bypass signaling pathways allow the cells to grow independently of ALK<sup>[17,18]</sup>. 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<sup>[17]</sup>. 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<sup>[19]</sup> (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<sup>[20]</sup>, 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<sup>[21]</sup>. In addition to gatekeeper mutants, several other mutations have been described in patients, as well as in preclinical models<sup>[14,17,22-24]</sup>, spanning the ALK kinase domain from the region immediately aminoterminal to the  $\alpha$ C 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<sup>TM</sup>; Novartis, Switzerland) showed great efficacy in ALK+ NSCLC patients, both Crizotinib-resistant and naïve<sup>[25]</sup>, 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<sup>[26]</sup>. As a comparison, OS from metastatic diagnosis in a comparable group of ALK-wild-type controls were approximately 24 mo<sup>[9]</sup>. Unfortunately, Ceritinib-resistant mutants do arise under treatment<sup>[27]</sup>. In particular, substitutions at F1174 and G1202 residues have been observed in lung cancer patients progressing on Ceritinib.

Alectinib (CH5424802, Alecensa<sup>TM</sup>) 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<sup>[28,29]</sup>, 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)<sup>[30]</sup>. 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<sup>[31,32]</sup>.

Preliminary phase I - II results with Brigatinib (AP26113, ARIAD Pharmaceuticals) were recently presented at AACR 2015<sup>[33]</sup>. 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<sup>[34]</sup>. Only the new compound PF-06463922 was able to inhibit this mutant at low nanomolar doses<sup>[35]</sup>. PF-06463922 is a very potent and selective ALK/ROS1 inhibitor undergoing phase I evaluation, with a very large therapeutic window<sup>[36]</sup>. Indeed, recent preclinical *in vivo* data demonstrate potent PF-06463922 activity against G1202R mutant xenografts, as well as other mutations<sup>[37]</sup>.

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<sup>®</sup>,

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<sup>[14]</sup>.

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## 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

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**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<sup>[1]</sup>.

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<sup>[2]</sup>.

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<sup>[3]</sup>.

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<sup>[4]</sup>. 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<sup>[5]</sup>.

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<sup>[6]</sup>.

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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## ***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

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**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.

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## INTRODUCTION

Gastric cancer (GC) is the fourth most common cancer and the second leading cause of cancer-related death<sup>[1]</sup>. *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<sup>[2]</sup>.

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<sup>[3]</sup>. *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<sup>[4]</sup>. 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<sup>[5,6]</sup>. 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<sup>[7]</sup>.

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<sup>[8]</sup>. 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)<sup>[9]</sup>. 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)<sup>[10]</sup>.

*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<sup>[11,12]</sup>. *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<sup>[13,14]</sup>. 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<sup>[15]</sup>. CagA can affect epithelial cells by several mechanisms and may contribute to GC development<sup>[16]</sup>. 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<sup>[17]</sup>.

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<sup>[17,18]</sup>. 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<sup>[19]</sup>.

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

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<sup>[14]</sup>. 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<sup>[25-28]</sup>.

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<sup>[29]</sup>. 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- $\kappa$ B and AP-1<sup>[30]</sup>.

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- $\kappa$ B pathway in response to *H. pylori* peptidoglycan which is injected into the host cell cytoplasm *via* the T4SS<sup>[31]</sup>. NF- $\kappa$ B 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<sup>[32]</sup>. 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-1 $\beta$ , IL-6, and TNF- $\alpha$  in order to establish T and B lymphocyte-mediated adaptive immunity<sup>[24,33,34]</sup>. Indeed, TNF- $\alpha$  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-1 $\beta$  contributes to NF- $\kappa$ B pathway activation in myeloid cells<sup>[35]</sup>.

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



are involved in response to *H. pylori* infection<sup>[34]</sup>, but discussion is ongoing as whether *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<sup>[38]</sup>. When activated by bacterial LPS, TLR4 may recruit MyD88 and IRAK which subsequently activates NF- $\kappa$ B<sup>[39]</sup>.

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<sup>[34,40,41]</sup>. Infection with cagPAI+ strains was associated with an increased production of IL-23<sup>[35]</sup>. 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<sup>[42]</sup>.

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*<sup>[24]</sup>.

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<sup>[43]</sup>. 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<sup>[44,45]</sup>.

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<sup>[46]</sup>.

Gastrotrophin-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- $\kappa$ B by inhibiting the degradation and phosphorylation of I $\kappa$ B and inactivating IKK $\alpha$ /IKK $\beta$ <sup>[47,48]</sup>. 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<sup>[49]</sup>. 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<sup>[50]</sup>. More recently, Yoon *et al.*<sup>[51]</sup> demonstrated that CagA reduces GKN1 expression and that GKN1 transfection suppresses the carcinogenic effects of CagA. GKN1 may also influence cytokine production, NF- $\kappa$ B pathway and COX-2 expression<sup>[52]</sup>.

### 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<sup>[53]</sup>.

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- $\kappa$ B is a key regulator of inflammation and other cellular cascades and was identified as a molecular bridge between inflammation and cancer, since improper NF- $\kappa$ B activation transactivates several target genes harboring inflammatory (e.g., COX2, iNOS, TNF- $\alpha$ ), 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)<sup>[54]</sup>.

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- $\alpha$  have been implicated in cancer development. Interleukin-1B and TNF- $\alpha$  induce NF- $\kappa$ B 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<sup>[55]</sup>. IL-6 activates STAT3 (signal transducer and activator of transcription 3), enhancing cell growth and growth factor production<sup>[56]</sup>. Besides, IL-6 promotes COX-2 induction and increases ROS production<sup>[57]</sup>. COX-2 subsequently enhances cell growth and angiogenesis while ROS can modify protein function<sup>[24]</sup>.

TLRs may also lead to the production of inflammatory cytokines through AP-1 and NF- $\kappa$ B dependent transcription, playing a role in carcinogenesis through the activation of NF- $\kappa$ B and COX2<sup>[58-60]</sup>. 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<sup>[61,62]</sup>.

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<sup>[63,64]</sup>. 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 overexpressed in both *H. pylori*-infected tissues and in GC<sup>[65,66]</sup>. NF- $\kappa$ B 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.*<sup>[11]</sup> 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- $\alpha$  were found. However, that correlation disappeared in the presence of gastric atrophy and was inverse, for IL-6 and IL-8, in intestinal metaplasia<sup>[67]</sup>. Levels of miR-103, miR-375 and miR-200a were negatively correlated with IL-6, IL-8

and TNF- $\alpha$ , respectively. Let-7b was also found to be inversely correlated with IL-1b levels<sup>[67]</sup>.

*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<sup>[11,68]</sup>. 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<sup>[69]</sup>. Indeed, Teng *et al.*<sup>[69]</sup> demonstrated that let-7b inhibition lead to increased TLR4 protein levels, activation of NF- $\kappa$ B 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- $\kappa$ B 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<sup>[70]</sup>. 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- $\alpha$ , suggesting that miR-7 downregulation is related to the severity of inflammatory responses and possibly linked with gastric tumorigenesis<sup>[71]</sup>. 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<sup>[72]</sup>.

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<sup>[73]</sup>.

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

*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<sup>[76]</sup>. 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<sup>[35]</sup>. TLR signaling activation and inflammatory cytokines such as TNF- $\alpha$  and IL-1B have also been shown to upregulate miR-146 and miR-155 during *H. pylori* infection<sup>[77,78]</sup>.

miR-146 was found to be rapidly upregulated after LPS stimulation and after *H. pylori* infection in a CagA-independent and in a NF- $\kappa$ B-dependent manner through TLR signaling<sup>[79-81]</sup>. 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- $\kappa$ B pathway and the downstream proinflammatory signaling in response to bacterial products, thus avoiding the overproduction of proinflammatory IL-1B and TNF- $\alpha$  cytokines<sup>[79-82]</sup>. As a result, the expression of key elements of the proinflammatory innate and adaptive immune responses like IL-1B, IL-8, TNF- $\alpha$ , growth related oncogene alpha, and macrophage inflammatory protein is negatively regulated by miR-146a overexpression in *H. pylori* infection<sup>[80]</sup>, 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<sup>[83]</sup>, 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<sup>[84]</sup>.

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<sup>[77,85]</sup>. *H. pylori* was found to upregulate miR-155 expression also *via* a NF- $\kappa$ B- 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<sup>[86,87]</sup>. 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- $\kappa$ B activation and thus in decreased release of proinflammatory cytokines like IL-8 and GRO- $\alpha$ , suggest-

ing that miR-155 overexpression during *H. pylori* infection is also involved in the negative feedback regulation of the host inflammatory response through attenuating NF- $\kappa$ B activity<sup>[86,87]</sup>. Ceppi *et al.*<sup>[88]</sup> 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- $\kappa$ B. Other gene transcripts of the NF- $\kappa$ B pathway like IKK-epsilon (IKK), SMAD2 and Fas-associated Death Domains (FADD) were also described as miR-155 targets in one study<sup>[86]</sup>.

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- $\alpha$  and IL-8<sup>[64]</sup>. Th17 cell differentiation is promoted by TNF- $\alpha$  and IL-6 while Th1 responses are triggered by IL-12 and INF-gamma<sup>[89]</sup>. MiR-155 deficient mice showed decreased production of IFN- $\gamma$  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<sup>[90]</sup>. 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<sup>[91]</sup>. 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<sup>[92]</sup>.

Tang *et al.*<sup>[93]</sup> 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<sup>[93]</sup>.

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<sup>[94]</sup>.

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<sup>[95-97]</sup>. 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<sup>[24,98,99]</sup>. 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<sup>[23,27,100,101]</sup>.

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<sup>[102,103]</sup>. 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<sup>[26,96,98,104,105]</sup>.

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<sup>[106]</sup>. 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<sup>[107]</sup>.

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<sup>[96]</sup>. *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<sup>[108]</sup>. 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<sup>[96,109]</sup>. 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<sup>[110]</sup>.

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<sup>[96]</sup>. 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 <i>H. pylori</i> 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. cerevisiae*); 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<sup>[111]</sup>. 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<sup>[112,113]</sup>. 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<sup>[113]</sup>.

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<sup>[109]</sup>. 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 dimethyladenosine 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)<sup>[50,52,114]</sup>. 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<sup>[115]</sup>. 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<sup>[114]</sup>. Accordingly, miR-185 downregulation was demonstrated in GC and lower miR-185 levels were associated with lymph node metastasis (LNM) and poorer prognosis<sup>[116]</sup>.

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<sup>[26]</sup>. Shiotani *et al.*<sup>[117]</sup> 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.*<sup>[118]</sup> 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<sup>[11]</sup>.

miR-106b was implicated in TGF- $\beta$  and MAPK signaling pathways and miR-204 was related with calcium and neurotrophic signaling pathways and axon guidance<sup>[118]</sup>. 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<sup>[119]</sup>. miR-106b was associated with suppression of TGF- $\beta$ -induced cell cycle arrest and promotion of GC development in a previous study<sup>[120]</sup>. 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<sup>[121]</sup>. miR-106a is upregulated in GC and targets retinoblastoma protein (RB1), a tumor suppressor protein that inhibits transcription factors of the E2F family<sup>[65]</sup>. miR-106a, upregulated in GC, was correlated with lymphatic and distant metastasis<sup>[65,122]</sup>.

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<sup>[123]</sup>. 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<sup>[11,108,124]</sup>. Recently, Zhou *et al.*<sup>[124]</sup> 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<sup>[72]</sup>.

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<sup>[125]</sup>. 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<sup>[125]</sup>. 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.*<sup>[126]</sup> 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<sup>Kip1</sup> 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<sup>Kip1</sup> 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<sup>Kip1</sup> expression. The FoxM1 overexpression may reduce P27<sup>Kip1</sup> and thus increase cell proliferation and promotion of gastric carcinogenesis. Furthermore, transcription of

P27<sup>Kip1</sup> was inhibited by CagA via PI3K/Akt pathway in another study<sup>[127]</sup>. However, Lo *et al.*<sup>[128]</sup> 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- $\beta$  receptor II was identified as a target for miR-370 in this study and an inverse correlation was found between miR-370 and TGF- $\beta$ -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*<sup>[110]</sup>. 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<sup>[49,50]</sup>. 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<sup>[115]</sup>. 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)<sup>[129,130]</sup>. 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<sup>[131]</sup>. CD44v, a variant of CD44, was shown to interact with xCT (a glutamate-cystine transporter) and to contribute to ROS defense in cancer cells<sup>[132]</sup>. 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<sup>[133]</sup>. Ishimoto *et al.*<sup>[134]</sup> 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<sup>[134]</sup>. 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<sup>[135]</sup>. 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<sup>[135]</sup>.

## 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)<sup>[14]</sup>. *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<sup>[136]</sup>.

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<sup>[66]</sup>. 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)<sup>[137,138]</sup> and actin-binding protein<sup>[139]</sup>. 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<sup>[140]</sup>. 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<sup>[140]</sup>.

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- $\kappa$ B activity through negative regulation of IRAK1 and TRAF6<sup>[79,80]</sup>. It is also well established that TLR2, 4, 5 and 9 are involved in *H. pylori* recognition<sup>[62,141]</sup> and that NF- $\kappa$ B is a key molecule in inflammation-cancer link<sup>[142]</sup>. miR-146a upregulation was found in *H. pylori*-positive gastric mucosa and in GC tissues as compared with matched non-tumor adjacent tissues<sup>[143]</sup>. 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- $\kappa$ B activation, suggesting that miR-146a inhibits NF- $\kappa$ B activation thus reducing the expression of NF- $\kappa$ B-regulated tumor-promoting cytokines and growth factors and suggesting that in fact miR-146a have tumor suppressing properties<sup>[144]</sup>. Further supporting that miR-146a acts as a tumor suppressor, Hou *et al.*<sup>[145]</sup> 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<sup>[145]</sup>. 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<sup>[146]</sup>. 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<sup>[147]</sup>. 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<sup>[148]</sup>. 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<sup>[149]</sup>. 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<sup>[86]</sup>.

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<sup>[150,151]</sup>. However, some inconsistencies were found in the literature. Indeed, Okubo *et al.*<sup>[152]</sup> 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.*<sup>[153]</sup> 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.*<sup>[154]</sup> 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<sup>[155]</sup>. 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<sup>[156]</sup>. 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<sup>[157-159]</sup>.

*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<sup>[11,68]</sup>. 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)<sup>[160,161]</sup>. 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<sup>[71,162]</sup>. 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<sup>[162]</sup>. 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<sup>[18,163]</sup>. *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<sup>[164]</sup>. *H. pylori* CagA transfect into gastric epithelial cells results in miR-584 and miR-1290 upregulation, via NF- $\kappa$ B and Erk1/2 respectively<sup>[110]</sup>. 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<sup>[110]</sup>.

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)<sup>[165,166]</sup>. 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<sup>[167]</sup>. ZEB2 also represses cyclin D1 transcription, a cyclin that promotes G1/S transition and is induced *via* AP-1 in gastric epithelial cells during *H. pylori* infection and under CagA dependence<sup>[168]</sup>. 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<sup>[11]</sup> targets fibroblast growth factor receptor (FGFR), and overexpression of miR-141

leads to decreased FGFR2 expression and inhibition of proliferation<sup>[169]</sup>.

MiR-375 repression and B-catenin-activating mutation also was described in hepatocellular adenoma and carcinoma<sup>[170]</sup>. Ye *et al.*<sup>[171]</sup> 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<sup>[172]</sup>. 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<sup>[173]</sup>. 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- $\beta$  is involved in mucosal immunity and in the control of the physiological turnover of epithelial cells, and the downstream effectors of TGF $\beta$ -dependent cell cycle arrest and apoptosis are the CDK inhibitor p21<sup>CIP1/WAF1</sup> 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<sup>[174]</sup>. 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- $\beta$  by interfering with the expression of p21 and Bim, affecting both cell cycle and apoptosis<sup>[120,175]</sup>. Indeed, miR-106b-25 cluster was found to silence p21<sup>CIP1/WAF1</sup>, E2F1 and the proapoptotic factor Bim leading to a decreased response of gastric cells to the TGF $\beta$  tumor-suppressor activity and to impairment of p21 tumor suppressor activities<sup>[120,174]</sup>. MiR-25 was also found to target and negatively influence Bim and the CDK inhibitors p27 and p57<sup>[176]</sup>.

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



ted apoptosis by targeting RUNX3, a known tumor suppressor silenced by promoter hypermethylation in GC<sup>[178,179]</sup>. mir-301a was also reported to be upregulated in GC, and directly downregulates Runx3 expression<sup>[180]</sup>. 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- $\beta$  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<sup>[181]</sup> and it was suggested that miR-524-5p may also be involved in GC by regulating cell cycle and TGF- $\beta$  signaling pathway<sup>[118]</sup>. 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<sup>[182]</sup>. miR-449 was also found to target Met, geminin, and SIRT1, proto-oncogenes that may be related with proliferation, angiogenesis, invasion and metastasis<sup>[182]</sup>.

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)<sup>[183]</sup>. 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<sup>[183]</sup>.

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<sup>[184,185]</sup>. miR-29c is a tumor-suppressor miRNA significantly downregulated in GC tissues compared with non-tumoral gastric mucosa<sup>[186]</sup>. Treatment with celecoxib, a selective COX-2 inhibitor, significantly activates miR-29c expression suppressing anti-apoptotic Mcl-1<sup>[108,187]</sup>. 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<sup>[188]</sup>. 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<sup>[189,190]</sup>.

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)<sup>[191]</sup>. In another study, Li *et al*<sup>[192]</sup> 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<sup>[193]</sup>. 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<sup>[194-196]</sup>.

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<sup>[197]</sup>. In *H. pylori*-infected gastric mucosa miR-200bc/429 cluster is downregulated increasing expression of Bcl-2 and XIAP and thus inhibiting apoptosis<sup>[194,195,198]</sup>.

Another tumor suppressor miRNA, mir-218 is significantly decreased in both *H. pylori*-infected mucosa and in GC tissues<sup>[199]</sup>. 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- $\kappa$ B transcriptional activation and inhibition of COX-2 transcription, leading to an apoptotic response<sup>[199]</sup>. miR-218 downregulation in GC cells was also correlated with increased metastasis and invasion through SLIT/ROBO1 signaling pathway upregulation<sup>[65,199,200]</sup>. Thus it seems that downregulation of miR-218 in GC cause ECOP overexpression, activation of NF- $\kappa$ B activity and COX-2 transcription, ultimately inhibiting apoptosis and inducing cell proliferation<sup>[199]</sup>. 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<sup>[201]</sup>. 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	<i>H. pylori</i>	GC	Targets	Consequences/associations
miR-21	↑	↑	RECK PTEN ABP	Decreased apoptosis; cell proliferation, invasion MMP stimulation 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 <sup>CIP1/WAF1</sup> Bim	Decreased response to TGF-β Cell cycle progression; inhibition of apoptosis
miR-130b		↑	E2F1	
miR-301a			RUNX3   Bim	Proliferation (CDK2 activation) and invasion
miR-181b	↑	↑	RUNX3	Apoptosis inhibition
miR-27a	↑	↑	Timp3	Inhibition of apoptosis, cell proliferation, invasion and migration
			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-β: Transforming growth factor beta; RUNX3: Runt-related transcription factor 3; Timp3: TIMP Metalloproteinase Inhibitor 3; FoxO1: Forkhead box protein O1.

*pylori*<sup>[202]</sup>.

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<sup>[203]</sup>. 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<sup>[203]</sup>. 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<sup>[204]</sup>. 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<sup>[101]</sup>. However, Ando *et al*<sup>[96]</sup> 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*<sup>[117]</sup> 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<sup>[117]</sup>, further supporting the clinical finding that intestinal metaplasia is a less reversible stage in the gastric carcinogenesis.

In another study by Shiotani *et al*<sup>[205]</sup>, 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	<i>H. pylori</i>	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-β induced cell cycle arrest suppression)
miR-320	↓	↓	Mcl-1	Apoptosis inhibition; progression of preneoplastic conditions
				Relapse of tumors; chemotherapeutic resistance
miR-101,	↓	↓	Mcl-1	Apoptosis inhibition
miR-515-5p			SOC2; DNMT1	Let-7 attenuation
miR-490-3p	↓	↓	Cyclin B1	EMT; proliferation; colony formation; migration; invasion
			SMARCD1	Metastasis and poorer survival
				Decreased through the spectrum of gastric carcinogenesis
miR-370	↓	↓	FoxM1	↓p27 expression; cell cycle progression and proliferation
				Decreased through the spectrum of gastric carcinogenesis
miR-328	↓		CD44v9	Survival and proliferation of metaplastic cells
Let-7	↓	↓	Ras	Cell proliferation and colony formation
			c-myc	
			HMGA2	Migration and invasion
			Cthrc1	
miR-200,	↓	↓	ZEB1/2	Epithelial differentiation; EMT suppression
miR-141,				Decreased E-cadherin, inhibition of migration and invasion
miR-429			Cyclin D1	Proliferation
			Bcl-2   XIAP	Apoptosis inhibition
				Tumor size, lymphatic invasion and LNM
miR-141	↓		FGFR2	Proliferation
miR-375	↓	↓	MDM2	p53 inactivation
			JAK2/STAT3	Neoplastic transformation; proliferation and migration
			3/3/2014	Inhibition of apoptosis
			PDK1	PI3K/Akt signaling pathway activation
miR-524-5p		↓	Jagged-1; Hes-1	Cell proliferation and invasion
miR-449	↓	↓	Cyclin E2   Met	Proliferation, angiogenesis, invasion and metastasis
			Gemini   SIRT1	
miR-203	↓	↓	CASK	Cell growth, colony formation and cell invasion
miR-29a		↓	p42.3; Mcl-1	Cell cycle progression and proliferation
miR-29c				
miR-15b, 16, 34, 181b, 497		↓	Bcl-2	Apoptosis inhibition
miR-218	↓	↓	ECOP	Activation of NF-κB and increased COX-2; apoptosis inhibition
			SLIT/ROBO1	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-β: 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: Hair cell 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 to 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

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**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<sup>[1]</sup>, claiming over 50000 lives in 2014<sup>[1,2]</sup>. The 5-year survival rate for patients diagnosed with metastatic CRC is < 15%<sup>[1]</sup>. 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<sup>[2]</sup>. 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%<sup>[3,4]</sup>. 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<sup>[5-8]</sup>. 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<sup>[9]</sup>. 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<sup>[10]</sup>.

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<sup>[11]</sup>.

## 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%<sup>[1]</sup>, 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<sup>[12]</sup>.

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<sup>[13]</sup>.

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<sup>[13]</sup>. There is, however, some data suggesting a benefit for adjuvant RT in colon cancer in select patients with high-risk features for local recurrence<sup>[14]</sup>.

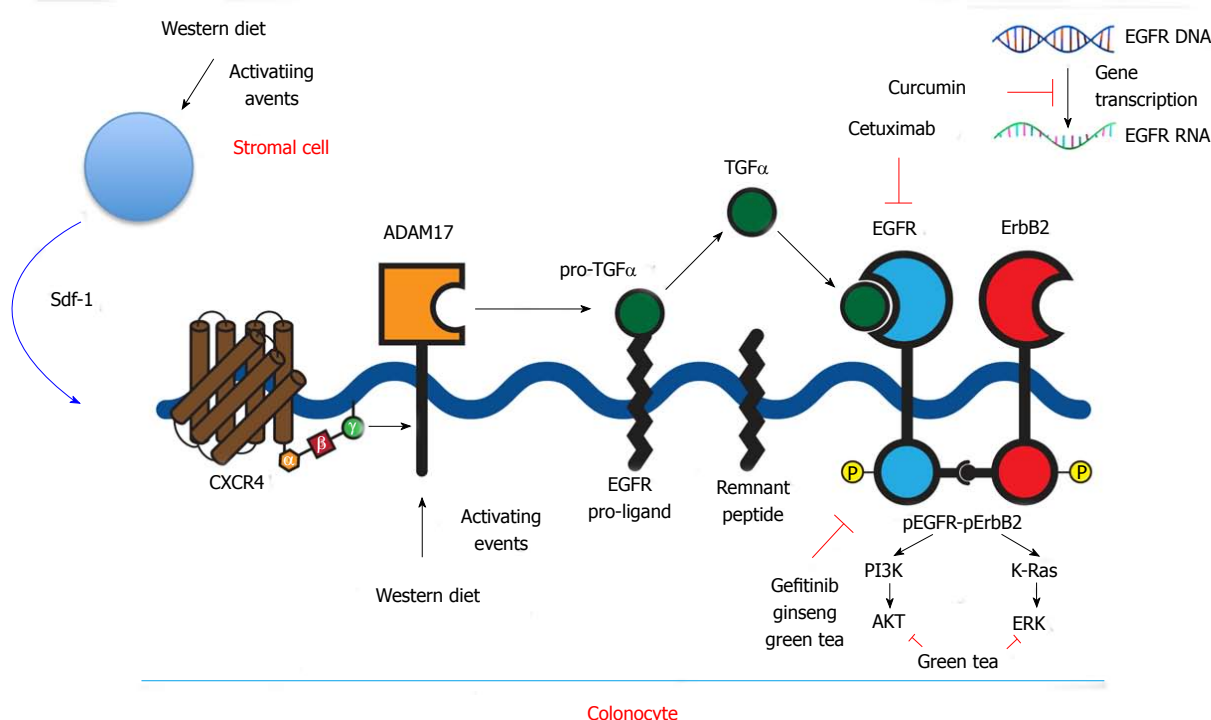
## EGFR PATHWAYS IN COLORECTAL CANCER

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-erbB 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 multi-functional receptor that plays a key role in cell division and apoptosis, cell differentiation and dedifferentiation, migration, and organogenesis<sup>[15]</sup>. 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-JNK-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 heterodimerization 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<sup>[16]</sup>.

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<sup>[17]</sup>.

EGFR is expressed in 60%-80% of CRCs<sup>[2]</sup>. 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<sup>[18-20]</sup>. 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<sup>[21]</sup>. 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<sup>[22]</sup>. 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<sup>[23]</sup>.

## 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<sup>[24]</sup>. 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  $\beta$ -catenin)<sup>[25]</sup>. In this model, EGFR is required for tumor promotion by Western diet<sup>[26,27]</sup>. 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*<sup>[7]</sup>. The proto-oncogenes CTNNB1, MYC, CNND1, and PTGS2 and the EGFR ligand TGF $\alpha$  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*<sup>[7]</sup>.

In more recent preliminary studies we showed that Western diet increases ADAM17 expression and up-regulates EGFR ligands TGF- $\alpha$  and amphiregulin<sup>[28]</sup>. Stroma-derived factor 1 alpha (Sdf1 $\alpha$ ) was also increased by WD. Sdf1 $\alpha$  is a ligand for the G-protein coupled receptor CXCR4. In other preliminary colon cancer studies we showed that Sdf1 $\alpha$  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<sup>[29]</sup>. 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<sup>[30]</sup>.

CTNNB1 codes for  $\beta$ -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*<sup>[31-33]</sup>. EGFR is an upstream regulator of  $\beta$ -catenin causing deacetylation that blocks  $\beta$ -catenin degradation and leads to nuclear localization of this molecule<sup>[34]</sup>. 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- $\rightarrow$  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  $\beta$ -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 TGF $\alpha$  was shown to correlate with tumor burden in both genotypes, with a stronger association with the wild type *Egfr* noted<sup>[7]</sup>.

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<sup>[35-38]</sup>.

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<sup>[7]</sup>.

### 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<sup>[39-41]</sup>. 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<sup>[42-45]</sup>. 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<sup>[46]</sup> (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<sup>[47]</sup>.

### 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<sup>[48]</sup> (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<sup>[49-51]</sup>. 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<sup>[49]</sup>.

### 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<sup>[52]</sup>. Recent studies have demonstrated that curcumin inhibits binding of the transcription factor

EGR-1 to the EGFR promoter as well as suppressing EGR-1 gene expression through the ERK signal pathway, thereby suppressing EGR-1 transactivation activity<sup>[53]</sup> (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<sup>[54]</sup>.

## 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 panitumumab, 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<sup>[55-58]</sup> (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<sup>[59,60]</sup>. 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<sup>[61]</sup>. Panitumumab is also used in metastatic CRC and also requires WT KRAS for efficacy<sup>[62]</sup>. 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<sup>[63]</sup>.

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<sup>[64]</sup>. A phase II RCT of FOLFIRI vs gefitinib plus FOLFIRI did not show any benefit and demonstrated high toxicity<sup>[65]</sup>. 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<sup>[66,67]</sup>, 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<sup>[55]</sup>. 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<sup>[68]</sup>.

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<sup>[60,69]</sup>. Mutations in other downstream signaling molecules such as BRAF have been shown to correlate with unresponsiveness to cetuximab and panitumumab<sup>[70]</sup>. 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<sup>[71,72]</sup>. 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<sup>[73]</sup>. 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<sup>[74]</sup>. 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<sup>[70]</sup>.

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<sup>[75]</sup>. This can occur through the amplification of ERBB2 itself or through the overexpression of heregulin,



one of the ERBB3 ligands. Increased c-Met signaling may be another mechanism for EGFR antibody resistance<sup>[76,77]</sup>. 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

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**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<sup>[1,2]</sup>. 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<sup>[3]</sup>. 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<sup>[4]</sup>. Surgical resection remains the gold standard and potentially curative treatment for CRLM<sup>[2,4]</sup>. 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<sup>[5-8]</sup>. 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<sup>[9]</sup>. The experience with preoperative PVE was first described more than 20 years ago by Makuuchi *et al*<sup>[10]</sup> 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<sup>[10]</sup>. This successful and relatively safe technique allowed more liver metastases removal<sup>[6,9]</sup>. 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<sup>[11]</sup>. 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<sup>[12]</sup>. 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<sup>[13-24]</sup>. 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<sup>[12,13]</sup>. 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<sup>[19,24]</sup>. 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<sup>[25]</sup>.

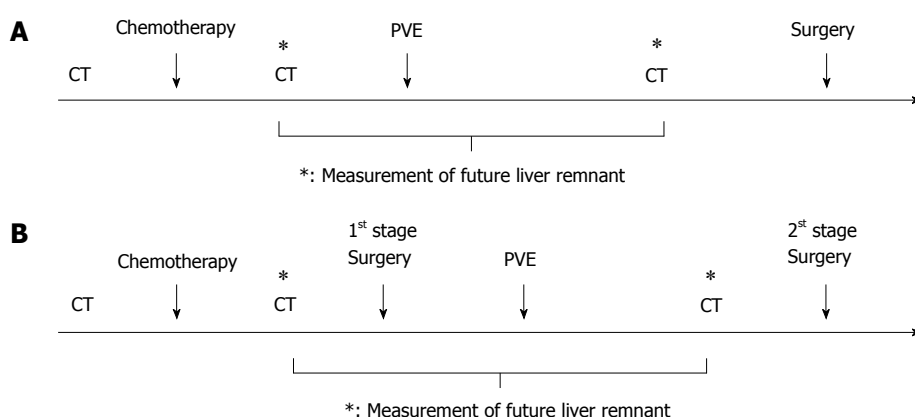
The increasing body of evidence emphasizing the superior contribution of PVE to the observed tumoral growth triggered interest during the past decade. Hoekstra *et al*<sup>[26]</sup> examined the relationship between PVE and enhanced tumor growth in a rabbit model, which mirrored the clinical setting 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 TGFβ 1). Additionally, Maggiori *et al*<sup>[27]</sup> 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<sup>[28]</sup>. 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<sup>[29]</sup>. Moreover, multiple clinical studies described concordant observations of tumor progression and higher recurrence rates in CRLM patients undergoing PVE<sup>[13-24]</sup>. A clinical study conducted by Pamecha *et al*<sup>[23]</sup> 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*<sup>[13]</sup> 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 <i>et al</i> <sup>[13]</sup>	<i>n</i> = 109	33.4% increase in TV in the right lobe ( <i>P</i> < 0.001) and 49.9% increase in TV in the left lobe ( <i>P</i> = 0.022) post-PVE
Elias <i>et al</i> <sup>[14]</sup>	<i>n</i> = 48	60% to 970% increase in TV post-PVE
Kokudo <i>et al</i> <sup>[15]</sup>	<i>n</i> = 18	+20.8% ( <i>P</i> = 0.016) increase in TV and 18.5% ( <i>P</i> = 0.014) increase in percent tumor volume post-PVE
Mueller <i>et al</i> <sup>[19]</sup>	<i>n</i> = 53	80.9% ( <i>n</i> = 17/53) of patients were unresectable due to tumor progression post-PVE
Pamecha <i>et al</i> <sup>[21]</sup>	<i>n</i> = 36	33% ( <i>n</i> = 12/36) of patients had tumor progression post-PVE
Hoekstra <i>et al</i> <sup>[22]</sup>	<i>n</i> = 28	25% ( <i>n</i> = 7/28) of patients developed new lesions in FLR and 42% of patients ( <i>n</i> = 8/19) had tumor recurrence in the liver on follow up post-PVE
Pamecha <i>et al</i> <sup>[23]</sup>	<i>n</i> = 22	TGR post-PVE was 0.36 ± 0.68 mL/d (-1) ( <i>P</i> = 0.06)
Lindner <i>et al</i> <sup>[24]</sup>	<i>n</i> = 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 2<sup>nd</sup> 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<sup>[13]</sup>. The study also showed a positive tumor growth rate (+0.07 cm<sup>3</sup>/d) in the PVE group compared to a negative growth rate (-0.06 cm<sup>3</sup>/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 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<sup>[30]</sup>. 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<sup>[25,31]</sup>. 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<sup>[29]</sup>. 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.*<sup>[12]</sup> 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<sup>[22,24-25,32]</sup>. 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<sup>[25]</sup>. 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<sup>[25,33]</sup>. Lastly, the use of systemic therapy (neoadjuvant or adjuvant chemotherapy) is now widely used in the management of these patients<sup>[25,34]</sup>. In fact, chemotherapy may protect against tumor progression post-PVE without disturbing liver hypertrophy especially in patients who initially respond adequately<sup>[6,25,35]</sup>. Fischer *et al.*<sup>[6]</sup> 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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## 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

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**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.

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## 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<sup>[1]</sup>. They also do not have organized data registries, thus they lack reliable data on breast cancer incidence, mortality, survival, and stage of presentation<sup>[2,3]</sup>.

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<sup>[4-6]</sup>. 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<sup>[7-11]</sup>.

In East Africa, the breast cancer incidence rate estimate is 19.3 per 100000 women<sup>[12]</sup>. Breast cancer is the most prevalent cancer among Kenyan women, and constitutes a major public health problem<sup>[13,14]</sup>. Although definite data are lacking for Kenya, estimates indicate that breast cancer accounts for about 23% of all cancers in the country<sup>[15]</sup>. Unfortunately, Kenya has not developed a comprehensive cancer surveillance system and there is no national population-based cancer registry<sup>[15]</sup>. 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<sup>[15]</sup>. 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<sup>[15]</sup>.

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<sup>[16]</sup>. 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)<sup>[17]</sup>. 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?" (Kwaza habisa, kwa maoni yako ni, dalili gani za mapema zinazotahadharisha kuhusiana na saratani ya matiti? Yani njia ambazo mtu anaweza kutambua mapema kuwa anaogua 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 (1.3)	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 <sup>1</sup>	25 (4.2)	5 (1.7)	13 (1.9)	43 (2.7)
No opinions expressed <sup>2</sup>	264 (44.2)	184 (61.9)	374 (55.7)	822 (52.5)

<sup>1</sup>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; <sup>2</sup>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				
Changes in breast size	1166 (40%)	552 (19%)	1192 (41%)	2910 (100%)
Pain, tingle or tenderness of the breast	207 (17.8)	109 (19.7)	266 (22.3)	582 (20.0)
Lump in 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 <sup>1</sup>	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)

<sup>1</sup>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 ( <i>n</i> = 481)	Mosoriot ( <i>n</i> = 277)	Turbo ( <i>n</i> = 577)	Total ( <i>n</i> = 1335)
		<i>n</i> (%)	<i>n</i> (%)	<i>n</i> (%)	<i>n</i> (%)
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<sup>[18-20]</sup>. 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<sup>[21]</sup>. 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<sup>[22]</sup> 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<sup>[23-26]</sup>. 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<sup>[27-30]</sup>. Additionally, the health care work force does not seem to be an active source of breast cancer information. For example, Oluwatosin's<sup>[31]</sup> (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<sup>[31]</sup>. 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<sup>[32]</sup>. 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<sup>[15]</sup>. 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<sup>[33]</sup>.

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<sup>[34]</sup> (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<sup>[34]</sup>. 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<sup>[2]</sup>. 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<sup>[6]</sup>. 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<sup>[15]</sup>. 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<sup>[15]</sup>.

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<sup>[6]</sup>. In Kenya, although cancer continues to burden many, cancer research is diminutive due to inadequate funding and limited training facilities<sup>[15]</sup>. 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<sup>[6]</sup>.

## 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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## 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<sup>2</sup> 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

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**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 chemoradiation 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)<sup>[1]</sup>. 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<sup>[2]</sup>. A meta-analysis reports that the addition of chemotherapy to radiation in this setting increases severe esophagitis rates from 4% to 18%<sup>[3]</sup>. Significant rates of high grade leukopenia and neutropenia have also been seen in the literature, with upper limits approximating 50%<sup>[4,5]</sup>.

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<sup>[6,7]</sup>. In addition, side effects including marked hypotension and the requisite IV route of delivery have precluded its widespread adoption in this setting.

Predclinical 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<sup>[8-17]</sup>. The dual properties of SLM to reduce normal tissue toxicity while increasing antitumor efficacy led to consideration<sup>[18]</sup> and implementation of early human studies combining chemotherapy with Se in a variety of tumors<sup>[19,20]</sup>. 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-III B squamous cell carcinoma, adenocarcinoma, large cell carcinoma, or NSCLC not otherwise specified; age  $\geq 18$ ; ECOG PS 0-1; weight loss  $\leq 5\%$  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  $\leq 1.5$  mg/dL; serum bilirubin and glutamic-oxaloacetic transaminase  $\leq 1.5$  times the upper limit of normal; hemoglobin  $\geq 8.0$  g/dL; absolute granulocyte count  $\geq 2000/\text{mm}^3$ ; and platelet count  $\geq 100000/\text{mm}^3$ . 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  $\geq 4$  patients experienced excessive toxicity. Stage 2 was planned to accrue an additional 20 patients, with the bar set at  $\geq 7$  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<sup>2</sup>) 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	<i>n</i> (%)	Characteristic	<i>n</i> (%)
Sex		Performance status	
Male	5 (31)	0	7 (44)
Female	11 (69)	1	9 (57)
Age		Stage	
Mean	63.25	III A	7 (44)
Median	61	III B	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<sup>[19]</sup>.

### 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  $\geq 50\%$  of the sum of the products of the perpendicular diameters of all measurable lesions. Stable disease (SD) required  $< 50\%$  reduction and  $\leq 25\%$  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  $\geq 3$  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

**Table 2 Adverse events**

<i>n</i> = 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

0.05 level was set for Type 1 error, and 95%CI were calculated using the Jennison and Turnbull method<sup>[21]</sup>. One-sided *P*-values were calculated. Median, overall, and failure-free survival rates were calculated using the Kaplan-Meier method, with 95%CI.

## 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

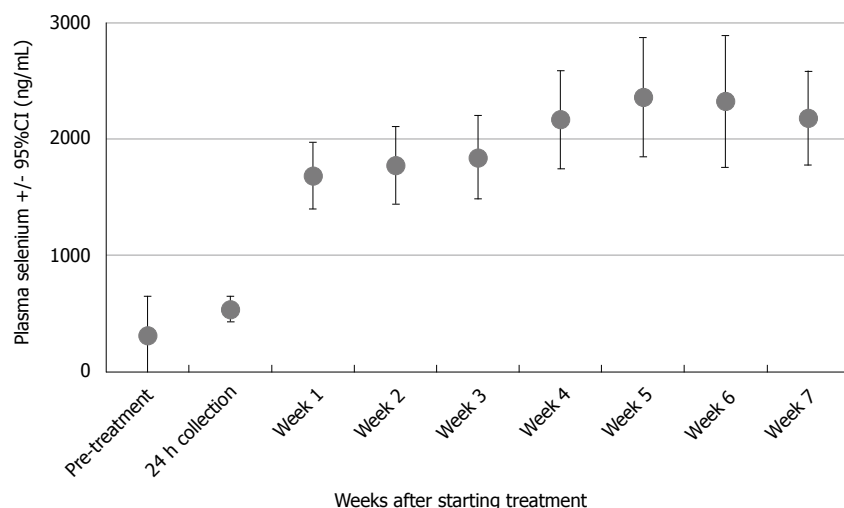


Figure 1 Serum selenium levels before and during concurrent chemoradiation.

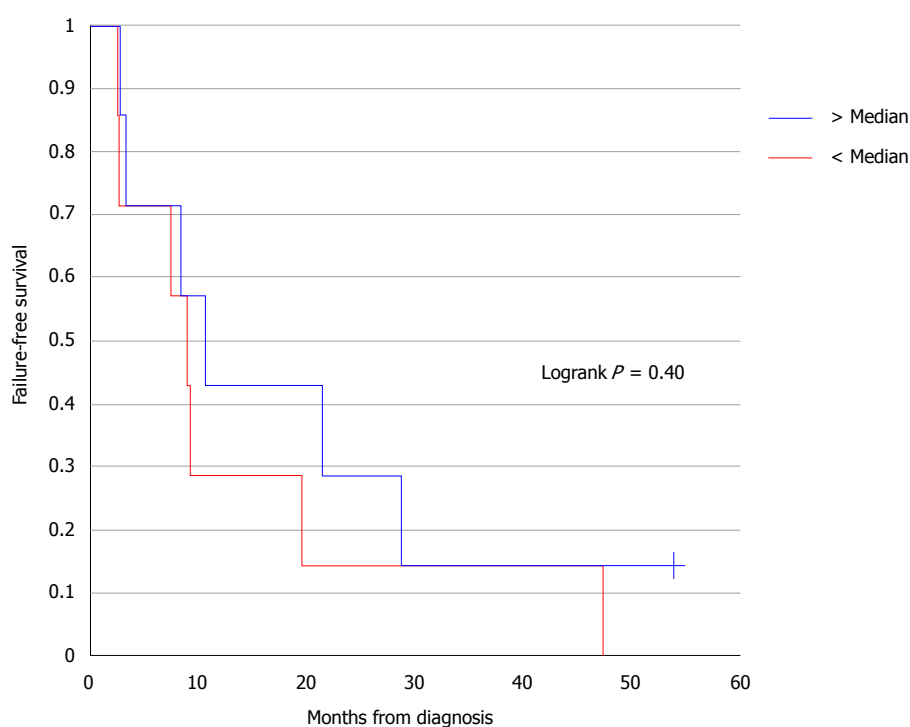


Figure 2 Failure-free survival stratified by baseline selenium level.

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 myelosuppression 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  $\mu\text{g}$  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 <i>et al</i> <sup>[27]</sup>	1999	Ind → RT CCRT	314	ENI	56 56 <sup>1</sup>	Cis/Vnd/Mit	3% 2%	- 1%
Zatloukal <i>et al</i> <sup>[28]</sup>	2004	Ind → RT CCRT	102	ENI	60	Cis/Vno	4% 18%	- 4%
Fournel <i>et al</i> <sup>[26]</sup>	2005	Ind → RT CCRT → Cons	205	ENI	66	Cis/Vno Cis/Eto → Cis/Vno	2% 32%	- 5%
Belani <i>et al</i> <sup>[4]</sup>	2005	Ind → RT Ind → CCRT CCRT → Cons	257	IFRT	63	Cbp/Pac	- 19% 28%	- 4% 16%
Vokes <i>et al</i> <sup>[2]</sup>	2007	CCRT Ind → CCRT	366	ENI	66	Cbp/Pac	28% 30%	4% 10%
Belderbos <i>et al</i> <sup>[25]</sup>	2007	Ind → RT CRT	158	ENI	66 <sup>2</sup>	Cis/Gem Cis	5% 14%	- 18%
Socinski <i>et al</i> <sup>[41]</sup>	2008	Ind → CCRT Ind → CRT	69	“ENI discouraged but allowed”	74	Cbp/Pac Cbp/Gem	16% 39%	16% 37%
Blumenschein <i>et al</i> <sup>[23]</sup>	2011	CCRT	87	“Selective nodal irradiation”	63	Cbp/Pac/ Cet	8%	22%
Curran <i>et al</i> <sup>[42]</sup>	2011	Ind → RT CCRT CCRT	407	ENI	63 63 69.6 <sup>3</sup>	Cis/Vnb Cis/Vnb Cis/Eto	4% 22% 45%	- 13% 15%
Hoang <i>et al</i> <sup>[5]</sup>	2012	CCRT CCRT + Thl	546	IFRT	60	Cbp/Pac Cbp/Pac/Thl	< 1% < 1%	1% 1%

<sup>1</sup>Split course; <sup>2</sup>2.75 Gy/d; <sup>3</sup>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 chemoradiotherapy; 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%<sup>[3]</sup>. Regarding the myelosuppressive endpoints, estimates based on similarly treated patients for leukopenia, anemia, and neutropenia, are 23%-51%, 3%-10%, and 15%-51%, respectively<sup>[2,4,5]</sup>. Given these estimates, the addition of selenium may have improved myelosuppression.

### 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*<sup>[2]</sup> at 28%. Based on the observation that ENI doesn't significantly reduce regional recurrence<sup>[22]</sup> 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%<sup>[5,23,24]</sup>. One such trial, however, revealed numerically-increased rates of esophagitis compared to ours, with grade 3-4 toxicity of 28%<sup>[4]</sup>. 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*<sup>[2]</sup> reported 67% CR/PR and 9% PD, while Blumenschein *et al*<sup>[23]</sup> 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 <i>et al</i> <sup>[4]</sup>	2005	CCRT → Cons	92	Cbp/Pac	10%	51%	26%
Vokes <i>et al</i> <sup>[2]</sup>	2007	CCRT	184	Cbp/Pac	5%	36%	15%
Hoang <i>et al</i> <sup>[5]</sup>	2012	CCRT	275	Cbp/Pac	3%	23%	51%
Blumenschein <i>et al</i> <sup>[23]</sup>	2011	CCRT	87	Cbp/Pac/Cet	"Blood/bone marrow": 48%		

Cbp: Carbo; Pac: Paclitaxel; Cet: Cetuximab; CCRT: Concurrent chemoradiotherapy; 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<sup>[2,4,25-28]</sup>. 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<sup>[29]</sup>. 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*<sup>[30]</sup> 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 ( $3.6 \pm 1.84$  vs  $5.3 \pm 2.2$  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<sup>[31]</sup>.

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<sup>[32]</sup>. 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<sup>[33]</sup>. 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<sup>[34,35]</sup>. 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<sup>[35]</sup>. Finally, a phase I study from our group has shown that SLM did not significantly impact irinotecan toxicity<sup>[19]</sup>.

### Combining radiotherapy and selenium

Other studies have examined the potential of Se to mitigate radiation-induced toxicity. Muecke *et al*<sup>[36]</sup>, 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  $\geq 2$  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*<sup>[37]</sup> 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<sup>2</sup> 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<sup>[38]</sup>.



### 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<sup>[39]</sup>. This fact suggests that there may be a correlation between toxicity and Se levels. A report from Eroglu *et al.*<sup>[40]</sup>, 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<sup>[19]</sup>. 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  $\geq 3$  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  $\mu\text{g/d}$  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 myelosuppression. 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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## 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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### 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<sup>2</sup> 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<sup>2</sup> 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

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**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<sup>[1]</sup>. Worldwide, approximately 350000 diagnoses are expected annually<sup>[2]</sup>. HNSCC is often related to tobacco and alcohol exposure<sup>[3]</sup>, human papilloma virus exposure<sup>[4]</sup>, 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%<sup>[5,6]</sup>. 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%<sup>[7-9]</sup>.

Pre-clinical literature suggested that organic selenium (Se) compounds including L-selenomethionine (SLM) might have both anti-tumor<sup>[10-15]</sup> and anti-toxicity<sup>[12,14,16-19]</sup> 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<sup>[20-22]</sup>.

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<sup>[23]</sup>. 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  $\geq 6$  MeV were utilized.

### **Cisplatin chemotherapy**

Cisplatin was dosed at 100 mg/m<sup>2</sup> intravenously over 3 h in 1000 mL of normal saline on days 1, 22, and 43 of radiation therapy. Institution-specific standard pre-medication 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<sup>2</sup>. 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<sup>2</sup>. 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<sup>[24]</sup>. Completion surgery to sites of remaining disease after CRT was performed if clinically appropriate.

### **Statistics**

Sample size calculations were based on a  $\geq$  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 II b 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<sup>[25]</sup>. With this design the chance of concluding there is an improvement in the proportion with  $\geq$  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  $\geq$  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	0.103
N stage	1	0	1	
	2	6	8	
	3	1	1	
	X	1	0	0.105
M stage	0	5	10	
	X	3	0	0.108
Stage group	IVA	7	8	
	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

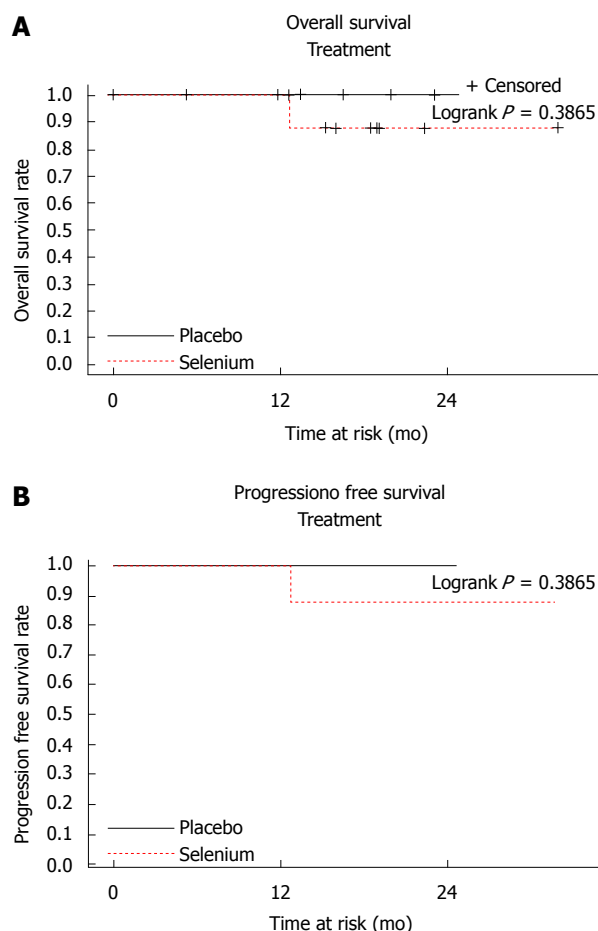


Figure 1 Overall and progression-free survival.

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.*<sup>[26]</sup>, 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<sup>[26]</sup>. Buntzel *et al.*<sup>[27]</sup> performed a randomized phase II trial of 39 patients with advanced head and neck cancer. Patients either received no Se substitution or 500  $\mu\text{g}$  sodium selenite orally on the days of radiotherapy and 300  $\mu\text{g}$  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<sup>[27]</sup>.

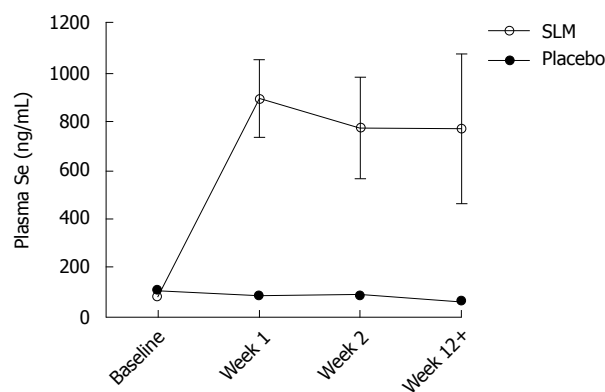


Figure 2 Mean ( $\pm$  SE) trough selenium concentrations in plasma prior to selenomethionine, after 1 and 2 wk of selenomethionine intake, and 1-2 wk after the end of treatment. SLM: Selenomethionine; Se: Selenium.

### 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.*<sup>[28]</sup>, 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<sup>[28]</sup>.

Jahangard-Rafsanjani *et al.*<sup>[29]</sup> found that oral Se 200  $\mu\text{g}$  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 ( $3.6 \pm 1.84$  d vs  $5.3 \pm 2.2$  d,  $P = 0.014$ )<sup>[29]</sup>. A series of randomized trials reported by Asfour *et al.*<sup>[30,31]</sup> 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  $\mu\text{g}$  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<sup>[32]</sup>.

In contrast, a prior phase I trial from our group has shown that SLM did not limit irinotecan toxicity<sup>[21]</sup>. 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  $\mu\text{g}/\text{d}$  or 800  $\mu\text{g}/\text{d}$  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 ( $P = 0.018$ )<sup>[33]</sup>.

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<sup>[34-37]</sup>. 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<sup>[38]</sup>. 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<sup>[10,11,14,15]</sup> and anti-toxicity<sup>[14,16-19]</sup> 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  $\mu\text{g}/\text{m}^2$  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 radioprotector.

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

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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

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**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.

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## INTRODUCTION

Breast cancer is the most common female malignancy worldwide<sup>[1]</sup>. 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<sup>2</sup>); 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).

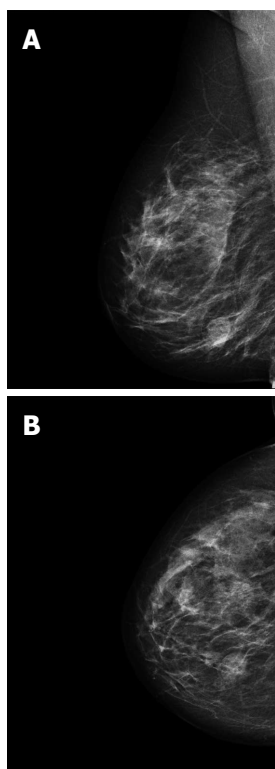
## DISCUSSION

Soft tissue sarcomas (STS) amount to less than 1% of all malignant tumors with an incidence estimated at 2-5 cases per 100000 yearly<sup>[2]</sup>. The exact diagnosis may pose a significant challenge since there are over fifty subtypes of STS, which determine their prognostic and therapeutic features<sup>[3]</sup>. Eight percent to 14% of all newly diagnosed STS have liposarcomatous differentiation making primary liposarcoma a common subtype. Lucas *et al*<sup>[4]</sup> 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.

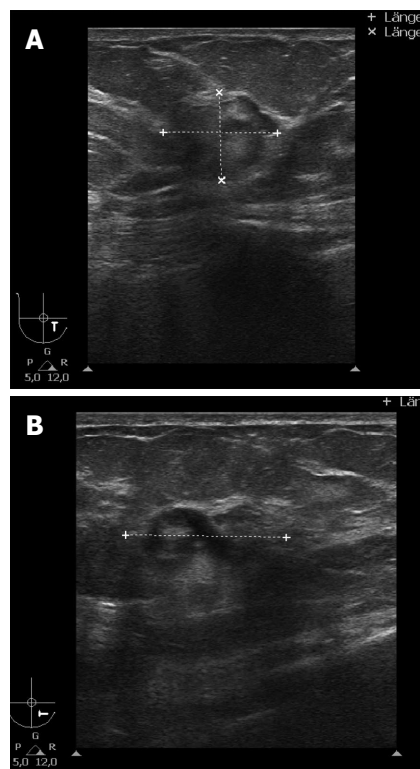
Primary sarcomas of the breast account for 0.1% of all malignant breast tumors. A thorough review on breast sarcomas, along with a series of 25 cases, was published by Adem *et al*<sup>[5]</sup>. The incidence of liposarcoma among breast sarcomas vary in the literature from 2% to 10%<sup>[6-10]</sup>. 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<sup>[11]</sup>. 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<sup>[12]</sup>. Further features associated with favorable survival include complete surgical excision of tumor with tumor-free margins<sup>[13]</sup>.

### Therapy of breast sarcomas

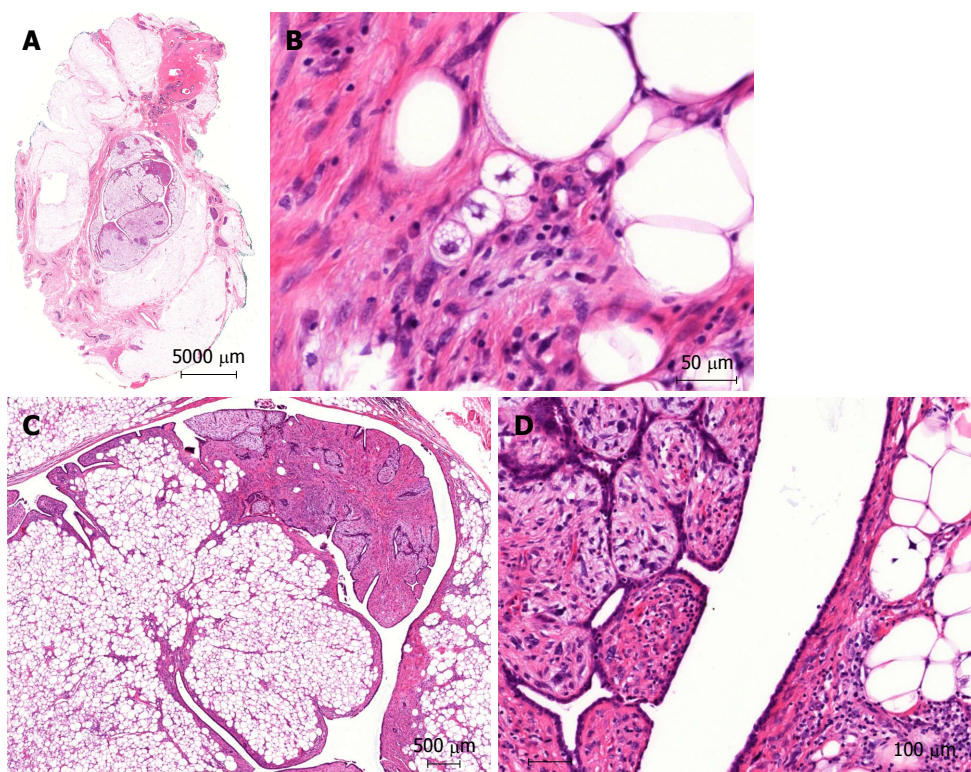
Given the rarity and heterogeneity of the disease, there are no prospective randomized trials on the surgical



**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<sup>[14]</sup> 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<sup>[15,16]</sup>. Since an adequate resection margin is the most important prognostic factor, negative margins are crucial for long-term survival<sup>[8,17]</sup>. Given clear margins of resection, survival rates after mastectomy and breast conserving surgery are similar<sup>[18]</sup>. 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<sup>[14,19,20]</sup>. In the retrospective analysis, Shabahang *et al.*<sup>[21]</sup> 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<sup>[9,19]</sup> while others reported no benefit of radiotherapy<sup>[18,20,22,23]</sup>. 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<sup>[14]</sup>. 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<sup>[14]</sup>.

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 breast, 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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## 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 out the pathological diagnosis, and critically revised the intellectual content of the manuscript and gave it final approval.

**Institutional review board statement:** Approved by Institutional Review board of Maharashtra Institute of Medical Sciences and Research, Medical College, Latur.

**Informed consent statement:** As we are not disclosing the identity of the patient, it was not needed.

**Conflict-of-interest statement:** All authors clear that they have no any conflicts of interests to be declared.

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### Abstract

A 16-year-old man had a swelling 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 spindle 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, and neurogenic 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 achieve the correct pathological diagnosis.

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

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**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.

Rao A, Ingle SB, Rajurkar P, Goyal V, Dokrimare N. Malignant

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<sup>[1,2]</sup>.

## CASE REPORT

A 16-year-old man was admitted to YCR Hospital Latur, with a peanut-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 sensation in the right 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 extend into the medullary cavity. Congruency of the knee joint was well maintained (Figure 3).

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, 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<sub>2</sub>O<sub>2</sub> 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<sup>[3,4]</sup>. Fifty percent of the cases occur in patients with NF-1<sup>[5-7]</sup>, and they usually occur in a pre-existing neurofibroma.

The genesis of MPNSTs is associated with genetic mutations in *p53* and *p16* genes<sup>[8-10]</sup>. *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%<sup>[6]</sup>, with exceptional cases seen in infants<sup>[11]</sup>.

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

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<sup>[14]</sup>.

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

## COMMENTS

### Case characteristics

A 16-year-old man presented with a peanut-size swelling, when first noticed, which progressively increased to its present size of 10 cm × 8 cm, and intermittent pain in the right proximal tibia and a tingling sensation in the right 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

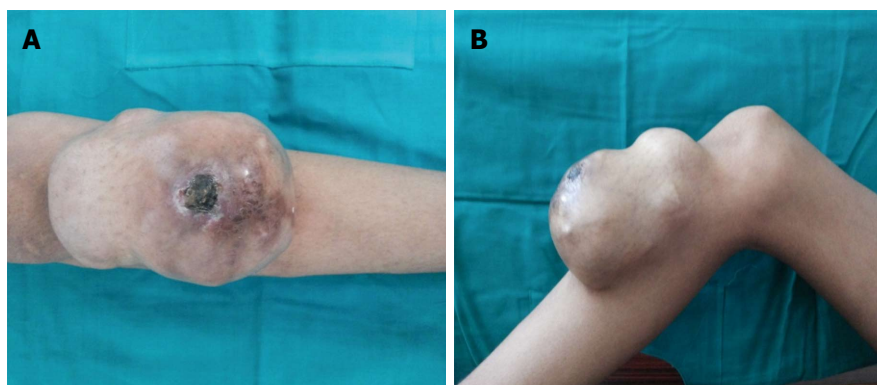


Figure 1 Preoperative clinical photographs (A and B).

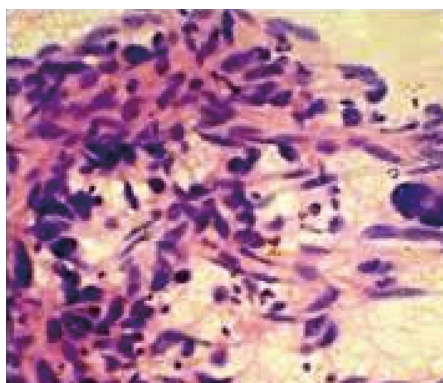


Figure 2 Fine needle aspiration cytology showing loosely scattered malignant spindle cells.



Figure 3 Preoperative X-ray.



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



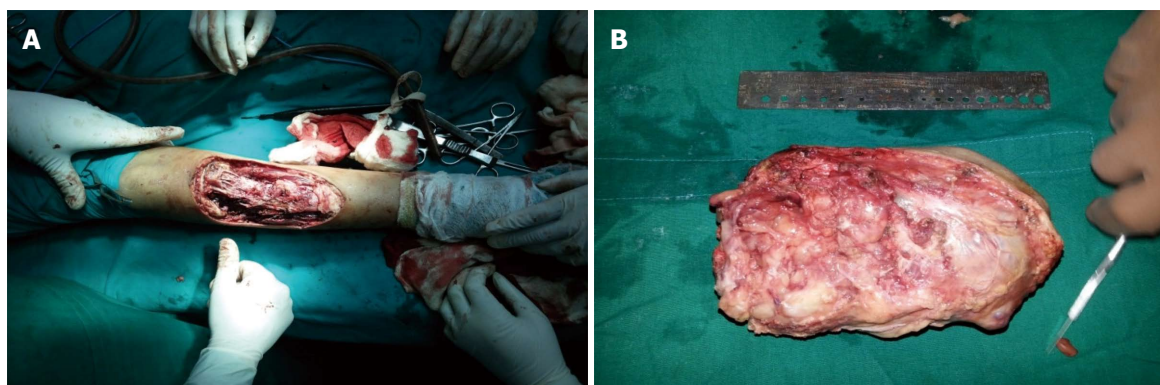


Figure 5 Intraoperative photograph showing excised mass (15 cm × 8 cm × 4 cm) (A and B).

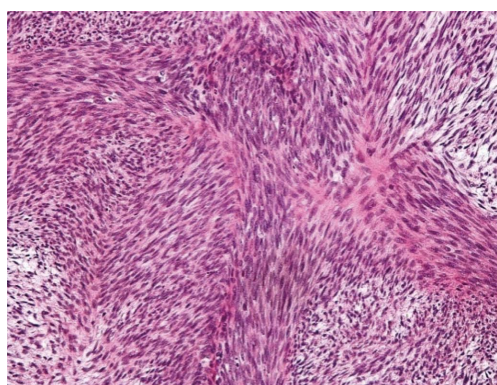


Figure 6 Malignant peripheral nerve sheath tumor on microscopy (LP 10 ×).

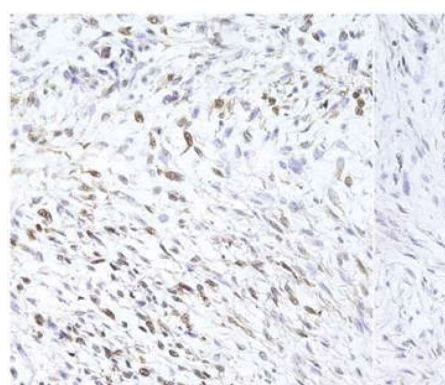


Figure 7 S-100 immunopositive tumor cells.

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 did not extend into the medullary cavity; congruency of the knee joint was well 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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## Current dichotomy between traditional molecular biological and omic research in cancer biology and pharmacology

William C Reinhold

William C Reinhold, Developmental Therapeutics Branch, Center for Cancer Research, National Cancer Institute, National Institutes of Health, Bethesda, MD 20892, United States

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### Abstract

There is currently a split within the cancer research community between traditional molecular biological hypothesis-driven and the more recent "omic" forms or research. While the molecular biological approach

employs the tried and true single alteration-single response formulations of experimentation, the omic employs broad-based assay or sample collection approaches that generate large volumes of data. How to integrate the benefits of these two approaches in an efficient and productive fashion remains an outstanding issue. Ideally, one would merge the understandability, exactness, simplicity, and testability of the molecular biological approach, with the larger amounts of data, simultaneous consideration of multiple alterations, consideration of genes both of known interest along with the novel, cross-sample comparisons among cell lines and patient samples, and consideration of directed questions while simultaneously gaining exposure to the novel provided by the omic approach. While at the current time integration of the two disciplines remains problematic, attempts to do so are ongoing, and will be necessary for the understanding of the large cell line screens including the Developmental Therapeutics Program's NCI-60, the Broad Institute's Cancer Cell Line Encyclopedia, and the Wellcome Trust Sanger Institute's Cancer Genome Project, as well as the the Cancer Genome Atlas clinical samples project. Going forward there is significant benefit to be had from the integration of the molecular biological and the omic forms or research, with the desired goal being improved translational understanding and application.

**Key words:** Omic; Molecular biology; Pharmacology; Cancer; Integration

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**Core tip:** This editorial describes the current split in approach, required expertise, and interpretation between the traditional molecular biological field, and the more recent "omic" approaches to cancer biology and pharmacology. The advantages and limitations of each of these disciplines are discussed and contrasted, highlighting their opposing approaches and mentalities.

The necessity of their efficient integration for the purpose of interpreting both cell line and clinical sample data is argued, especially when trying to project translationally into the clinic.

Reinhold WC. Current dichotomy between traditional molecular biological and omic research in cancer biology and pharmacology. *World J Clin Oncol* 2015; 6(6): 184-188 Available from: URL: <http://www.wjcnct.com/2218-4333/full/v6/i6/184.htm> DOI: <http://dx.doi.org/10.5306/wjco.v6.i6.184>

## PROBLEM OF INTEGRATION OF TRADITIONAL MOLECULAR BIOLOGICAL VS "OMIC" RESEARCH

The integration of the traditional molecular biological hypothesis-driven approach with the more recent "omic" forms or research for the purpose of providing translational insight is perhaps the premiere problem for cancer researchers today. How one views these two disparate forms of data impacts both the design and interpretation of biological and molecular pharmacological studies, and subsequently their prospective translational application. However, the two disciplines are by their nature in many ways mirror image opposites of one another, each with their own culture, assumptions, and requirements of expertise. This divergence has in the past, and continues at present to be an impediment to their successful merging.

## MOLECULAR BIOLOGICAL APPROACH

The molecular biological approach to research has been dominant for years. It has provided innumerable contributions in the fields of biology, molecular biology, pharmacology, and cancer<sup>[1-3]</sup>. The mindset of those in the field is rooted in their training, in which questions are ideally distilled down to single alteration-single response formulations that are addressed at the bench experientially. This approach typically requires years of carefully constructed, sequential, narrowly focused studies to explore, confirm, or repudiate their hypothesis. Addressing questions in this fashion allows one to provide quantitative assessments regarding the influence of a specific change on an outcome. The advantages of this approach include understandability, exactness, simplicity, and testability. Typically, improved understanding of one aspect of a pathway also generates testable hypothesis regarding up or downstream events.

However, the use of isogenic systems to focus on specific responses also has important limitations. As molecular events typically occur within the context of pathways, influential events that might occur either upstream or downstream within the salient pathway are typically ignored. Of course, the more complex integration of influences from disparate pathways is

also left out. If some single or small number of cell lines is being used to carry out the tests, then the results may be specific for those cell lines used, and less informative in other settings with significant variation. As one tries to apply these results translationally, one immediately encounters the inherent limitation of patients not being isogenic systems. For this reason, to propose that understanding either a patients cancer, or predicting their pharmacological response in the clinic can be successfully done based on an one-gene or one-molecular change type of analysis is likely to provide at best transient insight and benefit, in addition to being the exception to the (more complex) rule. A specific example of this using a dominant molecular event is provided by the BRAF V600E mutation, which provides a useful indicator for efficacious response to vemurafenib in melanoma<sup>[4,5]</sup>. However, even this unusually robust indication is typically short-lived in its usefulness, as alterations in the tumors undergoing treatment with BRAF inhibitors limit its affective treatment window to some period of months, generally followed by recurrence, often at the same locations<sup>[6]</sup>.

## "OMIC" APPROACH

The omic approaches to research, meanwhile, have their own set of advantages and disadvantages. On the positive side, data generated using technology such as array comparative genomic hybridization, transcript microarrays, mass spectrophotometrical proteomic analysis, exome sequencing, cell line screens, or broad spectrum patient sample compendiums view things in the broader context<sup>[7-13]</sup>. These more inclusive approaches provide the advantage of generating much larger amounts of usable data, allowing the consideration of multiple alterations simultaneously, both in those genes that one might expect to be altered, as well as in those whose involvement is completely unexpected. When the studies include multiple cell lines or patient samples, they also allow cross-sample comparisons to be made. This, of course, allows one to ask directed questions, while simultaneously making novel and potentially important discoveries and observations in totally unexpected areas. A single well-designed omic project can and does typically yield multiple potentially important observations and hypothesis, due to the large amount of data generated.

Unfortunately, there are multiple disadvantages inherent in these approaches as well. By their nature, the omic forms of data necessitate new forms of expertise just to process, and provide basic interpretation and access. These forms of expertise include computer science, statistics, mathematics, and more recently, bioinformatics. When added to the need to understand the results in the context of biology, including the detailed implications of the specific molecular alterations found, both the individual researcher as well the field in general are presented with the need for combinations of cross-disciplinary expertise that are rarely found. In the design

and implementation phase of studies, significant care needs to be taken with issues of quality control and reproducibility. This is necessary to assure the ability to meaningfully compare and interpret data across numerous samples harvested at different times, either from the clinic or cell lines. What cell line or clinical sample to select, their number, how they are handled, and what assay types to perform are all central considerations. For pharmacological studies, which compounds or drugs are selected, their number and type, the conditions under which they are used, and assay type are all key. Once the data assessment and interpretation phase is entered, there are multiple algorithmic approaches that may be used, with their choice being influenced by the data type, the question being asked, and the expertise and biases of the researcher. Correlations, linear regressions, classical statistics, information-theoretic algorithms, and machine learning all have contributions to make in the handling and interpretation of this data<sup>[4,14-20]</sup>. Additional complexity is then added as multiple forms of data are integrated<sup>[21-26]</sup>. Finally, algorithmic integration of biological knowledge into the mathematical approach is likely necessary, although this field is in its infancy<sup>[27,28]</sup>.

## MOVING FORWARD WITH THE INTEGRATION OF TRADITIONAL MOLECULAR BIOLOGICAL VS “OMIC” RESEARCH

So at the current time, integration of the molecular biological and omic disciplines is problematic. Increasing that tension is that the research community as it is constituted today, both at the bench and editorially, is dominated by those with traditional molecular biological training and understanding. This has led to some reluctance to either accept or understand the omic forms of research. It has even been proposed that the large-scale omic projects are jeopardizing progress in traditional molecular biology due to competition for the research dollar<sup>[29]</sup>.

However, attempts are ongoing throughout the research community to better interpret, integrate, and apply both these forms of data simultaneously<sup>[30]</sup>. Success may be had by starting with experimental data, and expanding its interpretation by overlaying omic data. This was done in the study of the effect of DNA methylation on E-cadherin expression using standard experimental approaches, and then assessing its influence in the context of multiple regulatory parameters using omic data<sup>[31,32]</sup>. Conversely, one may start with omic data, and verify its implications with standard experimental approach. This was done with the omic observation that SLFN11 transcript levels had a strong correlation to several drug activities, followed by the use of experimental approaches to prove its causality<sup>[33,34]</sup>.

Both the molecular biological and omic forms of research will be necessary in order to interpret the

results of the large cell line screens, including the Developmental Therapeutics Program's NCI-60, the Broad Institute's Cancer Cell Line Encyclopedia, and the Wellcome Trust Sanger Institute's Cancer Genome Project<sup>[10-12]</sup>. These screens are designed to provide the omic basis for improving the understanding of molecular pharmacology in cancer from the cell line level. Omic analysis has already provided multiple potentially important associations from these databases, including: (1) the association of MEK inhibitor efficacy with AHR expression in NRAS mutant cell lines; (2) a potential affect on the MET inhibitor PHA665752 by amplifications in MET; (3) sensitivity to PARP inhibitors in EWS-FLI1 translocation-containing cells; and (4) the activities of the DNA-damaging bleomycin, zorbaromycin, and peplomycin with ATAD5 mutations<sup>[4,5,35,36]</sup>. All of these omic associations will require traditional molecular biological experimental follow-up to verify or disprove whether they are causal. All insights gained from both the molecular pharmacological and omic approaches will be both useful and necessary for understanding the cells phenotypic differences and establishing a solid basis for drawing inferences. The cell line screens will certainly continue to provide hypotheses and useful study cases going forward. An example of this is the melanoma line LOXIMVI, which while containing the well studied BRAF V600E mutation, still has reduced sensitivity to vemurafenib when compared to the other cell lines containing the mutation, and is thus a potentially useful study case for patient relapse or resistance to that drug.

As one projects to patient samples, such as those found in The Cancer Genome Atlas (TCGA) both molecular biological and omic forms of research will again be necessary as one attempts to provide interpretation<sup>[13]</sup>. TCGA is designed to provide a base for omic analysis of clinical samples, providing data on some about 9939 patients from 33 cancer types. It provides both molecular and patient therapeutic information. Omic analysis of this data has already provided multiple potentially important associations, including: (1) targets for pharmacological intervention in squamous cell cancer including FAT1, MLL2, TGFBR2, HLA-A, and NFE212; (2) a potentially clinically relevant association between elevated levels of CX43 in glioblastoma tumor samples and temozolomide resistance; and (3) multiple FDA-approved drug targets of metabolic vulnerabilities<sup>[37-39]</sup>. As was the case for the cell line screens, these omic associations will require traditional experimental follow-up to verify or disprove their causality.

Going forward, considering the daunting set of challenges facing the researcher, it should be clear that all insights derived from both the traditional molecular biological and omic approaches will be both desirable and necessary to make sense of the complex and overlapping challenges that exist. As progress is made in these areas, one hopes that making patient treatment decisions based on that patient's complex



molecular profile will become the norm. An integrated vision for the molecular biological and omic approaches will be helpful if not necessary to that end.

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## Current role of spacers for prostate cancer radiotherapy

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### Abstract

Radiotherapy is an established curative treatment method for prostate cancer. Optimal tumor control rates can only be achieved with high local doses, associated with a considerable risk of rectal toxicity. Apart from already widely adapted technical advances, as intensity-modulated radiation therapy, the application of spacers placed between the prostate and rectum has been increasingly used in the last years. Biodegradable

spacers, including hydrogel, hyaluronic acid, collagen or an implantable balloon, can be injected or inserted in a short procedure under transrectal ultrasound guidance *via* a transperineal approach. A distance of about 1.0-1.5 cm is usually achieved between the rectum and prostate, excluding the rectal wall from the high isodoses. Several studies have shown well tolerated injection procedures and treatments. Apart from considerable reduction of rectal irradiation, a prospective randomized trial demonstrated a reduction of rectal toxicity after hydrogel injection in men undergoing prostate image-guided intensity-modulated radiation therapy. The results are encouraging for continuing evaluation in dose escalation, hypofractionation, stereotactic radiotherapy or re-irradiation trials in the future.

**Key words:** External-beam radiotherapy; Intensity-modulated radiotherapy; Brachytherapy; Spacer; Hydrogel; Biodegradable balloon; Hyaluronic acid; Collagen; Prostate cancer; Toxicity

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**Core tip:** Radiotherapy is widely used for the treatment of prostate cancer. Technical advances allow improved tumor control with increasing prescription doses, but rectal wall is known to be a dose-limiting organ. A new method that has been increasingly used in the last years is the application of a biodegradable spacer to increase the distance between the prostate and rectal wall. Clinical studies, including a prospective randomized trial, have reported considerable dosimetric advantages for the rectum, well tolerated insertion procedures and radiotherapy treatments.

Pinkawa M. Current role of spacers for prostate cancer radiotherapy. *World J Clin Oncol* 2015; 6(6): 189-193 Available from: URL: <http://www.wjgnet.com/2218-4333/full/v6/i6/189.htm> DOI: <http://dx.doi.org/10.5306/wjco.v6.i6.189>

## INTRODUCTION

Radiotherapy is an established curative treatment method for prostate cancer. Prospective randomized trials evaluating dose escalation have consistently shown significantly higher biochemical control rates for higher doses. However, significantly higher rectal toxicity rates resulted<sup>[1]</sup>. Rectal toxicity is regarded as the dose-limiting toxicity<sup>[2]</sup>. Rectal toxicity has been evaluated in a large number of studies and dose-volume correlations have been clearly established<sup>[3-5]</sup>. Apart from higher dose and larger volumes within specific isodoses, risk factors for toxicity after radiotherapy include history of prior abdominal surgery, advanced age, diabetes mellitus, concomitant use of androgen deprivation, hemorrhoids, and inflammatory bowel disease<sup>[6]</sup>.

Most of the randomized dose escalation studies applied three-dimensional conformal techniques<sup>[1]</sup>. Several further technical advances have been introduced in the last years. Intensity-modulated radiotherapy (IMRT) techniques, currently regarded as a standard for prostate cancer treatment in an increasing number of radiation oncology departments, result in improved dose conformity<sup>[7]</sup>. Image-guided radiotherapy (IGRT) is applied to show the prostate position before or even during each fraction, so that treatment margins and volumes can be reduced<sup>[8]</sup>. Using these techniques, new concepts as hypofractionated treatments or even stereotactic radiotherapy treatments have been introduced in the past, resulting in a considerable shortening of the external beam radiotherapy (EBRT) treatment period<sup>[9]</sup>.

As the prostate is usually situated without a relevant distance to the rectal wall and EBRT requires safety margins around the prostate of about 4-10 mm (depending on several factors as patient positioning and IGRT method), the anterior rectal wall is always included in the planning target volume and thus the prescription isodose. The insertion of a spacer between the prostate and rectum is an increasingly used method to create a considerable distance between the prostate and rectum and thus exclude the rectum from the high dose volume. A high dose can be delivered safely with adequate margins<sup>[10]</sup>.

## OPTIONS

Requirements for a spacer are a well tolerated insertion, a stable position during up to two months of radiotherapy and biodegradation. A spacer should not be allergenic or toxic. Studies in prostate cancer patients evaluated the effects of hyaluronic acid, human collagen, inflatable balloon or hydrogel as different spacer materials<sup>[11-14]</sup>. Hyaluronic acid is a natural polysaccharide component in connective tissue and extracellular matrix<sup>[15]</sup>. Human collagen is known from injections into the perineum to treat urinary incontinence<sup>[16]</sup>. An inflatable biodegradable balloon (PLCL, polylactide-co-ε-caprolactone) has been specifically introduced to be used as a spacer<sup>[17]</sup>. In the

past absorbable polyethylene glycol (PEG) hydrogels have been applied in surgical procedures as lung, dural and vascular sealants<sup>[14]</sup>. Hydrogels are injected as liquids and polymerize in situ within < 10 s following the mixture of two precursor solutions.

A transperineal approach with transrectal ultrasound (TRUS) guidance is used for spacer implantation/injection under local, spinal or light general anaesthesia. The actually selected anaesthesia will be chosen depending on the procedures planned (length and depth needed for incision, gold marker implantation, brachytherapy) and the respective local protocol. The approach is well known from prostate brachytherapy or gold marker implantation for IGRT. A needle is placed about 1-2cm anteriorly to the TRUS probe and forwarded to the prostatic apex. Prior hydrodissection facilitates spacer insertion. The spacer must be positioned between the rectal wall and the Denonvilliers' fascia<sup>[18]</sup>. In a series including 243 prostatectomy specimens, Villers *et al*<sup>[19]</sup> reported that prostate cancer invaded Denonvilliers' fascia in 19% of cases, but no patients presented a tumour progression through the full thickness of the fascia. Thus, the risk of tumour cell displacement can be regarded to be minimal.

Prada *et al*<sup>[13]</sup> performed hyaluronic acid injections without hydrodissection. Hydrogel or human collagen are injected following prior hydrodissection - the same-18 gauge spinal needle is used for hydrodissection and spacer injection<sup>[12,18]</sup>. Injection of fluid spacers is less invasive in comparison to the balloon implantation. However, a balloon can be deflated and repositioned if required.

An incision of 3-5 mm is required before implantation of a biodegradable balloon. The incision allows the dilatator and the introducer sheath to be inserted into the perineum. The dilatator is advanced towards the prostate base over the needle and the needle removed subsequently. The introducer sheath acts as a working channel for the introduction of the balloon. The balloon is filled with warm saline and sealed with a biodegradable plug following a full inflation<sup>[11,20]</sup>.

## TREATMENT PLANNING

As demonstrated in several studies, the injection or insertion of a spacer results in a distance of about 1.0-1.5 cm between the prostate and rectum, so that the rectal wall and planning target volume do not overlap<sup>[11-13,21]</sup>. The largest study included 100 patients after hydrogel injection<sup>[22]</sup>. A higher injected volume can result in a larger separation. Hydrogel is usually inserted in standardized 10-15 mL systems<sup>[14,22]</sup>. Comparably to the hydrogel studies, a mean separation of 12.7 mm was achieved with 20 mL human collagen injections in a pilot study<sup>[12]</sup>. The inflation of a balloon with nearly 20 mL of saline can result in mean prostate-rectum separation > 1.5 cm<sup>[11]</sup>. Though different injection volumes have not been compared in studies, an increasing volume can be potentially associated with toxicity related to



the pressure on the rectal wall or even the prostate. A volume of 10-15 mL and a resulting distance of 1.0-1.5 cm appear to be very effective and well tolerable for the patients<sup>[11-14,22]</sup>.

This separation results in a considerable dosimetric advantage for the rectum. In EBRT studies, relative rectal wall volume reductions of > 70% within the 90% isodose levels have been shown comparing treatment plans prior and following spacer insertion, *i.e.*, only < 5% of rectal volume is included in the 70Gy isodose when a prescription dose of 78-79Gy is used<sup>[11,15,21]</sup>. Guidelines recommend to limit this volume to 20%<sup>[23]</sup>, so that these recommendations can be met without problems. Thus, to reach an optimal dose distribution, treatment planning after spacer insertion must include much lower objectives for the rectum. The information from the dose-volume histogram indicates a low risk of rectal toxicity. On the other hand, it implicates the potential for safe delivery of new hypofractionated and stereotactic treatments or re-irradiation concepts without a relevant risk of higher grade rectal toxicity.

## CLINICAL EXPERIENCE

Spacer studies have been reported after several different treatment concepts, as low-dose rate<sup>[24]</sup> and high-dose rate (HDR) brachytherapy<sup>[22,25]</sup> with or without additional EBRT, hypofractionated EBRT concepts<sup>[26]</sup> or proton and heavy ion concepts<sup>[27,28]</sup>. Rare spacer-related complications have been reported in the literature, as focal rectal necrosis or ulceration as a result of unintentional injection of hydrogel into the rectal wall or urinary retention, usually resolving within a short time<sup>[14]</sup>.

Vanneste *et al*<sup>[29]</sup> calculated the cost-effectiveness of treating prostate cancer patients with and without a spacer, using a decision-analytic Markov model. According to the Dutch health costs, the spacer was found to be cost-effective for prostate cancer patients due to less severe toxicity and a reduction in treatment costs associated with side effects.

Taking into account a lack of long-term clinical experience with spacers, radiobiological models can be used to estimate long-term toxicity. They correlate prior data from the treatment plan and long-term toxicity<sup>[30]</sup>. Mean normal tissue complication probability (NTCP) for severe proctitis, necrosis, fistula or  $\geq$  grade 2 rectal bleeding was found to be reduced by  $\geq$  50% comparing data before vs after hydrogel spacer injection. A clear advantage was shown for conventional and IMRT techniques<sup>[31]</sup>.

The vast majority of published clinical studies have used hyaluronic acid or hydrogel. Studies with hyaluronic acid included smaller patient groups. Prada *et al*<sup>[25]</sup> did not observe grade 2 or higher toxicity after HDR brachytherapy as monotherapy (single 19Gy fraction) in an analysis of 40 patients and a median follow-up of 18 mo. Chapet *et al*<sup>[26]</sup> reported the acute toxicity of a hypofractionated IMRT with 3.1Gy fractions up to 62Gy

total dose in 36 patients, without any grade 2 or higher toxicities.

PEG hydrogel stability during treatment has been shown in studies, so that a constant prostate-rectum separation can be expected<sup>[32]</sup>. Hydrogel starts to liquify about 3 mo after injection, is absorbed within about 6 mo and cleared *via* renal filtration. Prostate position variability is similar with or without hydrogel, so that IGRT is still required with a spacer to keep safety margins small. However, in contrast to patients without a spacer, larger posterior displacements were not found with a spacer<sup>[32]</sup>.

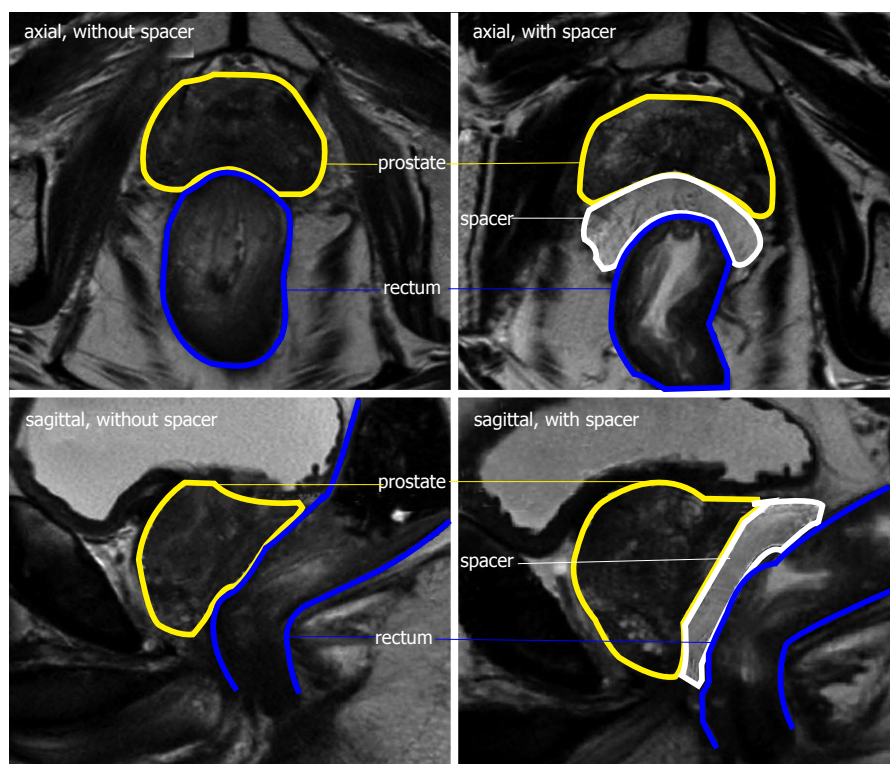
A learning curve has been reported in a study including 64 patients, showing an increasingly symmetrical hydrogel distribution and significantly larger prostate-rectum distances with the same hydrogel volume. As a consequence, an improved dosimetric rectum protection and smaller acute bowel quality of life changes resulted<sup>[10]</sup>.

Gastrointestinal toxicity (GI) was analyzed in a study including 48 patients in a multi-institutional prospective study. Grade 2 acute GI toxicity was reported in only 12% of patients (no grade 3-4 toxicity). Grade 1 late GI toxicity was found in 7% of patients within 12 mo after treatment (corresponding to two patients, one of them with grade 1 at baseline; no patients with grade 2-4 toxicity)<sup>[14]</sup>.

In a prospective randomized multicenter study 222 patients were randomized between a treatment with and without hydrogel (149 patients with and 73 without spacer). Patients were treated after fiducial marker placement (IGRT) with 1.8Gy fractions up to a total dose of 79.2Gy, using an IMRT technique. There were no device-related adverse events, rectal perforations, serious bleedings or infections within either groups<sup>[21]</sup>. Mean rectal volume within the 70Gy isodose was reduced from 12% to 3%. As also reported in a prior case control study<sup>[33]</sup>, similar acute rectal toxicity was observed in both patient groups. However, a significant reduction in late (3-15 mo) rectal toxicity in the spacer group was observed (2% vs 7%). There was no late rectal toxicity greater than grade 1 in the spacer group. At 15 mo, 12% and 21% of spacer and control patients experienced 10-point declines in bowel quality of life (EPIC questionnaire, Expanded Prostate Cancer Index Composite)<sup>[21]</sup>.

## CONCLUSION

The number of published studies reporting clinical data with spacer materials for prostate cancer radiotherapy is increasing. Hydrogel, hyaluronic acid, collagen or an implantable balloon, can be injected or inserted under transrectal ultrasound guidance. Most studies, including several studies with more than 50 patients treated with a spacer and a recently published prospective randomized study, evaluate the effects of a hydrogel spacer (Figure 1). A distance of about 1.0-1.5 cm is usually achieved between the prostate and rectum,



**Figure 1** T2 weighted magnetic resonance imaging without (left) and with (right) a hydrogel spacer. Spacer hyperintense, resulting in > 1 cm separation between prostate and rectum.

excluding the rectal wall from high isodoses. Procedure or spacer related complications are rare and treatments well tolerated. Reduced late toxicity rates have been shown in a prospective randomized study. Long-term results with a follow-up > 2 years are not available yet. Presently available results are encouraging for the design of further clinical studies.

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## Neuroendocrine tumors resistant to mammalian target of rapamycin inhibitors: A difficult conversion from biology to the clinic

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### Abstract

Deregulation of the phosphoinositide 3-kinase (PI3K)/protein kinase B (Akt) - mammalian target of rapamycin (mTOR) signaling pathway is one of the most commonly-

involved pathways in tumorigenesis. It has also been reported as altered in neuroendocrine tumors (NETs). mTOR inhibitors used in clinical practice are derived from rapamycin, an anti-cancer agent also used as an immunosuppressor after organ transplantation. Everolimus and temsirolimus are the two rapamycin-derived mTOR inhibitors used in NETs. Notably everolimus has been approved in advanced progressive well/moderately-differentiated pancreatic NETs (pNETs). It inhibits specifically the mTORC1 subunit of mTOR, not interacting with mTORC2. Although everolimus produced a significant prolongation of progression-free survival a number of patients with pNETs do not benefit from the drug due to early or late progression. Two supposed mechanisms of resistance to mTOR inhibitors are Akt and PI3K activation, by means of mTORC2 and insulin growth factor (IGF) - IGF receptor signaling, respectively. BEZ235 is a multi-targeted inhibitor binding to PI3K, mTORC1 and mTORC2, therefore potentially turning off all the supposed molecular targets of resistance to everolimus. The two clinical trials designed in pNETs were stopped early due to unmet statistical endpoint and the global clinical development of BEZ235 was also halted. Tolerability of this drug was challenging and conditioned the feasibility of therapy. The BEZ experience is an example of the huge difference between the preclinical and clinical setting and prompts us to pay more attention to the phase I step of clinical development and the design of phase II clinical trials.

**Key words:** Everolimus; BEZ235; Mammalian target of rapamycin; Phosphoinositide 3-kinase; Mammalian target of rapamycin C; Resistance; Mammalian target of rapamycin inhibitor

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**Core tip:** Although everolimus significantly prolongs progression-free survival in patients with advanced



pancreatic neuroendocrine tumors (NETs), some patients are refractory or progress early after an initial response. Mammalian target of rapamycin (mTOR) C2 and insulin growth factor (IGF) - IGF receptor signaling can mediate two supposed mechanisms of resistance to everolimus. BEZ235 is a multitargeted inhibitor binding to phosphoinositide 3-kinase, mTORC1 and mTORC2, therefore potentially turning off all the supposed molecular targets of resistance to everolimus. The two clinical trials designed in pancreatic NETs were stopped early due to unmet statistical endpoint and the global clinical development of BEZ235 was halted. Challenging tolerability probably conditioned the results. The BEZ experience is an example of the huge difference between preclinical and clinical setting and prompts us to pay more attention to the phase I step of clinical development and the design of higher-phase trials.

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## MAMMALIAN TARGET OF RAPAMYCIN PATHWAY

The mammalian target of rapamycin (mTOR) is a sort of intracellular metabolic switch, that physiologically regulates growth, proliferation and survival of normal cells integrating growth factors and nutrient signals<sup>[1]</sup>. It is an intracellular serine/threonine kinase activated by two main upstream factors, namely phosphoinositide 3-kinase (PI3K) and protein kinase B (Akt). This in turn activates downstream factors, including the ribosomal protein S6K and eukaryotic translation initiation factor 4E binding protein (4EBP-1). Based on the interaction of mTOR with other proteins, two functionally distinct subunits exist, mTORC1 and mTORC2; among others mTORC1 includes the regulatory-associated protein of mTOR, whereas mTORC2 includes the rapamycin-insensitive companion of mTOR. Activated mTORC1 activates in turn p70<sup>S6K</sup>, the kinase that phosphorylates the ribosomal protein S6, finally inducing protein synthesis. Activation of mTORC1 and S6K inhibits the tyrosine phosphorylation and signaling functions of insulin receptor substrates (IRS-1) through a negative feedback mechanism, resulting in the attenuation of PI3K-Akt signaling. Activated mTOR leads to protein synthesis also through 4EBP1 activation, inducing translation.

One of the main upstream stimulating factors of mTOR is the insulin-like growth factor (IGF) and its receptor (IGFR), activated by IRS-1; whereas phosphatase and tensin homolog, tuberous sclerosis complex and neurofibromatosis-1 factor are inhibitors of mTOR

signaling.

Deregulation of PI3K-Akt-mTOR signaling pathway is one of the most common mechanisms of tumorigenesis<sup>[2]</sup>. It has been reported as dysregulated also in neuroendocrine tumors (NETs), familial and sporadic<sup>[3,4]</sup>.

## mTOR INHIBITORS

The term mTOR derives from rapamycin, which is a macrolide, initially studied as an antifungal and antibiotic agent, known for its immunosuppressant activity, which has also demonstrated antitumor properties. Two derivatives of rapamycin have been used in NETs, everolimus and temsirolimus. Everolimus (RAD001) was approved by the FDA and EMA in progressing well-moderately differentiated pancreatic NETs (pNETs), based on the results of a randomized phase III study comparing it with placebo (RADIANT-3 trial).

## RESISTANCE TO mTOR INHIBITION

Some patients with pNETs show primary or secondary (acquired) resistance to everolimus. The precise mechanism is unknown, but some hypotheses have been postulated, including Akt activation by means of mTORC2 and IGF1/IGFR signaling activation due to inhibition of the S6K negative feedback<sup>[5,6]</sup>. On this basis, drugs inhibiting these supposed targets of resistance were preclinically studied.

## DUAL INHIBITOR BEZ235

BEZ235 is a potent oral multitargeted inhibitor of all four class I PI3K isoforms and the downstream effectors, mTORC1 and mTORC2<sup>[7]</sup>. In preclinical studies BEZ235 showed clearly higher activity than everolimus in NETs<sup>[8-10]</sup> and BEZ/everolimus combination was suggested as synergistic<sup>[8,9]</sup>. Furthermore BEZ235 reversed resistance to other anti-cancer therapies in a variety of tumor cell line<sup>[11-13]</sup>. However, conducting phase I studies with this agent has proved challenging. In the more than 200 patients treated, both by single agent and in combination, in several phase I / I b studies with different types of tumor, the formulation was changed moving from gelatine capsule to sachet<sup>[14]</sup>. Furthermore, the schedule was moved from QD (once per day) to BID (twice per day). High intra- and inter-patient pharmacokinetic variability was observed. In spite of these troubling premises, given the impressive preclinical activity, a world BEZ235 clinical development plan was launched for several types of tumor, including prostate, breast, renal cancers and pNETs. In pNETs two trials were designed with BEZ235 as single agent: One small multicentre phase II trial in pNETs resistant to everolimus and a large randomized phase II vs everolimus in pNETs not previously treated with mTOR inhibitors. Both were prematurely halted, the former after completion of the first stage with 30 patients

enrolled, due to unmet statistical endpoint, and the latter after randomization of 62 out of the 140 foreseen patients, due to unlikely superiority to everolimus at a first interim analysis of 35 patients<sup>[15]</sup>. In the phase II trial beyond everolimus the initial BEZ235 dose of 400 mg was amended to 300 mg due to intolerable toxicity. This agrees with a recently published phase I study of 33 patients with different types of malignancies who received BEZ235 administered twice daily as an oral sachet, where 300 mg BID resulted the recommended dose<sup>[14]</sup>.

The poor tolerability of BEZ235 negatively influenced both studies. Although its toxicity profile was confirmed without evidence of new toxicities, BEZ235 was less well tolerated than everolimus in the randomized study; furthermore in both studies a high percentage of adverse events led to frequent treatment discontinuation, in particular 39% in the BEZ arm of the randomized study (vs 16% for everolimus) and 36% in the phase II stage I study. Based on this experience, further clinical investigation of BEZ235 in cancer was halted.

## CONCLUSION

This is an example of the huge difference that sometimes exists between bench and bedside. Why would a drug such as BEZ235, which binds to the potentially correct targets for overcoming mTOR inhibition resistance and which is highly effective in preclinical investigations, meet with failure in clinical trials? A number of reasons may be advanced, both tumor-related and drug-related. Of course it is possible that the PI3K pathway is not the sole driver of resistance in pNETs and/or that the targets of BEZ235 do not represent the mechanism of activation of the PI3K pathway in some pNETs. However, the difficulty in managing the BEZ235 changeable toxicity in trials beyond phase I / I b strongly suggests that it is not only a matter of level of dose and therefore that the maximum tolerated dose concept is not suitable for all drugs. Other areas, including transportomics and metabolomics, which can strictly influence the tolerability of a drug, should be investigated. On the one hand, BEZ235 has a particular oral formulation susceptible to variable absorption while on the other hand its metabolism depends on CYP3A4, CYP1A2 and aldehyde oxidase activity.

Finally, the BEZ235 experience has taught that multidisciplinary could be useful for planning an anti-cancer agent clinical investigation, with clinicians dedicated to the specific tumor area and with pharmacologists who should work in close collaboration with phase I trial clinical researchers.

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## Inorganic phosphate in the development and treatment of cancer: A Janus Bifrons?

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### Abstract

Inorganic phosphate (Pi) is an essential nutrient to living organisms. It is required as a component of the energy metabolism, kinase/phosphatase signaling and in the formation and function of lipids, carbohydrates

and nucleic acids and, at systemic level, it plays a key role for normal skeletal and dentin mineralization. Pi represents an abundant dietary element and its intestinal absorption is efficient, minimally regulated and typically extends to approximately 70%. Maintenance of proper Pi homeostasis is a critical event and serum Pi level is maintained within a narrow range through an elaborate network of humoral interactions and feedback loops involving intestine, kidney, parathyroid gland and bone, and depends on the activity of a number of hormones, including parathyroid hormone, 1,25-dihydroxy vitamin D, and fibroblast growth factor 23 as major regulators of Pi homeostasis. Notably, Pi intake seemingly continues to increase as a consequence of chronic high-phosphorus (P) diets deriving from the growing consumption of highly processed foods, especially restaurant meals, fast foods, and convenience foods. Several recent reports have generated significant associations between high-P intake or high-serum Pi concentration and morbidity and mortality. Many chronic diseases, including cardiovascular diseases, obesity and even cancer have been proposed to be associated with high-P intakes and high-serum Pi concentrations. On the other hand, there is also evidence that Pi can have antiproliferative effects on some cancer cell types, depending on cell status and genetic background and achieve additive cytotoxic effects when combined with doxorubicin, illustrating its potential for clinical applications and suggesting that up-regulating Pi levels at local sites for brief times, might contribute to the development of novel and cheap modalities for therapeutic intervention in some tumours. Overall, the influence of Pi on cell function and the possible relationship to cancer have to be fully understood and investigated further.

**Key words:** Calcium-phosphate nanoparticles; Inorganic phosphate; Cancer; High-phosphorus diets; Phosphorus intake; Doxorubicin; Combination therapy; Naturally occurring molecule; Osteosarcoma



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**Core tip:** Many chronic diseases, including cancer have been proposed to be associated with high-phosphorus intakes and high-serum inorganic phosphate (Pi) concentrations. On the other hand, there is also evidence that Pi can have antiproliferative effects on some cancer cell types, depending on cell status and genetic background and achieve additive cytotoxic effects when combined with doxorubicin, illustrating its potential for clinical applications and suggesting that up-regulating Pi levels at local sites for brief times, might contribute to the development of novel and cheap modalities for therapeutic intervention in some tumors, including triple-negative breast cancer and osteosarcoma.

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## INORGANIC PHOSPHATE AND CANCER

One of most important nutrients to living organisms is Inorganic phosphate (Pi). It is required in the ATP formation, kinase/phosphatase signalling and in the synthesis of lipids, carbohydrates and nucleic acids. Furthermore, it plays a key role for normal skeletal and dentin mineralization<sup>[1]</sup>.

Diet represents the main source of Pi intakes, its intestinal absorption is minimally regulated and typically extends to approximately 70%. To maintain Pi levels within a proper range, an elaborate network, including intestine, kidney, parathyroid gland and bone, is involved in a feedback control in which hormones as parathyroid hormone (PTH), 1,25-dihydroxy vitamin D, and fibroblast growth factor 23 (FGF-23) are major regulators of Pi homeostasis<sup>[2]</sup>.

Diets always richer in phosphorus, due to a highly processed food, especially restaurant meals, fast foods, and cheap foods, have increased Pi intake<sup>[3,4]</sup>.

For example, in the United States the consume of phosphorus daily in meals is typically around 1400 mg, as inorganic phosphate (Pi) salts or as a part of organic molecules, that is almost doubled compared to the adult recommended dietary allowance.

The kidney is one of the major regulators of Pi homeostasis and can increase or decrease its capacity to reabsorb Pi; the increased cumulative use of ingredients containing Pi in food processing is now being shown to be potential toxic when it exceeds nutrient needs.

Several recent studies have underlined the relationship between high-Pi intake/high-Pi serum concentration and morbidity and mortality<sup>[3,4]</sup>.

A variety of conditions and diseases, especially

cardiovascular diseases, has been spotted in individuals with high-Pi intakes, resulting from chronic high-Pi diets. Other chronic diseases, including type 2 diabetes mellitus, obesity and even cancer have also been proposed to be associated with high-Pi intakes and high-Pi serum concentrations<sup>[3-5]</sup>.

As far as the mechanisms by which high Pi concentrations are linked to tissue damage and/or possibly to influence tumour growth, they are not completely understood and could very likely include a mixture of cell autonomous as well as autocrine, paracrine, and/or endocrine signals.

In particular, although both PTH and FGF-23 are stimulated to decrease the post-meal serum Pi concentration rise, approximately 1 h through the interruption of renal Pi reabsorption, it is hypothesized that if cells are exposed to even a brief high-serum Pi concentration there could be some signal alterations in cell functions leading to negative effects. Moreover, the increase of serum levels of FGF-23 or PTH might be toxic to particular cell types<sup>[3,4,6]</sup>.

Numerous recent studies have reinforced a long-standing hypothesis that there could be a phosphate-sensing mechanism capable of detecting serum and local phosphate variations and of informing the body, the local environment or the individual cell<sup>[7,8]</sup>. Because of the fact that the intracellular environment is electronegative compared to the extracellular one, the Pi transit into the cell does not happen by simple diffusion, but is mediated by Na<sup>+</sup>-coupled Pi cotransporters, which is a regulated event<sup>[9]</sup>. In addition, Pi is coming out as an essential signalling molecule capable of modifying a lot of cellular functions by varying signal transduction pathways, gene expression and protein levels in many cell types<sup>[8,10-12]</sup>.

It has been shown that high tissue phosphate concentrations increase oxidative stress in endothelial cells<sup>[13]</sup>. In human vascular smooth muscle cells it has been demonstrated that inorganic phosphate has effects on cell cycle and apoptosis, as well as, in the same cells, the increase of phosphate levels influences cellular and matrix elements promoting calcification<sup>[14,15]</sup>.

Moreover, it has also been supposed that high inorganic phosphate value speeds up senescence process in mouse models<sup>[16]</sup>. Recent data have confirmed that diets with a high intake of Pi enlarge tumorigenesis in the two-stage skin carcinogenesis model and K-ras lung cancer model in mice<sup>[17,18]</sup>.

In addition, inorganic phosphate has been demonstrated to promote the activation of distinct pathways like ERK1/2 and Akt kinases, as well as it stimulates cell growth in specific cell types, such as preosteoblastic MC3T3-E1 cells, human lung cells, epidermal JB6 cells, proposing Pi as a mitogenic molecule in these cells<sup>[17-21]</sup>.

Recently, a large scale transcriptomics and proteomics research has evidenced that many pro-angiogenic genes and proteins are upregulated by raised Pi levels in preosteoblasts cells<sup>[22]</sup> as osteopontin (OPN), a secreted cytokine, and forkhead box protein C2 (FOXC2), a

forkhead box transcription factor, both proteins recently associated with tumour angiogenesis. Lately, it has been demonstrated that in cancer cells Pi encourages tube formation and endothelial cells migration in vitro if exposed to elevated extracellular Pi levels, with FOXC2 and OPN as possible proteins involved in this mechanism<sup>[23]</sup>.

Notably, the pro-tumors and proliferative effects of Pi are not possible to extend to all cell types, in fact, it has been related that in MO6-G3 odontoblast-like cells Pi induces apoptosis<sup>[23,24]</sup>.

Previously, in the last years, we published a succession of articles, in which the aim has been to study the effects of elevated Pi on human osteosarcoma cell line U2OS and to know possible molecular mechanisms involved<sup>[25-28]</sup>.

Initially, we demonstrated that inorganic phosphate inhibits cell growth and reduces aggressiveness of human osteosarcoma cell line U2OS, identifying adenylate cyclase, beta3 integrin, Rap1, ERK1/2 as proteins whose expression and function are influenced by Pi<sup>[25,26]</sup>.

Later on, we proved also that Pi is capable of increasing the sensibility of osteosarcoma cells to doxorubicin in a p53-dependent manner and through down-regulation of ERK1/2 pathways<sup>[27,28]</sup>.

More recently, we described initial evidence of a strong antiproliferative action of Pi in MDA-MB-231 cell line, an extremely aggressive human triple negative breast cancer model, enlarging the hypothesis of Pi as a novel signalling molecule capable of modifying the function and survival of specific cell types<sup>[11]</sup>.

As part of our continuing effort to extend the knowledge on the role of inorganic phosphate as a "naturally occurring molecule" acting also as a "sensitizer" to increase the therapeutic index of clinical antitumor drugs, in a current study we describe that Pi induces strongly sensitization to doxorubicin by apoptosis induction in MDA-MB-231. We also show that Pi increases doxorubicin-induced cytotoxicity and that this mechanism involves ERK1/2 and STAT3 down-regulation<sup>[29]</sup>.

It is important to underline that in our studies we use a very low doxorubicin dose (until 0.1  $\mu\text{mol/L}$ ) that it is known to be a bearable dose because related to minimal side effects in patients, thus suggesting the possible clinical relevance of this positive pharmacological interaction<sup>[30,31]</sup>.

Latterly, new drug delivery system, called Calcium-phosphate nanoparticles, has been built up. Moreover, it is important to remember that hydroxyapatite nanoparticles release inorganic phosphate and that its retention, most likely, modifies Pi concentration at local sites<sup>[32,33]</sup>.

Furthermore, phosphate is the richest anion in the intracellular environment, with a concentration of 100 mmol/L, so it is easy to find an increase of extracellular Pi as a consequence of cell death induced by chemotherapy.

Maintenance of Pi systemic levels remains a crucial point, because an increase of serum values, even if moderate, and polymorphisms in genes implicated in Pi

homeostasis may have effects on ageing process and lifetime<sup>[2]</sup>.

The quantities of inorganic phosphate continue to rise in the diet, in particular way in the western countries, and an increase of the morbidity and mortality in the exposed population has been linked to this habit<sup>[3,4]</sup>.

In Particular, it is known that diet is an environmental element which can be manipulated; it has important consequences on genomics and proteomics functions and it is strongly connected to cancer<sup>[34,35]</sup>.

Inorganic phosphate, as a common dietary element, might modify cells behaviour. However, the possibility that Pi can modify cell functions and its relationship to cancer have to be fully understood and investigated further<sup>[36,37]</sup>.

By the way, the findings that inorganic phosphate, a simple "naturally occurring molecule", can have antiproliferative actions on some cancer cell types, depending on cell status and genetic background (p53, estrogen receptors, caspases expression, etc.) and can increase cytotoxic effects when combined with doxorubicin, show its potential for clinical applications, suggesting that up-regulating Pi levels at local sites for brief times might contribute to the development of novel and cheap modalities for therapeutic intervention in some tumors, including triple-negative breast cancer and osteosarcoma.

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## Sentinel lymph node metastasis after neoadjuvant treatment in breast cancer: Any size matters?

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### Abstract

One of the advantages of neoadjuvant chemotherapy (NAC) treatments is its ability to convert patients who need a mastectomy in breast conservative surgery. NAC has also increased the conversion of node positive patients into node negative in around 40% allowing the use of sentinel node biopsy (SLN) in this setting. Timing of SLN biopsy after NAC has been a subject

of debate. In patients with clinically node negative before NAC, rates of success and false negative rates of SLN after NAC are similar to those in the adjuvant setting, so SLN after NAC in previous negative axilla has been incorporated in the staging of the axilla. More controversial is its use in patients with positive axillary nodes before NAC who convert to node negative after NAC. Several randomized studies have reported the identification rates and the false negative rates of the SLN after NAC, concordant in the importance of surgical technique. As there is an agreement in the abandon of the immunohistochemistry (IHC) for SLN in the adjuvant setting as SLN IHC detected metastasis appear to have no impact on overall survival, in patients with SLN after NAC the inclusion of isolated tumor cell (ITC) as positive nodes lowers the false negative rates of the technique, suggesting the importance of assessing the SLN by IHC after NAC and considering it as residual disease. Longer follow up is needed to determine the prognostic implications of ITC in the SLN after NAC.

**Key words:** Sentinel node; Metastasis; Neoadjuvant treatment; Breast cancer

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**Core tip:** One of the advantages of neoadjuvant chemotherapy treatment in breast cancer is to downstage positive axillary nodes to negative. Postneoadjuvant sentinel lymph node (SLN) has been increasingly used and randomized studies in patients with positive axillary nodes who convert to node negative have shown that false negative rates are highly influenced by the surgical technique. Information from these studies has shown that isolated tumor cells in the SLN, when considered as positive nodes, lower false negative rates. Whether any residual disease in the SLNs may have prognostic implications warrants further research.

Rubio IT. Sentinel lymph node metastasis after neoadjuvant



treatment in breast cancer: Any size matters? *World J Clin Oncol* 2015; 6(6): 202-206 Available from: URL: <http://www.wjgnet.com/2218-4333/full/v6/i6/202.htm> DOI: <http://dx.doi.org/10.5306/wjco.v6.i6.202>

## RATIONALE FOR NEOADJUVANT TREATMENT IN BREAST CANCER

Neoadjuvant chemotherapy (NAC) is an accepted treatment for locally advanced and early stage breast cancer as it has shown many advantages. It allows *in vivo* determination of an individual tumor's chemosensitivity, it reduces micrometastatic disease and it can downstage tumors, allowing for breast conserving surgery in previously ineligible patients for conserving surgery<sup>[1]</sup>. Randomized studies have reported rates of downstaging after NAC between 49%-94% and 20%-40% of patients achieve a complete pathologic response<sup>[2-6]</sup>.

There is clear evidence that NAC downstages positive axillary nodes in a proportion of patients. Early studies have shown that NAC can completely clear axillary metastases in approximately 23% of patients with locally advanced breast cancer<sup>[6]</sup>, rates that have increased to 40%-60% with the use of targeted therapies<sup>[7]</sup>. Axillary complete downstaging after NAC has been correlated with better prognosis and assessment of residual disease after NAC is important not only in determining the prognostic information but also in selecting candidates for further systemic and radiation therapy treatment<sup>[7,8]</sup>.

## SENTINEL LYMPH NODE AFTER NEOADJUVANT TREATMENT

Timing of sentinel lymph node (SLN) in breast cancer patients undergoing NAC has been subject of continuous debate. An advantage of performing SLN after NAC is a single surgery and that patients with downstaging axillary nodes after NAC may potentially spared an axillary lymph node dissection (ALND). Most authors have taken the position to do it after NAC<sup>[9-14]</sup>, and results from meta-analysis and prospective studies have reported a success rate of SLN identification after NAC of 90% and rates of false negative around 10.5%<sup>[9,10]</sup>. In patients with clinically negative axilla, rates of success and false negative rates are similar to those in the adjuvant setting, so SLN after NAC in previous negative axilla has been incorporated in the staging of the axilla<sup>[15]</sup>. Recently, in a population-based study of SLN before (980 patients) or after NAC (203 patients) in clinically node negative patients of the Netherlands Cancer Registry, the SNL identification rate was higher in the SLN pre NAC group vs after NAC (98% vs 95%;  $P = 0.032$ ). Significantly, a lower proportion of patients had a negative SNB pre NAC compared to after NAC. In 67% of patients with SNB after NAC no axillary treatment

was given, compared to 55% of the patients with SNB before NAC. The authors conclude that SNL after NAC appears to lower surgical procedures and can benefit patients with downstaging of the axilla from less axillary treatment<sup>[16]</sup>.

In those patients with clinically positive axilla previous to NAC, three recently published prospective studies, ACOSOG Z1071, SENTINA and SN FNAC have shown that SLN false negative rates are directly related to the technique, the number of SLNs excised and the size of the SLN metastases after NAC<sup>[17-19]</sup>. In the ACOSOG Z0071, in 525 women who met the eligibility criteria, the SLN identification rate was 92.5%. The use of dual technique (radioisotope and blue dye) and the excision of  $\geq 2$  SLNs lower the false negative rates to 10.8% and 12.8% respectively. Because the FN rate was higher than the pre-established 10%, additional analysis of factors that influences the FN rates should be assessed<sup>[17]</sup>. The SENTINA trial, a four arm prospective multicenter cohort study, included patients with SLN before NAC and after NAC. In 592 patients with clinically positive axillary nodes before NAC who downstaged to node negative after NAC underwent SLN biopsy plus ALND. In this group, FN rates dropped to 9.6% when  $\geq 2$  SLNs were removed and to 8.6% when the dual technique (blue + radioisotope) was used<sup>[18]</sup>. The third study, the SN FNAC study included 153 patients with biopsy proven positive axillary nodes before NAC. Rates of FN were 9.6% with an identification rate of 87.6%. Similarly to the other studies, when 2 or more SLNs were removed the FN dropped to 4.9% that improves significantly the FN rates compared to the previous studies. Interestingly, this study analyses the FN rates related to the inclusion or not of isolated tumor cell (ITC) in the SLN as positive or negative staging. In those patients where ypN0(i+) were considered negative nodes, the FN increased to 13.3%, indicating the importance of including any residual tumor burden in the SLN as a positive node<sup>[19]</sup>.

## MINIMAL SLN INVOLVEMENT IN THE SLN (ISOLATED TUMOR CELLS)

Since the introduction of the SLN, we have learnt that the more thoroughly examination of the SLNs has increased the detection of minimal metastasis in the SLNs. Traditionally, routine hematoxyline-eosine (H and E) staining has been used to identify lymph node metastasis, and with the introduction of immunohistochemistry (IHC) staining the detection of ITC has come into the scenario. Recent studies showed a 10% increased in detection of micrometastasis in the SLN when using more extensive examination<sup>[20]</sup>. The outcome of histopathological analysis has implications in the surgical and adjuvant treatment of breast cancer patients. Staging breast cancer relies heavily on the status of the lymph nodes and the 6<sup>th</sup> edition incorporated the ITCs and micrometastasis

**Table 1 False negative rates in the randomized trials of sentinel lymph node after neoadjuvant chemotherapy in patients with axillary metastasis before neoadjuvant chemotherapy**

	ACOSOG Z1071	SENTINA	FN SNAC
No. of patients	756	592	153
FNR with 1 SLN	31.5%	24.3%	18.2%
FNR with > 2 SLNs	12.6%	9.6%	4.9%
FNR with single tracer	20.3%	16%	16%
FNR with dual tracer	10.8%	8.6%	5.2%
FNR with N0(i+) as positive	8.7%	-	8.4%

FNR: False negative rates; SLN: Sentinel lymph node.

into their classification. As the size of SLN metastasis increases, the rate of non-SLN metastasis size also increased from around 4% in the ITC, to 5%-19% in the micrometastasis and around 50%-60% in the macrometastasis<sup>[20-22]</sup>.

The prognostic implications of minimal lymph node involvement (*i.e.*, isolated tumor cells, micrometastasis) in early breast cancer have been long debated. The impact of finding this minimal metastasis in the SLN in the adjuvant setting has been reported extensively with different outcomes due to the great variability in patient population, tumor characteristics, histology assessment and so on<sup>[22,23]</sup>. Even more, the significance of micrometastasis in patients with ALND seems to be worst than in patients with SLN, making more difficult to establish its real significance<sup>[24]</sup>. It is important to consider that the studies that reported improved disease free survival in patients with SLN micrometastasis or ITCs are the ones where the majority of patients receive systemic treatments, and in this can also influence how to manage the axilla surgically<sup>[22]</sup>.

To shed light to this subject, the ACOSOG Z10 trial with 5184 patients, showed that IHC detected metastasis in neither the SLN ( $P = 0.66$ ) nor bone marrow ( $P = 0.08$ ) were independent predictors of overall survival, although bone marrow status showed a strong trend on multivariate analysis. SLN IHC detected metastasis appear to have no impact on overall survival<sup>[25]</sup>, because in the Z0010 trial treatment decisions were not based in the IHC results, the significance of ITCs may be better determined. Since the report of this trial, in many centers IHC has been abandoned for the assessment of SLN in the adjuvant setting.

Despite the knowledge of the prognosis of minimal involvement of SLNs in the adjuvant setting, this cannot be extrapolate to the neoadjuvant setting and actually, there is no such studies in the NAC setting. Rates of positivity of non sentinel nodes with a micrometastasis in the SLN in patients with NAC have been reported to be between 12% to 50%<sup>[13-15]</sup> and the SLN is the only positive node in around 50% of cases, rates lower than the adjuvant setting<sup>[15]</sup>.

It is likely that micrometastasis in the SLN in patients after NAC has a different meaning than micrometastasis

in the SLN in adjuvant therapy. Micrometastasis or ITC in the SLN in NAC patients could represent the presence of minimal nodal disease pretreatment which did not respond to therapy or the remnants of macroscopic nodal disease which has had a partial response to the treatment and in this way it has been addressed in the 7<sup>th</sup> edition of the AJCC<sup>[26]</sup>, where ypN0(i+) is considered residual disease in the SLN. Maybe, the classification of ITC after NAC under N0 should be revised although follow up on these patients is required to assess the real prognostic value of the ITC after NAC.

The number of residual metastatic axillary nodes after NAC has been established as an important prognostic factor for disease free survival<sup>[6]</sup>. Axillary response after NAC is a better prognostic factor than response of the primary tumor<sup>[6,8,27]</sup>.

Because most of these studies included patients with ALND, ITC in the axillary nodes are not reported. But one of the most important finding of the SN FNAC trial is that metastasis in the SLN after NAC of any size influences the rate of FN results, so ITC in the SLN after NAC should be considered positive<sup>[19]</sup>. In the ACOSOG Z0071, SLNs were not examined by IHC and positive SLNs were defined as those with metastasis higher than 0.2 mm, so ITC when reported were considered as node negative<sup>[17]</sup>. Data from the trial presented at the San Antonio Breast cancer Conference suggested that FN rate could be improved when ITC were included in the analysis as positive nodes, in these cases, FN rates decreased to 8.7% (Table 1). Also, our group presented data at the Society of Surgical Oncology assessing the overall survival (OS) of patients depending on the response to NAC treatment. A SLN biopsy was performed in 118 patients (32.5%). Eleven (9.3%) patients had residual ITCs in the SLN. When analyzing OS by axillary response, patients with ypN0(i+) who had a clinically negative axilla at diagnosis (cN0) had similar OS than those with pathologic complete response in the axilla, while those with ypN0(i+) who had a clinically positive axilla before NAC treatment (cN+) had a worse OS. This results suggest the importance of the ITCs in the SLN after a proven axillary metastasis before NAC, although these results need to be regarded with caution as the number of patients with ypN0(i+) were low in our study<sup>[27]</sup>.

In conclusion, SLN after NAC in patients with biopsy proven positive axillary nodes before NAC is feasible and accurate when surgical technique is improved by excising 2 or more SLNs, and by using a dual technique. False negative rates can be lowered when considering ITCs as positive nodes, suggesting that any size of metastasis in the SLN after NAC is important. Further follow up on this group of patients is needed to know the prognostic implications of the ITCs in the SLN after NAC.

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## Microenvironment and endocrine resistance in breast cancer: Friend or foe?

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### Abstract

Breast cancer affects one in eight women around the world. Seventy five percent of these patients have tumors that are estrogen receptor positive and as a consequence receive endocrine therapy. However, about one third eventually develop resistance and cancer reappears. In the last decade our vision of cancer has evolved to consider it more of a tissue-related disease than a cell-centered one. This editorial argues that we are only starting to understand the role the tumor microenvironment plays in therapy resistance in breast cancer. The development of new therapeutic strategies that target the microenvironment will come when we clearly understand this extremely complicated scenario. As such, and as a scientific community, we have extremely challenging work ahead. We share our views regarding these matters.

**Key words:** Breast cancer; Tumor microenvironment; Endocrine resistance; Tamoxifen; Stroma; Estrogen receptor; Aromatase inhibitors; Cancer stem cells

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**Core tip:** Resistance to endocrine therapy in breast cancer is an important clinical problem that requires further insight to develop a solution. We here discuss a paradigm shift, where the interplay of the tumor cells with the microenvironment, and the role of cancer stem cells are discussed as key targets in the development of novel therapeutic strategies.

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## WHY DOES ENDOCRINE RESISTANCE DEVELOP?

Breast cancer is the most frequent cancer in women in the Western world and one of the main causes of death. Seventy five percent of breast cancer patients have estrogen receptor-alpha (ER $\alpha$ ) positive tumors and endocrine therapy is the adjuvant treatment of choice in this scenario. However, a high percentage of patients develop resistance and cancer reappears; up to one third of patients recur within 15 years of the initial diagnosis<sup>[1]</sup>. Resistance to endocrine therapy is considered as *de novo* when there is no primary response to treatment. However, when therapy is initially successful but cancer eventually recurs, endocrine resistance is considered as acquired<sup>[2]</sup>.

The most widely used endocrine therapy for breast cancer patients has been tamoxifen, a selective ER modulator. Tamoxifen was developed in the 70's and is considered the first targeted therapy for cancer, as it specifically targets the ER<sup>[3]</sup>. Other endocrine treatments include selective ER down modulators such as Fulvestrant and aromatase inhibitors like Letrozole and Anastrozole<sup>[4]</sup>.

A number of mechanisms have been proposed as responsible for inducing acquired tamoxifen resistance. In particular a great number of papers have historically dealt with alterations in growth factor receptor pathways, in particular the HER family of growth factor receptors, as the responsible for this phenomenon<sup>[5]</sup>. A quick search in PubMed while we are writing this editorial shows that when we use the key words "breast cancer and tamoxifen resistance" we find 1869 publications; if the word "growth factor" is added, the number is reduced to 503 and "Her-2" leads to 236. However, if we look into other plausible mechanisms very few papers are found: For example adding the word "microenvironment" leads to 17 citations, "inflammation" accounts for 4, "integrins" 4, "stroma" 15, "fibroblasts" 40 and "stem cells" leads to 40. However, when we look at the first publications related to these topics we find that the first paper listed in PubMed related to growth factors and tamoxifen resistance was published in 1988<sup>[6]</sup>, whereas "stem cells", for example, dates to 1985<sup>[7]</sup>. So evidently researchers have been thinking about other mechanisms but probably the means to carry out these investigations were not available, or very few researchers thought that mechanisms other than autocrine loops within the tumor cell population could be responsible for the progression of the disease. We know today, however, that tumors are not only composed of neoplastic cells themselves, but that other cell types and extracellular components are critical both to tumor progression and response to treatment<sup>[8]</sup>. Thus, considering these as key players in the development of endocrine resistance is critical. The following paragraphs aim at highlighting some of the main findings related

to endocrine resistance through mechanisms that need extensive research to lead us to the development of novel strategies for the treatment of breast cancer.

## INFLAMMATION AND ENDOCRINE RESISTANCE

A growing body of evidence supports the role of the immune system as a regulator of tumor development and dissemination. Infiltrating immune cells produce cytokines, proteinases, chemokines and growth factors that promote extracellular matrix remodeling and angiogenesis. In particular gene-profiling studies have linked inflammation related gene clusters to resistance in patients treated with tamoxifen<sup>[9]</sup> and the aromatase inhibitor Anastrozole<sup>[10]</sup>. Moreover, a number of cytokines have been associated to suppression of ER $\alpha$  in breast cancer cells such as TNF, IL1 $\beta$ , IL6 and amphiregulin<sup>[11]</sup>; ER negative tumors are associated to more aggressive and invasive phenotypes. Epithelial to mesenchymal transition can be induced by factors such as IL6 with the upregulation of stem-related transcription factors<sup>[12,13]</sup>. Moreover, increased IL6 serum levels are correlated with decreased response to endocrine therapy in breast cancer and poorer survival<sup>[14,15]</sup>. In the ER $\alpha$  negative scenario IL1 $\beta$  is correlated with increased invasiveness and poor prognosis<sup>[16]</sup>. In ER $\alpha$  expressing breast tumors, IL1 $\beta$  has been shown to activate ER's transcriptional activity<sup>[17,18]</sup> and to modulate the response to 4-OH-tamoxifen; in particular in the presence of IL1 $\beta$  tamoxifen acts as an agonist instead of an antagonist<sup>[19]</sup>. The SDF-1-CXCR4 axis has also been implicated in breast tumor progression<sup>[20,21]</sup>. CXCR4 overexpression is correlated with worse prognosis and decreased survival in both the ER positive and negative scenario<sup>[21,22]</sup>. Moreover, using MCF-7 cells, treatment with SDF-1 induced tamoxifen and fulvestrant resistance in cells overexpressing CXCR4<sup>[21]</sup>.

Tumor associated macrophages (TAMs) are associated to increased angiogenesis and survival<sup>[23]</sup>. Results from experimental models in mice suggest that TAMs are key players in the progression to metastasis<sup>[24]</sup>. Moreover, experiments suggest that TAMs produce estrogens that directly stimulate the proliferation of ER $\alpha$  positive breast cancer cells<sup>[25]</sup>. CD68, a macrophage marker, has been associated to increased recurrence suggesting that in ER positive breast cancers the presence of macrophages may lead to endocrine resistance<sup>[26]</sup>.

## STEM CELLS AND ENDOCRINE RESISTANCE

Cancer stem cells have gained attention in the last years as responsible for tumor progression and resistance to therapy<sup>[27]</sup>. Experiments carried out using human samples have clearly shown that both chemo

and radiotherapy increase the percentage of breast cancer stem cells in a neo-adjuvant setting<sup>[28]</sup>. In the context of endocrine therapy our results together with those of other groups strongly suggest that endocrine treatment leads to enrichment in breast cancer stem cells. Our working hypothesis, based on the literature and our results, is that breast cancer stem cells express reduced levels or no ER- $\alpha$ , and would thus not be efficiently targeted by endocrine treatment<sup>[29]</sup>. A study testing the effect of neo-adjuvant treatment in patients with Letrozole shows that it leads to enrichment in cells with mammosphere forming capacity<sup>[30]</sup>. Simões *et al.*<sup>[31]</sup> analyzed the impact of estrogen signaling on MCF-7 mammosphere forming capacity. They plated MCF-7 cells straight onto nonadherent plates and treated the suspension cultures with hormones finding that estradiol decreased, and 4-OH-tamoxifen increased mammosphere forming capacity. The same results were true for suspension cultures of primary human normal and tumor breast cell suspensions, where treatment with 4-OH-tamoxifen led to an increase in Nanog and Sox-2. Ao *et al.*<sup>[32]</sup> on the other hand treated suspension cultures with 4-OH-tamoxifen and then passaged the cells to media without antiestrogen (still in suspension) and found that under these conditions a greater amount of mammospheres were formed. We showed that tamoxifen selects for cells with stem cell properties in the human MCF-7 cells line, as well as in mouse LM05-E cells and the M05 tumor from which they derive<sup>[33]</sup>. Mammosphere assays revealed that pretreatment of either cell line with 4-OH-tamoxifen leads to an increase in cells with increased clonogenicity in suspension. Additionally, we analyzed the gene expression of transcription factors associated to pluripotency and found that they were increased both in the mammospheres and in cells growing on 2D treated with 4-OH-tamoxifen for 5 d. *In vivo* studies using the M05 tumor showed similar results with an increase in the amount of cells with mammosphere forming capacity in tumors derived from mice treated with tamoxifen containing pellets. These tumors were enriched in CD29<sup>h</sup>/CD24<sup>l</sup> cells, in comparison to the parental tumor. Additionally, when passaged to untreated mice, those tumors that derived from mice that had been previously exposed to tamoxifen generated "secondary tumors" that grew at a faster rate compared to controls, and had a higher capacity of giving rise to mammospheres as well as maintaining an increased CD29<sup>h</sup>/CD24<sup>l</sup> cell population. Finally, M05 tumor passages that had progressed to hormone independence had a higher amount of cells with mammosphere forming capacity supporting the notion that increased aggressiveness and endocrine independence are correlated with an increase in cells with stem cell properties<sup>[33]</sup>. These results, in conjunction, strongly suggest that breast cancer stem cells are involved in endocrine resistance.

## THE EXTRACELLULAR MATRIX, CANCER ASSOCIATED FIBROBLASTS AND ENDOCRINE RESISTANCE

Cancer associated fibroblasts have long been believed to play a key role in cancer progression<sup>[34]</sup>. In breast cancer in particular, several lines of evidence strongly suggest that they are vital in determining tumor progression and the outcome of therapy<sup>[35]</sup>. A seminal paper by Finak *et al.*<sup>[36]</sup> identified distinct stromal signatures that corresponded to good and poor-outcome breast cancers. They were able to identify a 26 gene predictor that forecasted disease outcome with higher precision than predictors or signatures derived from whole tissues. In this line of evidence, but in the context of endocrine resistance, an extracellular matrix gene cluster has been associated to prognosis and response to treatment<sup>[37]</sup>. In particular they found that fibronectin, lysyl oxidase, SPARC and TIMP3 expression levels were associated to the prognosis of patients with breast cancer whereas levels of tenascin C were associated to resistance to treatment with tamoxifen. A recent paper by Holton *et al.*<sup>[38]</sup> using 3D cultures and Fourier transform infrared spectroscopic imaging shows that fibroblasts induce epithelial to mesenchymal transition in cancer cells together with a downregulation in ER $\alpha$  levels. Our work also suggests that stromal factors modulate response to endocrine resistance. In particular we showed that conditioned media derived from carcinoma associated fibroblasts induced tamoxifen resistance in otherwise sensitive cells, using a mouse model of estrogen dependent breast cancer<sup>[39,40]</sup>. Moreover, we found that fibronectin, which is mostly produced by fibroblasts in breast tumors, induces resistance in both LM05-E and MCF-7 cells. This effect is accompanied by an induction in ER $\alpha$  phosphorylation at serine-118. Interestingly, high levels of phospho-serine-118 has been previously associated to endocrine resistance in breast cancer<sup>[41]</sup>.

## BACK TO THE BEGINNING

The examples above are just a snapshot of the recent findings regarding endocrine resistance and microenvironment. So is the microenvironment a friend or a foe in this context? Clearly I believe that we are just beginning to unravel the complexity of cancer and that evidently there is no single culprit to failure when it comes to treatment. However, we definitely need a lot of work to be carried out to understand exactly what the role of each player is in this complicated challenge. Moreover, the work that needs to be done is extremely delicate given that it implies analyzing and understanding how tumor and tumor "associated" cells behave in different contexts. This type of work is time consuming, needs to be carried out in different model systems and is very expensive. The challenge I believe

is patience. The other key point here is that researchers that study different types of cancer need to interact more and companies must be willing to openly share new chemicals when requested even though they are not being developed for the purpose the scientist asking is wanting to explore.

The road ahead is exciting and may result frustrating at times. We are just beginning to understand that the microenvironment plays a key role in endocrine resistance in breast cancer. The numbers in PubMed are clear evidence that we are in the dawn of our understanding in this matter. As the poet Robert Frost would say, we are taking the road less travelled, and that should make the difference.

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## Combination therapies improve the anticancer activities of retinoids in neuroblastoma

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### Abstract

Most therapeutic protocols for child cancers use cytotoxic agents which have a narrow therapeutic index, and resulting in severe acute and chronic toxicities to normal tissues. Despite the fact that most child cancer

patients achieve complete remission after chemotherapy, death still occurs due to relapse of persistent minimal residual disease (MRD) which remaining after initial cytotoxic chemotherapy. Advanced neuroblastoma (NB) is a leading cause of cancer deaths in young children. Retinoids are an important component of advanced NB therapy at the stage of MRD, yet half of all patients treated with 13-*cis*-retinoic acid still relapse and die. More effective combination therapies, with a lower side-effect profile, are required to improve outcomes for NB. Fenretinide or N-4-hydroxyphenyl retinamide is a synthetic derivative of retinoic acid which works on cancer cells through nuclear receptor-dependent and -independent signalling mechanisms. Moreover, several histone deacetylase inhibitors have entered early phase trials, and, suberoylanilide hydroxamic acid has been approved for use in adult cutaneous T cell lymphoma. A number of studies suggest that retinoid signal activation is necessary for histone deacetylase inhibitor activity. A better understanding of their mechanism of actions will lead to more evidence-based retinoid combination therapies.

**Key words:** Retinoids; Histone deacetylase inhibitors; Combination therapies; Neuroblastoma; Fenretinide

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**Core tip:** Neuroblastoma (NB) begins in embryonal neural crest cells, which later give rise to the sympathetic nervous system, and is caused in part by factors which arrest differentiation. *In vitro*, retinoids force susceptible cancer cells down a pathway of terminal differentiation and, have been part of the routine treatment of advanced NB for number of decades. Synergistic anti-tumour activity between histone deacetylase inhibitors and retinoids has been observed in a variety of preclinical models. This editorial note discusses some of these findings on the combination therapies for improving the anticancer activities of

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## INTRODUCTION

Neuroblastoma (NB) is a tumor of the sympathetic nervous system and the most common extracranial solid tumour in childhood<sup>[1]</sup>. NB accounts for more than 7% of malignancies in patients younger than 15 years and around 15% of all paediatric oncology deaths<sup>[2]</sup>. Some infants experience spontaneous regression, whereas older patients have maturation of their tumor into benign ganglioneuromas. However, the outcome for children with a high-risk clinical phenotype has improved only modestly, with long-term survival still less than 40%<sup>[3]</sup>. The introduction of 13-*cis*-retinoic acid (13-*cis*-RA) in the therapy of NB has improved the prognosis of this disease. Currently, the standard treatment for high risk of NB consists of myeloablative therapy followed by autologous hematopoietic stem cell transplantation and maintenance with 13-*cis*-RA for the treatment of minimal residual disease (MRD), leading to a 3-year disease-free survival rate of about 50%<sup>[4]</sup>. Retinoids are an important component of advanced NB therapy at the stage of MRD, yet half of all patients treated with 13-*cis*-retinoic acid still relapse.

### Retinoid therapy in paediatric cancer

Retinoids are vital for the growth and differentiation of a variety of normal adult and embryonic tissues, and have potent antiproliferative effects on many malignant cell types<sup>[5]</sup>. Retinoids mediate their widespread effects on cells by regulating the transcription of target genes through a complex system of ligand-inducible nuclear transcription factors: The retinoic acid receptors and retinoid X receptors<sup>[6]</sup>. RA exists in several stereoisomeric forms: Predominantly *all-trans* retinoic acid (ATRA) and 13-*cis*-RA, but also as less-stable isomers such as 9-*cis* retinoic acid. In the last few decades they have been widely studied in cancer prevention and therapy because of their ability to induce differentiation of tumor cells<sup>[7]</sup>. Retinoids are successfully used for the treatment of one pediatric cancer: Acute promyelocytic leukemia. ATRA converts the PML-RAR- $\alpha$  fusion protein into activator of transcription and restores cell differentiation<sup>[8]</sup>. Retinoids have also been widely investigated in solid tumors, especially in NB. In a long-term study for children with high-risk NB treated on a randomized trial of myeloablative therapy followed by 13-*cis*-RA, which given after intensive therapy resulted in significant improvement in 5-year overall survival rates, regardless

of the type of consolidation<sup>[9]</sup>. However, as many high-risk patients still ultimately die due to relapse of persistent MRD after initial cytotoxic chemotherapy, novel therapies effective against MDR NB are needed.

### Fenretinide is an effective retinoid therapy

Retinoids are vitamin A analogues required for normal morphogenesis and maintenance of diverse embryologic and adult tissues, which act on cells by binding nuclear receptors<sup>[10]</sup>. Fenretinide or N-4-hydroxyphenyl retinamide (4-HPR) is a synthetic derivative of retinoic acid which works on cancer cells through nuclear receptor-dependent and -independent signalling mechanisms<sup>[11]</sup>. 4-HPR has a broad spectrum of cytotoxic activity against primary tumor cells, cell lines, and/or xenografts of various cancers, including NB<sup>[11-13]</sup> and has been tested in early phase clinical trials in recurrent NB<sup>[14,15]</sup>. 4-HPR was anti-angiogenic in multiple tumour types and cytopathic in some cancer cells which were resistant to other retinoids or chemotherapeutics<sup>[13]</sup>. Clinical trials have revealed that 4-HPR is a highly active therapeutic and chemo-preventive agent with minimal side-effects in NB<sup>[14]</sup>. A phase I / II trial of oral 4-HPR in children with high-risk, relapsed solid tumours demonstrated minimal 4-HPR toxicity, but only stable disease as the best clinical response<sup>[16]</sup>.

### Combination therapy improves the anticancer activity of histone deacetylase inhibitors and retinoids

Increased histone deacetylase activity is a common causal factor in human cancer that causes transcriptional silencing of tumour suppressor genes<sup>[17]</sup>. Histone deacetylase inhibitors prevent deacetylases removing acetyl groups from histone tails, thereby promoting gene transcription<sup>[18]</sup>. Several histone deacetylase inhibitors have entered early phase trials, and, suberoylanilide hydroxamic acid (SAHA) has been approved for use in adult cutaneous T cell lymphoma<sup>[19]</sup>. The histone deacetylase inhibitor side-effect profile is low when compared with cytotoxic chemotherapy<sup>[20]</sup>. Moreover, two unbiased preclinical screens identified retinoid signal activation as the most effective method of augmenting the histone deacetylase inhibitor anti-cancer signal<sup>[21,22]</sup>. Retinoic acid receptor  $\alpha$  (RAR $\alpha$ ), and, preferentially expressed antigen of melanoma, both repressor proteins for the retinoid signal, was shown to mediate resistance to histone deacetylase inhibitors<sup>[21]</sup>. Furthermore, RAR $\alpha$ -deficient cells showed enhanced sensitivity to histone deacetylase inhibitors *in vitro* and *in vivo*<sup>[22]</sup>. These studies suggest that retinoid signal activation is necessary for histone deacetylase inhibitor activity. Hahn *et al*<sup>[23]</sup> used an HDAC inhibitor (valproic acid) as an enhancer to screen a small-molecule library for compounds inducing NB maturation, the top hit identified in the screen was *all-trans*-retinoic acid. These studies demonstrated that investigation of HDAC inhibitors and retinoids in combination are warranted to improve the anticancer activities in cancer.

### Combination therapies improve the anticancer activities of retinoids in NB

Synergistic anti-tumour activity between histone deacetylase inhibitors and retinoids has been observed in a variety of preclinical models<sup>[24,25]</sup>. A study suggested that the HDAC inhibitor LAQ824 has a greater antitumor activity in combination with 13-*cis*-retinoic acid in melanoma tumors<sup>[24]</sup>. Another study showed that the intracranial tumors in ND2:SmoA1 mice treated with retinoid acid + SAHA + cisplatin showed a 4-fold increase in apoptosis over controls, and a 2-fold increase over animals receiving only SAHA or retinoid acid + SAHA<sup>[25]</sup>. We and others have shown that retinoids combined with histone deacetylase inhibitors are synergistic<sup>[26,27]</sup>. However, SAHA combined with 13-*cis*-retinoic acid, was well-tolerated in a phase I / II paediatric trial, but the best response for relapsed solid tumour patients was stable disease<sup>[28]</sup>. Recently, our study showed that 4-HPR+SAHA as a more effective therapy for NB than 13-*cis*-RA alone or with SAHA<sup>[29]</sup>. The 4-HPR + SAHA combination induced caspase-dependent apoptosis through activation of caspase 3, reduced colony formation and cell migration *in vitro*, and tumorigenicity *in vivo*. The 4-HPR and SAHA combination significantly increased mRNA expression of thymosin-beta-4 (T $\beta$ 4) and decreased mRNA expression of RAR $\alpha$ . Importantly, the up-regulation of T $\beta$ 4 and down-regulation of RAR $\alpha$  were both necessary for the 4-HPR + SAHA cytotoxic effect on NB cells. Moreover, T $\beta$ 4 knockdown in NB cells increased cell migration and blocked the effect of 4-HPR + SAHA on cell migration and focal adhesion formation<sup>[29]</sup>. This study demonstrates that T $\beta$ 4 is a novel therapeutic target in NB, and that 4-HPR and SAHA is a potential combination therapy for the disease.

### CONCLUSION

A therapeutic role for retinoids and HDAC inhibitors in several human cancer types, including NB, is well established. However, retinoids and HDAC inhibitors are not completely effective anti-cancer agents when used alone; thus, a better understanding of their mechanism of actions will lead to more evidence-based retinoid combination therapies. Because differentiation is aberrant in NB, compounds that modulate transcription and induce differentiation, such as HDAC inhibitors and retinoids, are of particular interest. Further studies to understand the mechanism of drug actions and the clinical trials with large cohort of patients to determine the efficacy of HDAC inhibitors and retinoids for patients with high-risk NB are warranted.

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## Direct therapeutic intervention for advanced pancreatic cancer

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### Abstract

Currently, chemotherapy is an accredited, standard treatment for unresectable, advanced pancreatic cancer (PC). However, it has been still showed treatment-resistance and followed dismal prognosis in many cases.

Therefore, some sort of new, additional treatments are needed for the better therapeutic results for advanced PC. According to the previous reports, it is obvious that interventional endoscopic ultrasonography (EUS) is a well-established, helpful and low-risky procedure in general. As the additional treatments of the conventional therapy for advanced PC, many therapeutic strategies, such as immunotherapies, molecular biological therapies, physiochemical therapies, radioactive therapies, using siRNA, using autophagy have been developing in recent years. Moreover, the efficacy of the other potential therapeutic targets for PC using EUS-fine needle injection, for example, intra-tumoral chemotherapeutic agents (paclitaxel, irinotecan), several ablative energies (radiofrequency ablation and cryothermal treatment, neodymium-doped yttrium aluminum garnet laser, high-intensity focused ultrasound), *etc.*, has already been showed in animal models. Delivering these promising treatments reliably inside tumor, interventional EUS may probably be indispensable existence for the treatment of locally advanced PC in near future.

**Key words:** Interventional endoscopic ultrasonography; Advanced pancreatic cancer; Radiofrequency ablation; Gemcitabine; Endoscopic ultrasonography guided-fine needle injection; Dendritic cells

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**Core tip:** Unresectable, advanced pancreatic cancer (PC) has been still showed treatment-resistance and followed dismal prognosis in many cases with conventional therapies. Therefore, some sort of new, additional treatments are needed for the better therapeutic results for advanced PC. In recent years, interventional endoscopic ultrasonography (EUS) has been developed, disseminated and used efficiently all over the world as indispensable therapeutic strategies for PC. Therapeutic trials by interventional EUS for advanced PC until now, and describe the possibilities and expectations of anti-

tumor therapy for advanced PC by interventional EUS to the future through this epoch-making deployment are summarized in this Editorial.

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## TEXT

In recent years, interventional endoscopic ultrasonography (EUS) has been developed, disseminated and used efficiently all over the world as indispensable therapeutic strategies for various diseases of digestive area, such as malignant tumors, drainage, pain relief and recurrent lesions. Besides, unresectable, advanced pancreatic cancer (PC) has been still showed treatment-resistance and followed dismal prognosis in many cases. Some sort of new, additional treatments are needed for the better therapeutic results. Because of the merit which approaches inside the pancreas directly through stomach or duodenum, interventional EUS may be a potential target of crucial treatment strategy. Different strategies of interventional EUS for advanced PC have been conducted, and will also be carried out in the future. We hope to summarize the therapeutic trials by interventional EUS for advanced PC until now, and describe the possibilities and expectations of anti-tumor therapy for advanced PC by interventional EUS to the future through this epoch-making deployment in this Editorial.

Since EUS techniques allow access to pancreas in a comparatively minimally invasive fashion, it is a feasible procedure for the potential of a targeted delivery of therapeutic agents for PC by fine needle injection (FNI) through gastric or duodenal wall. Hence, many therapeutic trials for advanced PC by EUS guided-FNI (EUS-FNI) with a curative intent have been conducted so far. EUS-FNI involves direct intra-tumoral delivery of anti-tumor agents under EUS guidance for local control of tumor growth in patients with unresectable PC. As opposed to systemic administration, direct treatment is able to effect the targeted lesion of cancer without many normal lesions. Therefore, the EUS-FNI technique offers theoretic potential to deliver high dose concentration while minimizing systemic side effects. In addition, immune-modulating cells such as mixed lymphocyte and dendritic cells (DCs) can also be injected into PC as a potential anti-tumor therapy. However these results were not fulfilled the expected level as well as conventional treatments for advanced PC.

Chang *et al.*<sup>[1]</sup> RFA conducted a phase I trial in which 8 patients with advanced PC were given intra-tumoral injections of activated allogenic mixed lymphocyte culture (cytoimplant) guided by EUS. In this report, no patient had treatment-related pancreatitis in the

procedures. However, the trial was suspended and final results have not been published. Irisawa *et al.*<sup>[2]</sup> reported a pilot study about EUS-FNI of immature DCs into advanced PC. In 7 patients with unresectable PC who previously failed a chemotherapeutic agent, gemcitabine. DCs are potent antigen-presenting cells which have ability to initiate CD4<sup>+</sup> helper and CD8<sup>+</sup> cytotoxic T lymphocytes (CTLs)-mediated anti-tumor immune responses<sup>[3]</sup>. In the report, injected immature DCs may intake apoptotic/necrotic pancreatic tumor cells and present tumor-associated antigenic peptides into MHC class I and II molecules on DCs, resulted in induction of antigen-specific CTLs. There were 3 partial responses (PR), 2 patients with stable disease (SD). Median survival was 9.9 mo without complication associated with EUS-FNI procedure. The results have not been achieved satisfactory level, however it is hopeful to publish the final results about the project. Apart from that, a combination therapy of chemotherapy (gemcitabine) with immunotherapy (OK-432-stimulated mature DCs) using EUS-FNI, followed by intravenous infusion of lymphokine-activated killer cells stimulated with anti-CD3 monoclonal antibody has reported<sup>[4]</sup>. In this report, 5 patients with inoperable locally advanced PC had been treated. No serious treatment-related adverse events were observed during the study period. One patient had PR and 2 had long-SD more than 6 mo in this regimen. Demonstrating in many more number of patients with locally advanced PC will be desired.

In locally tumors, induction of tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ), which is a pro-inflammatory cytokine can induce tumor necrosis and shrinkage. A phase I clinical trial using TNFerade *via* EUS-FNI in combination with radiation for patients with advanced PC therapy has been reported<sup>[5]</sup>. TNFerade is a replication-deficient adenovirus vector carrying the human TNF- $\alpha$  gene regulated by a radiation-inducible promoter (Egr-1). Intra-tumoral TNFerade with radiation has been shown to be safe in a phase I clinical trial of 30 patients with PC<sup>[5]</sup>. In addition, a phase I / II trial was conducted for 50 patients with advanced PC, using TNFerade in combination with chemoradiation therapy. In the study, TNFerade was delivered by EUS guidance for 27 patients without severe procedure-related complications<sup>[6]</sup>. Over a 5-wk treatment period, 1 patient had complete response, 3 had PR, and 12 patients had SD. The results showed a trend toward improved overall survival, however, it was not statistically significant. Moreover, the strategy is only suitable for patients with locally advanced PC. Although the clinical results suggest that TNF- $\alpha$  may be a useful candidate for locally advanced PC therapy, clinical benefits remain unknown. Subsequently, ONYX-015, an oncolytic attenuated adenovirus that preferentially replicates in malignant cells, leading to cell death had been introduced into PC<sup>[7]</sup>. Hecht *et al.*<sup>[8]</sup> completed a phase I / II trial of EUS FNI-guided intra-tumoral delivery of ONYX-015 combined with gemcitabine in 21 patients with advanced PC. Four patients developed comparatively severe complications, such as sepsis

and duodenal perforations which were attributed to the EUS procedures, in spite of no convincing efficacy of ONYX-015 was found. The median survival was 7.5 mo that has no significant difference with the conventional therapies.

Otherwise, EUS injectable anti-tumor agents, there are EUS-guided coagulative therapies. Radiofrequency ablation (RFA) therapy guided by EUS for advanced PC has not been actually clinical trial, because of the poorly accessible PC, in spite of the feasibility and effectiveness was confirmed in a porcine model<sup>[9]</sup>. Indeed, RFA provides localized tissue ablation within 1 cm zone from the FNI needle catheter. Another ablative technique is photodynamic therapy (PDT), which is more selective than RFA. The safety and efficacy of PDT guided by EUS for advanced PC was also demonstrated in a porcine model<sup>[10]</sup>. EUS-guided low-dose PDT may be safe and feasible for advanced PC, without no significant procedure-related complications. Moreover, brachytherapy using iodine-125 or palladium-103 has been successfully placed directly into tumors for the treatment of patients with PC. Pilot studies by Sun *et al.*<sup>[11]</sup> in 15 patients and by Jin *et al.*<sup>[12]</sup> in 25 patients with unresectable PC showed the safety and feasibility of EUS-guided brachytherapy. However, it may be needed to solve the mechanical difficulties of inserting solid seeds for contributing and disseminating worldwide.

Conceivable causes of the limited therapeutic effects by EUS-FNI for advanced PC are wide varieties. Primarily, advanced PC has extremely aggressive nature originally and increases momentarily. As the other conventional treatments, it is uneasy to overwhelm the progression of advanced PC. Secondly, many factors, such as genetic alterations, cellular dynamics and influences of intracellular or microenvironmental stress are intricately entangled in the development of PC. In existing state, it has never demonstrated the clinical effect by one kind of drug or tool alone for advanced PC. Thirdly, advanced PC has a feature of stubborn object because of the high density of fibrosis due to intense parenchymal inflammation. So that, it is incapable of piercing into PC without difficulty and penetrating injected solution adequately inside tumor in many cases. Lastly, even if the efficacy of injected solution is crucial, it is briefly uncontrollable the metastasis, invasion and angiogenesis of the PC, because EUS-FNI is only regional treatment. It may probably be needed to combine with some other systematic treatments for advanced PC in actual clinical application.

Currently, chemotherapy is an accredited, standard treatment for unresectable, advanced PC. According to the previous reports, it is obvious that interventional EUS is a well-established, helpful and low-risky procedure in general. The problems of interventional EUS as the decisive treatments for advanced PC that we must overcome are not the endoscopic procedures but how the therapeutic agents are delivered accurately and what are inserted directly inside the tumor. As the additional treatments of the conventional therapy

for advanced PC, many therapeutic strategies, such as immunotherapies, molecular biological therapies, physiochemical therapies, radioactive therapies, using siRNA, using autophagy have been developing in recent years. Moreover, the efficacy of the other potential therapeutic targets for PC using EUS-FNI, for example, intra-tumoral chemotherapeutic agents (paclitaxel, irinotecan), several ablative energies (RFA and cryothermal treatment, neodymium-doped yttrium aluminum garnet laser, high-intensity focused ultrasound), *etc.*, has already been showed in animal models<sup>[13-17]</sup>.

In conclusion, delivering these promising treatments reliably inside tumor, interventional EUS may probably be indispensable existence for the treatment of locally advanced PC in near future.

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## Tumor biology in estrogen receptor-positive, human epidermal growth factor receptor type 2-negative breast cancer: Mind the menopausal status

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### Abstract

Breast cancer is not one disease, but can be categorized into four major molecular subtypes according to hormone receptor [estrogen receptor (ER) and progesterone receptor (PgR)] and human epidermal growth factor

receptor type 2 (HER2) expression status. Ki67 labeling index and/or multigene assays are used to classify ER-positive, HER2-negative breast cancer into luminal A and luminal B (HER2-negative) subtypes. To date, most studies analyzing predictive or prognostic factors in ER-positive breast cancer have been performed in postmenopausal women, mainly using patients and samples in adjuvant aromatase inhibitor trials. In contrast, even the clinical roles of PgR and Ki67 have been little analyzed so far in premenopausal women. PgR is one of the estrogen-responsive genes, and it has been reported that plasma estradiol levels are related to expression levels of estrogen-responsive genes including *PGR* in ER-positive breast cancer. In this article, biological differences, especially differences in expression of PgR and Ki67 in ER-positive breast cancer between pre- and postmenopausal women are discussed. Clinical roles of PgR and Ki67 in ER-positive breast cancer differ between pre- and postmenopausal women. We suggest that the mechanisms of development and estrogen-dependent growth of ER-positive breast cancer might differ according to menopausal status.

**Key words:** Breast cancer; Progesterone receptor; Estrogen receptor; Ki67; Menopausal status

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**Core tip:** Progesterone receptor (PgR) is one of the estrogen-responsive genes, and it has been reported that plasma estradiol levels are related to expression levels of estrogen-responsive genes including *PGR* in estrogen receptor (ER)-positive breast cancer. In this article, biological differences, especially differences in expression of PgR and Ki67 in ER-positive breast cancer between pre- and postmenopausal women are discussed. Clinical roles of PgR and Ki67 in ER-positive breast cancer differ between pre- and postmenopausal

women. We suggest that the mechanisms of development and estrogen-dependent growth of ER-positive breast cancer might differ according to menopausal status.

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## INTRODUCTION

Breast cancer is not one disease, but a group of diseases that can be categorized into four major molecular subtypes according to their expression of hormone receptors (HR) [estrogen receptor (ER) and progesterone receptor (PgR)] and human epidermal growth factor receptor type 2 (HER2). Thus they are classified as: HR+/HER2-, HR+/HER2+, HR-/HER2+, and triple negative (HR-/HER2-). Treatments need to be tailored to a patient's particular subtype, so that endocrine therapies for HR-positive breast cancer and anti-HER2 therapies for HER2-positive breast cancer are recommended as first choice regardless of whether the disease is in the early stages or has become metastatic.

Expression of ER, PgR, HER2 and the proliferation marker Ki67 in breast cancer tissues is routinely assessed by immunohistochemistry, and multigene assays have recently been introduced for estimating prognosis and treatment efficacy<sup>[1]</sup>. The choice of appropriate drug therapies, especially the indication of adjuvant chemotherapy for ER-positive, HER2-negative early breast cancer, the subtype which is diagnosed in almost 80% of breast cancer cases, is sometimes controversial. Ki67 labeling index and/or multigene assays, such as 21-gene recurrence score (Oncotype Dx), 70-gene signature (Mammaprint) and PAM50 risk of recurrence score, that classify ER-positive, HER2-negative breast cancer into luminal A and luminal B (HER2-negative) subtypes are commonly used in practice, and adjuvant chemotherapy in addition to endocrine therapy is recommended for luminal B subtype<sup>[2]</sup>.

To date, most studies analyzing predictive or prognostic factors in ER-positive breast cancer have been performed in postmenopausal women, mainly using patients and samples in adjuvant aromatase inhibitor trials<sup>[3-5]</sup>. In contrast, even the clinical roles of PgR and Ki67 have been little analyzed so far in premenopausal women.

PgR is one of the estrogen-responsive genes, and it has been reported that plasma estradiol levels are related to expression levels of estrogen-responsive genes including *PGR* in ER-positive breast cancer in both pre- and postmenopausal women<sup>[6,7]</sup>. We previously investigated the expression of estrogen-responsive

genes (PgR and TFF1), a progesterone-responsive gene (*RANKL*), ER-related genes and Ki67 in ER-positive, HER2-negative breast cancer samples, and compared the correlations between expression levels of these molecular markers and clinicopathological factors, including prognosis, between pre- and postmenopausal women. Our results suggested that the mechanisms of development and estrogen-dependent growth of ER-positive breast cancer might differ according to menopausal status<sup>[8]</sup>. Thus, host factors, such as serum levels of estrogen and progesterone might affect the expression of multiple genes in ER-positive breast cancer tissues.

In this article, biological differences, especially in PgR expression and Ki67 labeling index in ER-positive, HER2-negative breast cancer between pre- and postmenopausal women are discussed.

## ***PgR expression in ER-positive breast cancer tissues correlates with serum estrogen levels***

*PgR* is an estrogen-responsive gene, and its expression, together with that of ER, is routinely examined in breast cancer tissues. We previously reported that expression levels of PgR in pretreatment biopsies were not predictive of the response to the neoadjuvant aromatase inhibitor exemestane, and that expression levels of PgR were decreased in posttreatment tumors compared to their levels in pretreatment specimens regardless of the treatment response<sup>[9]</sup>. It is clear that PgR expression does not fully reflect estrogen dependence: Many PgR-negative tumors respond to tamoxifen or aromatase inhibitors<sup>[9-12]</sup>. Furthermore, it has been reported that plasma estradiol levels are related to expression levels of estrogen-responsive genes, such as *PGR* and trefoil factor 1 (*TFF1*)/*pS2*, in ER-positive breast cancer in both pre- and postmenopausal women<sup>[6,7]</sup>. Dunbier *et al*<sup>[7]</sup> examined mRNA expression of estrogen-responsive genes including *PgR* in pretreatment tumor biopsies from postmenopausal patients with ER-positive breast cancer treated with the neoadjuvant anastrozole, and pretreatment plasma estradiol levels were determined by highly sensitive radioimmunoassay. They demonstrated that plasma estradiol levels were significantly associated with expression of estrogen-responsive genes in ER-positive breast cancer.

In premenopausal women, Haynes *et al*<sup>[6,13]</sup> reported significant differences in the expression of estrogen-related genes including PgR in ER-positive breast tumors across the menstrual cycle: Gene expression of estrogen-related genes was higher when serum estradiol levels were high. They also demonstrated that expression of the progesterone-regulated gene *RANKL* was almost three-fold higher when serum progesterone levels were at their highest point of the menstrual cycle.

The study of neoadjuvant endocrine therapy in premenopausal women with ER-positive breast cancer showed that positive PgR expression status by immunohistochemistry dramatically decreased in post-treatment specimens (34.4%) compared to the values

in pretreatment biopsies (98.9%) in patients treated with neoadjuvant anastrozole plus the LHRH agonist goserelin for 24 wk, whereas the percentage of patients with positive PgR status did not change significantly from baseline (91.9%) to 24 wk (89.5%) in patients treated with neoadjuvant tamoxifen plus goserelin<sup>[14]</sup>.

Taken together, these data suggest that expression levels of PgR in ER-positive breast cancer tissues are associated with serum estrogen levels in both pre- and postmenopausal women.

### **Biological differences between pre- and postmenopausal women with ER-positive, HER2-negative breast cancer – PgR**

A study analyzing clinicopathological characteristics of breast cancer in patients registered to the Japanese Breast Cancer Registry in 2011 showed that the ER-positive rate was approximately 90% in patients in their 40s and approximately 80% in those over 50 years old, while the PgR-positive rate was approximately 85% in patients in their 40s but less than 70% in those over 50 years old<sup>[15]</sup>. We previously showed that the incidence of ER-positive, PgR-negative breast cancer in women aged 50 years or younger and in those older than 50 years were 6% and 15%, respectively, whereas for ER-positive, PgR-positive tumors, incidences were 81% and 64%, respectively<sup>[16]</sup>. Moreover, most tumors had high PgR expression in women aged 50 or younger or in premenopausal women, while the distribution of PgR expression levels was evenly spread in tumors in women over 50 years of age or in postmenopausal women<sup>[16]</sup>. This suggests that reduced circulating estrogens after menopause could be the cause in the incidence of ER-positive/PgR-negative or ER-positive/low-PgR tumors in postmenopausal women<sup>[17]</sup>.

PgR expression has been reported to be a prognostic factor for postmenopausal ER-positive breast cancer patients in adjuvant aromatase inhibitor trials<sup>[3-5]</sup>. Our retrospective studies also demonstrated that high expression of PgR significantly correlated with improved disease-free survival in postmenopausal women with ER-positive, HER2-negative breast cancer<sup>[8]</sup>. In contrast, in premenopausal women, PgR expression was not associated with disease-free survival<sup>[8]</sup>.

### **Biological differences between pre- and postmenopausal women with ER-positive, HER2-negative breast cancer – Ki67**

Ki67 is a nuclear protein that is expressed during all phases of the cell cycle except the G0 phase, and is a marker of tumor proliferation<sup>[18]</sup>. Recent studies have shown that the so called “luminal A” subtype-characterized by low histological grade, low proliferation as measured by Ki67, high hormone receptor status, and negative HER2 status – is less responsive to chemotherapy, and that no preferable chemotherapy regimen could be defined for treatment of this subtype<sup>[2]</sup>.

The prognostic significance of Ki67 was examined

in postmenopausal women who were treated with letrozole or tamoxifen in the BIG1-98 trial<sup>[19]</sup>. It was reported that higher values (> 11%) of Ki67 labeling index were associated with worse disease-free survival. Our previous study showed that when the cutoff point for determining the division between low and high Ki67 labeling index was set at 14%, low Ki67 labeling index was strongly associated with increased disease-free survival in postmenopausal women with ER-positive breast cancer<sup>[8]</sup>. We also indicated that high expression of Ki67 ( $\geq 14\%$ ) was significantly associated with decreased disease-free survival in postmenopausal patients treated with adjuvant aromatase inhibitors<sup>[20]</sup>. In contrast, the best cutoff points of Ki67 labeling index for disease-free survival were 30% for premenopausal women with ER-positive breast cancer<sup>[8]</sup>.

In terms of a predictive value for Ki67, Dowsett et al<sup>[21]</sup> measured the expression of Ki67 in tumor biopsy samples taken before and after 2 wk of presurgical endocrine treatment in postmenopausal hormone receptor-positive breast cancer. They showed that a change in Ki67 labeling index between levels before and after 2 wk of endocrine treatment was significantly associated with clinical response. On the other hand, we demonstrated that Ki67 level in a tumor biopsy before treatment with the neoadjuvant aromatase inhibitor exemestane did not correlate with response to the therapy<sup>[9,22]</sup>.

In contrast, in premenopausal women, overall tumor response was better in patients who had a baseline Ki67 index of  $\geq 20\%$  compared with those whose baseline Ki67 index was  $< 20\%$  in a study of patients treated with neoadjuvant anastrozole or tamoxifen who also received goserelin for 24 wk<sup>[14]</sup>. It is possible that Ki67 may be positively stained in ER-positive breast cancer cells with estrogen-dependent growth, and that neoadjuvant endocrine treatment may be effective for Ki67-positive, estrogen-dependent tumor cells in premenopausal women.

## **CONCLUSION**

Clinical roles of PgR and Ki67 in ER-positive breast cancer differ between pre- and postmenopausal women. Of the available multigene assays, PgR and Ki67 are included in Oncotype DX and PAM50, and genes related to ER-signaling are included in EndoPredict. Care should be taken when these assays are introduced for premenopausal women, because most studies involved in the development of multigene assays for ER-positive breast cancer were performed in postmenopausal women. We previously analyzed genetic and environmental factors, endogenous hormones and growth factors to identify risk factors for ER-positive breast cancer, and showed that risk factors differ between women of different menopausal status<sup>[23]</sup>. We therefore suggest that the mechanisms of development and estrogen-dependent growth of ER-positive breast cancer might differ according to menopausal status.



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## Adjuvant chemotherapy for rectal cancer: Is it needed?

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### Abstract

Adjuvant chemotherapy has become a standard

treatment of advanced rectal cancer in the West. The benefits of adjuvant chemotherapy after surgery alone have been well established. However, controversy surrounds the use adjuvant chemotherapy in patients who received preoperative chemoradiotherapy, despite it being recommended by a number of international guidelines. Results of recent multicentre randomised control trials showed no benefit of adjuvant chemotherapy in terms of survival and rates of distant metastases. However, concerns exist regarding the quality of the studies including inadequate staging modalities, out-dated chemotherapeutic regimens and surgical approaches and small sample sizes. It has become evident that not all the patients respond to adjuvant chemotherapy and more personalised approach should be employed when considering the benefits of adjuvant chemotherapy. The present review discusses the strengths and weaknesses of the current evidence-base and suggests improvements for future studies.

**Key words:** Rectal cancer; Adjuvant chemotherapy

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**Core tip:** Adjuvant chemotherapy for rectal cancer is a contentious issue despite its widespread use. Recent randomised controlled trials have shown no benefit in survival of adjuvant chemotherapy in patients treated with preoperative chemoradiotherapy. It is becoming evident that not all patients benefit from adjuvant chemotherapy and identification of these patients should be the focus of future studies. The present review discusses the current evidence-base for adjuvant chemotherapy in rectal cancer and provides directions for future research.

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## INTRODUCTION

The role of adjuvant chemotherapy in advanced rectal cancer in combination with preoperative chemoradiotherapy is controversial. Colorectal cancer is a major cause of morbidity and mortality worldwide. It is the third most common cancer worldwide and the fourth most common cause of cancer-related death<sup>[1]</sup>. Rectal cancer is defined as carcinoma arising in the distal 15 cm from the anal verge. It is estimated that approximately 40000 new cases of rectal cancer were diagnosed in the United States and 14226 in the United Kingdom in 2014<sup>[2,3]</sup>. Surgical treatment is the cornerstone of curative therapy for rectal cancer. Indeed, patients with early disease (stage I, T1/2, node negative) can be effectively treated with surgical resection and 90% are expected to survive at 5 years<sup>[4]</sup>. Therapeutic approach and prognosis differs significantly in more advanced rectal cancers (stage II and III, T3/4, node negative or positive). Local recurrence rates are significantly higher with more advanced lesions compared to early disease (13% vs 5%) and 5-year survival is markedly decreased (35% vs 90%)<sup>[4,5]</sup>. As a result, a more aggressive approach combining radical surgical resection with total mesorectal excision (TME), radiotherapy and chemotherapy is used to treat locally advanced rectal cancers. Neoadjuvant chemoradiotherapy has now become a standard practice in the United States and Europe after the seminal German rectal cancer trial, which showed lower local recurrence rates in neoadjuvant chemoradiotherapy group compared to postoperative chemoradiotherapy<sup>[6]</sup>. Neoadjuvant chemoradiotherapy has led to an increase in sphincter sparing operations and better quality of life as a result of pre-operative downstaging, and decreased risk of local recurrence<sup>[7]</sup>.

Although mortality and local recurrence rates have improved dramatically over the past decades as a result of more accurate preoperative staging modalities [magnetic resonance imaging (MRI), endoscopic ultrasound] and surgical techniques (TME), the rate of systemic relapse is still unacceptably high and contributes significantly<sup>[8]</sup>. About a third of patients with advanced rectal cancer will eventually develop distant metastases<sup>[6]</sup>. In order to prevent this, postoperative adjuvant chemotherapy has been employed in the management of locally invasive treatment of rectal cancer and is now incorporated into most treatment protocols in the west. Various national and international guidelines (National Comprehensive Cancer Network, American Society of Clinical Oncology, European Society of Medical Oncology, National Institute of Clinical Excellence) recommend postoperative chemotherapy with either capecitabine or 5-FU for a total of 6 mo for stage II and III rectal cancers irrespective of surgical pathology results<sup>[9]</sup>. Despite

the widespread use of this approach, the evidence for beneficial effects of postoperative chemotherapy is conflicting. Indeed, the long-term results (10 years of follow-up) of the European Organisation for Research and Treatment of Cancer (EORTC) 22921 randomised trial published in 2014 showed no benefit of postoperative adjuvant chemotherapy after preoperative chemoradiotherapy prompting the authors to question the validity of current recommendations<sup>[10]</sup>. Whether or not postoperative chemotherapy should be given is an important clinical dilemma for healthcare professionals, as chemotherapy is associated with significant systemic toxicity, which may lead to diminished quality of life<sup>[11]</sup>. The present review provides an update on the current evidence-base for treatment of rectal cancer with adjuvant chemotherapy, discusses the strengths and pitfalls of recent research and suggests improvements for future studies.

## DIFFERENCES BETWEEN COLON AND RECTAL CANCER

Current recommendations for adjuvant chemotherapy treatment of rectal cancer are based on the evidence, which is largely extrapolated from studies in colon cancer<sup>[12-14]</sup>. However, it is now known that clinical course and biology of colon and rectal cancers differ significantly. Rectal cancers have distinct gene expression profile, fewer BRAF mutations and less microsatellite instability<sup>[15-17]</sup>. Furthermore, colon and rectum possess distinct embryological origins as well as anatomical and physiological characteristics. Clinically, rectal cancers have a worse prognosis in the early stages of disease, but longer survival in more advanced stages compared to colonic tumours of the same stage<sup>[18]</sup>. Finally, it is more difficult to achieve complete resection of rectal cancers with circumferential margin involvement, due to multi organ involvement, compared to colonic cancers<sup>[19]</sup>. As a result, it is scientifically justifiable to consider colonic and rectal cancers as distinct diseases and therefore the benefits of adjuvant chemotherapy cannot be assumed to be equal in both conditions.

## POSTOPERATIVE CHEMOTHERAPY IN COMBINATION WITH SURGERY ALONE

The value of postoperative chemotherapy in patients treated only with curative surgery has been investigated in a large number of trials. The Cochrane systematic review and meta-analysis (2012) of 21 RCTs comparing postoperative chemotherapy with observation alone found significant improvement in both overall (HR = 0.83, 95%CI: 0.76-0.91) and disease-free survival (HR = 0.75, 95%CI: 0.68-0.83)<sup>[20]</sup>. Data, pooled from almost 10000 patients, showed that 5-FU based postoperative chemotherapy was associated with risk reduction of 17% and 25% in overall and disease-free survival respectively. Only 5 out of 21 trials showed significantly



positive results, which implies that large numbers of study participants are needed to discern a small, but clinically important, difference. Of note, nine trials were conducted in Japan. Despite considerable differences in populations and treatment practices of rectal cancer in the west and in Asian countries (infrequent use of neoadjuvant chemoradiotherapy and different surgical technique in Japan), the authors of the Cochrane review found similar results both for Western and Japanese studies. It is unclear which groups of patients benefit most from chemotherapy, as only three trials reported results based on TNM stage. The QUASAR trial ( $n = 3239$ , 948 with rectal cancer) found significantly prolonged overall and disease survival in patients with stage II (node negative) disease<sup>[12]</sup>. In contrast, a subgroup meta-analysis of three trials, which included patients with stage III disease showed no significant improvement in overall survival, but longer disease-free survival<sup>[21-23]</sup>.

The results should be interpreted with caution as the heterogeneity of the studies was high, most likely due to variable TNM stages (Duke's stages A to C). In addition, the studies were conducted over the course of the past three decades, during which surgical and oncological treatment practices have changed considerably. Indeed, there is an argument that postoperative chemotherapy without preoperative treatment was only found beneficial in older studies not employing TME surgery<sup>[24]</sup>. No RCTs in the TME era have evaluated the value of postoperative chemotherapy and are unlikely to be performed as neoadjuvant treatment has become a "gold standard" approach. Finally, postoperative radiotherapy was administered alongside chemotherapy in some of the studies, hence individual contribution of chemotherapy to increased survival is difficult to determine.

## POSTOPERATIVE CHEMORADIATION AFTER SURGERY ALONE

Therapeutic utilisation of the synergistic effects of radiation and chemotherapy has dominated the treatment of cancer for many decades. The benefits of chemoradiation for rectal cancer was established in a number of trials (NSABP R-01, GITSG-7175, NCCTG-794751, GITSG-7180) in 1980s and early 1990s and is now a recommended optimal treatment modality in patients undergoing curative surgical resection<sup>[25-28]</sup>. The GITSG-7175 study ( $n = 227$ ) was the first trial to show lower recurrence rates (33% vs 55%), but no effect on overall survival in patients treated with radiotherapy and fluorouracil with semustine (methyl-CCNU) compared to surgery alone and had to be terminated prematurely as a result of these findings<sup>[25]</sup>. The following NCCTG trial randomised 204 patients to either chemoradiation (FU-semustine) or radiotherapy. In contrast to GITSG trial, chemoradiotherapy was found to be associated with significant reduction (46%) in cancer related deaths compared to radiotherapy alone<sup>[27]</sup>.

Based on these studies National Institute of Health in 1990 produced the guidance recommending that all rectal cancers with stages II and III should be treated with a combined pelvic irradiation and concomitant chemotherapy<sup>[29]</sup>.

To date, only the GITSG-7175 trial compared postoperative chemotherapy alone vs chemoradiotherapy and found no significant difference in survival and local recurrence rates<sup>[25]</sup>. The results of the NASPB R01 trial ( $n = 555$ ) showed that chemotherapy, when compared to surgery alone or radiotherapy, is associated with significantly prolonged disease-free survival<sup>[28]</sup>. Since postoperative radiotherapy has not been shown to prolong survival in rectal cancer, it is reasonable to believe that chemotherapy when combined with radiotherapy is responsible for reducing the risk of systemic dissemination of rectal cancer. The seminal trial, which compared preoperative with postoperative chemoradiation showed that patients achieve significantly better local control and have lower levels of systemic toxicity, although overall survival is similar in both approaches<sup>[6]</sup>. As a result of the findings of this trial, preoperative chemotherapy has gradually become a mainstay approach to treatment of locally advanced rectal cancer.

## POSTOPERATIVE CHEMOTHERAPY AFTER NEOADJUVANT (CHEMO)RADIO THERAPY AND SURGERY

Although postoperative chemotherapy with or without radiotherapy prolongs survival in patients treated with surgery alone, the evidence is much more conflicting in the setting of neoadjuvant treatment. Since majority of the patients in the West nowadays receive neoadjuvant chemoradiotherapy, the most pertinent question regarding the benefit of postoperative chemotherapy remains unanswered by the studies described above. In light of several systematic reviews reporting no benefit of neoadjuvant chemoradiotherapy when compared to radiotherapy alone in terms of disease free and overall survival, the role of postoperative chemotherapy has come into question<sup>[30,31]</sup>. Five recent European trials (CHRONICLE, QUASAR, EORTC 22921, PROCTO-SCRIPT, I-CNR-RT) enrolling 3143 patients with stage II and III rectal cancer investigated the benefits of postoperative chemotherapy after neoadjuvant chemoradiotherapy and surgery (Table 1)<sup>[10,12,32-34]</sup>. Four out of five trials reported negative results and only QUASAR study found significantly increased survival in the postoperative chemotherapy group. EORTC 22921 trial ( $n = 1011$ ) employed  $2 \times 2$  factorial design comparing the effectiveness of postoperative 5-FU and leucovorin based chemotherapy after preoperative chemoradiation or radiotherapy alone<sup>[10]</sup>. No difference in overall and disease-free survival was reported at 5 and 10 years of follow up. In the Italian trial (I-CNR-RT), 635 patients were treated with preoperative chemoradiotherapy

Table 1 Trials comparing adjuvant chemotherapy with observation after neoadjuvant treatment

	Sample size	Accrual period	Total mesorectal excision	Backmann		Adherence (%)	Overall survival (adjuvant vs observation)	Disease-free survival (adjuvant vs observation)	Local recurrence (adjuvant vs observation)
				Preoperative treatment	Adjuvant treatment				
EORTC 22921	1011	1993-2003	36.80%	25 doses of 1.8 Gy and fluorouracil-based chemotherapy	Four courses every 3 wk of fluorouracil and folinic acid	42%	51.8% vs 48.4%, $P = 0.32$	47% vs 43.7%, $P = 0.29$	11.7% vs 11.8%
CHRONICLE	113	2004-2008	NR	45 Gy and fluorouracil-based chemotherapy	Six courses every 3 wk of oxaliplatin and oral capecitabine	48.10%	89% vs 88%, $P = 0.75$	78% vs 71%, $P = 0.56$	Not reported
PROCTOR-SCRIPT	437	2000-2013	All patients	25 doses of 1.8-2.0 Gy and fluorouracil-based chemotherapy	Six courses of fluorouracil and folinic acid OR 12 courses of fluorouracil and folinic acid OR eight courses every 3 wk of oral capecitabine	73.60%	80.4% vs 79.2%, $P = 0.73$	62.7% vs 55.4%, $P = 0.13$	7.8% vs 7.8%, $P = 0.69$
I-CNR-RT	634	1992-2001	NR	25 doses of 1.8 Gy and fluorouracil-based chemotherapy	Six courses of fluorouracil and folinic acid	58.50%	70% vs 69.1%, $P = 0.77$	62.8% vs 65.3%, $P = 0.88$	4.5% vs 6.4%
QUASAR	3239 (948 with rectal cancer)	1994-2003	NR	Radiotherapy (21%)	Thirty doses of intravenous FU with high or low dose folinic acid	58.00%	HR = 0.8 (0.6-1.07) <sup>1</sup>	HR = 0.69 (0.51-0.94) <sup>1</sup>	19.8% vs 27.2%

<sup>1</sup>Hazard ratios were obtained from Cochrane review by Petersen *et al*<sup>[30]</sup>. HR: Hazard ratios; FU: Fluorouracil; NR: Not reported.

and then were randomised into observation and postoperative chemotherapy groups<sup>[33]</sup>. The investigators found no difference in 5-year survival and the distant metastases rates. PROCTO-SCRIPT trial ( $n = 437$ ) patients treated with preoperative chemoradiotherapy were randomised into observation and treatment arms, which consisted of 5-FU plus leucovorin or capecitabine regimens<sup>[34]</sup>. The trial was stopped prematurely due to slow accrual and showed no benefit of postoperative chemotherapy in terms of overall survival. Another trial (CHRONICLE,  $n = 112$ ), which was also terminated early due to slow accrual, found no survival advantage in patients treated postoperatively with capecitabine and oxaloplatin (XELOX)<sup>[32]</sup>. QUASAR trial was the only study to show borderline significant benefit of adjuvant chemotherapy after preoperative radiotherapy, however only 21% of patients with rectal cancer or both (rectal/colon) had radiotherapy<sup>[12]</sup>.

In all of the studies above, adjuvant chemotherapy was associated with only marginal benefit, which was not statistically significant. None of the trials were large enough to detect a 5% difference in 5-year survival, hence the likelihood of type II error was high<sup>[35]</sup>. As a result, Breugom *et al*<sup>[36]</sup> performed a meta-analysis of available studies using patient-level data. Unfortunately, the authors were not able to obtain the data from the QUASAR trial investigators. The analysis of 1196 patients with stage II and III rectal cancer with R0 resection showed no significant effect of adjuvant chemotherapy on overall survival, disease-free survival and distant metastases. In subgroup analysis, patients with tumours located 10-15 cm from the anal verge seemed to benefit from adjuvant chemotherapy as disease-free survival was significantly prolonged (HR = 0.59, 95%CI: 0.40-0.85;  $P = 0.005$ ) and rates of distant metastases were lower (HR = 0.61, 0.40-0.94;  $P = 0.025$ ). There was no survival difference between stages II and III.

A meta-analysis performed by Petrelli *et al*<sup>[37]</sup>, which included 16 randomised and non-randomised studies (a total of 5457 patients) found that overall adjuvant chemotherapy had significantly positive effects on disease-free and overall survival and distant metastasis rates. However, the validity of the results is limited due to significant bias of non-randomised studies. Indeed, in stratified analyses significant benefit was observed only in the non-randomised studies. Study participants who received chemotherapy were often younger, had node negative disease and showed good response to preoperative chemotherapy. In addition, median follow up rates were often shorter than 5 years, which could have exaggerated overall and disease-free survival in the short term.

The findings of these studies beg two questions: Are current recommendations for adjuvant chemotherapy in rectal cancer valid? Or, are the findings of the

studies reliable enough to change current practice?

## POTENTIAL PITFALLS OF THE CURRENT EVIDENCE

Although the RCTs described above are generally held to have robust designs, there are some important considerations to be made when interpreting the results. Poor adherence to postoperative chemotherapy is a well-recognised problem in the treatment of colorectal cancer. Of the patients assigned to the adjuvant chemotherapy group in the EORTC 22921 trial 25% did not start the adjuvant treatment, with similar figures in other studies. The numbers are even smaller for completion rates of chemotherapy with only around half of the patients fully complying with the treatment. Although this may reflect a real life scenario, it is pertinent to determine the effects of optimal chemotherapy treatment so that clinicians and patients can make the informed decision regarding the need for adjuvant chemotherapy. Breugom *et al.*<sup>[34,36]</sup> argued that the results of the trials could have not been affected by poor adherence as PROCTOR-SCRIPT trial showed no benefit of chemotherapy for patients who completed all cycles. Unfortunately the number of patients in this group ( $n = 159$ ) is too small to detect the clinically meaningful difference.

Another important consideration is a change of surgical practices over long accrual periods. Most trials commenced recruitment in the early 1990s (EORTC 22921, I-CTR-RT, QUASAR). Surgical practices have changed considerably since then and the type of surgery patients received in the trials poorly reflect current standards. For instance, in the EORTC 22921 trial TME was performed in only 36.8% of patients, which contrasts with the contemporary practices where TME is performed virtually in all patients with locally advanced rectal cancers<sup>[38]</sup>. Furthermore, abdominoperineal resection rate was 47.2% in the intervention arm in the CHRONICLE study, which is significantly higher proportion compared to the United Kingdom National Bowel Cancer Audit Programme (24%)<sup>[39]</sup>. These deviations from current treatment practices raise concerns about the applicability of the study findings to today's management of rectal cancer.

One of the most important shortcomings of the present studies is the use of inadequate imaging modalities. All of the RCTs relied on CT staging before the commencement of neoadjuvant treatment. Endoscopic ultrasound was only performed in 67% of patients in the EORTC 22912 trial and only in a selected proportion in I-CNR-RT study. The accuracy of the CT based-TNM staging is not perfect and the risk of overstaging is high<sup>[40]</sup>. Hence, it is likely that many patients were over-treated. Furthermore, CT does not enable accurate assessment of circumferential resection margin (CRM), which is an independent prognostic factor for disease-free survival<sup>[41-43]</sup> (Figure 1). The best modality to assess the extent of CRM is MRI, however no chemotherapy

trials have reported the use of MRI<sup>[44]</sup> (Figure 1).

Furthermore, lymph node status was determined using pathological staging. Earlier studies have indicated that preoperative chemoradiotherapy may reduce the number of lymph nodes available for pathological examination and thus may affect the accuracy of staging<sup>[45-49]</sup>. There is a theoretical risk that some patients with metastatic lymph nodes are not identified on pathological staging and are at risk of systemic dissemination<sup>[49]</sup>. In particular, proximal node involvement carries a significant risk of distant metastasis<sup>[49]</sup>. Advanced imaging modalities, such as PET and MRI may enable accurate assessment of lymph node involvement before neoadjuvant chemoradiotherapy and would subsequently guide clinicians in deciding whether or not adjuvant therapy is necessary (Figure 2).

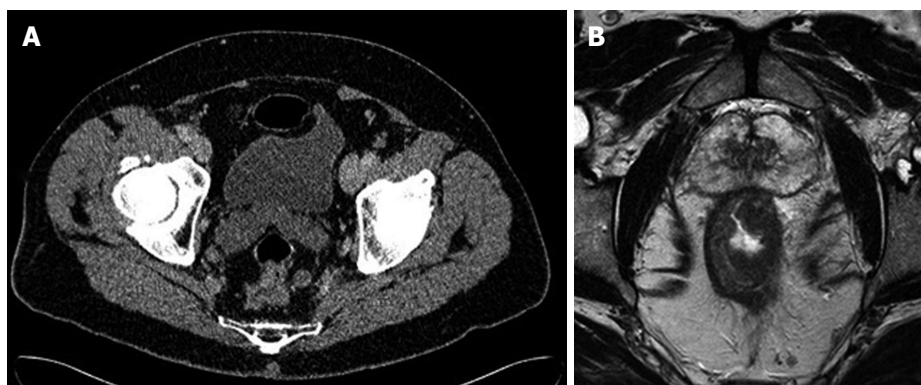
The timing of adjuvant chemotherapy may also have a considerable effect on survival outcomes and has been largely overlooked in the present studies. Several meta-analyses showed that the longer the chemotherapy is delayed the shorter survival is in patients with colorectal cancer<sup>[50,51]</sup>. One of the reasons why colon cancer responds to adjuvant chemotherapy and rectal does not may be prompt administration of adjuvant chemotherapy<sup>[9]</sup>. Stoma and prolonged preoperative radiotherapy especially in combination with chemotherapy for rectal cancer means that adjuvant chemotherapy may not start until months later. Adverse consequences of delayed chemotherapy are also supported by animal studies, in which surgery was shown to increase the number of circulating neoplastic cells and promote metastatic growth<sup>[52]</sup>. In addition, surgery has been shown to enhance the production of oncogenic growth factors, such as transforming growth factor -  $\alpha$ <sup>[53,54]</sup>.

Finally, the most informative analysis of these trials by Breugom *et al.*<sup>[34,36]</sup> is not without limitations. Out of 2195 patients available from four trials, only 1196 were included. These included patients only with stage II and III disease who had R0 resection, hence the meta-analyses does not address the question whether responders to neoadjuvant chemoradiotherapy achieve any benefit from adjuvant chemotherapy (see below). In addition, QUASAR, which was one of the largest trials and showed positive effects, was not included in the analysis.

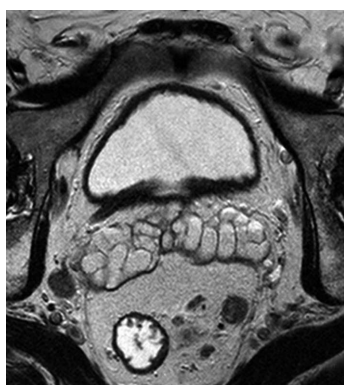
## WHO MIGHT BENEFIT FROM POSTOPERATIVE CHEMOTHERAPY?

Several studies have suggested that not all the patients with rectal cancer benefit from adjuvant chemotherapy and that only certain groups may respond to treatment. The degree of bowel wall penetration and nodal involvement has been shown to be one of most important predictive factors for local relapse, distant metastasis and survival<sup>[4,5,55]</sup>. For example, in a pooled analysis





**Figure 1** Computed tomography (A) and magnetic resonance imaging (B) of the T3 rectal cancer. Note poor quality of circumferential margin on the computed tomography scan compared to the magnetic resonance imaging.



**Figure 2** Magnetic resonance imaging of the T3 rectal cancer showing lymph node involvement.

of five randomised control trials in the United States, which included 3791 patients with rectal cancer, 5-year overall survival for T1-2N0 stage was 90%, for T3-4N0 60%, T4N1 30%<sup>[4]</sup>. Many studies have been conducted to investigate the benefits of chemotherapy in certain subgroups of patients, however the results have been rather conflicting. Most of the evidence comes from post-hoc subgroup analyses of randomised control trials or retrospective/prospective non-randomised studies, hence is subject to inherent weaknesses of these designs.

The exploratory analysis of the early results (5 years of follow up) of the EORTC 22921 trial has showed that only patients downstaged to ypTN0-2 benefit from adjuvant chemotherapy, while patients with ypTN3-4 do not<sup>[56]</sup>. In line with this, two other studies by De Stefano *et al*<sup>[57]</sup> and Janjan *et al*<sup>[58]</sup> found that patients who responded to preoperative chemoradiotherapy benefited from adjuvant chemotherapy, however no benefit was observed in the non-responders group. Such observations also have sound scientific basis, since rectal cancers are highly heterogenous tumours and preoperative chemotherapy may enable to predict favourable tumour biology, which may respond to subsequent adjuvant chemotherapy.

On the other hand, there have been several reports

to suggest that downstaged ypTNM0-2 tumours follow a more indolent course postoperatively and do not require additional chemotherapy. Three studies have shown that patients with good response to preoperative chemotherapy had excellent 5-year survival (90% survival) irrespective whether adjuvant chemotherapy was given or not<sup>[59-61]</sup>. Hence, additional chemotherapy may not be necessary and potentially harmful. This is also supported by the results of the long term outcomes of EORTC 22921 trial<sup>[10]</sup>. The investigators showed that although there appeared to be a survival advantage in patients with downstaged tumours in the short term, this benefit was transient and the survival curves equalised after 10 years.

Unfortunately, in a majority of patients a highly favourable response to preoperative chemotherapy is not observed and they are at greater risk of local and distant recurrence as well as shorter survival<sup>[62]</sup>. As a result, it appears logical to treat these patients aggressively with adjuvant chemotherapy<sup>[55,59,63-65]</sup>. Unfortunately, Breugom *et al*<sup>[36]</sup> in the meta-analysis of five trials (described above) showed no benefit of adjuvant chemotherapy neither in stage II nor in stage III, however there was no data available for stage 0 and I disease. The differing results in the studies above may reflect variations in chemotherapy regimes used. Poor response to neoadjuvant treatment, which is usually fluoropyridine-based, indicates unfavourable tumour pathology and, unsurprisingly, administration of fluoropyridines during postoperative period may bring no benefit due to tumour resistance. In these cases, more aggressive combined therapy may have a role. A retrospective analysis of 160 rectal cancers with ypN0 stage showed that patients with T3-4 disease have significantly longer disease-free and overall survival if adjuvant FOLFOX (Oxaliplatin with fluorouracil and folinic acid) or XELOX (capecitabine with oxaliplatin) regimens were given, while those with T0-2 appeared to show no benefit from adjuvant chemotherapy<sup>[64]</sup>. Randomised controlled trials are needed to determine whether non-responders may benefit from a more aggressive adjuvant treatment.



Location of rectal cancer in relation to anal verge was also found to have significance when aiming to predict which patients may benefit from adjuvant chemotherapy. In the subgroup analysis, Breugom *et al* reported that tumours occurring 10-15 cm from the anal verge have longer disease-free survival if adjuvant chemotherapy is administered (HR = 0.59, 95%CI: 0.40-0.85,  $P = 0.005$ ). No significant interaction between distance from the anal verge and treatment group was found for more distal tumours. The authors proposed that this observation might be as a result of the arbitrary definition of rectum and that tumours in proximal rectum are in fact biologically similar to colonic ones. Bujko *et al*<sup>[35]</sup> suggested several anatomical reasons why low lying rectal cancer may have poor prognosis compared to higher ones. The authors argued that higher proportion of low lying rectal cancers involve a circumferential margin. In addition, lower cancers receive both systemic and portal venous drainage and hence are at risk of systemic dissemination. Finally, internal iliac and obturator nodes are at risk of involvement in low lying rectal cancers, which are not routinely removed in the West.

## ADJUVANT CHEMOTHERAPY AGENTS: PAST, PRESENT AND FUTURE

### Fluoropyrimidine-based agents

Fluoropyrimidine-containing agents have formed the basis of adjuvant chemotherapy in rectal cancer. 5-FU can be administered either bolus or by continuous intravenous infusion. The NCCTG trial involving 660 patients with locally advanced rectal adenocarcinoma showed that protracted venous infusion of 5-FU (PVI FU) alongside pelvic irradiation was associated with significantly reduced distant metastases rate (31% vs 40%) and increased overall survival<sup>[66]</sup>. In contrast, a larger study ( $n = 1917$ ) by Smalley *et al*<sup>[67]</sup> found no significant differences between three trial arms (5-FU bolus plus leucovorin, 5-FU bolus plus infusion, 5-FU only) (United States intergroup study). There appears to be limited evidence to favour PVI FU over simple bolus FU in rectal cancer bearing in mind higher costs, inconvenience and requirement for a central line.

An attractive alternative to PVI FU is an oral agent called capecitabine. Capecitabine requires 3-step enzymatic activation *in vivo*, one of which preferentially occurs in tumours, hence capecitabine offers a highly targeted approach. Trials, mainly investigating the effectiveness of capecitabine in the neoadjuvant setting, show non-inferiority to intravenous 5-FU regimens in terms of disease-free and overall survival and distant and local recurrences<sup>[68,69]</sup>. A phase III German trial randomised 392 patients to receive either capecitabine or intravenous 5-FU in the perioperative period (231 patients received postoperative adjuvant chemotherapy). The results showed significantly lower distant metastases rates with capecitabine compared to 5-FU (19% vs 28%),

however similar 5-year survival (76% vs 67%)<sup>[70]</sup>. Aside from higher risk of hand-foot syndrome, capecitabine offers a great substitute to intravenous 5-FU regimens and obviates the need for a central line and is already recommended by the National Comprehensive Cancer Network guidelines.

### Oxaliplatin-based regimens

Oxaliplatin is a third-generation 1,2 diaminocyclohexane platinum analogue, which prevents replication and transcription of DNA. The MOSAIC and NSABP C07 trials showed significant improvement in overall survival in patients with advanced colon cancer<sup>[71,72]</sup>. Based on these encouraging results, several trials were carried out to determine the benefits of oxaliplatin in addition to standard fluoropyridine-based regimens in rectal cancer (ACCORD12/0405-Prodige, CAO/ARO/AIO-04, ADORE, STAR-01, NSAPB R-04, PETACC-6)<sup>[73-78]</sup>. While some studies reported significant improvement in pathological response and disease-free survival<sup>[76,78]</sup>, others found no superiority of oxaliplatin, but instead an increased risk of acute toxicity<sup>[74,75,77,79]</sup>. Only four trials reported the data on survival. Recently published results of ADORE trial showed significant improvements in 3-year disease-free survival in FOLFOX group (5-FU, oxaliplatin, leucovorin) compared to (5-FU and leucovorin) (71.6% vs 62.9%, HR = 0.657, 95%CI: 0.434-0.994;  $P = 0.047$ )<sup>[78]</sup>. Toxicity was more commonly seen in FOLFOX group, however there was no difference in frequency of grade 3 and grade 4 events. Similar results were reported in the CAO/ARO/AIO-04 trial, which showed significant increase in the proportion of patients achieving pathological complete response (17% vs 13%) and improved 3-year disease survival<sup>[76]</sup>. In contrast, interim results of PETACC-6 trial reported in a conference abstract did not show survival advantage in FOLFOX group<sup>[79]</sup>. CHRONICLE trial reported no benefit of oxaliplatin, however the study was considerably underpowered<sup>[32]</sup>. Three trials did not find improvement in pathological complete response (NSAPB R-04, ACCORD 12/0405-Prodige, STAR-01), however no data on survival were available. Although the evidence for oxaliplatin use in rectal cancer is limited, adjuvant chemotherapy incorporating oxaliplatin is widely used and is recommended by a number of international guidelines.

### Irinotecan and biological agents

Irinotecan is a plant alkaloid, which inhibits DNA replication and repair by blocking topoisomerase I. Although irinotecan has been used with success in metastatic colon cancer, no benefit was found for stage III<sup>[80,81]</sup>. Only one trial investigated the benefits of irinotecan in rectal cancer<sup>[82]</sup>. The study recruited only 225 patients out of expected 3250 and was terminated because of the competing trial on bevacizumab. The investigators reported no benefit of addition of irinotecan to fluorouracil and leucovorin in neoadjuvant or adjuvant settings. Hence, currently irinotecan has no proven role

in treatment of rectal cancer.

Biological agents such as anti-VEGF agent, bevacizumab, and monoclonal antibodies, cetuximab and panitumumab, which target epidermal growth factor receptor have been successfully used in metastatic colon cancer in patients who failed on first line chemotherapy regimens<sup>[83-85]</sup>. Although approved by FDA, NICE currently does not support their use<sup>[86]</sup>. The role of bevacizumab in non-metastatic rectal cancer is unknown. The on-going phase II BACCHUS trial is comparing FOLFOX with bevacizumab vs FOLFOXIRI with bevacizumab in the neoadjuvant setting in patients with locally advanced rectal cancer. However, the trial does not directly test the independent benefits of bevacizumab and its role in adjuvant setting is not under investigation.

## FUTURE DIRECTIONS AND TRIALS IN PROGRESS

Unfortunately, a definite answer regarding the effectiveness of adjuvant chemotherapy is unlikely to be forthcoming in the near future. Most on-going trials compare different chemotherapeutic agent combinations or intensification regimes (PETACC-6, NSAPB R04, AERO-R98) and do not include an observation arm. Hence, the fundamental issue of whether or not adjuvant chemotherapy is effective is unaddressed. The only phase III trial (NCT01941979) registered in the <http://clinicaltrials.gov.uk> website (accessed February 2015), which includes an observation arm is currently open and recruiting. The trial compares FOLFOX vs observation alone in patients with T3-4, N1, M0 who were treated with preoperative chemotherapy and showed poor response. The rationale of the study is based on the previous observations that only certain groups of patients with rectal cancer may benefit from adjuvant chemotherapy<sup>[57,87]</sup>.

Since rectal cancer is a highly heterogeneous disease, more trials are needed to take a targeted approach when evaluating the benefits of adjuvant chemotherapy. It is still unclear what role adjuvant chemotherapy has in patients who responded well to preoperative chemotherapy as the evidence is conflicting. Hence, ideally a separate trial investigating adjuvant chemotherapy is needed in this patient population. At the other end of the spectrum, the optimal management of patients who did not show improvement with preoperative chemoradiotherapy is also unclear. The use of adjuvant chemotherapy in non-responders appears to be unsupported by current evidence. However, there is scope for a more aggressive approach employing intensification regimens and combination treatments, including oxaliplatin and bevacizumab.

Reporting of the results based on stage may not be sensitive enough since there is high variability in prognosis within each TNM stage<sup>[88]</sup>. Valentini *et al.*<sup>[89]</sup> produced nomograms based on the data from five

major European RCTs on adjuvant chemotherapy in rectal cancer ( $n = 2795$ ), which take into account a large number of clinical and pathological variables. Using these nomograms to stratify patients with rectal cancer into low, intermediate and high risk groups may help identify with high accuracy patient subgroups, which would benefit from adjuvant chemotherapy, however a randomised control trial is needed to determine their benefit.

Accurate clinical staging before and after administration of preoperative chemotherapy is vital to avoid over-staging and subsequent overtreatment. CT and EUS assessment is far from adequate and instead MRI should be employed. Particular areas of interest are circumferential margin involvement and lymph node status, as these are the most important predictors of poor survival<sup>[43,49]</sup>.

## THE ROLE OF BIOMARKERS

It has been increasingly recognised that all cancers in general, including rectal cancer, are highly heterogeneous diseases requiring personalised therapies. Identification of reliable biomarkers could potentially aid clinical decision-making regarding the need for adjuvant chemotherapy. Many studies have identified dozens of biomarkers (microsatellite instability, p53, KRAS, BRAF, thymidylate synthase) in colon cancer and a 12-gene recurrence score assay (Oncotype DX Colon Cancer Assay) has been validated in the QUASAR trial as a reliable predictor for distant recurrence<sup>[90,91]</sup>. Whether similar assays can be used in rectal cancer is not known due to biological differences of colon and rectal cancer and requires separate validation. Biomarker analysis of the PROCTO-SCRIPT trial specimens is planned, which will hopefully help to identify patients who would benefit from adjuvant chemotherapy<sup>[34]</sup>.

## CONCLUSION

Adjuvant chemotherapy for rectal cancer has been a subject of controversy in recent years. The results of major trials, published in the last couple of years, do not support the use of postoperative chemotherapy after neoadjuvant chemoradiotherapy, however many clinicians throughout the world are understandably reluctant to abandon adjuvant chemotherapy. Concerns exist regarding the quality of the studies including inadequate staging modalities, out-dated chemotherapeutic regimens, non-TME surgical approaches and small sample sizes. It is becoming evident that not all patients with rectal cancer need adjuvant treatment. Identification of groups at risk using advanced imaging modalities, nomograms and biomarkers is the future of personalised treatment of rectal cancer. Hopefully, these questions will be answered in the near future. In the meantime, patients should be informed of benefits and risks of postoperative chemotherapy and the decision regarding the need for further treatment should be made

on individual basis.

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## Therapeutic role of template-based lymphadenectomy in urothelial carcinoma of the upper urinary tract

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### Abstract

Lymphadenectomy for urothelial carcinoma of the upper urinary tract has attracted the attention of physicians. The mapping study of lymphatic spread has shown that a relatively wide area should comprise the regional nodes for tumors of the right renal

pelvis or the right upper two-thirds of the ureter. A prospective study showed that an anatomical template-based lymphadenectomy significantly improved patient survival in tumors of the renal pelvis. This benefit was more evident for patients with pT2 stage tumors or higher. The risk of regional node recurrence is significant reduced by template-based lymphadenectomy, which is likely to be associated with improved patient survival. The removal of lymph node micrometastases is assumed to be the reason for therapeutic benefit following lymphadenectomy. The number of resected lymph nodes can be used to assess the quality of lymphadenectomy, but not to determine the extent of lymphadenectomy. The guidelines currently recommend lymphadenectomy for patients with muscle-invasive disease, even though the current recommendation grades are still low. The present limitation of lymphadenectomy is the lack of standardization of the extent of lymphadenectomy and the randomized trials. Further studies are warranted to collect the evidence to support lymphadenectomy.

**Key words:** Lymphadenectomy; Lymph node excision; Urothelial carcinoma; Treatment outcome; Therapeutic uses; Diagnosis; Guideline

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**Core tip:** The role of lymphadenectomy in urothelial carcinoma of the upper urinary tract had examined. A prospective study showed that anatomical template-based lymphadenectomy significantly improves patient survival in tumors of the renal pelvis. This benefit is demonstrated more clearly for patients with pT2 tumors or higher. The risk of regional node recurrence is significant reduced by template-based lymphadenectomy, which is likely to be associated with improved patient survival. The guidelines currently recommend lymphadenectomy for patients with muscle-invasive disease. Further studies are warranted to

collect the evidence to support lymphadenectomy.

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## INTRODUCTION

About 20%-30% of patients with urothelial carcinoma develop lymphatic metastases, and thus, it is known to confer a high risk of developing lymphatic metastases<sup>[1,2]</sup>. Thus, controlling lymphatic spread may be an important strategy to improve patient survival. Lymphadenectomy may be a possible strategy for surgically treating cancer that spread to the lymph nodes. The standard surgical treatment for muscle-invasive bladder cancer is radical cystectomy<sup>[3]</sup>. Concomitant lymphadenectomy provides a better outcome than no lymphadenectomy, and an extension in the lymphadenectomy template may possibly result in higher patient survival<sup>[4,5]</sup>. Thus, guidelines currently recommend lymphadenectomy as an integral part of radical surgery for bladder cancer<sup>[3]</sup>.

Most carcinomas arising from the upper urinary tract are pathologically urothelial carcinomas, which are similar to bladder cancer. It is well known that there is a high risk of metastases to the lymph nodes in urothelial carcinomas of the upper urinary tract (UCUT)<sup>[6,7]</sup>. Moreover, stage and grade migration toward more aggressive disease has been reported in UCUT<sup>[8]</sup>. Thus, one can speculate that controlling metastases to the lymph nodes is more important in UCUT.

In this review article, we summarize the current understanding of the role of lymphadenectomy in UCUT. Unfortunately, the evidence regarding lymphadenectomy in UCUT is small as compared with that for bladder cancer. A recent study by the cancer registry shows that lymphadenectomy is rarely performed<sup>[9]</sup>. In addition, patient survival did not improve with radical nephroureterectomy over a period of 18 years<sup>[10]</sup>. The role of lymphadenectomy needs to be discussed to improve the outcome of surgery.

## THE HISTORY OF LYMPHADENECTOMY IN UCUT

The high incidence of lymphatic metastases in UCUT was reported as early as the 1970s<sup>[6,7]</sup>. Thus, the inclusion of lymphadenectomy as a standard procedure was suggested for radical nephroureterectomy indications<sup>[11]</sup>. However, the role of lymphadenectomy was not examined sufficiently until the 1990s because UCUT is a very minor disease among malignancies<sup>[12]</sup>.

In the 1990s, 2 studies shed new light on the importance of lymphadenectomy. Komatsu *et al.*<sup>[13]</sup>

reported the outcomes of relatively wide lymphadenectomy. Lymph node metastases that were pathologically confirmed by lymphadenectomy (pN0), were not significantly associated with higher patient survival than those with pathologically confirmed lymphatic metastases (pN+). This result supports the use of lymphadenectomy for staging. Another study by Miyake *et al.*<sup>[14]</sup> showed that lymphadenectomy improved survival in selected patients without lymph vessel invasion. However, the small number of patients in these studies precluded widespread discussion. Thereafter, no new information regarding the benefits of lymphadenectomy was available until 2007.

## THE EXTENT OF LYMPHADENECTOMY

In the 1980s, some investigators examined the primary sites of lymphatic metastases in UCUT<sup>[7,15,16]</sup>. Their results showed that metastases spread primarily to the renal hilar, abdominal para-aortic, and paracaval nodes from the renal pelvis and to the abdominal ureter and the intrapelvic nodes from the distal ureter. Current descriptions in the Union for International Cancer Control TNM classification is based on results reported more than 30 years ago<sup>[17]</sup>. However, the location or laterality of primary tumors was not taken into account when considering the anatomical extent of the regional nodes. Therefore, the aforementioned results could not be used to determine the extent of lymphadenectomy in clinical practice.

In 2007, we conducted more detailed mapping studies of lymph nodes. In this study, we examined 42 patients with lymph node metastases confirmed by pathological examination of surgical specimens or radiological methods<sup>[18]</sup>. Sites of primary nodal metastases were identified according to the location of the tumors, for example, the renal pelvis, the upper and middle ureter, and the lower ureter. Our results showed that primary metastatic sites were located in a larger area than previously thought for tumors of the right renal pelvis and the upper two-thirds of the right ureter. We reanalyzed the pattern of lymphatic metastases by increasing the number of the patients with lymph node metastases to 75, but the results were similar (Table 1)<sup>[19]</sup>. In tumors of the right renal pelvis, lymphogenous metastases spread primarily to the right renal hilar, paracaval, retrocaval, and interaortocaval nodes. Primary metastatic sites in right upper and middle ureter tumors also include the right renal hilar, retrocaval, and interaortocaval nodes. Tumors of the left renal pelvis or the left upper/middle ureter primarily metastasized to left renal hilar and para-aortic nodes. The lower boundary of the metastatic sites was at the level of the inferior mesenteric artery for tumors of the renal pelvis and at the aortic bifurcation for tumors of the upper and middle ureter. Primary metastatic sites for tumors of the lower ureter included the ipsilateral common iliac, external iliac, obturator, and internal iliac



Table 1 The incidence of primary nodal involvement in each lymph node sites according to the location of the tumor in urothelial carcinomas of the upper urinary tract

Location of the primary tumor (No. of patients with nodal metastasis)	Ipsilateral			Ipsilateral				
	Suprahilar	Ipsilateral renal hilar	Para-caval	Retro-caval	Interaorto-caval	Para-aortic	Common iliac	External iliac
Right RP (22)	-	14 (64%)	8 (36%)	9 (41%)	3 (14%)	-	-	-
UU (3)	-	1 (33%)	-	1 (33%)	2 (66%)	-	-	-
MU (5)	-	-	-	1 (20%)	4 (80%)	-	-	-
LU (7)	-	-	-	-	-	-	4 (57%)	1 (14%)
Left RP (25)	-	20 (80%)	-	-	1 (4%)	11 (44%)	-	2 (29%)
UU (0)	-	-	-	-	-	-	-	-
MU (5)	-	-	-	-	-	5 (100%)	-	-
LU (8)	-	-	-	-	-	-	4 (50%)	2 (25%)
								3 (38%)
								1 (13%)

R-RP: Right renal pelvis; R-UU: Right upper ureter; R-MU: Right middle ureter; R-LU: Right lower ureter; L-RP: Left renal pelvis; L-UU: Left upper ureter; L-MU: Left middle ureter; L-LU: Left lower ureter.

nodes. Our first study did not reveal presacral nodes as a primary site, but a revised study showed that 14% of patients had primary metastases to this site.

Based on these results, we thought that nodal sites at more than 10% risk of metastasis, for example, regional lymph nodes, should be dissected. The proposed anatomical extent of lymphadenectomy is shown in Figure 1<sup>[19]</sup>. The suggested template for renal pelvic cancer is very similar to that for renal cell carcinoma, which is based on several studies<sup>[20]</sup>. For the right kidney, the paracaval, retrocaval, and precaval nodes should be included from the adrenal vein to the level of the inferior mesenteric artery, and for the left kidney, the para-aortic and pre-aortic nodes should be included from the crus of the diaphragm to the inferior mesenteric artery<sup>[20]</sup>. Interaortocaval nodes should always be removed despite the laterality of tumors when extended Lymphadenectomy (LND) is sought, but this is different from our results in which dissection of interaortocaval nodes can be ignored for tumors of the left renal pelvis. The template for lower ureteral cancer is also similar to that proposed for bladder cancer<sup>[21,22]</sup>.

Our nonrandomized prospective study showed the therapeutic benefit of lymphadenectomy for tumors of the renal pelvis, confirming the rationale of this template<sup>[23]</sup>. However, our prospective study did not support the therapeutic role in ureteral cancer tumors. It remains to be determined whether the currently proposed anatomical template of ureteral cancer is appropriate.

Recently, another multi-institutional mapping study was reported, where a similar pattern of lymphatic metastases was observed for renal pelvic cancer. However, tumors below the crossing of the common iliac artery were more likely to spread cranially than expected, with an incidence of 33%-40%<sup>[24]</sup>. This might suggest that the template we propose for lower ureteral cancer is not adequate to cover primary metastatic sites. Further studies are warranted to standardize the extent of lymphadenectomy for UCUT.

## DOES LYMPHADENECTOMY BENEFIT ACCURATE STAGING?

One of the major roles of lymphadenectomy is to provide accurate staging of lymphatic metastases. Lymphadenectomy could allow better stratification of patients to determine the indication of adjuvant therapy. In bladder cancer, lymphadenectomy has a role in staging. Extended lymphadenectomy reportedly improves staging accuracy because the incidence of pathological node metastases is increased by extending the extent of lymphadenectomy<sup>[5,25-27]</sup>.

Several studies have examined the benefits of staging in UCUT. Komatsu *et al.*<sup>[13]</sup> reported the role of relatively wide lymphadenectomy in 1997. Their results showed significantly higher cancer-specific survival (CSS) in patients without lymphatic metastases (pN0) as confirmed by lymphadenectomy compared to those with pathological node metastases (pN+), suggesting a role for wide lymphadenectomy in staging<sup>[13]</sup>. Roscigno *et al.*<sup>[28]</sup> reported results for a similar extent of lymphadenectomy as Komatsu *et al.*<sup>[13]</sup>. They compared patient survival between 3 groups, including patients without lymphatic metastases as confirmed by lymphadenectomy (pN0), those with pathological node metastases (pN+), and those without lymphadenectomy (pNx). Five-year CSS was highest in pN0 patients, moderate in pNx patients, and lowest in pN+ patients (73%

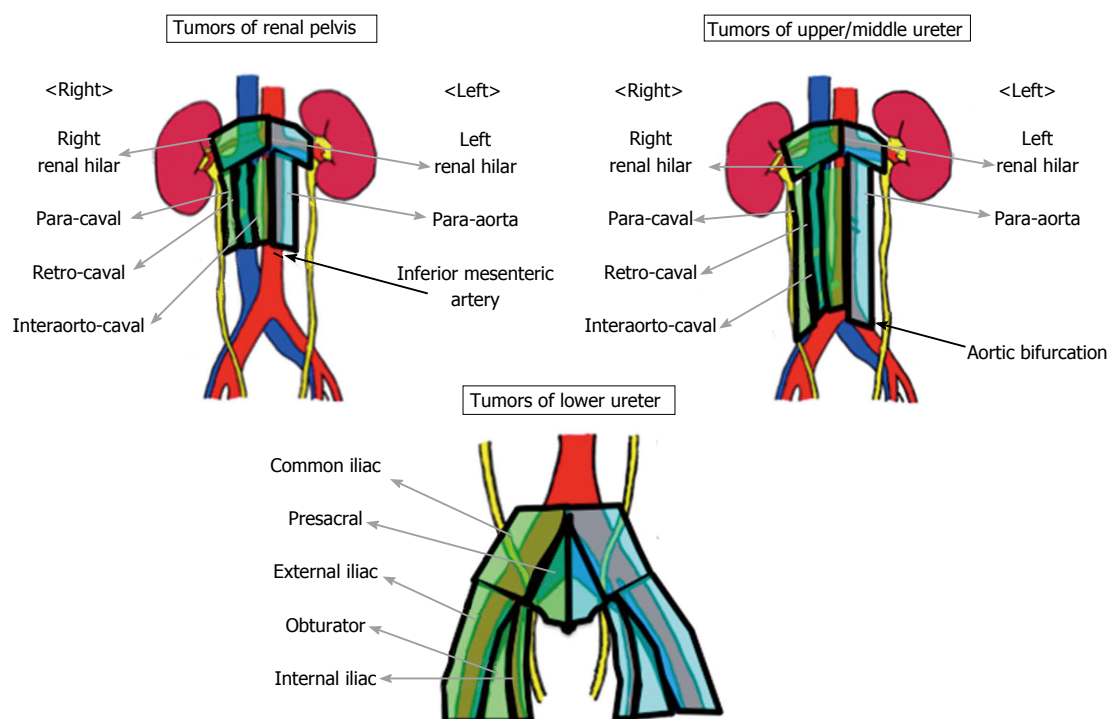


Figure 1 The extent of lymphadenectomy currently proposed for urothelial carcinoma of the upper urinary tract.

vs 48% vs 39%)<sup>[28]</sup>. Although there was no difference between pNx and pN0 patients ( $P = 0.476$ ), the difference between pN0 and pNx patients was significant ( $P < 0.001$ ). They concluded that lymphadenectomy is likely to provide better stratification of pN0 patients just like Komatsu *et al.*<sup>[13]</sup>.

Thereafter, several multi-institutional studies have been reported. The results are summarized in Table 2. Roscigno *et al.*<sup>[29]</sup> conducted a multi-institutional study to further examine patient survival according to the lymph node status. CSS was stratified according to the nodal status in patients with a staging of pT1 or higher. Five-year CSS was 77% in pN0, 69% in pNx, and 35% in pN+. For patients with pT2 staging or higher, this difference was demonstrated more clearly (5-year CSS: 70% vs 58% vs 33%). Abe *et al.*<sup>[30]</sup> also reported the results of a similar analysis. Recurrence-free survival was significantly higher in pN0 patients than in pNx patients with pT2 stage tumors or higher, but not in those with pT1. These 2 studies confirm the role of lymphadenectomy in stratifying patients with a favorable prognosis (pN0). This benefit is more prominent in those at the pT2 stage or higher. The extent of lymphadenectomy was described in these 2 studies, and they utilized a relatively wide template.

However, another 3 studies failed to demonstrate better stratification of pN0 by lymphadenectomy than that achieved without lymphadenectomy (pNx). On the other hand, these studies showed that lymphadenectomy could stratify patients with unfavorable prognosis by identifying pathological metastases to the lymph nodes (pN+)<sup>[31-34]</sup>. Lughezzani *et al.*<sup>[31]</sup> collected the most number of patients by using for a population-based

study by using a surveillance, epidemiology, and end results database. Lymphadenectomy could discriminate between pN+ patients with a poor prognosis, and pN0 or pNx patients. However, this benefit was limited to patients with a pT3 stage tumor or higher. Burger *et al.*<sup>[32]</sup> also reported that stratification of pN+ patients with a significantly poor prognosis was observed only in locally advanced disease. Mason *et al.*<sup>[33]</sup> also reported similar results to those by Lughezzani *et al.*<sup>[31]</sup> and Burger *et al.*<sup>[32]</sup>. Ouzzane *et al.*<sup>[34]</sup> failed to demonstrate the benefit of staging in patients with a tumor of stage pT2 or higher, when examining 714 patients from multiple institutions in France. However, the extent of lymphadenectomy was not described in these 4 studies where the survival was similar between pN0 and pNx patients.

As mentioned above, there is a difference in the stratification of patients; pN0 stratification is better than pNx, and pN+ stratification is worse than pNx. One possible reason is the extent of lymphadenectomy. The latter 4 studies included all types of lymphadenectomy, whereas the first 2 studies had a relatively wide extent for dissection. We also examined the benefit of lymphadenectomy-based staging in our patient cohort. From 1988 to February 2015, we treated 314 nonmetastatic patients who underwent radical nephroureterectomy. Of these, 158 patients (53%) underwent lymphadenectomy, including 126 patients with lymphadenectomy based on the anatomical template (Figure 1, complete LND) and 42 where all regional sites were not dissected (incomplete LND). Our result was very similar to that reported by the others<sup>[29,30]</sup>. Five-year CSS, according to the status of lymph node metastases,

**Table 2** Reports on staging benefit of lymphadenectomy in urothelial carcinoma of the upper urinary tract

Authors	Year	Institute	Template of LND	Subject	No. of patients	Results	Staging benefits	Ref.
Roscigno	2009	Multi	Not well described	≥ pT1	1130	5 yr-CSS: pN0 77% > pNx 69% ( $P = 0.032$ ) > pN+ 35% ( $P < 0.001$ )	Yes	[29]
				≥ pT2	813	5 yr-CSS: pN0 70% > pNx 58% ( $P = 0.017$ ) > pN+ 33% ( $P < 0.001$ )		
Abe	2010	Multi	Not well described	pT1	66	RFS: pN0 = pNx ( $P = 0.702$ )	Yes	[30]
				≥ pT2	227	RFS: pN0 > pNx ( $P < 0.001$ ) = pN+ ( $P = 0.134$ )		
Burger	2011	Multi	Not well described	Organ-confined	519	CSS: pN0 = pNx = pN+	Yes	[32]
Lughezzani	2010	Multi	Not described	Locally advanced pT1, pT2	266	CSS: pN0 = pNx ( $P = 0.633$ ) > pN+ ( $P < 0.001$ )	In locally advanced disease Yes	[31]
					1324	CSS: T1 pN0 = pNx ( $P = 0.4$ ) = pN+ ( $P = 0.1$ ) T2 pN0 = pNx ( $P = 0.8$ ) = pN+ ( $P = 0.1$ )		
				pT3, pT4	1382	CSS: T3 pN0 = pNx ( $P = 0.9$ ) > pN+ ( $P < 0.001$ ) T4 pN0 = pNx ( $P = 0.3$ ) > pNx ( $P < 0.001$ )		
							In ≥ pT3	
Mason	2012	Multi	Not described	All patients	1029	OS: pN0 66.1% = pNx 66.0% ( $P = 0.617$ ) > pN+ 22.3% ( $P < 0.01$ )	Yes	[33]
Ouzzane	2013	Multi	Not described	All patients	714	5 yr-CSS: pN0 81% = pNx 85% ( $P = 0.6$ ) > pN+ 47% ( $P < 0.001$ )	Yes	[34]
				≥ pT2	337	CSS: pN0 = pNx ( $P = 0.44$ ) = pN+ ( $P < 0.15$ )		
TWMU	2015	Single	Well described	All patients	314	5 yr-CSS: pN0 84% > pNx 70% ( $P = 0.02$ ) > pN+ 31% ( $P < 0.001$ )	Yes	-
				≥ pT2	212	5 yr-CSS: pN0 79% > pNx 59% ( $P < 0.007$ ) > pN+ 31% ( $P < 0.004$ )		

LND: Lymphadenectomy; CSS: Cancer-specific survival; RFS: Recurrence-free survival; LNs: Lymph nodes; CompLND: Complete lymphadenectomy; DFS: Disease free survival; OS: Overall survival.

was 84.9% in pN0, 70.2% in pNx, and 31.5% in pN+ patients (Figure 2). The difference between the groups was statistically significant. This trend was demonstrated more clearly in patients with pT2 stage tumors or higher. Five-year CSS according to the status of lymph node metastases was 79.6% in pN0, 59.1% in pNx, and 31.5% in pN+ patients (Figure 2). Thus, we believe that the extent of lymphadenectomy influences the staging benefits.

Collectively, most studies agree that there are benefits from lymphadenectomy-based staging. In addition, this benefit is likely to be demonstrated more clearly in patients with advanced disease.

## DOES LYMPHADENECTOMY IMPROVE SURVIVAL?

### Retrospective study

In bladder cancer, extended lymphadenectomy where the cranial boundary of the template is at the level of aortic bifurcation has shown improvement in not only staging accuracy but also patient survival<sup>[4,5]</sup>. A

therapeutic benefit of lymphadenectomy is expected in UCUT as well as bladder cancer because of histological similarity. However, no one had examined the role of lymphadenectomy in improving patient survival until 2007, except for Miyake *et al.*<sup>[14]</sup> who showed that LND benefited only selected patients. The therapeutic benefits of lymphadenectomy are summarized in Table 3.

Three retrospective studies from single institutes were published in 2007. We identified an anatomical template of lymphadenectomy from the mapping study (Figure 1)<sup>[18]</sup>. Thus, we hypothesized that the extent of lymphadenectomy was an important factor that influences patient survival. In this study, we subclassified 169 patients into 3 groups, and compared the patient survival among groups<sup>[35]</sup>. The 3 groups include the patients for whom the regional nodes were all dissected [complete lymphadenectomy (CompLND)]; those in whom lymphadenectomy did not include all regional sites [incomplete lymphadenectomy (IncompLND)]; and those without lymphadenectomy (No-LND). CSS was lower in the No-LND group than in the CompLND or IncompLND groups, but the difference was not

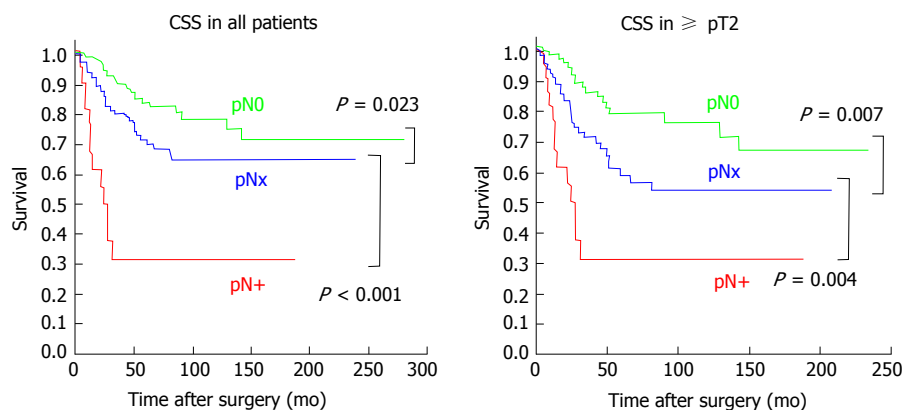


Figure 2 Benefit of staging lymphadenectomy by stratification of patients according to lymph node status in our institute. CSS: Cancer-specific survival.

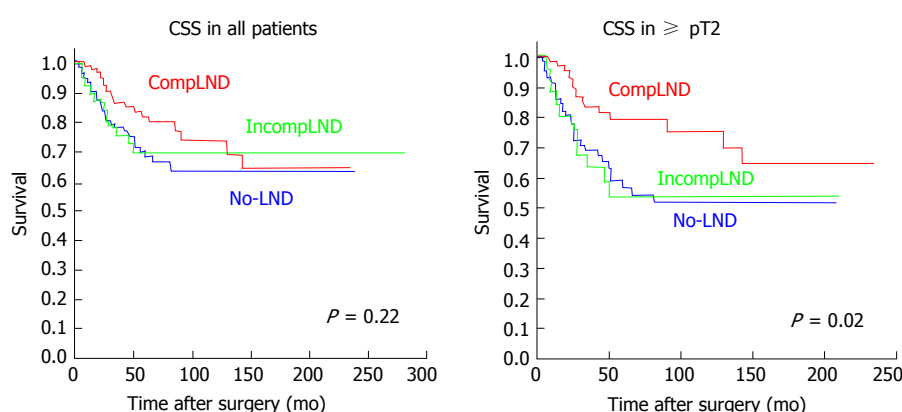


Figure 3 Therapeutic benefit of lymphadenectomy according to the extent of lymphadenectomy in our institute. LND: Lymphadenectomy; CSS: Cancer-specific survival.

statistically significant. However, for patients with pT3 stage tumors or higher, the survival rate increased incrementally from No-LND to IncompLND to CompLND. The difference between the CSS in the No-LND and CompLND groups, but not the IncompLND group, showed statistical significance. Multivariate analysis showed that CompLND was a significant independent factor for reducing the risk of cancer-specific mortality. Figure 3 shows the results from our current database, which includes 314 nonmetastatic patients, which is almost double that in our previous report. The results are similar to what we reported in 2007. A significant improvement in patient survival is observed in the CompLND group in patients with pT2 stage tumors or higher. In contrast, CSS in the IncompLND group was similar to that of No-LND even in patients with advanced stage cancer. Thus, our results suggest a therapeutic benefit of lymphadenectomy; however, lymphadenectomy should be performed based on the anatomical template.

Results from other retrospective studies have been reported. Brausi *et al.*<sup>[36]</sup> reported the influence of relatively wide lymphadenectomy in 82 patients with pT2 stage tumors or higher. Lymphadenectomy included the following lesions: The para-aorta or vena cava between the renal hilum and the inferior mesenteric

artery for tumors of the renal pelvis or the upper ureter; the para-aorta or vena cava between the renal hilum and the bifurcation of the common iliac artery for tumors of the mid-ureter; and the pelvic nodes on the ipsilateral side for lower ureteral tumors. The lymphadenectomy groups showed significantly higher disease-specific survival than those without lymphadenectomy in patients with pT2 stage tumors or higher (81.6% vs 44.8%,  $P = 0.007$ ). Roscigno *et al.*<sup>[28]</sup> also examined the influence of lymphadenectomy with an extent similar to that used by Brausi *et al.*<sup>[36]</sup> on patient survival. Patients who underwent lymphadenectomy showed significantly higher CSS than those who did not undergo lymphadenectomy for advanced disease at the pT2 stage or higher (5-year CSS: 57% vs 40%,  $P = 0.01$ ). These 2 studies from Italy also supported a therapeutic role for lymphadenectomy and emphasized the disadvantage of ignorance regarding lymphadenectomy.

Thereafter, multi-institutional retrospective studies were conducted to confirm the therapeutic benefit of lymphadenectomy. However, a major limitation of these multi-institutional studies is the lack of a standardized lymphadenectomy template among institutes and surgeons. Thus, we should carefully interpret these results. The largest study was reported by Roscigno *et al.*<sup>[29]</sup>, in which 1130 patients from 13 international



Table 3 Reports on therapeutic benefit of lymphadenectomy in urothelial carcinoma of the upper urinary tract

Authors	Year	Institute	Property	Template of LND	Subject	No. of patients	Survival results	Independent factors in Multivariate analysis?	Therapeutic benefit?	Ref.
Kondo	2007	Single	Retrospective	Clearly described	All patients	169	CSS: ComplLND = IncomplLND = No-LND ( $P = 0.06$ )	Yes: ComplLND for CSS	Yes	[35]
Kondo	2012	Single	Retrospective	Clearly described	$\geq pT3$	88	CSS: ComplLND > No-LND ( $P = 0.01$ )	Not determined	In $\geq pT3$	Yes
						191	5 yr-CSS: ComplLND 77.9% > IncomplLND 54.0% = No-LND 59.0% ( $P = 0.03$ )			
						140	5 yr-CSS: ComplLND 73.2% > IncomplLND 43.7% = No-LND 47.3% ( $P = 0.01$ )			
Brausi	2007	Single	Retrospective	Described	$\geq pT2$	82	DFS: RPLN 81.6% > No-LND 44.8% ( $P = 0.007$ )	Yes: RPLD for OS	Yes	[36]
Rosignio	2008	Single	Retrospective	Described	$\geq pT2$	132	5 yr-CSS: LND 57% > No-LND 40% ( $P = 0.01$ )	Yes: LND and pN0 for CSS	in $\geq pT2$	Yes
						95	pN0 72% > pNx 39% ( $P < 0.001$ ) 7 LNs > less than 7 ( $P < 0.001$ )			
Rosignio	2009	Multi	Retrospective	Not well described	$\geq pT2$	1130	5 yr-CSS: LND 66% = No-LND 69% ( $P = 0.23$ )	Yes: No. of LNs for CSS	In $\geq 7$ LNs removed	Yes
Rosignio	2009	Multi	Retrospective	Not well described	$\geq pT1pN0$	412	5 yr-CSS: 8 LNs or more 84% > less than 8 73% ( $P = 0.038$ )	Yes: pN0 for CSS	No	[29]
Abe	2010	Multi	Retrospective	Not well described	All patients	293	RFS: pN0 > pNx ( $P < 0.001$ ) > pN+ ( $P = 0.004$ )	Yes: No. of LNs for CSS	Yes in $\geq 8$ LNs removed	Yes
Burger	2011	Multi	Retrospective	Not well described	Organ-confined	519	CSS: pN0 = pNx	No	Yes but limited only in locally advanced disease	[32]
Lughezzani	2010	Multi	Retrospective	Not described	All patients	266	CSS: pN0 = pNx ( $P = 0.633$ )	Yes: pN0 for CSS in locally advanced		
						2824	No: CSS is pN0 = pNx			
						1029	OS: pN0 66.1% = pNx 66.0% ( $P = 0.617$ )			
						714	5y-CSS: pN0 81% = pNx 85% ( $P = 0.6$ ) > pN+ 47% ( $P < 0.001$ )			
Kondo	2014	Multi	Prospective	Clearly described	Renal pelvis	337	CSS: pN0 = pNx ( $P = 0.44$ ) = pN+ ( $P < 0.15$ )	Yes in CSS in $> pT2$	Yes in renal pelvic cancer in $\geq pT2$	[23]
						90	$\geq pT2$			
							3 yr-OS: LND 86% > No-LND 48% ( $P = 0.01$ )			
							3 yr-CSS: LND 89% > No-LND 51% ( $P = 0.01$ )			
							3 yr-DFS: LND 77% > No-LND 50% ( $P = 0.06$ )			
Kondo	2014	Multi	Prospective	Clearly described	Ureter	76	$\geq pT2$	No		
							3 yr-OS: LND 46% = No-LND 71% ( $P = 0.57$ )			
							3 yr-CSS: LND 54% = No-LND 71% ( $P = 0.99$ )			
							3 yr-DFS: LND 54% = No-LND 59% ( $P = 0.79$ )			

LND: Lymphadenectomy; LNs: Lymph nodes; ComplLND: Complete lymphadenectomy; IncomplLND: Incomplete lymphadenectomy; DFS: Disease-free survival; RPLD: Retroperitoneal lymph node dissection; RFS: Recurrence free survival; OS: Overall survival.

institutes were analyzed. Disappointingly, CSS was not significantly different between patients who underwent lymphadenectomy and those who did not (5-year CSS: 66% vs 69%,  $P = 0.23$ )<sup>[29]</sup>. Moreover, lymphadenectomy benefited pN0 patients. The number of lymph nodes removed significantly correlated with the improvement of CSS<sup>[37]</sup>. A

cutoff level of 8 lymph nodes improved CSS significantly (84% vs 73%,  $P = 0.038$ ), and the number of lymph nodes removed was an independent factor for predicting CSS. Thus, it was suggested that lymphadenectomy should be performed to an adequate extent, which is in accordance with our principles for anatomical template-based lymphadenectomy<sup>[19,35]</sup>.

Other studies were not a direct comparison between patients who did and did not undergo lymphadenectomy. They tried to find the therapeutic benefits of lymphadenectomy by comparing the survival of pN0 and pNx patients. This reflects the benefits of staging, but may also reflect therapeutic benefit. Abe *et al.*<sup>[30]</sup> also reported improved CSS in pN0 patients compared to pNx patients with pT2 stage tumors or higher from multiple institutions. These studies also demonstrated that ignorance of lymphadenectomy (pNx) was an independent factor for predicting a poor patient outcome. Multivariate analysis by Burger *et al.*<sup>[32]</sup> also showed an increased risk of recurrence and death in pNx patients with locally advanced disease. Thus, these 2 studies also support the therapeutic role of lymphadenectomy in patients with advanced disease.

However, 3 studies demonstrated no difference in patient survival between pN0 and pNx patients as mentioned in section 4<sup>[31,33,34]</sup>. In addition, multivariate analysis in a population-based study based on the surveillance, epidemiology, and end results database showed that omitting lymphadenectomy did not pose a disadvantage to patient survival<sup>[31]</sup>. They concluded that no therapeutic benefit was obtained from lymphadenectomy.

Thus, retrospective studies examining the therapeutic benefit of lymphadenectomy show large discrepancies among studies. One of the major reasons for this is the lack of standardization of the extent of lymphadenectomy. We need a prospective study to resolve this issue.

### Prospective study

We conducted a prospective study in 2 Japanese institutes<sup>[23]</sup>. This study was initiated in 2006. At that time, we were not aware that the presacral lymph node was a regional node in lower ureteral cancer. Thus, dissection of presacral nodes was not necessary for inclusion in this study. In principle, template-based lymphadenectomy was performed at the time of radical nephroureterectomy in all patients irrespective of preoperative staging, except for patients over 75 years old or with significant comorbidities. Thus, this study was considered to be a nonrandomized prospective study. Lymphadenectomy was performed for 77 patients, while 89 patients did not undergo lymphadenectomy.

Figure 4 shows recurrence-free, cancer-specific, overall survival of patients. In patients with renal pelvic cancer, CSS and overall survival were significantly higher in the lymphadenectomy group compared to the no lymphadenectomy group, although the difference

in recurrence-free survival was marginally significant. Multivariate analysis showed that template-based lymphadenectomy was a significant independent factor for reducing cancer mortality in patients with renal pelvic cancer. In contrast, lymphadenectomy did not improve patient survival in ureteral cancer. A similar trend was observed for patients with pT2 stage tumors or higher.

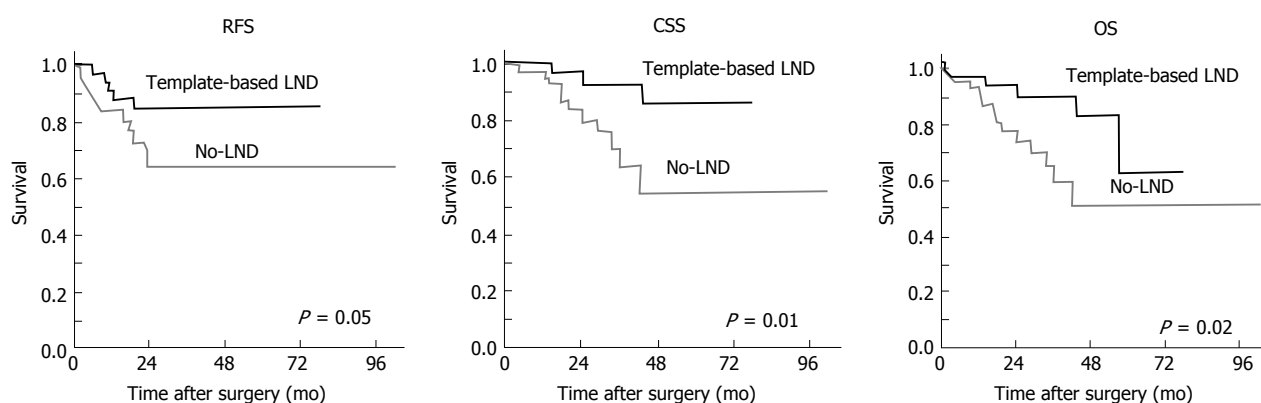
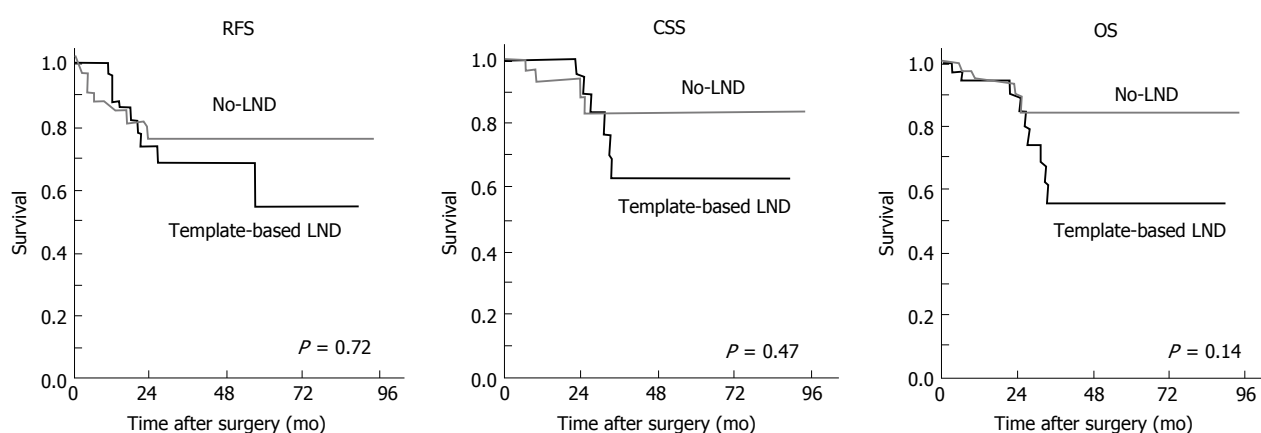
Thus, our bi-institutional, nonrandomized prospective study further supports a therapeutic benefit for lymphadenectomy in patients with renal pelvic cancer, but not in those with ureteral cancer. This study also confirms the rationale of using our anatomical lymphadenectomy template for renal pelvic cancer. Again, our prospective study failed to show the survival benefit of lymphadenectomy in ureteral cancer. However, our recent retrospective study shows that lymphadenectomy is also likely to improve survival in patients with upper/middle ureteral cancer, but not in those with lower ureteral cancer (prepared for submission). The template of lymphadenectomy for upper/middle ureteral cancer is similar to that for renal pelvic cancer. I believe that the benefit of lymphadenectomy will be confirmed in upper/middle ureteral cancer in the future. The reason why patients with lower ureteral cancer did not benefit from lymphadenectomy needs to be determined. Some possible explanations include an inadequate template and the higher malignant potential of lower ureteral cancer.

To the best of our knowledge, this is the only published prospective study that examines the role of lymphadenectomy. Another ongoing prospective study analyzed a preformed super-extended template<sup>[38]</sup>. They only reported the safety and feasibility of utilizing this template, not the patient survival. We definitely need a randomized trial to confirm the therapeutic benefit of lymphadenectomy.

### Does lymphadenectomy reduce the risk of regional node recurrence?

There is a dearth of evidence to support the survival benefits of lymphadenectomy. One possible way of improving patient survival by lymphadenectomy may be the prevention of regional node recurrence.

Our prospective study shows significantly improved patient survival following anatomical template-based lymphadenectomy in renal pelvic cancer. In order to further examine the role of template-based lymphadenectomy, we analyzed how the extent of lymphadenectomy influences the recurrence pattern in renal pelvic cancer<sup>[39]</sup>. We collected the data of 180 patients with nonmetastatic (cN0M0) urothelial carcinoma of the renal pelvis from 2 institutions, and compared the sites of tumor recurrence between template-based lymphadenectomy, incomplete lymphadenectomy, and no lymphadenectomy. Recurrence in the regional nodes was significantly decreased in the complete template-based group (2.9%, 2/67) compared to the incomplete lymphadenectomy (18.1%, 4/22) and

**A** Renal pelvic cancer**B** Ureteral cancer

**Figure 4** Patient survival in a non-randomized prospective study according to the location of the primary tumor. LND: Lymphadenectomy; CSS: Cancer-specific survival; RFS: Recurrence-free survival; OS: Overall survival.

no lymphadenectomy (10.9%, 10/91) groups ( $P = 0.03$ ; Figure 5). We should emphasize that 75% (3/4) of regional node recurrences in the incomplete lymphadenectomy group were found outside the dissected sites. Complete lymphadenectomy was a predictive factor for a reduced risk of regional node recurrence. Thus, this study shows the role of template-based lymphadenectomy in reducing the risk of regional node recurrence in renal pelvic cancer, which may in turn be associated with improved patient survival. At the same time, our study suggests that the prevention of regional node recurrence by lymphadenectomy is attributed to the dissection of tumor microdeposits in the regional lymph nodes.

Abe *et al.*<sup>[30]</sup> confirmed the above hypothesis by examining micrometastases to the lymph nodes using immunohistochemistry with an anticytokeratin antibody. They demonstrated that 14% of patients with no metastases, as diagnosed by regular hematoxylin-eosin staining, showed a positive immunohistochemical reaction for micrometastases. In addition, the majority of patients with micrometastases survived for a long time after lymphadenectomy.

We also examined lymph node micrometastases

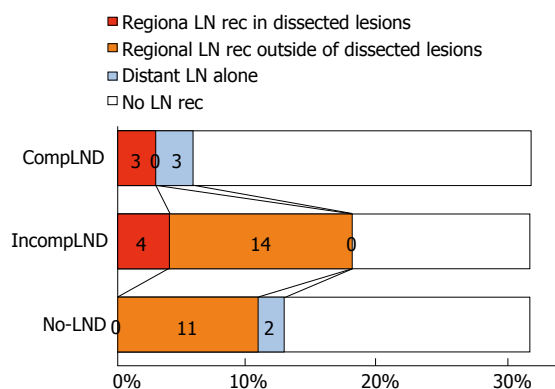
by examining the expression of urothelial carcinoma-specific markers in lymphadenectomy specimens using the quantitative reverse transcription-polymerase chain reaction<sup>[40]</sup>. We found that this technique detected micrometastases in about 10% of patients who had no metastases according to routine hematoxylin-eosin staining. Moreover, the prognosis of the patients was stratified well according to the metastatic status of the lymph nodes.

Collectively, these results show that the therapeutic benefit of lymphadenectomy is likely to be attributed to the surgical resection of microtumor deposits that spread to the lymph nodes in UCUT. Again, we should emphasize that the therapeutic benefit could not be obtained without anatomical template-based lymphadenectomy.

## UNDERLYING ISSUES REGARDING LYMPHADENECTOMY

### Minimum number of lymph nodes removed that influence patient survival

The number of lymph nodes removed is likely to be a good indicator for assessing the extent of lymphadenectomy.



**Figure 5** Recurrence pattern in regional nodes according to the extent of lymphadenectomy in patients with renal pelvic cancer. LND: Lymphadenectomy; LN: Lymph node.

denectomy in bladder cancer<sup>[41-43]</sup>. Reportedly, survival rates continued to rise as the number of resected lymph nodes increased<sup>[44]</sup>. The question is whether a minimum number of lymph nodes should be resected to influence patient survival in UCUT.

Roscigno *et al.*<sup>[28]</sup> reported that a minimum of 7 lymph nodes should be removed to significantly improve survival in patients with pT2 stage tumors or higher. A multi-institutional study showed that the removal of 8 lymph nodes or more resulted in higher CSS compared to the removal of less than 8 lymph nodes in patients with  $\geq$  pT1pN0<sup>[37]</sup>. In this patient cohort, the risk of cancer mortality continued to decrease as the number of lymph nodes removed increased, as in bladder cancer.

We also examined whether there is a minimum number of resected lymph nodes that can affect patient survival<sup>[45]</sup>. Our results showed that there was no cutoff value that significantly influenced patient survival (Figure 6). Eight lymph nodes were likely to be a minimum requirement for improving patient survival, but it was not a statistically significant value. In contrast, template-based lymphadenectomy was significantly associated with a higher CSS rather than incomplete lymphadenectomy where all regional sites were not resected. Thus, lymphadenectomy should be performed by following the anatomical template. We believe that the number of resected lymph nodes cannot be used to determine the extent of lymphadenectomy, but can be used for assessing the adequacy of lymphadenectomy.

### Indication of lymphadenectomy

It is important to determine the indication of lymphadenectomy in UCUT. Patients who benefit from lymphadenectomy have been examined. According to studies that analyzed the benefit of lymphadenectomy (Table 2), this role is limited in patients with pT2 stage tumors or higher. The therapeutic benefit of lymphadenectomy is also more clearly demonstrated in patients with pT2 stage tumors or higher (Table 3). Thus, an indication for lymphadenectomy is assumed in patients with pT2 tumors or higher. This is also supported by the results showing the incidence of

lymphatic metastases according to pathological stage. Our data shows that the incidence of lymph node metastases increases incrementally as the pathological stage becomes higher (Figure 7). The risk of lymphatic metastases was only at 1% in the patients with pT1 stage tumors or lower, whereas tumors at the pT2 stage show a 7% risk of lymph node metastases. This risk increases to 26% in pT3 tumors. It is reasonable to perform lymphadenectomy in patients with pT2 tumors or higher.

However, there is a major concern about accurate preoperative staging based on current radiological modalities. Although multidetector computed tomography might provide more accurate staging<sup>[46]</sup>, it is likely to be very difficult to distinguish stage 1 from 2. In other words, invasion of the muscle layer of the renal pelvis or the ureter is very difficult to diagnose according to our results<sup>[47]</sup>. Some tumors clinically diagnosed as carcinoma in situ may upstage to the muscle-invasive diseases pathologically<sup>[48]</sup>. Thus, we currently consider all patients with an indication of nephroureterectomy as candidates for lymphadenectomy. We omit lymphadenectomy for patients of an advanced age or with severe comorbidity<sup>[47]</sup>.

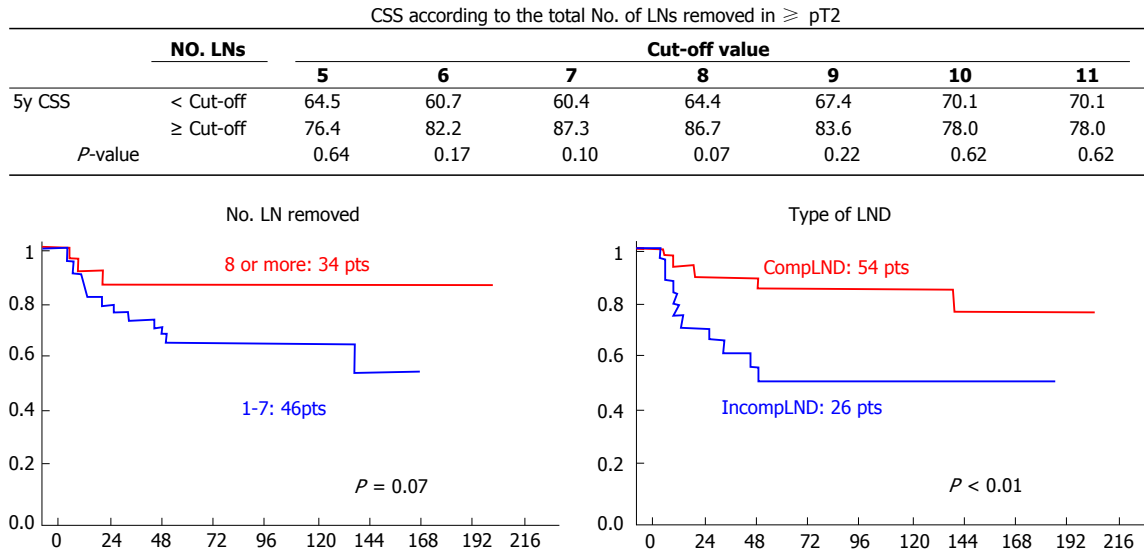
### Association with neoadjuvant or adjuvant chemotherapy

The role of neoadjuvant therapy has been discussed recently since a majority of patients is unfit for cisplatin-based chemotherapy after nephroureterectomy because of the development of chronic kidney disease<sup>[49,50]</sup>. In addition, adjuvant chemotherapy has little effect on improving survival<sup>[51,52]</sup>. Thus, the role of neoadjuvant chemotherapy has recently attracted physicians' attention.

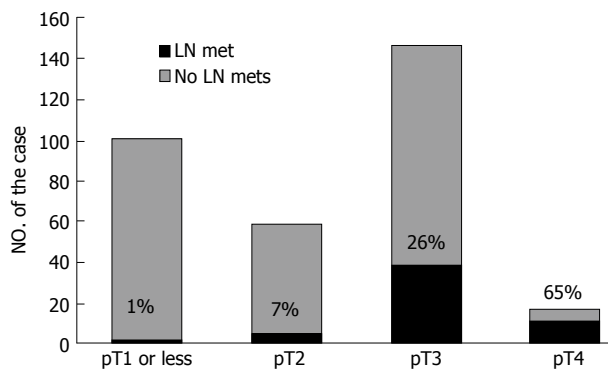
A recent retrospective study showed that cisplatin-based neoadjuvant chemotherapy significantly improved patient survival<sup>[53]</sup>. In addition, multivariate analysis showed that lymphadenectomies where more than 8 lymph nodes were resected were no longer a significant factor when neoadjuvant chemotherapy was included. Furthermore, it is difficult to draw a definitive conclusion from these results, which are from a single institute. However, further study is warranted to elucidate the association between the benefit of lymphadenectomy and neoadjuvant chemotherapy.

Adjuvant chemotherapy might enhance the therapeutic benefit of lymphadenectomy. Several studies examined the effect of adjuvant chemotherapy, but most failed to show an improvement in patient survival<sup>[51,52,54-56]</sup>. We examined the role of adjuvant chemotherapy in a retrospective study. Lymphadenectomy was a significant independent factor reducing the risk of cancer mortality, but adjuvant chemotherapy was not a significant factor, even in the univariate analysis (HR = 1.89, 95%CI: 0.677-5.43;  $P = 0.222$ )<sup>[35]</sup>. Our prospective study also showed that adjuvant chemotherapy does not influence either cancer-specific or disease-free survival on univariate analysis in patients with renal pelvic cancer<sup>[23]</sup>. Thus, these results suggest that the therapeutic benefit of lymphadenectomy





**Figure 6** The influence of the number of lymph nodes removed on cancer-specific survival. LND: Lymphadenectomy; CSS: Cancer-specific survival; LNs: Lymph nodes.



**Figure 7** The incidence of lymphatic metastases according to the primary tumor stage. LN: Lymph node.

is independent, but not synergistic with adjuvant chemotherapy.

#### Is laparoscopic or robotic lymphadenectomy feasible?

Lymphadenectomy was performed using an open procedure for all the patients in our study. The median yield of lymph nodes from template-based lymphadenectomy was 15 in renal pelvic cancer and 14 in ureteral cancer for our prospective study<sup>[23]</sup>. This number is believed to be the current standard, but it was only 7 in the lymphadenectomy cohort before 2006<sup>[35]</sup>.

Laparoscopic lymphadenectomy results were reported by Abe *et al.*<sup>[57]</sup> showing that the median number of resected lymph nodes was 10. They recently reported the prospective results for their current laparoscopic lymphadenectomy procedure<sup>[58]</sup>. The median number of lymph nodes removed increased to 14, which is very similar to the number from our prospective study for open lymphadenectomy<sup>[23]</sup>. Thus, experienced surgeons can perform laparoscopic lymphadenectomy as effectively as an open procedure. However, a long learning curve will be required.

Very few results of robotic lymphadenectomy with nephroureterectomy have been reported. Pugh *et al.*<sup>[59]</sup> reported that the median number of lymph nodes was 11. The mean number of resected lymph nodes was 14.1 according to Lee *et al.*<sup>[60]</sup>. These results are very similar to those from our prospective study. Our opinion is that robotic lymphadenectomy may be feasible. The appropriate procedure can be determined by the surgeons' experience. However, we believe that an open procedure is the most reliable and the experience of surgeons is not likely to influence its quality.

#### What are the disadvantages of lymphadenectomy?

The disadvantages of lymphadenectomy in UCUT should also be considered. In our prospective study, we compared the incidence of complications in the template-based lymphadenectomy group to that of the no lymphadenectomy group (Table 4)<sup>[23]</sup>. Patients who undergo template-based lymphadenectomy show a higher incidence of complications at all grades as well as grade 3 or higher, but without a significant difference. More frequent complications in the lymphadenectomy group include numbness in the thighs and lymphorrhea. Lymphorrhea including chyle fistulas occur at a higher incidence and grade in those who undergo lymphadenectomy than those who do not (5.2% vs 1.1%). One patient required percutaneous drainage, but conservative management spontaneously resolved the problem in other patients. Numbness in the thigh may be associated with lymphadenectomy for pelvic nodes (2.5% vs 0%).

Rao *et al.*<sup>[38]</sup> reported complications from a prospective study of super-extended lymphadenectomy that encompassed the area from the retroperitoneum to the pelvis. The morbidities in this study were transfusion (32%), ileus (5%), and chylous leakage (10%). Chylous leakage was managed with conservative treatment except for 1 patient for whom surgical intervention was

**Table 4 Perioperative complications of the template-based lymphadenectomy and the no lymphadenectomy group**

Template-based lymphadenectomy (77 patients)		No lymphadenectomy (89 patients )	
Morbidity	<i>n</i>	Morbidity	<i>n</i>
Grade 1		Grade 1	
Numbness of thigh	2	Atelectasis	1
lymphorrhea	1	Delirium	2
Wound infection	1	Wound infection	2
Grade 2		Lymphorrhea	1
Chylous leakage	1	Subcutaneous hematoma	1
Retroperitoneal abscess	1	Grade 2	
Lymphorrhea	1	Anemia	1
Gastric ulcer	1	Grade 4	
Grade 3a		Intraoperative massive bleeding	1
Lymphorrhea	1		
Grade 3b			
Rectal injury	1		
Ureteral injury	1		
Incidence (all grades)	11		9
	14.20%		10.10%
Incidence (≥ grade 3)	3		1
	3.90%		1.10%

needed.

We also compared intraoperative bleeding and operation time in patients who underwent template-based lymphadenectomy or no lymphadenectomy. The lymphadenectomy group showed more intraoperative bleeding and longer operation times (407 mL vs 321 mL, 323 min vs 288 min), but there was no significant difference<sup>[19]</sup>. The length of hospital stay after surgery did not differ between groups. A randomized prospective study examining the role of lymphadenectomy in renal cell carcinoma showed no increase in complications from extensive lymphadenectomy compared to no lymphadenectomy (26% vs 22%)<sup>[61]</sup>.

We performed lymphadenectomy in an open procedure with a retroperitoneal approach in all patients. Thus, we cannot comment on transperitoneal lymphadenectomy for UTUC. However, in the above randomized phase 3 trial for kidney cancer, all surgeries were done with a transperitoneal open procedure<sup>[61]</sup>. Thus, we believe that lymphadenectomy does not increase the risk of complications, irrespective of the approach used.

Thus, lymphadenectomy may result in a slight increase in complications including lymphorrhea or hemorrhage, but has no influence on patients' recovery from surgery. We should consider the complications of lymphadenectomy; however, they should not dissuade surgeons from performing lymphadenectomy except in patients with comorbidity or of an advanced age.

## CURRENT RECOMMENDATIONS FOR LYMPHADENECTOMY IN THE 2015 GUIDELINES

Four guidelines are currently available for UCUT. The latest European Association of Urology guidelines (2015 version) recommend lymphadenectomy for cases of

invasive disease<sup>[62]</sup>. The recommendation grade is still low at grade C. The National Comprehensive Cancer Network Clinical Practice Guidelines in Oncology Version 1.2015 state that lymphadenectomy should be a part of nephroureterectomy for high-grade tumors, or tumors that are large and invade the renal parenchyma<sup>[63]</sup>. The National Cancer Institute-Physician Data Query suggests that lymphadenectomy at the time of radical nephroureterectomy may offer prognostic information, but little, if any, therapeutic benefit<sup>[64]</sup>. The guideline of the Japanese Urological Association also supports the staging benefit, and recommends lymphadenectomy to improve survival in patients with advanced disease with suspected muscle invasion as a grade C recommendation<sup>[65]</sup>.

Thus, the current recommendation grade for lymphadenectomy still remains low; however, our nonrandomized prospective study is not incorporated<sup>[23]</sup>. The role of lymphadenectomy is expected to be supported by guidelines at a higher level than at present, especially in renal pelvic cancer.

## CONCLUSION

Herein, the current situation and issues of lymphadenectomy for UCUT have been summarized. There are some major problems underlying lymphadenectomy, including the lack of standardization of the extent of lymphadenectomy and a randomized prospective trial. However, we believe that lymphadenectomy is strongly recommended for tumors of the renal pelvis. Lymphadenectomy should follow the anatomical template. Further research is warranted to establish the role of lymphadenectomy in UCUT.

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## Biomarkers in triple negative breast cancer: A review

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### Abstract

Breast cancer is an intrinsically heterogeneous disease. In the world about 1 million cases of breast cancer are diagnosed annually and more than 170000 are triple-negative. Characteristic feature of triple negative breast

cancer (TNBC) is that it lacks expression of oestrogen, progesterone and human epidermal growth factor receptor-2/neu receptors. They comprise 15%-20% of all breast cancers. We did a systematic review of PubMed and conference databases to identify studies published on biomarkers in TNBC. We included studies with biomarkers including: Epidermal growth factor receptor, vascular endothelial growth factor, c-Myc, C-kit and basal cytokeratins, Poly(ADP-ribose) polymerase-1, p53, tyrosinase kinases, m-TOR, heat and shock proteins and *TOP-2A* in TNBC. We also looked for studies published on synthetic lethality and inhibition of angiogenesis, growth, and survival pathways. TNBC is a complex disease subtype with many subclasses. Majority TNBC have a basal-like molecular phenotype by gene expression profiling. Their clinical and pathologic features overlap with hereditary *BRCA1* related breast cancers. Management of these tumours is a challenge to the clinician because of its aggressive behaviour, poor outcome, and absence of targeted therapies. As the complexity of this disease is being simplified over time new targets are also being discovered for the treatment of this disease. There are many biomarkers in TNBC being used in clinical practice. Biomarkers may be useful as prognostic or predictive indicators as well as suggest possible targets for novel therapies. Many targeted agents are being studied for treatment of TNBC.

**Key words:** Triple negative breast cancer; Epidermal growth factor receptor; Vascular endothelial growth factor; p53; Cyclin

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**Core tip:** Triple negative breast cancer (TNBC) are type of breast cancer which lack of estrogen receptors, progesterone receptors and human epidermal growth factor receptor. It is a complex disease subtype with many subclasses. There are many biomarkers in TNBC used for its sub-classification. Clinically-practical assay/biomarkers that can reliably identify TNBC are

necessary. Biomarkers may be useful as prognostic or predictive indicators as well as suggest possible targets for novel therapies.

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## INTRODUCTION

Breast cancer is a complex disease entity with different biological characteristics and clinical behaviour. Many clinical and pathological features have been defined to predict outcome and treatment response in breast cancer. These features include: Patient age, tumour stage, axillary lymphnode involvement, lymphovascular invasion, histologic grade, hormonal and human epidermal growth factor receptor (HER-2/neu receptor) status. In the past chemotherapy was the only systemic therapy for triple negative breast cancer (TNBC) patients. Currently lot of research is going on to further characterise TNBC with different molecular markers and find targets for therapy in order to improve its outcome. Sørli *et al.*<sup>[1]</sup> has diversified five subgroups of breast cancer by gene expression profiling (GEP) using DNA microarrays. These are luminal A, luminal B, HER-2/neu over expressing, basal like (BL) and normal like breast cancer. BL breast cancer lacks estrogen receptors (ER), progesterone receptors (PR) and HER-2/neu receptors, thus contribute to 80% of TNBC<sup>[1,2]</sup>. The present review provides an insight into the different biomarkers in TNBC and its sub classification based upon the marker profile to understand molecular targets in each subtype.

## TNBC

TNBC<sup>[3]</sup> are type of breast cancer which lack ER, PR and HER-2/neu receptors. It has different and poor clinical and pathological features as compared to other subtypes of breast cancer. It is usually seen in young age, advanced stage at presentation, unfavourable histopathology, grade III, higher proliferative index, lack of tubule formation and higher rate of metastases<sup>[4-9]</sup>. It is associated with higher rate of local recurrence during 3 year after treatment and a high 5 year death rate<sup>[10]</sup>. Survival is poor after distant metastasis<sup>[11,12]</sup>. TNBC frequently affects younger patients (< 50 years) and has higher prevalence in the African-American women<sup>[13]</sup>. Patients with TNBC has inferior disease free survival (DFS) and overall survival (OS) as compared to age and grade matched controls of non-TNBC patients<sup>[11]</sup>. In TNBC metastatic rate is high to visceral organs<sup>[14,15]</sup> and lung and cerebral metastasis is more common<sup>[16-19]</sup>. Cytotoxic chemotherapy is the only treatment option<sup>[20-22]</sup>.

### TNBC subtypes

TNBC is a distinct breast cancer. It is classified into six

groups based upon the GEP and DNA microarray. This sub-classification is not only useful in understanding the disease better but also to find molecular targets for its treatment<sup>[23]</sup>.

**BL-1 and BL-2:** The BL-1 subtype was found to be composed rapidly dividing cells associated with increased proliferation and cell cycle checkpoint loss consistent with the increased expression of DNA damage response genes. Due to its high proliferation rate it has increased Ki67 mRNA expression and it is more responsiveness to antimetabolic agents targeting cell cycle. The BL-2 subtype on the other hand displayed unique gene ontologies involving epidermal growth factor signalling as well as glycolysis and gluconeogenesis pathway. On microarray it showed a higher expression of epidermal growth factor receptor (EGFR), TP63, MET, *etc.*

**Immunomodulatory subtype:** Immunomodulatory (IM) is composed of immune cell responses such as immune cell and cytokine signalling, antigen presentation and processing and signalling of immune transduction pathways. Its GEP substantially overlaps with the medullary breast cancer, histologically a rare distinct form of TNBC which carry favourable prognosis despite its high grade.

**Mesenchymal and mesenchymal stem like subtype:** On GEP these subtypes consists of epithelial-mesenchymal (M) transition and growth factor pathways. The mesenchymal stem like subtype is also expressed by genes involved in angiogenesis including VEGFR2 and was found to be highly responsive to dasatinib [tyrosine kinase (TK) inhibitor], and mTOR inhibitors.

**Luminal androgen receptor subtype:** This subtype is characterised by androgen receptor (AR) signalling. It is ER negative but gene ontologies were heavily composed of hormonally regulated pathways such as steroid synthesis, porphyrin metabolism and androgen/estrogen metabolism. AR mRNA expression was nine times higher than other subtypes therefore, these lines were found to be highly sensitive to AR antagonists eg bicalutamide. Patients with this subtype had decreased DFS and OS.

### Basal cell and TNBC

Among TNBCs 80%-90% falls into the category of BL molecular subtype when appropriately tested for IHC cancer biomarkers and GEP but these terms are nonsynonymous and are overlapping<sup>[10,24]</sup>. At present, there is no optimal IHC panel for identification of basal like breast cancer (BLBC). Therefore TNBC, despite having above limitations is considered as a BL cancer. In a study Thike *et al.*<sup>[9]</sup> with a tri-panel of cytokeratin-14 (CK-14), EGFR and 34βE12 in TNBC reported 84% to be BL tumors with a specificity and sensitivity of 100% and 78% respectively. In BLBC over expression of ID4 leads to the deregulation of *BRCA1*. BLBCs are also

**Table 1** Epidermal growth factor receptor expression in triple negative breast cancer

Ref.	Total number	No. of TNBC subjects	EGFR expression <sup>1</sup>
Thike <i>et al</i> <sup>[9]</sup> , 2010	7048	767	30%
Patil <i>et al</i> <sup>[10]</sup> , 2011	683	136	7.4%
Nielsen <i>et al</i> <sup>[24]</sup> , 2004	-	21 basal like tumours	57%
Rakha <i>et al</i> <sup>[45]</sup> , 2007	1726	282	37% in TNBC vs 15% in non-TNBC
Mehdizadeh <i>et al</i> <sup>[47]</sup> , 2012	1132	103	23.3%
Rydén <i>et al</i> <sup>[48]</sup> , 2010	564	48	41% TNBC vs 11% non-TNBC

<sup>1</sup>The expression is depicted as the percentage of patients expressing the marker. TNBC: Triple negative breast cancer; EGFR: Epidermal growth factor receptor.

known to have either p53 over expression or mutations in the gene<sup>[24]</sup>.

In array, BLBCs are characterised by low expression of ER and HER-2 related genes, so pathologically they are usually ER-negative, PR-negative and lack HER-2 over expression<sup>[8,9]</sup> or are < 1%; < 5%; 10%; 20% immunoreactive for the above receptors<sup>[24]</sup>. They stain positive for cytokeratins (CKs) 5/6 and 17, and over express EGFR (HER1). Furthermore they show a highly aggressive GEP with low Bcl-2 but high p53 and Ki67<sup>[25-29]</sup>.

## BRCA AND TNBC

Genetic instability leads to cancer predisposition. Genetic mutations in the *BRCA* genes in patients predisposes them to develop many cancers such as breast, ovarian, pancreatic and prostate. *BRCA 1* plays vital role in DNA repair by homologous recombination. Inactivation of this gene due to *BRCA* mutation should trigger cell cycle arrest but this too is inhibited by p53 mutations in TNBC<sup>[30]</sup>. Lack of a functional *BRCA1/2* in cells lead to loss of repair of DNA double-strand breaks (DSB). This mechanism leads to increased risk of cancer in these patients. Histologically and transcriptionally, TNBC share similarities with *BRCA1*-linked breast cancers, which means that dysfunction of *BRCA1* is seen in TNBCs<sup>[31,32]</sup>. TNBCs are heterogeneous with respect to GEP. TNBC is associated with cancers arising in *BRCA1* mutation carrier in young women as compared to those in their late forties. Both sporadic BLBCs and *BRCA1* associated breast cancers have evidence of genomic instability. More than 80% of breast cancers in women who carry germ-line *BRCA1* mutations are TN and 10% TN breast tumors have *BRCA1* mutation. The reasons for these associations are unclear but may ultimately provide avenues for prevention as well as targeted therapy with poly(ADP-ribose) polymerase (PARP) inhibitors and chemotherapy with DNA-damaging agents such as platinum compounds<sup>[33-35]</sup>.

## Biomarkers in TNBC

TNBC is characterised by the marked expression of certain biomarkers. The presence of these molecules though is not restricted to TNBC but somehow show increased prevalence in this subgroup. The following are the important biomarkers in TNBC.

**EGFR:** EGFR is one of the members of four closely related receptors each playing an important role in tumour cell survival. The four receptors being EGFR (or ErbB-1), HER-2/neu (ErbB-2), HER-3 (ErbB-3), and HER-4 (ErbB-4)<sup>[36,37]</sup>. The inactive monomer receptor dimerizes after ligand activation followed by TK, intracellular domain of the receptor is activated by autophosphorylation, leading to cascade of intracellular events. EGFR signal cascade is important for cell proliferation, angiogenesis, metastatic spread, and the inhibition of apoptosis<sup>[38]</sup>. Most of the TNBCs express EGFR, and poses a strong therapeutic challenge<sup>[39]</sup>. Studies with different methods of gene amplification have found variable expression EGFR in metaplastic breast carcinoma, a phenotypes of BLBCs<sup>[40-42]</sup>. However, Toyama *et al*<sup>[43]</sup> with real-time polymerase chain reaction have reported high *EGFR* gene copy number in TNBCs. EGFR expression is found in 40%-50% of patients with breast cancer and in 80% of TNBC; and is estimated to substitute major proliferation pathways of breast cancer induced by activation of HER-2, ER, PR proteins which are thereby absent in TNBC<sup>[25]</sup>.

In a study the authors found that 60% of patients with grade III and > 3 lymph nodes showed EGFR expression, indicating that EGFR expression is related to aggressiveness of the disease. They also concluded that patients with EGFR expression had worse DFS, distant disease free survival (DDFS), OS and cause specific survival<sup>[44]</sup>. EGFR expression in TNBC is associated with poor response to chemotherapy<sup>[45]</sup>. Nogi *et al*<sup>[46]</sup> observed that EGFR was expressed in 24% of the TNBC patients and was related to less favourable response to chemotherapy and poorer survival and on the contrary the luminal groups where EGFR expression showed good response to chemotherapy and better survival. Recently EGFR has been defined with other markers to differentiate BL subtype from TNBC<sup>[47]</sup>. This aids in segregating TNBC into subtypes and thus defining the prognostic difference and molecular target specification between the two. Non-uniformity of expression profiles in studies shown in Table 1 is due to absence of subtype consideration or BL subtype non segregation from core TNBC. So EGFR is a biomarker in TNBC and a target for cetuximab, a TK inhibitor<sup>[48]</sup>. Many studies have evaluated its response in TNBC<sup>[48-51]</sup>. In a recent study, EGFR expression was shown as prognostic factor for DFS



**Table 2** Vascular endothelial growth factor receptor expression in triple negative breast cancer

Ref.	Total number	No. of TNBC	VEGFR-2 expression <sup>1</sup>
Mehdizadeh <i>et al</i> <sup>[47]</sup> , 2012	1132	103	93.2%
Iosifidou <i>et al</i> <sup>[63]</sup> , 2009	-	73	77%
Chanana <i>et al</i> <sup>[63]</sup> , 2012	70	27	54% vs 23%
Linderholm <i>et al</i> <sup>[67]</sup> , 2008	679	87	Higher intratumour VEGF levels in TNBC
Andre <i>et al</i> <sup>[68]</sup> , 2009	69	35	34%

<sup>1</sup>The expression is depicted as the percentage of patients expressing the marker. TNBC: Triple negative breast cancer; VEGFR: Vascular endothelial growth factor receptor; VEGF: Vascular endothelial growth factor.

not only in univariate but also in multivariate analysis<sup>[52]</sup>.

**Vascular endothelial growth factor:** Angiogenesis is important for tumour growth and spread especially beyond a diameter of 2 mm as oxygen and nutrients cannot diffuse beyond this distance. Angiogenic signals are mediated by vascular endothelial growth factor (VEGF) to aid neovascularisation. VEGF A, B, C, D, E (viral factor) and placental growth factor is a family of six proteins. VEGF protein is found in 4 isoforms because of alternative splicing of its mRNA<sup>[53,54]</sup>. Among the different isoforms VEGF165, the 165-amino acid molecule is more common<sup>[55,56]</sup>. Its gene expression is controlled by many of stimuli such as hypoxia, nitric oxide, growth factors, oncogenes, tumour suppressor genes and HER-2<sup>[57]</sup>.

It causes proliferation and maintains structural and functional integrity of cells of the endothelium. It also regulates vascular permeability and migration of endothelial stem cells from the bone marrow<sup>[58]</sup>. Neovascularisation in the tumour is also regulated by VEGF by increasing the expression of the anti-apoptotic proteins such as Bcl2, XIAP, and survivin. In its absence the endothelial cells undergo apoptosis and newly formed vessels disintegrate<sup>[59-61]</sup>. Thus neovascularisation is dependent on VEGF expression throughout tumour development. VEGF shows multiple interactions with receptor TKs, such as VEGFR-1, VEGFR-2, and VEGFR-3. The angiogenesis is initiated by VEGF binding to VEGFR-2 which triggers the specific activation of TKs followed by multiple signalling cascades resulting in the endothelial cells survival, proliferation, migration, adhesion, actin remodelling and vessels permeability<sup>[62]</sup>.

VEGF expression is elevated in DCIS and invasive breast cancer. It has been also well utilised for prognosis in breast cancer<sup>[63,64]</sup>. Its quantification by IHC or immunoassay of tissue extracts has shown a significant co relation with micro vessels counts or density. High mean vascular density in breast cancer has been found to linked with more aggressive tumour behaviour and poor survival so intratumoral microvessels density is now considered as one of the important factors affecting survival<sup>[65]</sup>. According to recent studies<sup>[63,66]</sup> there was a direct co relation between serum and tissue levels of VEGF to grade III tumours, larger tumour size,

positive lymph node and negative hormone status and poor survival along with a substantial decrease in levels with chemotherapy. In TNBC higher VEGF levels are associated with shorter DFS, OS, and DDFS. Also VEGF levels have been significantly related to size of the tumour, grade and metastatic sites. In patients with higher VEGF levels disease progressed despite of therapy and such patients were associated with significantly lower progression free survival as compared to patients with lower levels. In TNBC patients it was found that VEGF level elevated from baseline to middle of the therapy significantly but showed a non significant increase from middle of the therapy to its end when patients were administered FAC<sup>[65-67]</sup>. VEGF is a target for bevacizumab in TNBC patients. Table 2 shows VEGF expression reported in different studies.

**C-kit and basal cytokeratins:** C-kit is a cytokine receptor present on the surface of hematopoietic stem cells and also in other cells. C-kit binds to stem cell factor and is a growth factor receptor that stimulates major cellular functions such as cell survival, proliferation, differentiation, adhesion and chemotaxis. It induces apoptosis and also increases the invasiveness of the cancer cells<sup>[68]</sup>. CKs are keratin-containing proteins of intermediate filaments found in the intracytoplasmic cytoskeleton of epithelial tissue. Different epithelial tissues express different CKs at the time of its terminal differentiation and the stage of development. This different CK expression helps in the classification of all epithelia. Similarly different cancers express specific CKs of that epithelium. Therefore the CK expression profile tends to remain constant when an epithelium undergoes malignant transformation.

The study of the CK profile by IHC techniques is very important for tumor pathologic classification<sup>[69]</sup>. These CKs were earlier used to distinguish malignant breast lesions from benign ones<sup>[70]</sup>, but later their prognostic value was ascertained and it was seen that expression of CK-5, CK-14 and CK-17 was related to poor prognosis, high grade tumours, ER negativity, short DFS and OS<sup>[71-73]</sup>. It is expressed in BLBCs. Since BLBC and TNBC show overlapping features therefore C-kit and basal CKs along with other markers and pathological features are used for the differentiating BLBCs from

**Table 3** C-kit expression in triple negative breast cancer

Ref.	Total number	No. of TNBC	C-kit expression <sup>1</sup>
Thike <i>et al</i> <sup>[90]</sup> , 2010	7048	767	CK 5/6 in 6%, CK-14 in 48%, CK-17 in 50%, C-kit in 45%
Nielsen <i>et al</i> <sup>[24]</sup> , 2004	-	21	CK 5/6 in 62% and C-kit in 29%
Kim <i>et al</i> <sup>[76]</sup> , 2009	625	147	CK5/6 in 35.4% and C-kit in 11.6%
Bryan <i>et al</i> <sup>[78]</sup> , 2006	66	4	75% of TNBC vs 29% of non-TNBC

<sup>1</sup>The expression is depicted as the percentage of patients expressing the marker. TNBC: Triple negative breast cancer; CK: Cytokeratin; EGFR: Epidermal growth factor receptor.

TNBC. Many studies have revealed that presence of CKs is higher in TNBC than non-TNBC and also among TNBC subgroup it is higher in the BL subclass (Table 3). BL subclass of TNBC was identified on the basis of CK and EGFR expression and when the clinicopathological features were compared between the basal and non-BL it was seen that BL subclass of TNBC were more aggressive<sup>[9,74-78]</sup>.

**p53:** It is a tumour suppressor protein which is encoded by the *TP53* gene (the tumour suppressor gene). It is also called the “guardian of genome” as it is important cell cycle regulator<sup>[79]</sup>. It regulates cell growth, multiplication, proliferation and apoptosis, and promotes chromosomal stability. Disruption of these functions by mutation in the gene producing p53 lead to carcinogenesis. p53 is activated in response to cellular stress by many pathways that are dependent on distinct upstream regulatory kinases. First, an ataxia-telangiectasia mutated proteins released in response to the DSB, second, a pathway dependent on *INK4* gene product, p14ARF activated by oncogenes, and finally, a pathway induced by chemotherapy drugs and ultraviolet light and is independent of the above two pathways<sup>[80,81]</sup>.

p53 mutations are seen in 18%-25% of primary breast carcinomas (Table 4)<sup>[82]</sup>. p53 plays an important role in breast cancer prognosis. p53 over expression leads to poor response to chemotherapy<sup>[83,84]</sup>. Many studies have reported that its activation is associated with aggressive form of breast cancer and significantly decreases DFS and OS in TNBC patients<sup>[85-88]</sup>. Also co existence with HER-2 was significantly related to early relapse and death within shorter period after surgery<sup>[87]</sup>. Along with EGFR and cytokeratins it is used for segregation of a subclass, *i.e.*, basal like from core TNBC<sup>[89]</sup>.

Tumours with p53 mutation are highly invasive, poorly differentiated and high grade tumours. In a study by Chae *et al*<sup>[90]</sup>, p53 mutation was associated with poor response to the chemotherapy in TNBC patients. Other proteins of p53 family are p63/p73 proteins. Tumors expressing these proteins are reported to have many folds higher sensitivity to platinum based chemotherapy. p63/p73 expression is seen in one-third of patients with TNBC<sup>[91]</sup>.

**TOP-2A:** This gene encodes topoisomerase II  $\alpha$  and

plays a crucial role in DNA transcription. This enzyme causes the temporary break of double strands of duplex DNA and rejoins them so that the strands cross through one another, therefore altering the topology of DNA. Mutation in cancer leads to deprivation of its functions and thus worsening of the situation. In TNBC or breast carcinoma the gene acts as a target for anthracycline therapy which is a topoisomerase II inhibitor<sup>[92]</sup>. So it is a marker for the evaluation of resistance to the anthracycline therapy. A study revealed a higher expression of *TOP-2A* in 2.7% to 8.8% of TNBC patients<sup>[93]</sup>. Its over expression in TNBC leads to the decreased sensitivity towards the anthracyclines and thus decreased response<sup>[94]</sup>.

**Ki67:** Also known as MKI67, Ki67 is a cellular marker for proliferation. Ki67 antigen is present inside the cell nucleus during interphase and during mitosis it is relocated to the surface of the chromosomes. Since it is a marker of proliferation it is found in all cells when they are in dividing phases of the cell cycle (G<sub>1</sub>, S, G<sub>2</sub>, and mitosis) and it is absent from cells during their resting phase (G<sub>0</sub>). Its absence in resting cells and generalised presence in dividing cells had made it a marker of cell proliferation<sup>[95]</sup>. Proliferation is a salient feature for the spread of cancer and can be assessed by the IHC measurement of the nuclear antigen Ki67. It's over expression also correlates with levels of bromodeoxyuridine uptake and S-phase fraction, other markers of proliferation.

Ki67 expression is less in normal breast tissue (< 3%). It has been reported in many studies that Ki67 antigen and steroid-receptor are expressed in different cells in normal human breast epithelium. Ki67 was over expressed particularly in ER-negative cells and its expression in carcinoma cells was much higher<sup>[96,97]</sup>. In breast cancer high Ki67 is associated with of poor outcome although these tumours show very good clinical response to combination chemotherapy. However, its independent significance is modest and does not merit measurements in routine clinical practice. With respect to treatment response in breast cancer, Ki67 expression was found to be independent predictor of pathologic complete response (pCR), clinical complete response, OS and DDFS and locoregional recurrence. It was also seen that patients without pCR still showed a decrease in Ki67 index post therapy<sup>[98-100]</sup>. In a recent meta-analysis

Table 4 p-53 expression in triple negative breast cancer

Ref.	Total number	No. of TNBC	p-53 expression
Patil <i>et al</i> <sup>[10]</sup> , 2011	683	135/683	47.8%
Nielsen <i>et al</i> <sup>[24]</sup> , 2004	11	11	82%
Rakha <i>et al</i> <sup>[45]</sup> , 2007	1726	282/1726	56% in TNBC vs 22% in non-TNBC
Chae <i>et al</i> <sup>[90]</sup> , 2008	135	32/135	40.6% in TNBC vs 42.7% in non- TNBC
Biganzoli <i>et al</i> <sup>[89]</sup> , 2011	-	(633 + 1026) from two separate sources	Divided TNBC into subclass BL which accounts for 89% of total TNBCs

TNBC: Triple negative breast cancer; BL: Basal like.

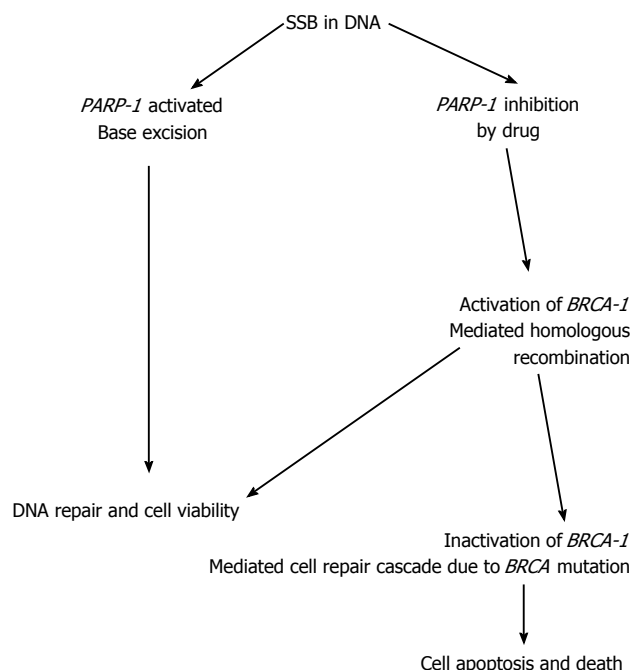
by de Azambuja *et al*<sup>[101]</sup> who retrieved DFS data from 29 studies, they concluded that high Ki67 levels was associated with poor prognosis irrespective of nodal status and whether patients undergo treatment or not at all.

In TNBC, it was found that Ki67 levels were significantly increased in ductal TNBC compared to other histologic types (80% in TNBC vs 10%-30% in other types). Its expression also represented a direct correlation with tumour size and grade in TNBC patients and higher levels (> 35% staining) were linked with an increased risk of death<sup>[102,103]</sup>. In TNBC patients Ki67 accumulation was associated with a higher pCR to chemotherapy but poor RFS and OS. Its expression was also used for subdivision of TNBC into two subtypes where only 26.7% of TNBC patients showed lower Ki67 expression<sup>[104]</sup>.

**PARP:** PARPs are a family of cell signalling enzymes present in eukaryotes, which catalyses the poly(ADP-ribosylation) of DNA binding proteins. Till now eighteen enzymes of PARPs has been detected, but PARP1 the most common isoform. PARP1 is responsible for majority of its functions. Main function of PARP1 is as DNA damage nick sensor. It forms polymers of ADP-ribose and nicotinamide with use of NAD<sup>+</sup>. Activation of PARP1 is important in tumours because of three interesting biological reasons: First, it plays a vital role in DNA repair through base excision repair pathway; second, it is capable of depleting cellular energetic pools, which results in cell dysfunction and necrosis; and third, its ability to promote the transcription of proinflammatory genes. PARP enzymes are involved in cellular response in inflammation, ischemia and oxidative stress. Carcinogenesis is a multistep process involving alterations in many cellular processes such as genomic stability, cell division, proliferation, growth, differentiation and cell death. PARP1 are involved in all these cellular processes, indicating possible link between PARP1 function and carcinogenesis<sup>[105]</sup>. PARP1 repairs DNA single strand breaks (SSB) by binding to the exposed ends of the damaged DNA strand and bring in important enzymes required for repair in SSBs<sup>[106-110]</sup>. The base excision repair pathway fails when PARP1 is inhibited; this leads to accumulation of SSBs. In a dividing cell entering S-phase, cell division is arrested at SSBs, leading to a DSB (Figure 1). In BRCA1 deficient cells excision repair pathway is dependent on

PARP1, inhibition of PARP1 leads to cell death through apoptosis<sup>[106,107]</sup>. BRCA2 operates through excision repair pathway like BRCA1, mutation of this gene make the cells susceptible to PARP inhibitors as well<sup>[109,110]</sup>. PARP also plays a vital role in DNA repair as BRCA. Unlike BRCA it recognises SSBs and repairs by base excision repair pathway<sup>[105]</sup>. PARP inhibitors are effective in TNBC because damage to one of the arms of the DNA could not be repaired by homologous recombination due to BRCA mutation and PARP inhibition in synergism will create a state of "synthetic lethality" - a process that occurs when inactivation of individual genes have no effect but mutations in both the genes lead to death of cancer cells<sup>[107]</sup>. So BRCA mutation is responsible for the action of many chemotherapeutic agents in TNBC. The inhibition of PARP1 is also known to potentiate the effect of ionizing radiation and many drugs such as DNA methylating agents, topoisomerase I inhibitors, and platinum compounds. Studies in mouse models have shown that the addition of PARP inhibitors with platinum compounds increases RFS and OS<sup>[35,105,107]</sup> while many of other studies on cell lines reveal that the activity of PARP inhibitors was increased in presence of BRCA mutations or dysfunction<sup>[105,108]</sup>. PARP1 has been targeted as therapeutic option in TNBC with drugs like iniparib, olaparib etc though not found to be independently helpful but their addition to cytotoxic agents have surely brought synergism to their activity and improvement in treatment response in TNBC patients.

**Heat shock protein 90:** It is a cellular chaperone (proteins that assist the assembly or disassembly of other macromolecular structures) protein that mediates the post-translational modification and stabilization of a number of conformationally labile proteins, steroid receptors, cyclin-dependent kinase 4, RAF-1, AKT and other proteins that are useful for sending proliferative signals<sup>[111]</sup>. Once function of heat shock protein (HSP) 90 is blocked, its dependent proteins are broken by proteasomes. Small HSP  $\alpha$ B-crystalline is expressed in BLBCs and is associated with shorter survival. Its' over expression is associated with neoplastic changes in mammary acini, increases cell migration and invasion in vitro. Geldanamycin and tanespimycin both are antibiotics and inhibitors of HSP. These have shown clinical benefit in HER2-positive metastatic breast cancer<sup>[112]</sup>. The PU-H71 another HSP blocker has shown complete response in TNBC models<sup>[113]</sup>.



**Figure 1 Mechanism of action of poly(ADP-ribose) polymerase-1 inhibitors in triple negative breast cancer.** PARP-1: Poly(ADP-ribose) polymerase-1; SSB: Single strand breaks.

**Cox-2:** Cox is a conversion enzyme of arachidonic acid and prostaglandin. It is a 74kDa protein located in the cell endothelium, reticulum and nuclear membrane. It is expressed by stimuli such as inflammatory response and tumor promoters. In a study by Liu *et al.*<sup>[114]</sup> they observed that 85% of transgenic mice with over expression of Cox developed breast cancer, suggesting the involvement of this enzyme in breast carcinogenesis. Other studies have correlated its expression with invasiveness and metastatic stimuli in breast cancer<sup>[115,116]</sup>. Approximately 40% of patient with breast cancer over expresses Cox-2. Cox-2 can also be used as a biomarker to assess response to neoadjuvant chemotherapy in breast cancer.

Lymph node status is major of prognostic significance in breast cancer patients. Studies have shown that Cox-2 expression is associated with positive lymph node involvement. So Cox-2 may have some role in lymphangiogenesis. Cox-2 expression has been also correlated to hormone receptors in breast cancer, negative hormone receptors with Cox-2 expression indicate worse prognosis. Cox-2 is correlated to HER2 through Ras/MAPK pathway and it is associated with HER2 over expression<sup>[117]</sup>. Cox-2 expression is also related to MDR-1, a multidrug resistance gene. Patients with expression of both these are least responsive to chemotherapy. So Cox-2 can be a good biomarker in breast cancer patients with its correlation with size of the tumour, number of nodes involved, hormone receptors and HER2 status<sup>[118]</sup>.

**TK:** TKs are regulatory proteins that help in the cell

growth and differentiation. These proto-oncogenes play an important role in progression and metastasis of cancer cells. They also increase sensitivity of cancer cells once the tumour has been exposed to radiation and chemotherapy through apoptosis<sup>[36]</sup>. Hence, TKs are of major interest and are subject of many active studies to look targets for therapeutic intervention in many solid tumours. HER2/neu and EGFR are also TKs receptors as discussed above. HER2/neu over-expression is seen in 20%-25% of invasive breast cancers and it is considered a poor prognostic factor. Other TKs over-expressed in carcinoma of the breast are BRK, c-Src, and EGFR<sup>[119]</sup>. Lack of expression of some of TKs such as Syk and C-kit are also linked to carcinogenesis of breast cancer. TK over-expression in women with breast cancer is have high risk of metastasis. There are many agents that target the phosphorylation of the receptor by acting at TK<sup>[120]</sup>. TK inhibitors such as imatinib, erlotinib, gefitinib and lapatinib are used for treatment of many solid tumours. Dasatinib and lapatinib are used in treatment of women with HER2/neu positive breast cancer.

**Mammalian target of rapamycin:** One of the pathway is commonly dysregulated in breast cancer is phosphatidylinositol 3-kinase/mammalian target of rapamycin (PI3K/mTOR). Over expression of the PI3K/mTOR is associated with poor response to treatment with hormones and trastuzumab<sup>[121]</sup>. To overcome endocrine resistance agents such as rapalogs, that efficiently block mTOR-raptor complex 1, can be used along with hormones. However, it has demonstrated variable results in hormone receptor positive metastatic breast cancer<sup>[122]</sup>.

Many targets such as  $\alpha$ V $\beta$ 6, cyclin E, C-kit, E-cadherin, O<sup>6</sup>MGMT, FOXp3,  $\beta$ -blockers, insulin like growth factors, glycoprotein NMB and mitogen-activated protein kinase pathway needs further exploration to dissect TNBC and may possibly identify new biomarkers and targets for therapy.

## CONCLUSION

TNBC is the most poorly understood and is refractory to current targeted therapies. It is a cause of significant breast cancer mortality because of very few treatment options. Biomarker may be useful as prognostic or predictive indicators as well as suggest possible targets for novel therapies. Targeted therapy directed against many biomarkers has not shown significant improvement in outcome in TNBC, therefore it is challenging for the clinicians to deal with this distinct disease. The emphasis should be put on research for effective drugs and targets for the treatment TNBC. So, to translate the present knowledge about TNBC into oncological practice, biomarkers/molecules/GEP assays that can truly classify TNBC and can be easily translated to the clinics are necessary.



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## Dynamic role of myofibroblasts in oral lesions

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### Abstract

Fibroblasts are the most abundant cellular components of connective tissue. They possess phenotypical heterogeneity and may be present in the form of smooth muscle cells or myofibroblasts (MFs). MFs are spindle-shaped cells with stress fibres and well-developed fibronexus, and they display  $\alpha$ -smooth muscle actin immunohistochemically and smooth-muscle myofilaments ultrastructurally. MFs play a crucial role in physiological and pathological processes. Derived from various sources, they play pivotal roles not only by synthesizing and producing extracellular matrix components, such as other connective tissue cells, but also are involved in force production. In the tissue remodelling phase of wound closure, integrin-mediated interactions between MFs and type I collagen result in scar tissue formation. The tumour stroma in oral cancer actively recruits various cell types into the tumour mass, where they act as different sources of MFs. This article reviews the importance of MFs and its role in pathological processes such as wound healing, odontogenic cysts and tumours, salivary gland tumours, oral preneoplasia, and oral squamous cell carcinoma. Research oriented on blocking the transdifferentiation of fibroblasts into MFs can facilitate the development of noninvasive therapeutic strategies for the treatment of fibrosis and/or cancer.

**Key words:** Myofibroblasts; Neoplasm; Fibroblasts; Precancerous lesions; Carcinoma-associated fibroblasts; Precancerous conditions

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**Core tip:** Myofibroblast (MFs) are spindle-shaped cells consisting  $\alpha$ -smooth muscle actin myofilament. They have a multicellular origin. MFs of the oral cavity have more contractile ability than dermal fibroblasts in physiologic wound healing. Recently, carcinoma-associated fibroblasts (CAFs) have received considerable attention because of their role in carcinogenesis. Mainly,

transforming growth factor- $\beta$  released from oral cancer cells is responsible for transforming fibroblasts into CAFs, which leads to tumour progression. However, the role of MFs in oral leukoplakia and oral submucous fibrosis is not completely understood. Understanding the implications of therapeutic approaches for the transdifferentiation of fibroblasts into MFs at different stages of carcinogenesis will facilitate in developing a treatment plan.

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## INTRODUCTION

Oral mucosa comprises stratified squamous epithelium, and its underlying connective tissue harbours various mesenchymal cells such as fibroblasts, endothelial cells, and pericytes<sup>[1,2]</sup>. These cells and their extracellular matrix (ECM) play pivotal roles in cell differentiation and proliferation during tissue development, wound healing processes, and pathological alterations<sup>[2,3]</sup>.

Fibroblasts, the most abundant cellular components of connective tissue, are phenotypically heterogeneous and are present in the form of smooth muscle cells or myofibroblasts (MFs)<sup>[4]</sup>. Gibbani was the first to observe fibroblasts under an electron microscope and coined the term "myofibroblast"<sup>[5]</sup>.

MFs may be defined as large spindle-shaped cells with stress fibres and well-developed fibronexus. They display  $\alpha$ -smooth muscle actin ( $\alpha$ -SMA) immunohistochemically and smooth muscle myofilaments ultrastructurally<sup>[6,7]</sup>. Therefore, MFs, which are found in normal skin tissue; pulmonary septa; and periodontal ligament<sup>[8]</sup>, are unique<sup>[4]</sup> and owing to their location, they are called as "juxtaparenchymal cells"<sup>[4]</sup>.

MFs show functional heterogeneity through various mechanisms. During mesenchymal-epithelial interactions, they play a pivotal role in organogenesis and morphogenesis. They secrete the components of the ECM and basement membrane<sup>[4]</sup>. Their myomechanical function is crucial during wound healing<sup>[9]</sup>. Their role in the carcinogenic process is well-recognised<sup>[10]</sup>. This article describes the cascade of events pertaining to the role of MFs in wound healing, oral squamous cell carcinoma (OSCC), odontogenic cysts and tumours, and salivary gland tumours.

## DISCUSSION

### Genesis and identification of MFs

MFs are multicellular in origin (Figure 1). Local fibroblasts, pericytes, endothelial cells, circulating hematopoietic precursor cells, and fibrocytes are various sources of

MFs. They are also derived from bone marrow and are known as bone marrow-derived MFs<sup>[11]</sup>. The main factor responsible for the transition (activation) of fibroblasts to MFs is transforming growth factor- $\beta$ 1 (TGF- $\beta$ 1)<sup>[12]</sup>. A previous study demonstrated that connective tissue factors (thrombin and endothelin) and platelet-derived growth factor (PDGF) are also responsible for the differentiation of fibroblasts into MFs<sup>[11]</sup>. According to Werner *et al.*<sup>[13]</sup>, keratinocytes play an important role by releasing interleukin-1, activin, and TGF- $\beta$ 1 during the differentiation of fibroblasts into promyofibroblasts into MFs. Thus, three local events are required to generate  $\alpha$ -SMA-positive differentiated MFs: (1) the accumulation of biologically active TGF- $\beta$ 1, which enhances the assembly of stress fibres and the formation of fibronexus adhesion complexes; (2) the accumulation of an ED-A splice variant of fibronectin, which are specialised ECM proteins; and (3) the mechanical properties of the ECM and cell remodelling activities, which are responsible for high extracellular stress<sup>[9-11]</sup>. Carthy *et al.*<sup>[14]</sup> demonstrated that Wnt3a promotes MF-like phenotype formation in cultured fibroblasts. Based on MF gene expression patterns, various studies have revealed that distinct subtypes of fibroblasts exist at different sites of the body<sup>[14,15]</sup>. Nevertheless, an inactive JunD, which protects the cell against oxidative stress, promotes MF differentiation<sup>[16]</sup>. Their presence at a site may either be pre-existing, or they may originate *denovo* from the surrounding subpopulation<sup>[15]</sup>.

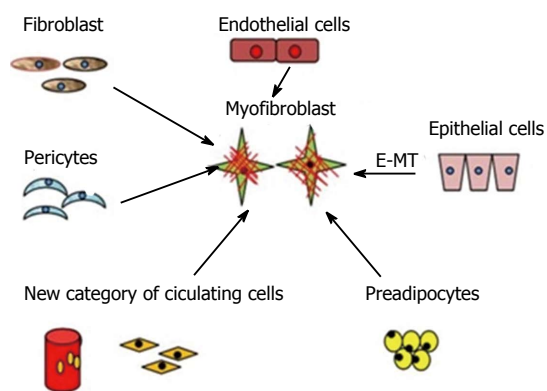
MFs possess several distinguishing morphologic features<sup>[17]</sup> and are characterised by the highly contractile  $\alpha$ -SMA apparatus<sup>[9]</sup>, which is the most significant marker of myofibroblastic cells. They may express smooth muscle myosin heavy chains and desmin<sup>[18]</sup>. They also express caldesmon, SM22, and tropomyosin. Under the electron microscope, MFs are large cells with abundant rough endoplasmic reticulum and fibronexus<sup>[5]</sup>, prominent cytoplasmic actin microfilaments (stress fibres), nonmuscle myosin, and vimentin<sup>[17]</sup>, which are connected to each other by adherens and gap junctions<sup>[4,9]</sup>.

Recently, the 4Ig isoform of the protein palladin in stress fibres was proposed as a new marker of MF differentiation<sup>[19]</sup>. Conversely, another study suggested that interferon- $\gamma$  reduces  $\alpha$ -SMA expression in smooth muscle cells<sup>[18]</sup>.

### Role of MFs in wound contraction

The breach of the epithelial layer is followed by changes that occur in the underlying connective tissue resulting in the loss of tissue homeostasis<sup>[1]</sup>. Normal wound healing is a well-known phenomenon. It involves a sequence of events including inflammation, proliferation, and tissue remodelling<sup>[18,20,21]</sup>. Wound closure involves connective tissue deposition, epithelization, and contraction<sup>[22]</sup> (Figure 2).

Wound contraction is mainly carried out by MF, a specialised contractile fibroblast<sup>[22]</sup>. Initially, small tractional forces exerted by the ECM facilitate the formation of protofibroblasts, which are composed



**Figure 1** Origin of myofibroblasts-multicellular origin. Multicellular origin of myofibroblasts: Fibroblast, pericytes, endothelial cells, circulating hematopoietic precursor cells and fibrocytes can transform into myofibroblasts. E-MT: Epithelial-mesenchymal transition.

of cytoplasmic actin components and devoid of the contractile apparatus and  $\alpha$ -SMA. Protofibroblasts migrate to the wound site by acquiring a migratory phenotype at the fibronectin-fibrin wound interface. At the site, protofibroblasts generate comparably small traction forces. Cytokines, such as PDGF, granulocyte-macrophage colony-stimulating factor (GM-CSF), heparin, integrin<sup>[20]</sup>, and TGF- $\beta$ , and existing tractional forces stimulate protofibroblast differentiation through  $\alpha$ -SMA expression, leading to their transformation into MFs. A study conducted on the role of tenascin in wound contraction demonstrated that increased tenascin expression correlated with MF differentiation<sup>[23]</sup>. However, interferon- $\gamma$ , a basic fibroblast growth factor (bFGF), prostaglandin E2, and high cell density inhibit the differentiation of protofibroblasts to MFs<sup>[20]</sup>. After activation, MFs initiate the synthesis of a new collagen-containing matrix that consists proteoglycans and glycosaminoglycans (highly hydrated molecules)<sup>[1,22,24]</sup>. They produce tractional forces at the margins for wound contraction, which is known as tractional remodelling<sup>[22]</sup>.

MFs generate forces in two ways. Initially, actin filaments present within the cell form a fibronexus by connecting intracellular actin and extracellular fibronectin fibrils using integrins. Integrins mediate the reorganisation and contraction of collagen matrices with the help of fibroblasts<sup>[20]</sup>. A study on  $\alpha$ 1 integrin knockout mice revealed impaired wound healing<sup>[20]</sup>. The assembly formed by MF with integrin and the actin filament is responsible for the "mechano-transduction system", which produces a high degree of tractional forces<sup>[9]</sup>. Later, MFs connect to each other through gap junctions to form a "multicellular contractile unit". They again exert a force on the ECM by implicating the use of this unit<sup>[9,22]</sup>. Both mechanisms exert a high level of tractional forces for wound closure.

After complete wound closure and re-epithelization, the number of MFs decreases either by reverting to the quiescent form or by undergoing apoptosis<sup>[1,9,25]</sup>. In the tissue remodelling phase, integrin-mediated interactions between MFs and collagen I results in scar tissue formation<sup>[20]</sup>.

Wound healing in the oral cavity essentially occurs without scarring and is faster than skin healing<sup>[20]</sup>. Fibroblasts in the oral mesenchyme possess a unique phenotypical character by constitutively expressing elevated  $\alpha$ -SMA levels, along with a higher capacity to contract collagen gel and a higher replicative potential than dermal fibroblasts<sup>[17]</sup>, ultimately leading to a "scar-free" healing process. Factors, such as epidermal growth factor, vascular endothelial growth factor, bFGF, and insulin-like growth factor, present in saliva and crevicular fluid are responsible for wound healing in the oral cavity<sup>[20]</sup>.

### MFs in oral leukoplakia

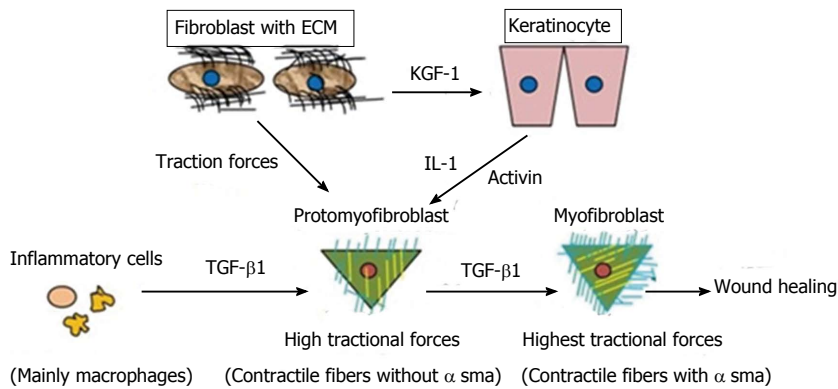
Leukoplakia is the most common potentially malignant disorder of the oral mucosa<sup>[26]</sup>. Studies have been conducted on various histopathological grades of oral leukoplakia (OL), but failed to establish a conclusive relationship between OL and MFs. MFs were not found in the stroma under dysplastic epithelium<sup>[16]</sup>. Myofibroblastic differentiation depends on the following factors: (1) Neoplastic microenvironment, which releases various growth factors<sup>[27]</sup>; (2) Genetically altered epithelium (carcinomatous epithelium), which is responsible for the inductive effect on the underlying stroma<sup>[28]</sup>; and (3) Epithelial-mesenchymal interaction (EMI), which plays a role in "epithelial invasion"<sup>[16,19]</sup>.

However, these factors were absent in various grades of epithelial dysplasia<sup>[16,19,27,29]</sup>. A study conducted by de-Assis *et al.*<sup>[29]</sup>, demonstrated that OL was not associated with MFs because MFs were not found in any of the samples. Therefore, it was hypothesised that myofibroblastic differentiation was entirely dependant on oral carcinoma development and, simultaneously, on the contact of cancer cells with stromal cells achieved by invading the epithelial cells<sup>[16]</sup>.

### MFs in oral submucous fibrosis

The most chronic and functionally hampering condition of oral cavity is oral submucous fibrosis (OSMF). OSMF is an abnormal healing process in response to the chronic mechanical and chemical irritation caused by chewing areca nuts<sup>[30]</sup>. The cellular mediator responsible for fibrosis is MF, which serves as collagen-producing cells when activated<sup>[31]</sup>. Continuous MF presence stimulates an abnormal repair mechanism, leading to excessive contraction and ECM secretion, subsequently causing fibrosis<sup>[30]</sup>. It was proposed that in fibrosis, MFs acquire an immune-privileged cell phenotype, which helps them to evade apoptosis and allows their uninterrupted accumulation<sup>[31]</sup>. A study suggested that OSMF could represent failed wound healing after chronic and sustained injury. Studies have proposed that fibrosis in OSMF could result from a hypersensitivity response caused by arecoline and a juxta-epithelial inflammatory response, which initiates a defective inflammatory response, activates fibroblasts, and culminates in fibrosis<sup>[30,32]</sup>. In addition, MFs could be used as potential markers for evaluating disease





**Figure 2 Differentiation of promyofibroblast to myofibroblast.** By getting stimulus from various cytokines profibroblasts (promyofibroblasts) transforms into myofibroblasts by expressing  $\alpha$ -smooth muscle actin. TGF- $\beta$ 1: Transforming growth factor- $\beta$ 1; ECM: Extracellular matrix; IL-1: Interleukin-1; KGF-1: Keratinocyte growth factor-1.

severity because a progressive increase in MFs from the early to the advanced stages was observed<sup>[30]</sup>.

The malignant transformation rate of OSMF ranges from 7% to 13%<sup>[33]</sup>. Dyavanagoudar<sup>[33]</sup> hypothesised that precancerous epithelial cells of OSMF acquire multiple genetic mutations in mesenchymal-epithelial crosstalk, and the associated stroma becomes activated and expresses SMA markers. These cells express ECM proteins and growth factors. These factors enhance and support tumour cell survival in an autocrine and paracrine manner, respectively. In contrary, Moutasim *et al.*<sup>[34]</sup> demonstrated that  $\alpha v \beta 6$  was markedly upregulated in OSMF by oral keratinocytes in an “*in vitro*” study. The results of the study also revealed that arecoline (the major alkaloid of areca nut) upregulated keratinocyte  $\alpha v \beta 6$  expression, which induced oral fibroblasts to transdifferentiate into MFs and resulted in the upregulation of genes associated with tissue fibrosis (Figure 3). Blocking these specific integrins can develop novel therapies for such fibrotic conditions<sup>[35]</sup>.

### MFs in OSCC

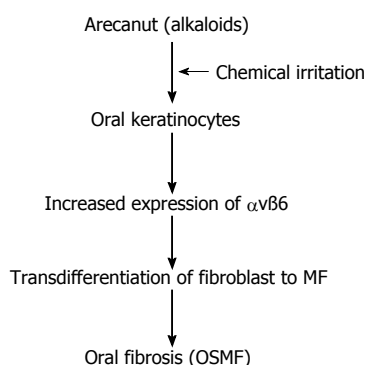
Stromal changes in wound healing and tumorigenesis depend on epithelial homeostasis<sup>[1]</sup>. Changes in epithelial homeostasis lead to changes in the underlying connective tissue stroma. This stroma is called reactive or desmoplastic stroma. Reactive stroma plays a significant role in the growth and progression of carcinoma because malignant epithelial cells require support from the surrounding stroma to promote tumorigenic progression<sup>[3]</sup>. Carcinoma-associated fibroblasts (CAFs) or tumour-associated fibroblasts or MFs are found in considerable quantities in such stroma<sup>[36]</sup> (Figure 4).

CAF origin is controversial. A study demonstrated that CAF originate from cancer stem cells, which are a small subpopulation of the tumour stroma<sup>[37]</sup>. Another study demonstrated that 60% CAF originate from bone marrow-derived mesenchymal cells<sup>[36]</sup>. Some additional sources may be resident fibroblasts, endothelial cells, pericytes, smooth muscle cells, and preadipocytes. Studies have demonstrated that the mutual paracrine effects between oral cancer cells and normal fibroblasts

are responsible for the transdifferentiation of normal fibroblasts into malignant fibroblasts. CAF genesis is also related to an EMI in the stromal population or a possible transdifferentiation of the malignant epithelial cells into MFs during oral carcinogenesis<sup>[1,19]</sup>. The tumour stroma continues to remodel itself during tumour progression and actively recruits various cell types into the tumour mass where they act as different sources of MFs<sup>[3]</sup>. TGF- $\beta$  is the main factor responsible for fibroblastic differentiation leading to activated tumour MFs. It is a main mediator found in the saliva and is expressed by cancer cells. A study demonstrated that the tension exerted in the tumour stroma is also responsible for the transformation of fibroblasts into MFs<sup>[1]</sup>. Keratinocytes also seem to be responsible for forming tumour-associated fibroblasts<sup>[33]</sup>.

Morphologically tumour fibroblasts differ from normal MFs in their abundant rough endoplasmic reticulum, Golgi apparatus, fibronectin fibrils, and fibronexuses on the cell surface<sup>[5]</sup>. They also differ in their contractile property, MFs because they can exert more contractile force which is responsible for stiffness in the advanced stages of the neoplasm<sup>[1]</sup>. MFs secrete several enzymes such as stromelysins and matrix metalloproteinases (MMPs- 1, 2, 3, 9, 13, and 14)<sup>[1]</sup>, which cause ECM degradation. They also release different growth factors such as PDGF, bFGF, keratinocyte growth factor, stem cell factor, epidermal growth factor, GM-CSF, and other cytokines<sup>[25]</sup>. They also secrete matricellular proteins including CCN2; collagens; tenascins C and FN; and elastins<sup>[1]</sup>. MFs promote tumour cell migration on tenascin<sup>[38]</sup>. Hence, MFs play an important role in tumour progression by invading the tumour stroma and consequently remodelling the ECM by forming more desmoplastic responses<sup>[25,38]</sup>. Most importantly, they secrete fibroblast-associated proteins (FAP). The loss of FAP is associated with inhibition of tumour cell progression and decrease in MF quantity and blood vessel density in the tumour<sup>[16]</sup>.

MFs function as “sentinel cells” by acting as immunoregulatory cells in the stroma, by reducing the physical contact between cancer and immune cells, which is



**Figure 3 Transdifferentiation of fibroblasts into myofibroblasts.** Major alkaloid of areca nuts, up-regulates keratinocyte  $\alpha v \beta 6$  expression which induced transdifferentiation of oral fibroblasts into MF. OSMF: Oral submucous fibrosis.

imperative for cancer cell destruction<sup>[25]</sup>.

Several MFs in the tumour stroma are often associated with high-grade malignancies. They promote tumour progression through “neo-angiogenesis” by releasing growth factors, such as fibroblast growth factor-2<sup>[3]</sup>. A study demonstrated that the most migratory and invasive behaviour of tumour cells correlated with the presence of MFs at the invasive tumour front in OSCC because this precedes the invasive stage of the cancer<sup>[39,40]</sup>. In addition, abundant MFs in the tumour stroma correlates with a higher tumour incidence, specifically in patients aged below 60 years<sup>[19]</sup>.

MFs are present in the OSCC stroma in two dominant patterns, spindle pattern (MFs are arranged in rows with a few cells around the neoplastic islands) and network pattern (several abundant layers of MFs around the neoplastic islands). The network pattern fibroblasts are exceptionally abundant and occupy almost the entire tumour stroma, whereas the spindle pattern includes spindle cells that are located at the periphery in 1-3 concentric layers<sup>[16]</sup>. Studies conducted by Seifi *et al.*<sup>[39]</sup> and Kawashiri *et al.*<sup>[41]</sup> have demonstrated that the presence of MFs and the arrangement of MFs in the tumour stroma play a role in invasion. They observed that MFs arranged in the network pattern caused tumour invasion and not those arranged in the spindle pattern. They also noted that tumour desmoplasia is associated with aggressive cancer.

### Functional similarities and differences between wound healing and tumorigenesis

Morphological similarities exist between the tumour stroma and granulation tissue following wound healing. A wound is disruption of an anatomical structure, such as an epithelial membrane, and healing is the restoration of structure and function<sup>[41]</sup> (Figure 5).

Wound healing and tumour progression are loosely related in terms of MFs<sup>[1]</sup>. In wound healing, changes in the underlying stroma occur with a breach in the normal lining epithelium. The appearance of a break causes changes in the underlying connective tissue stroma. Inflammatory cells release TGF- $\beta 1$ , which stimulate the

differentiation of fibroblast into proto-myofibroblast and finally into MFs<sup>[9]</sup>. MFs later release MMPs and growth factors, which lead to matrix degradation and new blood vessel formation (angiogenesis), respectively. MFs also cause wound contraction and undergo apoptosis. However, in the last stage of wound healing, a defect in the apoptosis of MF and the persistence of MF results in an hypertrophic scar tissue<sup>[42]</sup>.

A tumorigenic process involves acquiring multiple genetic mutations. In the normal cell ecosystem, a continuous cross-talk between epithelial cells and stroma occurs. Epithelial cells acquiring neoplastic properties result in altered stromal compartment. Neoplastic cells release TGF- $\beta 1$  and PDGF, which result in the emergence of CAF<sup>[3,10,43]</sup>. The petite concentration of TGF- $\beta 1$  increases (femtomolar to picomolar) as the fibroblasts approach the cancer cells and transdifferentiate into MFs<sup>[43]</sup>. CAF release growth factors and cause angiogenesis similar to wound healing<sup>[10]</sup>. However, in carcinogenesis, MMPs (1, 2, 9 and 13) liberated by CAF are approximately double than the normal fibroblasts<sup>[44]</sup>. These factors together promote tumour progression and invasion<sup>[43]</sup>.

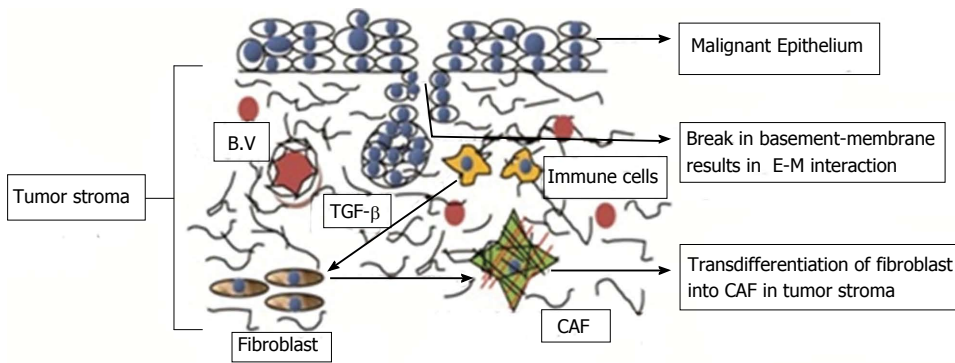
Thus, the loss of epithelial homeostasis is a common trigger for the stromal reaction against tumours and for normal wound healing<sup>[1]</sup>. In both processes, growth factors and MMPs liberated by MFs are responsible for progression.

In wound healing, MFs transiently acquire the phenotype<sup>[1]</sup>, which persists during fibrosis in the tumour environment because of an imbalance between the apoptosis and the proliferation of transformed cells leading to disease progression<sup>[40]</sup>.

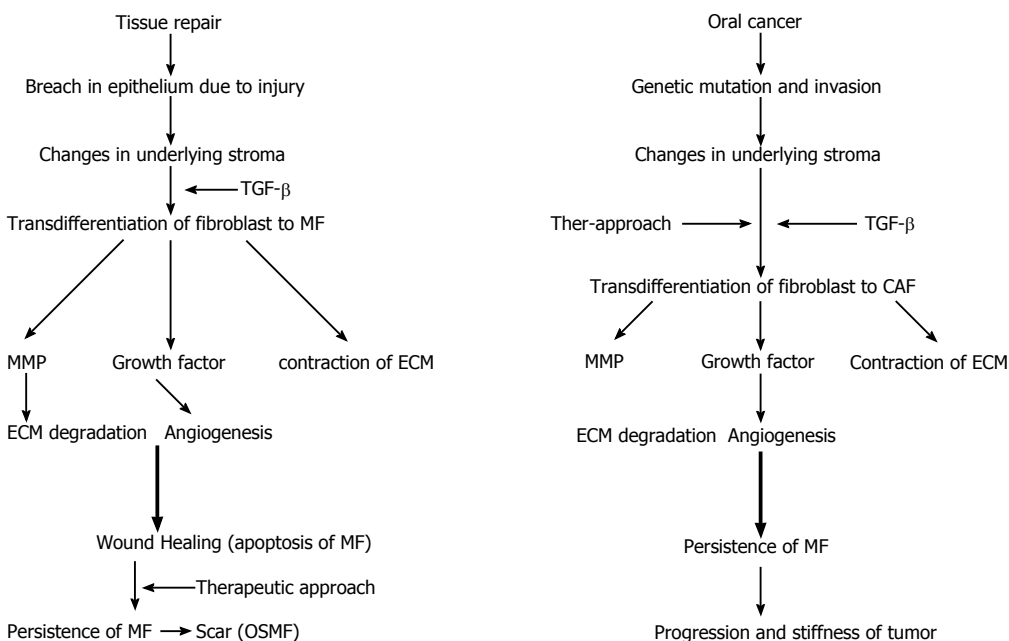
### MFs in odontogenic cyst and tumour

Smith was the first to question the relationship between MFs and the aggressive behaviour of a neoplasm<sup>[45]</sup>. Rothhouse reviewed the ultrastructural features of ameloblastoma and found that the stromal component of an ameloblastoma is composed of MFs along with associated collagen and basal lamina material<sup>[46]</sup>. Vered *et al.*<sup>[43]</sup> studied the number of MFs in a solid ameloblastoma and parakeratinised odontogenic keratocyst to find that the mean number of MFs in the odontogenic lesions was the same as that in OSCC<sup>[43]</sup>. The number of MFs was high in these lesions, which were responsible for aggressive and invasive behaviour. They explained that the epithelium of parakeratinised odontogenic keratocyst and solid ameloblastoma behave like the OSCC epithelium by releasing more TGF- $\beta 1$  and simultaneously modulating stromal MFs, making the tumour more aggressive and invasive<sup>[43]</sup>. This explains the biologic behaviour of these odontogenic lesions. Another study demonstrated the invasive behaviour of a recurrent infiltrative ameloblastoma because of a high number of MFs in stroma<sup>[46]</sup>. Thus, the presence and the frequency of MFs in the stroma possibly determine the biologic behaviour of the lesions.

Odontogenic myxoma, which is considered to be a slow-growing invasive tumour, showed abundant MFs in



**Figure 4 Transdifferentiation of fibroblast into carcinoma associated fibroblast in oral squamous cell carcinoma.** Mutual paracrine effect between oral cancer cells and normal fibroblasts is responsible for transdifferentiation of the latter into malignant fibroblasts. CAF: Carcinoma associated fibroblast; TGF- $\beta$ : Transforming growth factor- $\beta$ ; B.V: Blood Vessels; E-M: Epithelial-mesenchymal.



**Figure 5 Functional similarities and differences between wound healing and tumorigenesis.** Wound healing and tumour progression phenomenon in relation with MF. TGF- $\beta$ : Transforming growth factor- $\beta$ ; ECM: Extracellular matrix; CAF: Carcinoma-associated fibroblasts; OSMF: Oral submucous fibrosis; MMP: Matrix metalloproteinase; MF: Myofibroblast.

its stroma associated with its invasive behaviour. Effiom *et al.*<sup>[47]</sup> hypothesised that MFs present in the stroma modify the ECM by releasing various cytokines, which are responsible for epithelial invasion.

Various authors have studied the relationship between different cysts and MFs in their stroma. The increased MFs in the stroma directly related to a more aggressive behaviour of the odontogenic cysts<sup>[48]</sup>, radicular cysts<sup>[49]</sup>, dentigerous cysts<sup>[49]</sup>, and keratocystic odontogenic tumours<sup>[49]</sup>. The results indicated that MFs were present in a decreasing order in the cyst stroma of keratocystic odontogenic tumours, dentigerous cysts, and radicular cysts. However, the results were not statistically significant. Some authors suggest that the presence of MFs in the cyst wall might be part of a homeostatic response to the distension caused by cyst enlargement<sup>[50]</sup>.

### MFs in salivary glands

The tumorigenic role of MFs in the salivary gland is controversial. Studies conducted on MFs in the tumour stroma of several salivary gland tumours, such as carcinoma ex pleomorphic adenoma, mucoepidermoid carcinoma (MEC), and adenocarcinoma, revealed the presence of tumour MFs at the tumour front in all the malignant lesions, which further correlated with the invasiveness of the cancer cells<sup>[40]</sup>. Gupta *et al.*<sup>[51]</sup> demonstrated that a high MF density in adenoid cystic carcinoma and MEC at tumour front, contributing to the aggressiveness of the lesions, whereas a moderate MF density was observed in polymorphous low grade adenocarcinoma. Conversely, Soma *et al.*<sup>[52]</sup> demonstrated that MFs inhibit tumour growth because they found more MF presence at the tumour border in benign lesions (pleomorphic adenoma) than that

in malignant lesions. Thus, they postulated that the MFs present at the periphery of benign lesions were responsible for containing the tumour, whereas the absence of MFs in malignant tumours was responsible for the progression of the tumour cells owing to no containment of the tumour<sup>[52]</sup>. Sobral *et al.*<sup>[53]</sup> reported similar findings. They demonstrated that MF was higher in low grade MEC than the intermediate and high grade suggesting that the inflammatory infiltrate in the tumour stroma causes the cessation of MF differentiation. With the increasing grades of MEC, the inflammatory infiltration increased, and therefore, the number of MF decreased, leading to a poor prognosis<sup>[53,54]</sup>.

The role of MFs in the pathogenesis of mucocele or chronic sialadenitis was not found. It was postulated that MFs may play a "supportive muscular role" around the cystic wall of the mucous retaining cyst and distended excretory duct<sup>[55]</sup>.

## CONCLUSION

MFs are known to contribute to the biological behaviour of various lesions. They actively participate in diseases characterised by tissue fibrosis because of their ability to secrete and degrade ECM components. MFs also play a role in ECM remodelling induced by tumour cells. It, thus, creates a permissive or suitable environment for tumour progression. Therefore, MFs are unique contractile cells that play a role in not only growth, development, and wound healing but also in inflammation, fibrosis, and tumour progression. Benefitting from their advantages in physiologic processes and blocking the processes leading to the causation and progression of a disease are the need of the hour. Additional knowledge and clinical studies involving this unique cell may provide us with an effective target for cancer therapy. Limited studies on oral lesions calls for further research for understanding the molecular mechanisms of MFs in the progression of these lesions.

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## Pelvic radiation disease: Updates on treatment options

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### Abstract

Pelvic cancers are among the most frequently diagnosed neoplasms and radiotherapy represents one of the main treatment options. The irradiation field usually

encompasses healthy intestinal tissue, especially of distal large bowel, thus inducing gastrointestinal (GI) radiation-induced toxicity. Indeed, up to half of radiation-treated patients say that their quality of life is affected by GI symptoms (*e.g.*, rectal bleeding, diarrhoea). The constellation of GI symptoms - from transient to long-term, from mild to very severe - experienced by patients who underwent radiation treatment for a pelvic tumor have been comprised in the definition of pelvic radiation disease (PRD). A correct and evidence-based therapeutic approach of patients experiencing GI radiation-induced toxicity is mandatory. Therapeutic non-surgical strategies for PRD can be summarized in two broad categories, *i.e.*, medical and endoscopic. Of note, most of the studies have investigated the management of radiation-induced rectal bleeding. Patients with clinically significant bleeding (*i.e.*, causing chronic anemia) should firstly be considered for medical management (*i.e.*, sucralfate enemas, metronidazole and hyperbaric oxygen); in case of failure, endoscopic treatment should be implemented. This latter should be considered the first choice in case of acute, transfusion requiring, bleeding. More well-performed, high quality studies should be performed, especially the role of medical treatments should be better investigated as well as the comparative studies between endoscopic and hyperbaric oxygen treatments.

**Key words:** Pelvic radiation disease; Radiation-induced proctopathy; Radiotherapy; Gastrointestinal toxicity; Sucralfate; Metronidazole; Probiotics; Argon plasma coagulation; Hyperbaric oxygen; Formalin

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**Core tip:** Radiotherapy is frequently employed as part of the multimodal treatment of pelvic cancers. Despite recent advances in irradiation techniques, acute and late-onset radiation-induced gastrointestinal toxicity, also known as pelvic radiation disease, is still being frequently reported. This review provides an up-to-

date summary on medical and endoscopic approaches that have been evaluated with treating intent, focusing on the best available evidence, primarily randomized controlled studies.

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## INTRODUCTION

Pelvic cancers are among the most frequently diagnosed neoplasms<sup>[1]</sup>. The employment of radiation therapy as part of a multidisciplinary treatment for pelvic malignancy has progressively increased in recent years<sup>[2]</sup>, as it is estimated that over 200000 patients in the United States receive pelvic or abdominal radiation therapy annually. The irradiation field usually encompasses healthy intestinal tissue, especially of distal large bowel, thus inducing gastrointestinal (GI) radiation-induced toxicity. Indeed, up to half of radiation-treated patients say that their quality of life is affected by gastrointestinal symptoms<sup>[3]</sup>. Recently, the constellation of gastrointestinal symptoms - from transient to long-term, from mild to very severe - experienced by patients who underwent radiation treatment for a pelvic tumor have been comprised in the definition of pelvic radiation disease (PRD)<sup>[3]</sup>. Radiation toxicity is defined as acute when occurring during radiotherapy or within 3 mo, while it is considered as chronic when developing after longer period of time. Among the most frequently reported symptoms are diarrhea, urgency, rectal bleeding and fecal incontinence<sup>[4]</sup>.

The type of irradiation technique has been recognized as an influential factor for the development of PRD<sup>[5]</sup>. It is important to notice that even the most recent radiation procedures, such as intensity-modulated radiotherapy, have reduced but not completely annulled the occurrence of GI radiation-related toxicity<sup>[6]</sup>. Moreover, the prolonged survival of this category of patients will undoubtedly increase the risk of developing PRD over time. Thus, a correct and evidence-based therapeutic approach of patients experiencing gastrointestinal radiation-induced toxicity is mandatory. Therapeutic non-surgical strategies for PRD can be summarized in two broad categories, *i.e.*, medical and endoscopic. Over years, a number of medical treatments have been investigated, such as aminosaliclates, sucralfate, antibiotics, probiotics, steroids and hyperbaric oxygen therapy. Endoscopic treatments have been explored too, including argon plasma coagulation, formaline application, radiofrequency, cryotherapy and band ligation.

In the current review, we provide a critical appraisal of the efficacy of the treatment options for radiation-

induced gastrointestinal toxicity.

## Pathogenesis

The occurrence and severity of radiation-induced gastrointestinal toxicity depends upon several factors. Therapy-related factors include radiation dose, volume of irradiated bowel, time- and dose-fractioning parameters and concomitant employment of chemotherapy. Patient-related factors include smoking, body mass index, previous abdominal surgery and comorbidities like inflammatory bowel disease, diabetes and collagen vascular disease<sup>[7-13]</sup>.

Traditionally, the development of radiation enteropathy was explained through the "target cell" theory, which addressed early pathology to the epithelial injury, while fibroblast and endothelial cell damage was accounted for late-onset harm<sup>[14]</sup>. In recent years, the above-mentioned theory has been questioned, and other factors have been taken into account. For instance, the enteric nervous system is the second largest nervous system of human body, and it has been pointed out as capable to regulate radiation enteropathy development<sup>[15]</sup>. It has also been demonstrated that the gut microbiota, consisting of about 100 trillion bacteria, influences radiation-induced damage<sup>[16]</sup>. Thus, the understanding of PRD pathogenesis has gone far beyond the single "target cell" concept, and considers intestinal toxicity as the result of multiple interactions between epithelial injury, gut microvasculature, enteric nervous system, and gut microbiota<sup>[17]</sup>.

Acute and chronic gastrointestinal toxicity have a different pathogenesis<sup>[18]</sup>. Indeed, acute PRD is due to an acute inflammatory response, whilst chronic, late-onset disease is mainly mediated by vascular sclerosis and fibrosis<sup>[19]</sup>. However, acute and chronic radiation toxicities are not independent events, as it is underlined by the consequential late effect theory: indeed, late injury is more likely to develop when severe acute toxicity exists<sup>[20,21]</sup>. Recent studies have added complexity to these models<sup>[17]</sup>, however a deeper discussion on the pathological basis of PRD is beyond the purpose of this review and we invite to consider for this purpose the review by Hauer-Jensen *et al*<sup>[17]</sup>.

## Treatment options

Medical treatment, hyperbaric oxygen therapy and endoscopic approaches represent the mainstay for treating pelvic radiation-induced disease. However, the existing evidence on such approaches for treating PRD cannot be judged as of high quality, due to few and low-quality randomized controlled trials (RCTs), high clinical and methodological heterogeneity, small sample sizes and short periods of follow-up<sup>[22]</sup>.

## MEDICAL TREATMENT

Medical therapy should represent the first step in the management of radiation-induced pelvic radiation disease. Over years, a number of medical treatments

**Table 1** Medical strategies for treating pelvic radiation disease

Medical treatments	Acute PRD	Chronic PRD	Notes
Topical sucralfate	N	Y	Twice-daily enema with two 1 g sucralfate tablets mixed with 4.5 mL of water is effective for chronic rectal bleeding
Metronidazole	N	Y	3 × 400 mg/d of metronidazole for up to 12 wk is effective for chronic rectal bleeding and diarrhea
Probiotics	Y	N	3 sachets/d of <i>Lactobacillus rhamnosus</i> for at least 1 wk is effective for acute diarrhea
Mesalazine	N	N	No recent RCTs available; one prospective study showed that combined oral and topic mesalazine was effective for chronic rectal bleeding
Corticosteroids	N	N	RCTs have not shown a substantial improvement with steroids administration
Hyperbaric oxygen	N	Y	At least 30 sessions (up to 100) are effective for chronic rectal bleeding not responding to medical treatment

Y: Evidence supports treatment; N: Evidence does not support treatment; PRD: Pelvic radiation disease; RCT: Randomized controlled trial.

have been investigated, such as aminosalicylates, sucralfate, antibiotics, probiotics, steroids and hyperbaric oxygen therapy (Table 1).

Radiation-induced injury has been misleadingly referred to as *proctitis*, though inflammation has a non-central role in the pathogenesis of the disease. Thus, anti-inflammatory agents (steroids and 5-aminosalicylic acid) have traditionally been proposed as first-line treatment, with inconsistent results confirmed by a recent systematic review<sup>[23]</sup>. In fact, only sucralfate and metronidazole have clearly shown to be effective for treating symptoms of PRD, and the role of probiotics is supported by one RCT only.

### Sucralfate

**Rationale:** Sucralfate is an alkaline aluminum hydroxide of sulfated sucrose. The rationale for the administration of sucralfate in the treatment of PRD lies on its supposed property to protect mucosa by forming a viscous superficial coating and to stimulate mucosal healing by its angiogenic action<sup>[24,25]</sup>.

**Evidences:** According to published prospective studies - including one small, non-placebo controlled randomized controlled trial - topical sucralfate is effective in the treatment of PRD, as it significantly reduces the entity of rectal bleeding<sup>[26-29]</sup>. Indeed, patients experiencing symptoms improvement ranged from 73% to 100% of considered cohorts, after a follow-up period between four and six weeks. However, when surveillance interval was expanded, symptoms recurred in 10%-20% of patients<sup>[27,28]</sup>. Oral sucralfate was evaluated by one randomized, placebo-controlled trial and did not show to improve symptoms of PRD when added to endoscopic argon plasma coagulation<sup>[30]</sup>.

Based on the available evidence, topical administration of sucralfate should be considered as one of the first-line treatments of radiation-induced rectal bleeding. The topical administration is the only way of assumption that showed to be effective for PRD<sup>[26,27,29]</sup>. Sucralfate can be administered twice daily as a retention enema prepared by patients themselves, using two 1 g sucralfate tablets mixed with 4.5 mL of water in an enema applicator and producing a low-volume paste<sup>[29]</sup>.

### Metronidazole

**Rationale:** Metronidazole is a bactericidal agent that kills anaerobic and microaerophilic bacteria, which contribute to hypoxia, and also has an immunomodulator effect; these two actions may reduce the risk of rectal bleeding and help in the management of PRD.

**Evidences:** According to RCT-based evidence, metronidazole is effective in treating chronic rectal bleeding and diarrhea<sup>[31,32]</sup>. Cavčić *et al.*<sup>[31]</sup> randomized 60 patients with radiation-induced rectal bleeding, diarrhea and ulcerations to receive metronidazole (3 × 400 mg/d orally), mesalazine (3 × 1 g/d orally) and betamethasone enema (once a day during 4 wk) or only the combination of mesalazine and betamethasone. After 12 mo of follow-up evaluation, a significant reduction in the incidence of rectal bleeding and diarrhea was found in the metronidazole group.

Sahakitrungruang *et al.*<sup>[32]</sup> enrolled in a RCT 50 patients with chronic radiation-induced PRD; patients were randomized to daily colonic irrigation plus metronidazole (3 × 500 mg/d orally) and ciprofloxacin (2 × 500 mg/d orally) for a week, or to receive 4% formalin by using proctoscopy. Outcomes were evaluated after 8 wk, showing a significant improvement in rectal bleeding, urgency, diarrhea in patients treated with metronidazole.

At the present day, these two studies represent the only RCTs showing the efficacy of metronidazole in the management of pelvic radiation disease. Other studies are recommended in order to confirm the results already achieved.

Based on the existing RCT-based evidence, metronidazole can be administered orally (3 × 400 mg/d) from 1 wk up to 12 wk. Metronidazole can be considered as a safe drug. Skin rash, nausea and vomiting are the most frequently reported side effects<sup>[32]</sup>.

### Probiotics

**Rationale:** Probiotics are defined as living microorganisms that confer a health benefit to the host when administered in adequate amounts<sup>[33]</sup>. They mainly include lactobacilli and bifidobacteria strains. The possible mechanism of action has been investigated



in various studies. Probiotics seems to have a strong immunomodulation effect by acting on epithelial cells, dendritic cells, monocytes/macrophages and lymphocytes. They also have antimicrobial activity against pathogenic bacterial strains, which is mediated by the reduction of pH, secretion of antimicrobial peptides, inhibition of bacterial invasion and adhesion to the gut epithelium<sup>[34]</sup>. They enhance barrier integrity and function, also by improving the production of short chain fatty acids, in particular butyrate<sup>[35]</sup>.

In conclusion, probiotics have the potential to maintain or restore the gut microflora during and after radiation therapy, especially reducing the incidence of radiation-induced diarrhea<sup>[36]</sup>.

**Evidences:** Up to now only one RCT has been performed and showed that probiotics are effective in treating acute diarrhea<sup>[37]</sup>. More in details, Urbancsek *et al*<sup>[37]</sup> performed a randomized, placebo-controlled, double-blind trial recruiting 205 patients with diarrhea lasting for at least 2 wk and developed within 4 wk from radiotherapy for pelvic cancers. The efficacy was inferred through the need of rescue medication per patient. After a 1 wk period of treatment, the active group required antidiarrheal drugs less frequently than placebo group, although the difference was not statistically significant. Number of bowel movements, diarrhea grading and stool consistency were also evaluated as secondary end-points, and the active group showed a significant improvement in patients' diarrhea rating and in stool consistency.

According to Urbancsek *et al*<sup>[37]</sup> *Lactobacillus rhamnosus* can be administered orally as 1.5 g sachets, three time a day, for at least one week. Probiotics, regarded as drugs or only food supplementation, can be considered as safe. No serious adverse drug reactions were reported<sup>[37]</sup>.

### Aminosalicylates

**Rationale:** Aminosalicylates are compounds that contain 5-aminosalicylic acid (5ASA), which is a potent inhibitor of the synthesis and release of proinflammatory mediators (e.g., nitric oxide, leukotrienes, thromboxanes, and platelet activating factor) and also inhibits the function of several cells implicated in the acute inflammatory and immune response (e.g., natural killer cells, mast cells, neutrophils, mucosal lymphocytes, and macrophages)<sup>[38]</sup>. Aminosalicylates are currently available as pro-drugs (sulfasalazine) and active compound (mesalazine). As eicosanoid inflammatory mediators are the main mediators in the pathophysiology of acute, early-onset PRD<sup>[39]</sup>, the administration of aminosalicylates might be effective in reducing inflammation and therefore improve radiation-induced symptoms.

**Evidences:** Current evidence on the role of mesalazine in the treatment of PRD is scanty. Indeed, only one

randomized, controlled trial has been performed so far, showing that mesalazine significantly improved symptoms such as diarrhea, abdominal pain and flatulence<sup>[40]</sup>. However, radiotherapy techniques have completely changed since the 70 s, thus the results of the above mentioned trial might not be suitable to present day. One prospective study assessed the efficacy of combined oral and topical mesalazine in 23 patients with chronic PRD, and found that mesalazine significantly improved rectal bleeding, but not other radiation-related symptoms (i.e., pain, tenesmus and stool frequency) after 4 wk of treatment<sup>[41]</sup>.

Current evidence does not support mesalazine routine use for the treatment of acute nor chronic PRD. However, a 4 wk treatment of mesalazine, once daily as a 1 g rectal suspension, might be considered in patients referred for chronic rectal bleeding developed after radiation treatment, as second-line therapy<sup>[41]</sup>.

### Corticosteroids

**Rationale:** Corticosteroids have many metabolic and physiological effects. In fact, they wield anti-inflammatory action by inhibiting the arachidonic acid cascade, blocking cytokine release and production, inhibiting histamine release and activation of macrophages and finally by stabilizing cell membranes<sup>[42]</sup>. Since the first phase of PRD development is an inflammatory based process, all the effects of corticosteroids might play a role in the early phases.

**Evidences:** As far as RCT-based evidence is considered, corticosteroids have not clearly shown to induce substantial benefits for treating pelvic radiation disease<sup>[31,42,43]</sup>. Cavcić *et al*<sup>[31]</sup> found that the addition of oral metronidazole to mesalazine and betamethasone enema significantly improved rectal bleeding and diarrhea, therefore suggesting that metronidazole may have synergistic effects with steroids.

Kochhar *et al*<sup>[26]</sup> performed a double-blind controlled trial comparing sulfasalazine (500 mg three times a day) plus prednisolone (20 mg) enemas vs sucralfate enemas (2 g twice a day) plus oral placebo. Thirty-seven patients were enrolled and the treatment was continued for 4 wk. After the follow-up period, the sucralfate group showed a significantly better response as assessed clinically (94% vs 53%), thus the authors concluded that both treatment regimens were effective in the management of radiation proctopathy, though sucralfate enemas were better tolerated and had a better clinical response<sup>[26]</sup>. However, this study had a small sample size, with a follow-up period of 4 wk only, therefore detracting from any relevant conclusion.

Rougier *et al*<sup>[42]</sup> compared two different corticosteroid enemas, randomizing patients to receive either betamethasone enema (5 mg twice a day) or hydrocortisone acetate foam (90 mg twice a day). At the end of the treatment period, there was a non-significant reduction of rectal bleeding (38% vs 21%) in

**Table 2** Endoscopic approaches for treating pelvic radiation disease

Endoscopic approaches	Rectal bleeding	Notes
Argon plasma coagulation	Y	Treatment of choice when clinically significant rectal bleeding occurs
Formalin	Y	Alternative to APC, but more prone to complications and requires more skilled endoscopist
Radio frequency ablation	N	No RCT available; possibly effective but more expensive than other treatments
Cryoablation	N	No RCT available; risk of cecal perforation
Rectal band ligation	N	Anecdotal case report

Y: Evidence supports treatment; N: Evidence does not support treatment; RCT: Randomized controlled trial; APC: Argon plasma coagulation.

favour of hydrocortisone, and betamethasone enemas were poorly tolerated in 10 of 14 patients compared with 2 of 16 patients in the hydrocortisone group. However, no firm conclusion can be drawn from this study, as patients in the betamethasone group suffered from a more severe disease and the follow-up period was too short.

### Hyperbaric oxygen

**Rationale:** As the pathogenesis of chronic, late-onset pelvic radiation disease is mainly mediated by mucosal ischemia due to vascular sclerosis and fibrosis, and by oxidative stress, hyperbaric oxygen (HBO) therapy has been proposed. Indeed, HBO acts by inducing regrowth of injured vascular endothelial cells and epithelial cells, both directly and through stimulation of connective tissue elements<sup>[43]</sup>. HBO also improves the activity of radioprotective antioxidant enzymes and reduces free-radical damage<sup>[44,45]</sup>.

**Evidences:** A systematic review of several case-series concluded that HBO therapy improved symptoms of radiation-induced GI toxicity in nearly 60% of patients and induced symptoms remission in 35% of patients<sup>[46]</sup>. So far, only one randomized controlled trial has been performed, comparing HBO therapy at 2.0 absolute atmospheres to normal air at 1.1 absolute atmospheres in 120 patients with chronic rectal bleeding refractory to medical treatment<sup>[47]</sup>. In this study, Clarke *et al.*<sup>[47]</sup> found that HBO therapy significantly improved late-onset rectal bleeding, yielding a 32% absolute risk reduction and a number needed to treat equal to 3. However, the crossover design of the trial did not allow concluding whether symptom improvement was maintained long-term in the HBO therapy arm.

HBO should be regarded as the treatment of choice in case of chronic, radiation-induced rectal bleeding not responding to medical treatment or as second-line option in case of endoscopic failure. HBO can be considered as a relatively safe therapy, as its reported side effects were mild, transitory and self-limiting. The most frequently reported side effects are otic barotrauma, confinement anxiety and temporary myopia<sup>[47,48]</sup>. Of note, none of these side effects led patients to stop therapy<sup>[47]</sup>.

## ENDOSCOPIC TREATMENT

Several endoscopic techniques have been evaluated,

however only argon plasma coagulation (APC) and formalin application have consistently proved to be effective for treating severe rectal bleeding. Other approaches, such as radiofrequency ablation (RFA), cryoablation and band ligation should not be considered of choice in the clinical setting (Table 2). ND: RAG laser treatment should be considered as an obsolete treatment, fully replaced by APC treatment.

### Argon plasma coagulation

**Rationale:** Argon plasma coagulation is a noncontact technique with a governable depth of coagulation (0.5-3 mm), which applies a high-frequency current to the tissue and burns bleeding vessels, thus stopping rectal hemorrhage. As compared to ND: YAG laser therapy, APC is much more easier to use and safer; however, RCTs matching the two techniques have not been performed so far.

**Evidences:** The evidence supporting the employment of APC for treatment of clinically significant, intractable rectal bleeding cannot be judged as of high quality. Indeed, evidence comes from several retrospective and prospective case-series and observational studies, while only a few, small-sized RCTs comparing APC to formalin application have been conducted<sup>[49-58]</sup>. A systematic review focusing on studies published upon 2011 found that APC improved or completely resolved symptoms in 50% to 100% of patients<sup>[59]</sup>. Since then, a prospective observational study and an RCT have been published. Sato *et al.*<sup>[60]</sup> performed a prospective observational study considering 65 patients with chronic rectal bleeding, and found that APC was successful in improving symptoms in 60 (94%) of them after a mean follow-up of 35 mo. Yeoh *et al.*<sup>[61]</sup> randomized 30 patients with intractable rectal bleeding to receive APC or formalin endoscopic treatment, and concluded that APC was effective in treating symptoms in 94% of patients. Indeed, only one patient required further intervention after a follow-up of 111 mo.

Argon plasma coagulation should be considered as the treatment of choice when clinically significant bleeding occurs. As APC burns not only the bleeding vessels, but also mucosa and submucosa, it can lead to ulcerations, sometimes associated with chronic pain and slow healing<sup>[62]</sup>. Thus, APC should be performed reducing argon flow rates ( $\leq 2$  L/min) and wattage ( $\leq 40$  watt). Adverse events are mild in most cases, and have been

reported in up to 18% of patients<sup>[55]</sup>. Abdominal cramps are the most frequently described side effects, occurring due to the colonic distention induced by argon gas; thus, two-channel endoscopes should be employed in order to insufflate and contextually remove argon gas during the procedure. Ulcerations have been often reported too<sup>[62]</sup>. Severe complications have been rarely described, including gas explosion and perforation, fistula, stricture, and long-term pain<sup>[51,58,62]</sup>. Notably, colonic explosion mostly occurred when the endoscopic procedure was performed after inaccurate, local bowel cleansing with enemas, instead of gold-standard oral preparation<sup>[53,57]</sup>.

### Formalin

**Rationale:** Formalin is an aldehyde commonly used to preserve or fix tissues by cross-linkage of primary amino groups in proteins with other nearby nitrogen atoms in proteins or DNA through a CH<sub>2</sub>-linkage. As formalin is highly irritant to biologic tissues, when directly applied to radiation-damaged tissues it induces local chemical cauterization that scleroses and seals fragile neo-vasculature<sup>[63]</sup>. Thus, formalin has been proposed as a treatment for refractory severe rectal bleeding.

**Evidences:** Formalin might be considered as alternative to thermal coagulation therapy with argon plasma in patients with severe rectal bleeding. However, the existing evidence upon the role of formalin in PRD is not completely satisfactory: Indeed, three randomized controlled trials have been conducted so far, two of which are published in abstract form only<sup>[50,54,61]</sup>. Yeoh *et al*<sup>[61]</sup> randomized 30 patients suffering from severe rectal bleeding to receive either argon plasma coagulation or formalin application, and found that both treatments were not differently effective, as control of rectal bleeding was achieved in all patients.

Topical formalin therapy can be performed with an operating sigmoidoscope under general anesthesia. It is important to smear the anus and buttocks with petroleum jelly, in order to prevent direct contact with the formalin solution. Standard gauze pledgets soaked in 4% formalin solution have to be applied to the affected areas under direct vision, starting proximally. Each pledget needs to be held in place for 1 min for each affected area until all areas distally had been treated<sup>[61,64]</sup>. Endoscopic application of formalin is more frequently associated with complications and requires more skilled endoscopists than argon plasma coagulation therapy<sup>[65]</sup>. The most frequently reported adverse events include ano-rectal pain, fecal incontinence, severe diarrhea, fever and the severe formalin-induced colitis<sup>[66]</sup>. Other complications include anal or rectal strictures, rectal perforation or ulceration.

### RFA

**Rationale:** RFA is an endoscopic procedure in which a target tissue is ablated using the heat generated from

high frequency alternating current<sup>[67]</sup>. RFA, performed with the BARRx Halo90 system used to treat Barrett's esophagus, has been recently proposed for severe intractable rectal bleeding. In comparison with APC, RFA allows broader areas of tissues to be treated and induces prompt squamous re-epithelialization with prevention of re-bleeding; furthermore, RFA is restricted to the superficial mucosa, thus it could represent a safer alternative to traditional endoscopic treatments<sup>[63]</sup>.

**Evidences:** Up to now, the role of RFA as an alternative endoscopic treatment for severe intractable rectal bleeding has yet to be defined. Indeed, no randomized controlled trial has been performed, thus the quality of evidence supporting the use of RFA is poor<sup>[68-72]</sup>. Rustagi *et al*<sup>[72]</sup> performed the largest observational study concerning RFA technique in PRD. Thirty-nine patients were enrolled, and all of them experienced complete resolution of rectal bleeding during a mean follow-up of 28 mo. Furthermore, treatment with RFA led to discontinuation of blood transfusion and iron therapy in 92% and 82% of patients, respectively. As far as the existing, unsatisfactory evidence is concerned, RFA can be regarded as a relatively safe procedure<sup>[68-72]</sup>. Indeed, the most frequently reported side effects were mild-to-moderate anorectal pain, transient fecal incontinence, asymptomatic perianal ulceration and difficult evacuation of stool<sup>[70,72]</sup>.

### Cryoablation

**Rationale:** Cryoablation is a non-contact therapy that employs liquid nitrogen to apply extremely cold temperatures to a targeted area, resulting in tissue destruction. Effects are both immediate and delayed, due to the induction of ischemic necrosis.

**Evidences:** Up to now, the evidence supporting cryoablation as a therapeutic option for PRD is absolutely scanty. Indeed, only a few small-sized case-series have been reported<sup>[73-75]</sup>. Thus, cryoablation might not be considered as a feasible alternative to other established endoscopic treatments. The largest case series was enrolled by Hou *et al*<sup>[75]</sup> who treated with cryoablation ten patients with chronic hemorrhagic PRD and found it to significantly improve rectal bleeding. However, this was a non-powered case series pilot study, therefore these results, though attractive, are not sufficient to draw any firm conclusion. As cryoablation has not yet been performed in an adequately large sample of patient, it cannot be still considered as a safe procedure. In fact, the major risk associated with the procedure consists of colonic over-insufflation resulting in cecal perforation<sup>[75]</sup>.

### Rectal band ligation

A case report described the use of rectal band ligation in a patient with radiation-induced rectal bleeding not responsive to endoscopic conventional treatment, *i.e.*,

APC. Five bands were placed in two separate sessions, with nearly total eradication of rectal teleangiectasias and without complications<sup>[76]</sup>. Obviously, though encouraging this result is anecdotic, thus further studies are warranted to define the role of rectal band ligation for treating PRD.

## CONCLUSION

The management of pelvic radiation disease may be challenging; several treatment options exist and the choice should be based on the best available evidences. Most of the studies have investigated the management of radiation-induced rectal bleeding. Patients with clinically significant bleeding (*i.e.*, causing chronic anemia) should firstly be considered for medical management (*i.e.*, sucralfate enemas, metronidazole and HBO), in case of failure, endoscopic treatment should be implemented. This latter should be considered the first choice in case of acute, transfusion requiring, bleeding. Alternative treatments, such as embolisation or surgery, should be considered in case of acute severe bleeding once endoscopy has failed. More well performed, high quality studies should be performed, especially the role of medical treatments should be better investigated as well as the comparative studies between endoscopic and HBO treatments.

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## Cervical cancer screening in developing countries at a crossroad: Emerging technologies and policy choices

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### Abstract

Cervical cancer (CC) represents the fourth most common malignancy affecting women all over the world and is the second most common in developing areas. In these areas, the burden from disease remains important because of the difficulty in implementing cytology-based screening programmes. The main obstacles inherent to these countries are poverty and a lack of healthcare infrastructures and trained practitioners. With the availability of new technologies, researchers have attempted to find new strategies that are adapted to low- and middle-income countries (LMIC) to promote early diagnosis of cervical pathology. Current evidence suggests that human papillomavirus (HPV) testing is more effective than cytology for CC screening. Therefore, highly sensitive tests have now been developed for primary screening. Rapid molecular methods for detecting HPV DNA have only recently been commercially available. This constitutes a milestone in CC screening in low-resource settings because it may help overcome the great majority of obstacles inherent to previous screening programmes. Despite several advantages, HPV-based screening has a low positive predictive value for CC, so that HPV-positive women need to be triaged with further testing to determine optimal management. Visual inspection tests, cytology and novel biomarkers are some options. In this review, we provide an overview of current and emerging screening approaches for CC. In particular, we discuss the challenge of implementing an efficient cervical screening adapted to LMIC and the opportunity to introduce primary HPV-based screening with the availability of point-of-care (POC) HPV testing. The most adapted screening strategy to LMIC is still a work in progress, but we have reasons to believe that POC HPV testing makes part of the future strategies in association with a triage test that still needs to be defined.

**Key words:** Low- and middle-income countries; Cervical cancer screening; Human papillomavirus testing

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**Core tip:** Cervical cancer (CC) burden in developing countries remains important because of the difficulty in implementing cytology-based screening programmes. With the introduction of new technologies, researchers have attempted to find new strategies for CC screening adapted to these countries. Rapid human papillomavirus (HPV) tests are one of these advantageous methods. However, HPV testing has a low positive predictive value for CC, so a triage test is needed. Visual inspection tests, cytology and novel biomarkers are some options. We provide an overview of current and emerging screening approaches for CC. We discuss the challenge of implementing an efficient CC screening adapted to developing countries and the opportunity to introduce primary HPV-based screening with the availability of point-of-care tests.

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## INTRODUCTION

The incidence of cervical cancer (CC) varies greatly worldwide. There is a large difference between developing and developed countries, where CC cases have been significantly reduced since the implementation of effective screening programmes. However, in developing countries, the burden from CC remains because of the difficulty in implementing cytology-based screening programmes. According to the latest world cancer statistics<sup>[1]</sup>, CC is the fourth most common cancer in women globally (528000 new cases each year) and the second most common in developing areas (445000 new cases each year). CC is also the fourth most lethal cancer in women worldwide (266000 deaths) and the third cause of cancer-related death in developing countries (230158 deaths)<sup>[1]</sup>, which means that more than 80% of the global burden occurs in developing areas.

In addition, the incidence and mortality of CC is variable within low- and middle-income countries (LMIC). In India, there are 20.2 per 100000 new cases of CC diagnosed and 11.1 per 100000 deaths annually, accounting for more than one fifth of the global CC deaths<sup>[2]</sup>. In sub-Saharan Africa, 34.8 per 100000 women are diagnosed with CC annually and 22.5 per 100000 women die from this disease<sup>[1]</sup>. In contrast, in western Asian countries, only 3.8 per 100000 new

cases are diagnosed per year and 1.6 per 100000 die from CC<sup>[1]</sup>. Therefore, if the chances to survive CC are considered, a woman in Thailand will have an approximately 58% chance of survival, while in India she will only have a 42% chance. This survival is even more critical in Sub-Saharan Africa, where women only have a 21% chance to survive CC<sup>[3]</sup>. Overall, the mortality to incidence ratio of CC is 52%<sup>[4]</sup>.

Human papillomavirus (HPV) is a major co-factor of CC. Development of vaccines against HPV has been a major advance for prevention of this cancer. Nevertheless, large-scale implementation of HPV vaccination is still lacking in developing countries and will not replace the need for CC screening.

In LMIC, there are several issues and challenges associated with CC screening. The main failure to implement an effective screening programme is related to the complexity of the screening process and the obstacles inherent in these countries. Poverty, limited access of the population to information, lack of knowledge of CC, the absence of sustained prevention programmes, lack of healthcare infrastructure required and lack of trained practitioners are the main obstacles to implementation of CC screening programmes<sup>[5]</sup>. Socio-religious and cultural barriers may also play an important role, as shown in an attempt to screen for CC in Peru<sup>[6]</sup>. Finally, government resources may be allocated to competing public health programmes with higher visibility and international attention than CC screening.

In this review, we discuss the challenge of implementing an efficient cervical screening adapted to LMIC and the opportunity to introduce a primary HPV-based screening with availability of a rapid HPV test.

## ACTUAL SCENARIO AND DIFFICULTIES FOUND IN LOW-RESOURCE AREAS FOR CC SCREENING

At the present, very few developing countries have been able to implement CC screening programmes. To screen successfully in LMIC different requirements are important. The programme shall ensure wide coverage of the target population; it must guarantee screening, management and adequate follow-up of patients; it shall be provided on-site and be low-cost, with minimum infrastructure requirement that can lead to immediate treatment if abnormal. CC screening should be planned in line with other national programmes for cancer control. Moreover, in order to implement CC screening policies in these countries, a support and funding from the Ministry of Health is indispensable.

In the Middle East and North Africa, the first steps to implement national screening programmes based on visual inspection tests are being currently completed<sup>[7]</sup>. In contrast, in Sub-Saharan Africa, it is estimated that less than 5% of women at risk have ever been screened<sup>[8]</sup>. In India's case, guidelines for population-



**Table 1 Obstacles to cervical cancer screening in low- and middle-income countries**

Practical/logistical reasons
Widespread poverty
Lack of healthcare infrastructure
Absence of sustained prevention programmes
Lack of trained practitioners
Lack of laboratory supplies
Lack of patient management guidelines
Limited physical access of the population
Knowledge, religion and beliefs
Lack of knowledge of cervical cancer
Limited access of the population to information
Women disempowerment
Socio-religious and cultural barriers to routine pelvic screening
Political
Lack of support from the Ministry of Health
Competing healthcare priorities
War and civil strife
Others
High temperatures in tropical countries with lack of proper climatisation
Particularities about the screening test
VIA
Significant number of unnecessary and unsustainable treatment
Cytology
Need important health-care resource and infrastructure
Need important laboratory supplies
Screening requires more than one visit (important drop out)
Further testing with colposcopy wouldn't be possible, leading to unnecessary aggressive and unsustainable treatment
HPV
Need important healthcare infrastructure
Need important laboratory supplies

VIA: Visual inspection tests with 3%-5% acetic acid; HPV: Human papillomavirus.

based screening programmes for cervical cancer are established for about 10 years<sup>[9]</sup> and are based on visual inspection tests. However, despite the introduction of these national guidelines, screening coverage is still very low<sup>[10]</sup>. Several obstacles are responsible for the failure to implement an effective screening program in LMIC. A summary of these obstacles is represented in Table 1.

### Cytology screening

Cytology screening (Pap test) for CC, especially as part of organised screening programmes, is the oldest and most widespread cancer screening technique. This technique has led to effective reduction in the incidence and mortality from CC in many developed countries<sup>[11-13]</sup>. CC screening is one of the most successful disease-prevention programmes. However, this approach has failed to attain the same results in developing areas. A cytology-based screening programme requires repeat testing and visits to identify women who need treatment. Besides a cytopathologist, a colposcopy specialist and a pathologist should also be involved. To guarantee the success of a screening programme, training and continuing education are essential<sup>[14]</sup>. Previous experience has shown no decline in the

incidence and/or mortality of CC, and this is probably because of low-quality cytology smears<sup>[8]</sup>. Consequently, implementation and execution of the whole process is too complex and expensive.

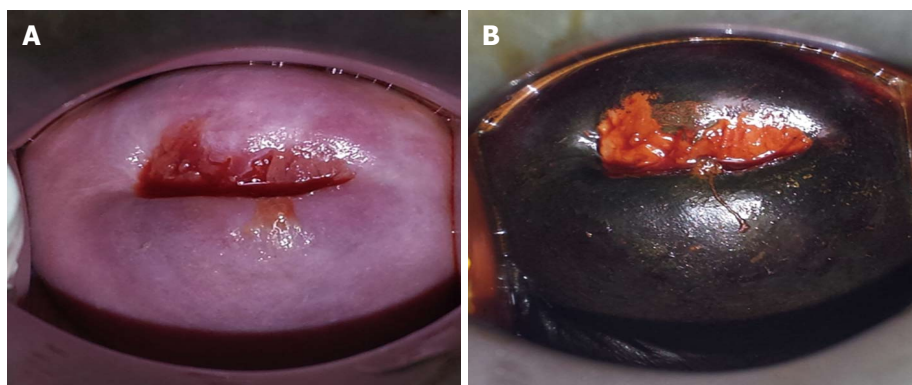
Moreover, even if implementing a high-quality cytology programme in these countries is possible, it would only be moderately effective. This is because the currently used Pap test misses approximately 50% of high-grade precursor lesions and cancers with a single screening<sup>[15]</sup>. Additionally, in low-resource settings, women would probably only be screened once or twice in their lifetime.

### Visual inspection tests

Visual inspection tests with 3%-5% acetic acid (VIA) and/or Lugol's iodine (VILI) appear to be a satisfactory alternative screening approach to cytology. These tests have been used since the 1990s, mainly in poor resource settings. They are simple, cost-effective with relative ease of use<sup>[16-19]</sup>, and may be performed by different healthcare workers (physicians, nurse, midwives and technicians). Moreover, this approach does not require high technology or infrastructure and has been shown to reduce mortality in developing countries<sup>[20,21]</sup>. The visible changes that occur in the cervix after application of acetic acid are immediate, and can be categorised as negative or positive for cervical neoplasia. These immediate results facilitate a same-day screen and management strategy. Therefore, this allows most of the eligible women to participate in the programme by minimising repeat visits. Evidence shows that this single-visit approach leads to the most significant decrease in high-grade cervical intraepithelial neoplasia (CIN)<sup>[22]</sup> and it is regarded safe, acceptable and fairly effective in India and Sub-Saharan Africa<sup>[17,23]</sup>. Despite the limitations of the concept of "screen and treat", it helps to overcome barriers of time, distance and loss to follow-up. This is relevant, because in a low-resource context, recalling patients for additional testing or treatment can be a critical component to a programme's success (Figure 1).

The performance of VIA has been evaluated in numerous studies<sup>[18,19,24-26]</sup>. An extensive meta-analysis by Sauvaget *et al.*<sup>[19]</sup> pooled data from 26 studies that were conducted in different high- and low-income countries. They found an overall sensitivity of 80% and a specificity of 92% for the VIA method, although sensitivities greatly varied between studies. Close values were found in a meta-analysis where pooled data from 11 studies that were performed in Africa and India showed a sensitivity for VIA of 79% (range: 73%-85%) and a specificity of 85% (range: 81%-89%) for CIN2 lesions or worse (CIN2+)<sup>[18]</sup>. With regard to VILI, its use appears to increase VIA's sensitivity by 10%, without affecting the specificity<sup>[18,24,26]</sup>.

VIA and VILI also have some drawbacks that need to be addressed. Interpretation of a visual test of the



**Figure 1 Visual inspection tests.** A: Visual inspection test with 3%-5% acetic acid; B: Visual inspection test with Lugol's iodine.

cervix has limited value in older women because of degenerating cervical epithelium and partial or lack of visibility of the transition zone with ageing. Indeed, studies have shown that VIA sensitivity declines substantially in women aged 40 years or older<sup>[27,28]</sup>. VIA-based screening is also healthcare provider dependent and lacks reliable quality assurance control. As a consequence and to maintain high quality, implementation of VIA screening at primary and secondary facilities would require close supervision, which is difficult to attain at a national level.

More importantly, reported sensitivity for detecting CIN2+ widely varies in different studies (37%-96%), as does specificity (49%-98%)<sup>[27,29]</sup>, which makes it dependent on the skill of the provider. Finally, studies that were conducted under screening conditions to assess the sensitivity and specificity of VIA used the gold standard of colposcopy, and this technique has been proven to yield error in the recognition of disease<sup>[30]</sup>. Because of these drawbacks, alternative methods need to be developed to improve, complement, or even replace VIA.

## HPV TESTING FOR PRIMARY SCREENING

In recent years, there has been overwhelming evidence that HPV testing is more effective than cytology for CC screening, providing increased reassurance and allowing longer screening intervals to be adopted<sup>[31]</sup>. Highly sensitive tests have been developed and are currently used to replace cervical cytology for primary screening<sup>[32]</sup>.

Currently, in the worldwide market, there are at least 150 different HPV tests available for the detection of alpha-HPVs and over 95 variants of the original tests. However, only some commercial HPV tests have documented clinical performance compared with the standard HPV test. According to guidelines, a candidate test should present a clinical sensitivity for CIN2+ of at least 90%, and a clinical specificity of at least 98% of that of the reference assays<sup>[33,34]</sup>. Regardless, the number of assays for HPV that have been approved by the Food and Drug Administration is increasing over

time<sup>[33,35,36]</sup>.

Moreover, among HPV tests, there is an important difference concerning the choices of primers to be used. Because of this overwhelming amount of choice available, choosing which HPV test is more suitable given a certain context can be difficult. Furthermore, and paradoxically, clinicians are generally not involved in choosing the HPV test.

Evidence shows that HPV tests should not only be type specific but also viral region specific (specific regions in the HPV genome are L1, E1/E2 and E6/E7). Indeed, during integration of HPV in the human genome, L1 expression is sometimes lost, but E6/E7 expression always remains present, which explains why there are not E6- or E7-negative cancers<sup>[37]</sup>. A test designed only for L1 will miss approximately 10% of all invasive cancers. This is why an HPV test is not recommended by some authors as a stand-alone test in CC screening programmes<sup>[37]</sup>.

Current HPV tests are able to detect the presence of viral markers by signal amplification techniques, such as the Digene Hybrid Capture® II assay or by amplification of nucleic acid with polymerase chain reaction. When combined with Pap smears, HPV tests can achieve nearly 100% sensitivity and a specificity of 93% in women aged 30 years and older, with a negative predictive value of almost 100%<sup>[38]</sup>.

Several studies support that HPV testing is feasible in low-resource settings and appears to be the best strategy for CC in this context<sup>[17,24,39]</sup>. A large-cluster randomised trial from rural India showed that a single round of HPV screening could reduce the incidence and mortality from CC of approximately 50%, whereas approaches based on VIA and cytology had little effect on these outcomes<sup>[40]</sup>.

Until recently, the greatest limitations of HPV testing were the need for expensive laboratory infrastructure and the 4-7 h time to process the test. The development of rapid molecular methods for detecting HPV DNA (e.g., care HPV® - Qiagen, GeneXpert® - Cepheid) for screening or other POC type of tests is a milestone in CC screening in low-resource settings. This is because these new options may make screening more feasible in

the future and reduce the infrastructural requirements of previous screening programmes.

In a cohort of unscreened women aged 30 and over from South Africa, HPV testing followed by the treatment of HPV-positive women at the second visit was the most effective option (27% reduction in the incidence of CC) at a cost of 39 USD/years of life saved (YLS)<sup>[41]</sup>. VIA combined with the immediate treatment of women who tested positive at the first visit was cost saving and was the next most effective strategy, with a 26% decrease in the incidence of CC<sup>[41]</sup>. In another cost-effectiveness analysis in a rural Chinese population, where the careHPV® test (Qiagen, Gaithersburg, MD, United States) was directly compared with VIA, a once-per-lifetime screening at the age of 35 years would reduce CC mortality by 8% combined with VIA (cost of 557 USD/YLS), compared with 12% with the careHPV test (cost of 959 USD/YLS)<sup>[42]</sup>.

### **Self-vaginal sampling for HPV testing**

HPV - based screening requires that a sample be taken using a swab or brush by a healthcare provider or by the patient herself. The greatest advantage of HPV-based testing is obvious in that it allows sample collection to be performed by the patient herself, not requiring trained personnel and infrastructure to perform a pelvic examination. The criteria for a good quality sample are less rigorous with HPV testing compared with cytology. Many studies have shown that offering self-sampling for HPV testing (Self-HPV) can improve attendance to a CC screening programme and it is well accepted among women<sup>[39,43-45]</sup>. This strategy can not only be more appealing to non-attendees in developed countries, but also makes CC screening accessible to women in LMIC<sup>[46,47]</sup>. Evidence from multiple prospective studies has shown that the accuracy of Self-HPV versus clinician-collected specimens to detect precancerous lesion is comparable for the detection of precancerous and cancerous lesions<sup>[39,48,49]</sup>. Because of the numerous advantages of self-HPV, it will become a major focus of CC screening programmes worldwide in the near future.

## **TARGETED AGE FOR INITIAL SCREENING**

The most relevant approach to identify women at risk for CC or pre-cancer is by age restriction. The World Health Organization (WHO) recommends targeting HPV screening to women who are 30 years of age and older because of their higher risk of CC, and that priority should be given to screening women aged 30-49 years (WHO screening recommendation update 2014). In addition, VIA is less effective in women aged older than 50 years because the squamocolumnar junction is less visible in menopausal women. If HPV is used as primary screening, recent evidence supports its use in women aged 30 years and over<sup>[50,51]</sup>. Most HPV infections are transient at an age younger than 30 years. Therefore, the screening of young women leads to unrequired

assessment and potentially to treatment of cervical lesions that might have regressed spontaneously<sup>[52,53]</sup>. However, even in women aged  $\geq 30$  years, most of HPV infections are transient, and only a small fraction of cases with persistent infection are at risk of CC<sup>[54]</sup>. Therefore, selecting HPV-positive women aged older than 30 years who are most likely to have or to develop a CC precursor in the future and require treatment is necessary for further evaluation (triage).

## **TRIAGE OF HPV-POSITIVE WOMEN**

HPV-based screening has a low positive predictive value for CC because it does not directly test for cancer, but for HPV infection instead. A negative HPV test only indicates a low probability for the patient to develop CC within 5-10 years, and a positive result is only an indication of the presence of an essential risk factor. Therefore, women who test positive for HPV must be further evaluated to determine the optimal management. At the present time, three candidates can potentially be used as triage test: (1) visual methods (VIA/VILI; (2) cytology; and (3) molecular testing. To date, there is no clear evidence to determine which strategy should be prioritised. Therefore, the choice of test essentially depends on the available health resource (Figure 2).

### **Triage with VIA/VILI**

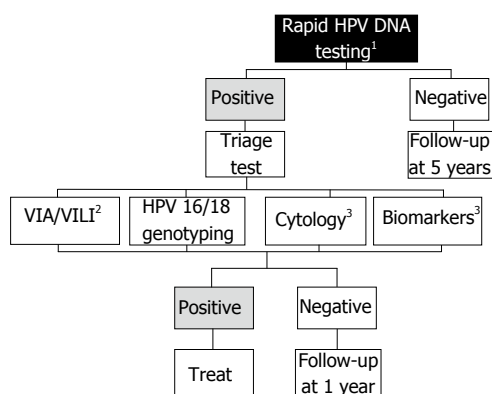
Triage with VIA/VILI offers the dual benefit of HPV screening to maximise detection of the disease and VIA/VILI for triage. In low-resource areas where the necessary equipment is lacking, VIA/VILI following an HPV-positive test is probably a good option, offering the possibility to adopt a "see and treat" approach. VIA/VILI will identify women with a precancerous change requiring immediate treatment by cryotherapy or cold coagulation, and those women in which cancer is suspected who should be referred to a specialised centre to receive aggressive multimodal treatment. Women with a negative VIA/VILI will be followed without treatment.

### **Triage with cytology**

Triage with cytology is proposed in developing (middle income) countries where infrastructure exists with experience of screening<sup>[55]</sup>. However, healthcare providers should be aware that cytology is associated with multiple clinic visits and delays between screening, laboratory results, colposcopy and ultimately treatment, which are major barriers to the success of this method.

### **Triage with molecular tests**

Cervical carcinogenesis is characterized by the integration of HPV DNA into the host cell genome resulting in abnormal proliferation of basal and parabasal cells due to the deregulated expression of viral oncoproteins, leading ultimately to the development of CC<sup>[56]</sup>.



**Figure 2** Decision making algorithm for human papillomavirus triage.

<sup>1</sup>HPV testing done on a self-taken sample by women aged 30-50 years; <sup>2</sup>Triage tests suitable for same-day Screen and Treat; <sup>3</sup>Triage tests requiring a second visit for treatment. HPV: Human papillomavirus; VIA: Visual inspection test with 3%-5% acetic acid; VILI: Visual inspection test with Lugol's iodine.

Therefore, the detection of HPV DNA is used by many assays and is the only molecular marker fully developed and approved for primary CC screening. These tests can be based on the detection of specific types of oncogenic HPV that identify women at a higher cancer risk (e.g., HPV genotypes 16 and 18)<sup>[36]</sup>. However, many other molecular mechanisms associated with HPV infection are necessary for CC development, such as chromosomal abnormalities, expression of oncogenes<sup>[57]</sup>, epigenetic regulation (hypermethylation)<sup>[58]</sup> and apoptotic markers, which covers a large number of potential biomarkers. Molecular tests have been lately under intensive study as a potential alternative and triage tests for CC screening<sup>[59]</sup>.

**Expression of oncogenes:** Oncoproteins expressing viral oncogenic activity could potentially be used as biomarkers in the triage of HPV-positive women or directly as a primary screening method. When HPV-infected cervical cells undergo precancerous or cancerous changes, oncoprotein E6 is expressed in cervical cells at elevated levels. Only E6 protein from high-risk HPV types promotes carcinogenesis by binding to a human PDZ domain. This allows E6 protein to bind to cellular molecules and deregulate cellular proliferation and differentiation, which may lead to the development of cancer<sup>[60]</sup>. An HPV E6 test using lateral flow (OncoE6™, Arbor Vita Corporation) has been developed to detect E6 protein of HPVs 16, 18 and 45<sup>[61]</sup>. Weaknesses of the OncoE6™ Cervical Test are low sensitivity (approximately 45%)<sup>[62]</sup> because it only detects HPV 16, 18 or 45. Additionally, specimens stored in buffers/transport medium used for HPV DNA testing cannot be used, and thus new cervical collection is always required. The oncogenic activity of E7 protein may also be tested indirectly by the host cyclin-dependent kinase inhibitor p16Ink4a. This kinase inhibitor decelerates the cell cycle by inactivating the cyclin-dependent kinases (CDK4/CDK6) involved in retinoblastoma protein phosphorylation<sup>[63]</sup>. Overexpression of p16INK4a in

almost all cervical precancer (High-grade lesions) and invasive CC<sup>[64,65]</sup> has been shown to be directly linked to the transforming activity of E7 oncoprotein, which is produced by HPV<sup>[66]</sup>. Cellular accumulation of p16INK4a can be measured by cytochemistry using ELISA assays, which are commercially available (CINtec® p16, Roche mtm laboratories, Mannheim, Germany).

### Modulation of host microRNAs and methylation status of protein-coding genes:

HPVs modulate expression of host microRNAs (miRNAs)<sup>[67]</sup> via deletion, amplification, or genomic rearrangement. Recent studies have explored the role of the miRNAs in the development of CC. They found that several miRNAs are dysregulated in CC, such as miR-21, miR-127, miR-143, miR-145, miR-155, miR-203, miR-218 and miR-214, among others<sup>[68-72]</sup>. The miRNA-203 is downregulated in HPV-positive cells and its repression leads to maintenance of increased levels of p63 in infected suprabasal cells, maintaining cells in an active state in the cell cycle<sup>[73]</sup>. Other well studied miRNA is the miRNA-21, whose upregulation has been associated with aggressive progression and poor prognosis in CC<sup>[74]</sup>. Also miRNA-143 and -145 were found to be less expressed in CC<sup>[67,70]</sup>. Despite being a hot-spot topic, some discordance exists between studies concerning miRNAs, therefore further studies need to be conducted before these molecular biomarkers can be safely introduced in CC screening routine.

Epigenetic silencing of tumor suppressor genes is also responsible for cervical carcinogenesis<sup>[58]</sup>. Quantification of DNA methylation can be easily done and has been drawing attention in the recent years, making it a promising biomarker in CC<sup>[75]</sup>. L1 genes from HPV16 and 18 L1 are always highly methylated in CC<sup>[76,77]</sup>. A recent study using a rapid and sensitive technique<sup>[77]</sup>, methylation-sensitive high-resolution melting analysis, has shown that L1 HPV16 methylation was highly associated with cervical pre-cancer and cancer and can be used as a triage test for women positive for HPV16 who are at greater risk to develop invasive cancer. Another study on HPV DNA methylation<sup>[78]</sup> tested 14 methylated candidate genes (ADRA1D, AJAP1, COL6A2, EDN3, EPO, HS3ST2, MAGI2, POU4F3, PTGDR, SOX8, SOX17, ST6GAL2, SYT9, and ZNF614) and found that POU4F3 gene methylation had the highest area under the ROC curve (0.86; 95%CI: 0.78-0.95) in detecting CIN3+, which makes it a potential molecular tool for triage in HPV-positive women.

**Other protein biomarkers:** Promising additional molecular markers for triage of HPV-positive women are molecular markers expressing aberrant S-phase induction (BD ProEx™ C reagent), including two proteins: Topoisomerase II A and minichromosome maintenance protein. Both proteins are overexpressed in HPV-infected cells as a result of the uncontrolled activation of the gene transcription and are linked to severity of cervical lesions<sup>[79,80]</sup>. Moreover, carcinoma embryonic antigen



has found to be a good biomarker for CC prognosis and disease management<sup>[81]</sup>, though it is elevated in different non-cancerous and cancerous conditions. Many other biomarkers, such as integral membrane protein CD44, enzyme cyclooxygenase-2, cytokine vascular endothelial growth factor and membrane protein caveolin-1 might be useful in CC screening, by being more or less associated with cervical lesions severity, disease progression and prognosis<sup>[82-85]</sup>.

## CONCLUSION

Emerging technology places CC screening in developing countries at a crossroad and a choice of new policies is warranted. Primary HPV testing is widely accepted as being more effective than cytology for CC screening. Primary HPV testing increases sensitivity for the detection of CIN2+ compared with cytology and its high negative predictive value allows screening intervals to be extended. However, HPV testing has a mediocre specificity and positive predictive value. Additionally, HPV testing could be impractical in developing countries without a triage strategy to further characterise and evaluate the risk of an HPV-positive woman. Therefore, follow-up and management should be carried out. The emergence of rapid POC HPV tests that are performed in self-obtained vaginal samples will permit not only first-line screening, but also a triage of HPV-positive women during the same visit. As a result, a new concept can be achieved in a single visit, consisting of self-HPV testing, triage and treatment. This could allow most of the eligible women living in low-resource settings to participate in a CC screening programme by minimising repeated visits.

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## Neoadjuvant chemotherapy followed by surgery in gastric cancer patients with extensive lymph node metastasis

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### Abstract

Gastric cancer with extensive lymph node metastasis (ELM) is usually considered unresectable and is associated with poor outcomes. Cases with clinical enlargement of the para-aortic lymph nodes and/or bulky lymph node enlargement around the celiac artery and its branches are generally dealt with as ELM. A standard treatment for gastric cancer with ELM has yet to be determined. Two phase II studies of neoadjuvant chemotherapy followed by surgery showed that neoadjuvant chemotherapy with S-1 plus cisplatin followed by surgical resection with extended lymph node dissection could represent a treatment option for gastric cancer with ELM. However, many clinical questions remain unresolved, including the criteria for diagnosing ELM, optimal regime, number of courses and extent of lymph node dissection.

**Key words:** Extended lymph node metastasis; Gastric cancer; Neoadjuvant chemotherapy; Gastrectomy; Lymph node dissection

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**Core tip:** Gastric cancer with extensive lymph node metastasis (ELM) is usually considered unresectable and associated with poor outcomes. Phase II studies of neoadjuvant chemotherapy followed by surgery have shown the efficacy of this multimodal therapy for this pathology, but many clinical questions remain unresolved, including the criteria for diagnosing ELM, optimal regime, number of courses and extent of lymph node dissection.

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## INTRODUCTION

Surgical resection represents the most important step in the treatment of gastric cancer and is the only approach offering complete cure. Systemic chemotherapy is administered for gastric cancer patients with liver, peritoneal, or other distant metastases, but extensive lymph node metastasis (ELM) stands on the borderline between surgical resection and systemic chemotherapy. The development of treatment for gastric cancer with ELM is a difficult and challenging task. This article reviews previous reports and looks to the future of treatment for gastric cancer with ELM.

## ELM IN GASTRIC CANCER

No widely accepted consensus has been reached regarding the definition of ELM. In most reports, cases with clinical enlargement of the para-aortic lymph nodes (PAN) but no other distant metastases have generally been dealt with as ELM<sup>[1-3]</sup>. The range of PAN that are subject to surgical resection extends from the caudal end of the celiac axis to the cranial side of the inferior mesenteric artery, and is taken to be No. 16a2-b1 in the Japanese classification of gastric cancer<sup>[4]</sup>.

Most reports consider positivity for PAN metastasis with enlargement of  $\geq 1$  cm in the long axis as ELM<sup>[1-3]</sup>. In the most recent response evaluation criteria in solid tumors (RECIST)<sup>[5]</sup>, enlargement of  $\geq 15$  mm in the short axis is considered to represent measurable and assessable lymph nodes. Using multislice computed tomography, Marrelli *et al.*<sup>[6]</sup> diagnosed clinical metastasis with enlargement of  $\geq 8$  mm in the short axis of PAN in gastric cancer, and reported positive and negative predictive values of 73% and 97%, respectively, with 93% accuracy.

Indications for surgical intervention do not always need to follow RECIST criteria, as the purpose of these criteria is to objectively evaluate treatment effect. However, further investigation of the criteria for diagnosing ELM is needed in the future.

In a series of Japan Clinical Oncology Group (JCOG) studies by Yoshikawa *et al.*<sup>[3]</sup> and Tsuburaya *et al.*<sup>[1]</sup>, bulky lymph node enlargement around the celiac artery and its branches (bulky N), in addition to clinical enlargement of PAN, is treated collectively as ELM. The reasons are that patients with such metastases are commonly considered to be inoperable and, similar to PAN-positive patients, prognosis is poor even if curative resection is possible. According to the report by Tsuburaya *et al.*<sup>[1]</sup>, survival outcomes are equivalent in PAN-only patients and bulky N-only patients. Treating PAN metastasis

and bulky N as a single disease group has thus been considered reasonable.

## STANDARD TREATMENT FOR GASTRIC CANCER WITH ELM

From the 1980s to 1990s, PAN dissection was actively performed at some institutions in Japan. As a result, PAN metastasis was seen in about 20% of patients at maximum, with long-term survival achieved in about 10%-20% of these patients<sup>[7,8]</sup>. Similar reports have recently been seen from Western countries<sup>[9]</sup>.

On the other hand, JCOG 9501 was conducted to verify the significance of prophylactic PAN dissection, but no meaningful impact was found<sup>[10]</sup>. As a result, while PAN were categorized as regional lymph nodes in past Japanese classifications<sup>[4]</sup>, the new classifications categorize them as distant lymph nodes<sup>[11]</sup> that are no longer considered a target of curative resection. PAN are also taken to be distant lymph nodes in Western guidelines<sup>[12]</sup>, and are again not considered a target of curative resection.

However, it must be noted that the results of JCOG 9501 show the ineffectiveness of prophylactic PAN dissection. In that sense, a standard treatment for gastric cancer with clinical PAN metastasis has yet to be determined.

No comprehensive investigations of gastric cancer with bulky N have been reported. In some cases, curative resection may be achieved, but many cases are judged as unresectable because of direct invasion of major blood vessels.

## TREATMENT OUTCOMES OF NEOADJUVANT CHEMOTHERAPY FOR GASTRIC CANCER WITH ELM

The JCOG 0001 was a phase II clinical study for gastric cancer patients with ELM<sup>[3]</sup>. After excluding micrometastases to the liver and peritoneum by staging laparoscopy, irinotecan plus cisplatin combination therapy (IP) was administered as neoadjuvant chemotherapy. This was followed by gastrectomy with extended lymph node dissection including PAN. As a result of three treatment-related deaths, the study was discontinued. However, a subsequent follow-up and survival analysis showed a median survival time (MST) of 14.6 mo and a 3-year survival rate of 27% (95%CI: 15.2%-38.8%), exceeding the 3-year survival rate threshold (15%) established in the initial protocol. Although careful management of adverse events and appropriate patient selection are essential, this treatment could be recommended for gastric cancer patients with ELM.

In the JCOG 0405 study<sup>[1]</sup>, S-1 plus cisplatin combination therapy (SP) was used for neoadjuvant chemotherapy in similar patients, and the primary

endpoint was the percentage of complete resections with clear margins in the primary tumor (R0 resection). Fifty-three patients were enrolled, and among the 51 who proved eligible, R0 resection was performed in 42 patients (82.4%). A subsequent survival analysis showed an unexpectedly good 3-year survival rate of 58.5% (95%CI: 44.1%-70.4%).

Some reports have shown better survival results in gastric cancer patients who had only abdominal lymph node metastases. Yoshida *et al.*<sup>[13]</sup> investigated cases of long-term survival in patients who underwent chemotherapy for advanced gastric cancer, and reported that the 2- and 5-year survival rates of patients with metastasis to the abdominal lymph nodes only were 14.3% and 10.4%, respectively. Park *et al.*<sup>[14]</sup> reported a 3-year survival rate of 13.1% in gastric cancer patients with isolated involvement of PAN.

In those reports, clinically metastases to the liver and peritoneum that were not obvious may not have been excluded, as such metastases could not be excluded with laparotomy or staging laparoscopy. In addition, the extent of abdominal lymph node involvement in those studies may have partly exceeded that of the two neoadjuvant studies. Accordingly, direct comparison of survival rates between these chemotherapy and neoadjuvant studies is inappropriate. Despite this, the 3-year survival rate seen in the JCOG 0405 study is notably high. Furthermore, Yoshida *et al.*<sup>[13]</sup> and Park *et al.*<sup>[14]</sup> reported that some kind of local therapy had been used in many cases of long-term survival in their articles. In view of these results, neoadjuvant chemotherapy followed by surgical resection could represent a useful treatment option for gastric cancer with ELM. However, Tsuburaya *et al.*<sup>[1]</sup> described a lower 5-year survival rate for patients with both bulky N and PAN. The indication of neoadjuvant chemotherapy followed by surgery for this target is controversial.

## OPTIMAL CHEMOTHERAPY REGIME FOR GASTRIC CANCER WITH ELM

From the results of the above-mentioned JCOG 0001 and JCOG 0405 studies, 2 or 3 courses of SP is currently recommended for gastric cancer patients with ELM. For unresectable or recurrent gastric cancer, on the other hand, several triplet regimes such as docetaxel/cisplatin/5-fluorouracil<sup>[15]</sup> or docetaxel/cisplatin/S-1 combination therapy (DCS)<sup>[16-19]</sup>, have been developed and are reported to provide markedly high response rates. The JCOG 1002 study was therefore undertaken with DCS as neoadjuvant chemotherapy<sup>[20]</sup>, and the results are scheduled to be published soon.

The optimal number of cycles for neoadjuvant chemotherapy has not been established, but 2 or 3 cycles of therapy have been adopted in most neoadjuvant studies. The COMPASS-D trial, a randomized phase II trial with a factorial design comparing 2 and 4 courses of SP and DCS in neoadjuvant chemotherapy,

is underway for curable gastric cancer with serosal invasion<sup>[21]</sup>. Informative results are expected in terms of optimal regime and number of courses of neoadjuvant chemotherapy for gastric cancer from that trial.

No detailed reports have been published regarding the optimal interval between neoadjuvant chemotherapy and surgery, but patients ordinarily receive surgery if they meet adequate organ functions according to laboratory testing within 14 d before surgery<sup>[20]</sup>.

## SIGNIFICANCE OF EXTENDED DISSECTION FOR GASTRIC CANCER WITH ELM

In the above-mentioned JCOG 0001 and JCOG 0405 studies, gastrectomy with D2 plus PAN dissection was performed following neoadjuvant chemotherapy. This strategy is based on the high rate of PAN metastasis seen not only in patients with clinical PAN metastasis, but also in patients with bulky N. In addition, complete elimination of cancer cells can hardly be expected with a few courses of neoadjuvant chemotherapy. Inoue *et al.*<sup>[22]</sup> evaluated the efficacy and feasibility of neoadjuvant chemotherapy with SP in initially unresectable locally advanced gastric cancer, and reported 3-year survival rates of 31.0% and 53.8% in all and curative cases, respectively. However, they also reported that the most common site for initial recurrence after R0 resection was the PAN. Wang *et al.*<sup>[2]</sup> reported an MST of 29.8 mo after performing gastrectomy with D2 dissection following capecitabine plus oxaliplatin combination therapy in patients with clinical PAN metastasis. Those results should be interpreted with caution, since selection bias for curative cases may have had an effect. The good survival outcomes in the JCOG 0405 and JCOG 0001 studies were obtained with PAN dissection as well as gastrectomy plus D2 lymph node dissection. Given these findings, gastric cancer with ELM should be treated using concurrent PAN dissection not only in patients with PAN metastasis, but also in bulky N-only patients. However, further investigation is needed regarding the optimal extent of lymph node dissection for gastric cancer patients with ELM.

## CONCLUSION

A certain level of outcome is expected with multimodal therapy combining neoadjuvant chemotherapy and extended lymph node dissection in gastric cancer patients with ELM. At the same time, many questions remain to be unresolved, including the criteria for diagnosing ELM, optimal regime, number of courses and range of lymph node dissection.

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## Carcinoma of unknown primary and paraneoplastic dermatomyositis

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### Abstract

Dermatomyositis is known to be associated with neoplastic disorders, however the presentation of carcinoma of unknown primary as dermatomyositis is rare. We describe a case index of 50-year-old female who presented with enlarged inguinal lymph nodes accompanied with symmetric proximal muscle

weakness and erythematous plaques. Conventional basic work-up did not reveal the diagnosis, however, positron emission tomography-computed tomography and re-staining of the pathology specimen suggested the ovaries as the primary site. Chemotherapy including carboplatin paclitaxel and bevacizumab led to complete response of disease and improvement in the dermatomyositis. The present case emphasizes the importance of a thorough directed evaluation for the underlying cancer in patients with carcinoma of unknown primary presenting as dermatomyositis. We further provide an up-to-date detailed review of published data describing these clinical entities.

**Key words:** Paraneoplastic; Dermatomyositis; Cancers of unknown primary; Positron emission tomography

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**Core tip:** The presentation of carcinoma of unknown primary as dermatomyositis is rare. Positron emission tomography-computed tomography and pathology case oriented evaluation may identify the site of origin. We provide an up-to-date detailed review of published data describing these clinical entities.

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### INTRODUCTION

Cancer of unknown primary origin (CUP) is a group of metastatic tumors for which the site of origin cannot be detected at the time of diagnosis. In most of the cases, the source of the cancer will never be determined.

According to the European society of medical oncology, CUPs account for up to 5% of all malignancies<sup>[1]</sup>. The biology of these tumors is not fully elucidated although mechanism of metastatic spread in the absence of growth of the primary tumor can occur through site-specific transformation of disseminated cells, or oncogene induction at metastatic stroma.

Dermatomyositis is a connective-tissue disease characterized by progressive, proximal muscle weakness and pathognomonic cutaneous findings. Malignancy is associated with dermatomyositis in up to 40% of patients, representing a paraneoplastic phenomenon<sup>[2,3]</sup>. We describe here a rare case of a female who presented with carcinoma of unknown origin accompanied with symmetric proximal muscle weakness and erythematous plaques.

## CASE DESCRIPTION

A 50-year-old woman with carcinoma of unknown origin was admitted to the E.R because of intense weakness. She was evaluated one month earlier when she underwent biopsy from enlarged left inguinal lymph node. The biopsy revealed poorly differentiated carcinoma of unknown origin (performed out of our institute). On arrival, her physical examination revealed proximal weakness, which was profound in all extremities. Skin manifestations included peri-orbital edema and erythematous plaques on the extremities. She had enlarged left inguinal lymph node and signs of biopsy from the right lymph node. Biochemical analysis demonstrated elevated creatine kinase 5600 u/L (normal range 26-192), elevated AST 147(normal range 0-40) and ALT 130 (normal range 0-35). A computed tomography (CT) scan was unremarkable except enlarged left inguinal lymph node. Indirect immunofluorescence for anti-Jo-1 and ANA were negative. Tumor markers showed CEA-4.16 (0-4) CA15-3 -60 (0-30) CA125 80 (0-30). The patient underwent another biopsy from the left lymph node for re-pathology evaluation and staining which showed poorly differentiated Carcinoma. Staining for keratins: CK7 - positive strong, CK20 - weak, CK5/6 - negative, WT1 - positive and TTF - negative. Gynecological evaluation, colonoscopy and endoscopy were unremarkable. Since dermatomyositis is associated with gynecological - ovarian cancer there was a clinical decision to look for findings at the urogenital system. Gynecologist examination and vaginal US were unremarkable. It was then decided to perform positron emission tomography (PET)-CT scan which demonstrated increased standardized uptake values uptake in the left pelvis in an ovarian cyst and there was also high standardized uptake values uptake in paraaortic and cervical lymph node (Figure 1). These observations have led us to re-staining the specimen for CA125 and p53 which were both positive suggestive



**Figure 1** Positron emission tomography-computed tomography scan which demonstrated increased standardized uptake values uptake in the pelvis in an ovarian cyst and uptake in para-aortic and cervical lymph node (arrows).

of ovarian origin. The patient begun chemotherapy treatment with protocol directed to ovarian cancer including carboplatin [Area under the curve (AUC) 6] paclitaxel 175 mg/m<sup>2</sup> and bevacizumab 15 mg/kg every three weeks. After 2 cycles dose reductions in both carboplatin (to AUC 4-5) and paclitaxel (to 80 mg/m<sup>2</sup> weekly) were needed due to neuropathy and neutropenia. She also received steroids (prednisone 60 mg which was gradually tapered off and replaced with methotrexate) for dermatomyositis. This treatment (chemotherapy and/or the steroids) led to complete response in the disease and improvement in the dermatomyositis.

## LITERATURE REVIEW

Few studies demonstrated that dermatomyositis and polymyositis are association with different cancers<sup>[4-6]</sup>. A pooled analysis from Scandinavian repositories, confirmed that both dermatomyositis and polymyositis are associated with malignancy (dermatomyositis more than polymyositis)<sup>[3]</sup>. This study confirmed that ovarian, lung, gastric, colorectal, and pancreatic cancers were the cancers most strongly associated with dermatomyositis but other cancers were associated with dermatomyositis as well. Cancer treatment, local (surgery) or systemic (chemotherapy) usually results in remission of the dermatomyositis, and recurrence of symptoms can represent relapse of the malignant disease, further supporting its paraneoplastic origin<sup>[7,8]</sup>. Due to the increased risk of some cancers in patients with dermatomyositis, further examinations which may include whole body imaging, mammography and gynaecological evaluation are justified.

CUPs diagnosis requires pathology evaluation<sup>[1]</sup>. CUPs are categorized by pathological evaluation into: Differentiated carcinomas (well, moderately, or poorly); undifferentiated neoplasms; squamous cell carcinomas and carcinomas with neuroendocrine differentiation. In

the past CUP was characterized as an individual entity with dismal prognosis, while as our understanding of cancer biology evolved it became clear that CUP may retain the underpinnings of the primary origin<sup>[9]</sup>. Staining for keratins, may mislead the clinicians if interpreted incorrectly. For example our patients was CK7 positive and CK20 - weak - if weak is interpreted as negative, lung breast and thyroid cancers are more suspected. If CK20 - weak staining is interpreted as positive ovarian and pancreas cancers are suspected and WT-1, p53 and CA125 may add information as shown in our case. Full physical examination, blood and biochemistry analysis, and CT scans of thorax, abdomen and pelvis constitute the basic work-up in CUPs. Other evaluation should be only clinically guided. The minority of patients with CUP (less than 20%) belong to subsets with more favorable outcomes and treatment response and it is therefore crucial to identify this patients during the work-up<sup>[1,10,11]</sup>. Peritoneal carcinomatosis in females represent one example of this favorable risk CUP. Our patient pathology did not reveal serous papillary but rather poorly differentiated carcinoma. Moreover the disease distribution according the PET-CT was not classic for ovarian cancer except uptake in the left ovary. However the decision to treat her with chemotherapy used for ovarian cancers seems rational.

PET-CT is one of the most sensitive imaging techniques to detect malignant lesions and has been used in different cancer conditions with quite success<sup>[12-15]</sup>. Selva-O'Callaghan *et al*<sup>[16]</sup> prospectively evaluated the role of PET-CT in the diagnosis of occult tumors in patients with dermatomyositis/polymyositis. Using PET-CT they evaluated prospectively 55 patients with myositis. 9 out of the 55 patients were diagnosed with cancer. PET-CT identified 6 out of the 9 patients. The authors concluded that PET-CT for diagnosing CUP in patients with myositis was an option comparable to other multi diagnostic tests.

As the radiotracer doses administered using PET-CT are relatively small, the risk is very low compared with the potential benefits. There are no known long-term adverse effects from such low-dose exposure and the potential benefits from PET-CT outweighs the risks which may include allergic reactions (rare and mild), and exposure to low amount of radiation<sup>[17]</sup>.

## CONCLUSION

In conclusion this rare case of patient with carcinoma of unknown primary emphasizes the importance of a thorough directed evaluation and the usage of PET-CT for the underlying cancer in patients with carcinoma of unknown primary presenting with dermatomyositis.

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## Basic Study

## Fluoxetine induces cytotoxic endoplasmic reticulum stress and autophagy in triple negative breast cancer

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### Abstract

**AIM:** To investigate the mechanism of action of lipophilic antidepressant fluoxetine (FLX) in representative molecular subtypes of breast cancer.

**METHODS:** The anti-proliferative effects and mechanistic action of FLX in triple-negative (SUM149PT) and luminal (T47D and Au565) cancer cells and non-transformed MCF10A were investigated. Reverse phase protein microarray (RPPM) was performed with and without 10  $\mu$ mol/L FLX for 24 and 48 h to determine which proteins are significantly changed. Viability and cell cycle analysis were also performed to determine drug effects on cell growth. Western blotting was used to confirm the change in protein expression examined

by RPPM or pursue other signaling proteins.

**RESULTS:** The FLX-induced cell growth inhibition in all cell lines was concentration- and time-dependent but less pronounced in early passage MCF10A. In comparison to the other lines, cell growth reduction in SUM149PT coincided with significant induction of endoplasmic reticulum (ER) stress and autophagy after 24 and 48 h of 10  $\mu$ mol/L FLX, resulting in decreased translation of proteins along the receptor tyrosine kinase/Akt/mammalian target of rapamycin pathways. The increase in autophagy marker, cleaved microtubule-associated protein 1 light chain 3, in SUM149PT after 24 h of FLX was likely due to increased metabolic demands of rapidly dividing cells and ER stress. Consequently, the unfolded protein response mediated by double-stranded RNA-dependent protein kinase-like ER kinase resulted in inhibition of protein synthesis, growth arrest at the G1 phase, autophagy, and caspase-7-mediated cell death.

**CONCLUSION:** Our study suggests a new role for FLX as an inducer of ER stress and autophagy, resulting in death of aggressive triple negative breast cancer SUM149PT.

**Key words:** Inflammatory breast cancer; Endoplasmic reticulum stress; Autophagy; Apoptosis; Fluoxetine

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**Core tip:** Our study demonstrates for the first time the complex but selective actions of Food and Drug Administration-approved, well-tolerated antidepressant drug known as fluoxetine (FLX) in malignant triple negative breast cancer (TNBC) cells. The significant reduction in cell growth of inflammatory TNBC line SUM149PT was a consequence of unfolded protein response induced by FLX and subsequent induction of autophagy and mitochondrial apoptosis, demonstrating the intricate crosstalk between endoplasmic reticulum and mitochondria in response to cellular stress. Combination of low dose FLX with existing regimen for TNBC may provide dual benefit of alleviating psychological distress, including depression and anxiety, and inducing death in aggressive tumor cells.

Bowie M, Pilie P, Wulfkühle J, Lem S, Hoffman A, Desai S, Petricoin E, Carter A, Ambrose A, Seewaldt V, Yu D, Ibarra Drendall C. Fluoxetine induces cytotoxic endoplasmic reticulum stress and autophagy in triple negative breast cancer. *World J Clin Oncol* 2015; 6(6): 299-311 Available from: URL: <http://www.wjgnet.com/2218-4333/full/v6/i6/299.htm> DOI: <http://dx.doi.org/10.5306/wjco.v6.i6.299>

## INTRODUCTION

A major roadblock to effective breast cancer therapy is development of de novo or acquired resistance. Triple-

negative breast cancer, which lacks the expression of steroid estrogen and progesterone receptors as well as overexpressed HER2, accounts for 15%-20% of all breast cancers. Majority of triple negative breast cancers (TNBCs) are basal-like, among the most aggressive types, likely to develop chemotherapy resistance, and lack suitable targeted therapeutics<sup>[1]</sup>. Resistance to apoptosis is often the mechanism by which these cancers evade death. Thus, an alternative approach to trigger cell death is greatly needed.

Autophagy is an example of alternative mechanism of cell death. However, this evolutionarily conserved process in response to metabolic stress typically leads to cell survival. Autophagy is a process in which damaged or long-lived proteins and organelles are encapsulated in double-membraned vesicles called autophagosomes, targeted for lysosomal degradation, and released into the cytosol as intermediate metabolites for nutrient recycling and ATP production<sup>[2]</sup>. While evidence has been limited, autophagic cell death has been shown in cells with deficient apoptotic proteins<sup>[3,4]</sup>, upregulated mitochondrial cell death protein BNIP3<sup>[5]</sup>, and deficient tumor suppressor Von Hippel-Lindau<sup>[6]</sup>. The pro-death function of autophagy is believed to be due to prolonged digestion of cellular components or selective digestion of survival (over death) factors.

NF $\kappa$ B regulates diverse cellular processes in response to numerous stimuli, including unfolded protein response (UPR) as a result of oxidative and metabolic stress<sup>[7,8]</sup>. UPR is induced when there is a buildup of unfolded, misfolded or damaged proteins within the endoplasmic reticulum [*i.e.*, endoplasmic reticulum (ER) stress]. The goal of UPR is to stop general protein synthesis but allow selective synthesis of ER chaperones, such as binding immunoglobulin protein (BiP), to restore balance<sup>[9]</sup>. ER stress can directly induce autophagy through upregulation of BiP, which is required for autophagosome formation<sup>[10]</sup>. The repressive effect of BiP on UPR signal transducers, such as double-stranded RNA-dependent protein kinase-like ER kinase (PERK), inositol-requiring enzyme 1  $\alpha$  and activating transcription factor 6, is released during ER stress<sup>[11]</sup>. If proper protein folding capacity is not restored, then all three arms of UPR induce CCAAT/enhancer binding protein-homologous protein (CHOP) and growth arrest and DNA damage 34 (GADD34) to stimulate apoptosis. In some situations, autophagy is induced to promote cell survival by removal of accumulated ubiquitinated proteins and aggregates<sup>[12]</sup>. Together, these studies demonstrate the integration of signals from autophagy, ER stress/UPR, and apoptosis in regulating cell survival or cell death.

The anti-cancer properties of widely used antidepressants, specifically the selective serotonin reuptake inhibitors (SSRIs), have received attention in the last two decades. Fluoxetine (FLX) was the first approved SSRI for depression, and it is still used today across a diverse population, including in many cancer patients, for the treatment of anxiety and/or depression. FLX is a well-tolerated drug with a mild side effect profile,

safe in overdose, and almost no associated withdrawal symptoms, even when compared to other SSRIs<sup>[13]</sup>. Like most SSRIs, FLX blocks the reuptake of serotonin (5-HT) at the pre-synaptic membrane, enhancing the actions of 5-HT on serotonin receptors at the post-synaptic neuron<sup>[14]</sup>. But FLX is known to have various off-target interactions, resulting in modulation of cancer cell growth. For example, a single study in rodents suggested that FLX stimulates malignant cell growth<sup>[15]</sup>. However, multiple epidemiological studies have shown no association between SSRI use and breast cancer risk<sup>[16,17]</sup>. Previous studies have shown FLX-induced cell death in a variety of malignant cell lines, including those originating from the prostate, colon, lung, ovary, breast, brain, and the immune system<sup>[3,18-24]</sup>. One study has implicated the inhibition of the extracellular signal regulated kinase 1 and 2 (ERK1/2) as a potential consequence of FLX's anti-tumor effect<sup>[20]</sup>. However, the exact role of FLX in modulating ERK1/2 pathway in breast cancer subtypes is currently unknown.

In this study, distinct molecular subtypes of breast-derived cell lines, including triple-negative (SUM149PT) and luminal (T47D and Au565) breast cancer cells as well as non-transformed MCF10A, were evaluated for their response to FLX exposure in regards to protein expression of key components of signaling pathways that mediate cell growth, survival and death. Our study demonstrates that the cell growth inhibition in rapidly dividing TNBC SUM149PT is due to ER and metabolic stress that leads to decreased translation of proteins along the RTK/Akt/mTOR and MEK/ERK pathways. Excessive ER stress and autophagy induced by FLX in SUM149PT eventually leads to cell death mediated by executioner caspase-7. Given this proposed anti-proliferative mechanism and safety profile, FLX may prove an ideal part of a targeted regimen against TNBC in future *in vivo* and clinical studies.

## MATERIALS AND METHODS

### Reagents

Tissue cell culture media, FBS, horse serum, and 2X Tris-glycine SDS loading buffer were obtained from Life Technologies. Insulin, hydrocortisone, epidermal growth factor (EGF), FLX, MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide], and bovine serum albumin were from Sigma. Cholera toxin was obtained from Calbiochem. Mammary epithelial growth medium bullet kit was purchased from Lonza. Tissue protein extraction reagent (T-PER), bicinchoninic acid (BCA) assay, and SuperSignal West Dura chemiluminescent substrate were from Thermo Fisher Scientific. The complete protease and phosphatase (PhosSTOP) inhibitors were obtained from Roche Applied Science. Primary antibodies for Western blotting were as follows: LC3B (ab48394) from Abcam; ERK1/2 T202/Y204 (4370), AMPK $\alpha$  T172 (2535), p70 S6 Kinase (p70 S6K) T389 (9234), LC3B (3868), BiP (3177), PERK (5683), eIF2 $\alpha$  Ser-51 (3398), PARP (9542),  $\mu$ -Calpain (2556)

from Cell Signaling Technology;  $\beta$ -actin (sc-47778), GADD34 (sc-8327), GADD153/CHOP (sc-575) from Santa Cruz Biotechnology; caspase-12 (PRS3195) from Sigma-Aldrich.

### Cell culture

Most breast cancer cell lines were obtained from American Type Culture Collection (Manassas, VA). T47D cells were cultured in alpha MEM prepared as previously described<sup>[25]</sup>. SUM149PT cells were originally obtained from Dr. Stephen Ethier (Karmanos Cancer Institute, Detroit, MI) and are commercially available (Asterand, Detroit, MI). SUM149PT cells were maintained in Ham's F12 supplemented with 5% FBS, 5 mg/mL insulin, and 1 mg/mL hydrocortisone. Au565, BT474, and lapatinib-resistant BT474 (R-BT474) cell lines were maintained in RPMI 1640 supplemented with 10% FBS and 2 mmol/L L-glutamine. R-BT474 cell line was kindly provided by Dr. Neil Spector (Duke University Medical Center, Durham, NC). MCF10A lines were cultured in two different media. MCF10A late passage cells were maintained in HuMEC complete media, while MCF10A early passage cells (generous gift of Dr. David Beebe, University of Wisconsin, Madison, WI) were grown in DMEM-F12 supplemented with 5% horse serum, 20 ng/mL EGF, 0.5  $\mu$ g/mL hydrocortisone, 100 ng/mL cholera toxin, and 10  $\mu$ g/mL insulin. Normal human mammary epithelial cells (HMEC) were obtained from Lonza and grown in HuMEC complete media. DKAT cell line is unique to our laboratory and maintained in supplemented MEBM<sup>[26]</sup>. All cell lines were maintained in a humidified atmosphere of 5% CO<sub>2</sub> at 37 °C and have been authenticated by DNA fingerprinting at the Duke University Cell Culture Facility.

### Reverse phase protein microarray analysis

The aforementioned cell lines were grown in 100 mm dishes for 24 h, followed by addition of FLX at a final concentration of 10  $\mu$ mol/L and cell harvest after 24 h and 48 h of treatments. The indicated FLX concentration was previously tested in another breast cancer cell line<sup>[22]</sup> and served as a starting point for our proteomic study. Untreated (control) cells were run in parallel. This experiment was performed at least three different times. Briefly, adherent cells were washed twice in cold 1  $\times$  PBS and lysed directly in dishes on ice with modified T-PER buffer as previously described<sup>[27]</sup>. Following centrifugation at 3000 g for 5 min at 4 °C, each supernatant was transferred to clean microcentrifuge tubes. After determining the total protein content by BCA protein assay, samples were diluted in 2  $\times$  Tris-glycine SDS sample buffer with 2.5% 2-mercaptoethanol up to 2 mg/mL and boiled for 8 min. Samples were spun briefly and then stored at -80 °C until they were shipped in dry ice to George Mason University where subsequent lysate printing in triplicate, immunostaining, and reverse phase protein microarray (RPPM) analysis were performed. For this study, we examined the expression of 79 phosphorylated, total, and cleaved proteins that are thought to play a role in breast cancer cell proliferation,

survival, apoptosis, and metastasis. Enumerated are some antibodies used in the experiments: Akt S473 (9271), ERK1/2 T202/Y204 (9101), GSK-3 $\alpha/\beta$  S21/S9 (9331), AMPK $\beta$ 1 S108 (4181), mTOR S2448 (2971), p70 S6K T389 (9205), eukaryotic translation initiation factor 4G (eIF4G) S1108 (2441), NF $\kappa$ B p65 S536 (3031), Bax (2772), Bcl-2 S70 (2827), cleaved Casp-7 D198 (9491), cleaved Casp-3 D175 (9661), E-cadherin (4065), Vimentin (3295), Snail (4719), and SAPK/JNK T183/Y185 (9251) from Cell Signaling Technology; GSK-3 $\alpha/\beta$  Y279/Y216 (44-604) from BioSource; IkB $\alpha$  S32/S36 (551818) from BD.

### MTT assay

Cell viability or growth was measured by the MTT assay. Cells were seeded in triplicate at  $1.8 \times 10^4$  per well in 1 mL complete media in 12-well plates and grown at 37 °C for 24 h. Subsequently, 10  $\mu$ mol/L FLX was added in the cell media and incubated at the indicated time points. Day 0 reading was done at the same time as treatment was added. The MTT assay was carried out as follows: MTT solution was added at a final concentration of 0.5 mg/mL and incubated in the dark at 37 °C for 2 h. The reaction was stopped by adding Solubilization solution (95% DMSO/5% 1  $\times$  PBS), and absorbance values were determined at 560 nm on the Modulus microplate reader (Turner Biosystems). All MTT assays were performed at least two independent times.

### Western blotting

Cell lines were seeded in 100 mm dishes at  $2.6 \times 10^5$ , followed by treatment with and without fluoxetine after 24 h. Cells were grown at the indicated FLX concentration and time points, harvested and lysed in radio-immunoprecipitation assay buffer<sup>[28]</sup> containing phosphatase and protease inhibitor cocktails, and centrifuged at 14000 rpm for 10 min. The resulting supernatants (whole cell lysates) were assayed *via* BCA to determine total protein content and stored at -80 °C until use. Cell lysates were solubilized in reducing sample buffer, boiled, electrophoresed on Bis-Tris gel (Life Technologies), and transferred to polyvinylidene difluoride membranes (Bio-Rad Laboratories). The membranes were blocked and incubated with primary antibodies overnight at 4 °C, washed 3  $\times$  in TBS with 0.1% Tween 20, incubated with horseradish peroxidase-conjugated secondary antibody, and detected by enhanced chemiluminescence.

### Cell cycle analysis

Cells were seeded at  $2.6 \times 10^5$  in 100 mm dishes. After 24 h, FLX was added at a final concentration of 10  $\mu$ mol/L. Cells were harvested at indicated time points after treatment. Briefly, floating cells were retained and combined with trypsinized cells. Cells were spun down, washed with 1  $\times$  PBS, fixed in ice-cold 70% ethanol. Propidium iodide (PI) staining was performed as follows. Briefly, ethanol was removed and the cell

pellets were resuspended in 1  $\times$  PBS containing 15-25  $\mu$ g RNase A (Roche) and incubated for 30 min at 37 °C. PI stain (2 mg/mL) was added to each sample at a final concentration of 100  $\mu$ g/mL. Cells were sorted on a BD FACSCalibur using CellQuest software.

### Statistical analysis

Data were analyzed with SAS Enterprise Guide 5.1 software (Cary, NC) and represented as the mean  $\pm$  standard error. Two-sample *t*-test was used when comparing control and treated groups. To identify which proteins were differentially expressed in cell lines as a result of FLX treatment, changes in protein levels due to treatment were evaluated as percentages relative to control *via* 1-way ANOVA with Tukey adjustment for multiple comparison. *P*-values < 0.05 were considered statistically significant. Unsupervised hierarchical clustering analysis of the log 2-transformed proteomic data was carried out using the Ward method in JMP v5.1 (SAS Institute). GraphPad Prism 6 (San Diego, CA) was used to fit curves to concentration-dependent and time-dependent cell viability (growth) data, and the IC<sub>50</sub> values were determined from these generated curves.

## RESULTS

### Fluoxetine modulates the RTK/Akt/mTOR and RTK/ERK pathways

While the anti-proliferative and apoptotic effects of FLX in various malignant cell lines have been demonstrated, there appears to be no common signaling pathway(s) modulated by this drug. Given the heterogeneity of breast cancer, we hypothesized that the mechanism of action of FLX would be different for each subtype of breast cell line. Here, we examined the expression levels of several proteins encompassing the broad growth factor-mediated signaling and apoptotic pathways by high-throughput RPPM. We performed RPPM on basal normal (HMEC-15 and late passage MCF10A), triple-negative (SUM149PT and DKAT), luminal A (T47D), luminal B (BT-474 and R-BT474), and HER2+ (Au565) cell lines. The unsupervised hierarchical clustering analysis segregated the samples into distinct clusters of luminal (R-BT474, BT474, Au565, T47D) and basal-like cell lines (HMEC, MCF10A, SUM149PT, and DKAT) (Figure S1), which are consistent with the gene expression analysis of breast cancer cell lines<sup>[29]</sup>. There was also a distinct clustering of the HER2+ cell lines (R-BT474, BT474, and Au565) as previously described<sup>[30]</sup>. In contrast, there was no clear partitioning of samples into treatment groups (*i.e.*, control vs FLX).

Next, we determined the effects of 10  $\mu$ mol/L FLX treatment on protein expression changes across cell lines by one-way ANOVA. Here, we limit our statistical analysis to triple-negative SUM149PT, luminal T47D, HER2+ Au565, and normal late passage (Late) MCF10A. Given the mixed population of HMEC-15 (*i.e.*, cobblestone vs large, flattened morphology) that



we encountered during RPPM lysate preparation, Late MCF10A data were used in our analysis. As Figure 1A indicates, the FLX-induced changes in protein levels of Akt S473, p70 S6K T389, and eIF4G S1108 were significantly different across cell lines after 24 h. These proteins are components of the RTK/Akt/mTOR pathway, which plays a vital role in cell proliferation, growth, and survival.

The Akt S473 levels in SUM149PT consistently decreased after 24 h and 48 h of FLX treatment, while the expression in Late MCF10A cells increased (Figure 1). For both T47D and Au565 cells, treatment resulted in no change in baseline Akt S473 at 24 h, but a small decrease in protein levels after 48 h. Interestingly, SUM149PT showed an increase in activated glycogen synthase kinase 3 (GSK3) $\alpha/\beta$  Y279/Y216 after 48 h of treatment. This effect could be explained by a decrease in activity of Akt, which would otherwise inactivate GSK3. In contrast, the increased GSK3 $\alpha/\beta$  Y279/Y216 levels in Late MCF10A may be attributed to some other mechanism, which remains to be determined.

Downstream of Akt are important regulators of protein synthesis, namely the p70 S6K and eIF4G. In all cell lines, FLX induced a decrease in both proteins at 24 h, suggesting inhibition of translation (Figure 1A). Although not statistically significant, the activation of master regulator of cell growth, mTOR S2448, was inhibited by FLX in all cell lines, which is consistent with decreased p70 S6K activity. The translational inhibition was sustained up to 48 h as evidenced by a decrease in eIF4G S1108 (Figure 1B) as well as p70 S6K T389 by Western blot (Figure 1C).

To date, only few studies have shown FLX-mediated inhibition of ERK, and this effect appears to be cell type-dependent<sup>[20,31]</sup>. Here, we showed that after 48 h, FLX had different effects on ERK1/2 T202/Y204 level of each breast cell line (Figure 1B). SUM149PT and T47D showed inhibition of ERK1/2 after 48 h of treatment, while Late MCF10A and Au565 showed ERK1/2 activation, which we also confirmed by Western blot (Figure 1C).

### **Fluoxetine modulates mammary epithelial cell growth**

Several groups have shown that FLX treatment can lead to growth inhibition or death of cancer cells, although not much is known about its effect on breast cancer subtypes. We assessed the effect of FLX on cell viability or growth of the aforementioned cell lines, using various concentrations at different times. Although MCF10A originated from the same mastectomy fibrocystic diseased tissue, several variations of this cell line exist<sup>[32]</sup>. Initially, we obtained the Late MCF10A cells, which are spindle in shape (Figure 2A). A lower passaged MCF10A cells (Early MCF10A) were also obtained, and the derived epithelial cells have cobblestone morphology. In comparison to untreated (control) cells, FLX treatment reduces cell growth in a time-dependent and dose-dependent manner, with the biggest changes in IC<sub>50</sub> occurring between 24 h and 48 h for most cell lines

(Figure 2B-F). At 48 h, IC<sub>50</sub> ranges from 6.8-10.7  $\mu$ mol/L across cell lines. In our subsequent analysis and experiments, we used a fixed dose of 10  $\mu$ mol/L to assess the mechanism of action of FLX.

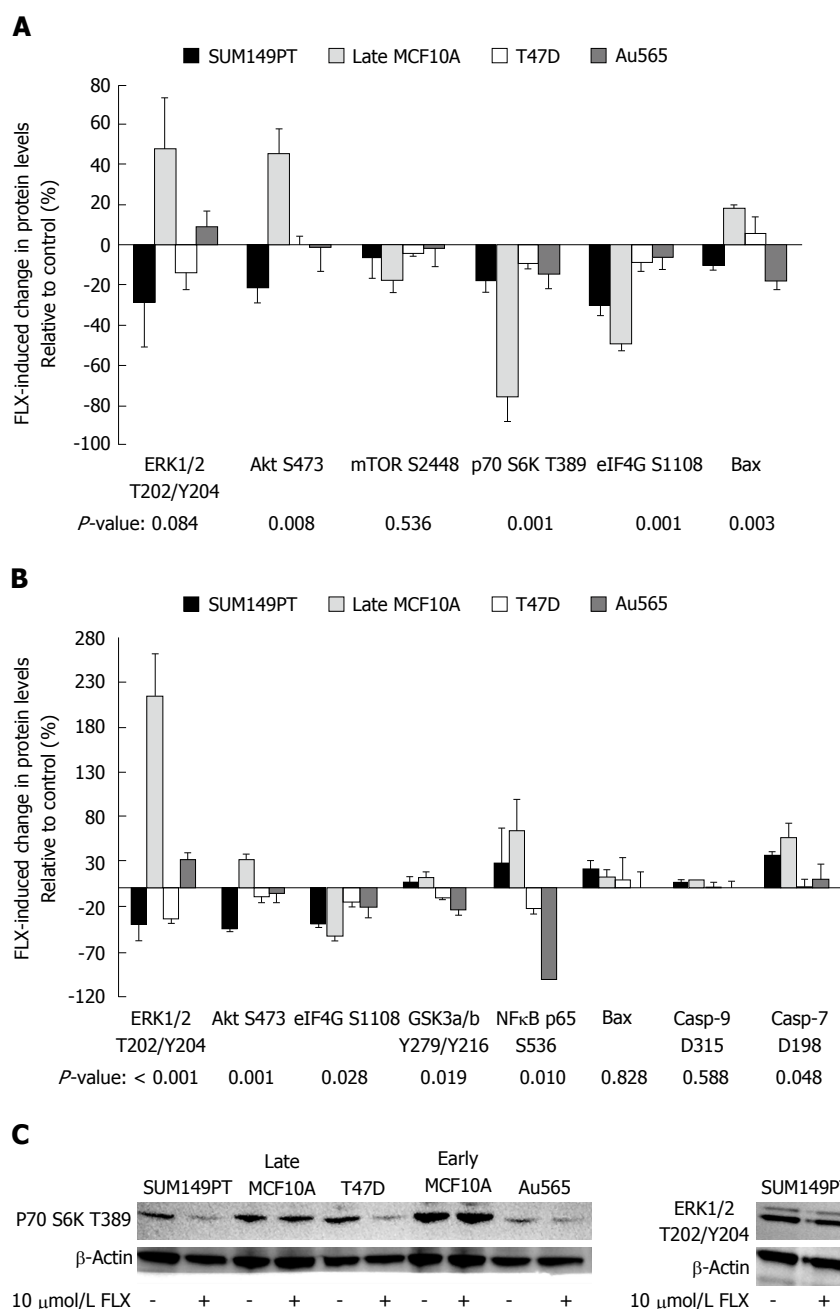
In comparison to control cells, the FLX-treated SUM149PT and Late MCF10A showed a decreased ability to reduce MTT to formazan crystals, suggesting significant cell growth inhibition by 48 h (Figure 3, red and blue solid lines vs red and blue dotted lines). In contrast, the treated Early MCF10A continued to grow albeit at a slower rate than control cells (green dotted vs solid lines) even after 48 h of treatment. The FLX-treated T47D and Au565 cells also showed cell growth reduction, which was greater than treated Early MCF10A (Figure 3, compare black and purple dotted lines vs green dotted lines). The effects of FLX on T47D and Au565 over time suggest that the drug is acting as a cytostatic rather than cytotoxic agent.

### **Fluoxetine induces autophagy and ER stress in rapidly dividing cells**

In this study, we showed that both rapidly dividing SUM149PT and Late MCF10A cells are most sensitive to FLX-induced cell growth inhibition (Figure 3). The decreased protein synthesis in both cell lines at 24 h of FLX treatment (Figure 1, p70 S6K and eIF4G) suggests altered energy metabolism, which can contribute to cell growth inhibition and even cell death.

Our RPPM data suggested that mTOR activity was inhibited by FLX by 24 h (Figure 1A). Inhibition of mTOR is mediated by adenosine monophosphate kinase (AMPK) during low cellular energy status or stress, which is then followed by autophagy<sup>[2,9,33]</sup>. We confirmed by Western blot that the central metabolic sensor AMPK was activated in FLX-treated SUM149PT and Late MCF10A as early as 2 h and up to 24 h, suggesting metabolic stress in these cells (Figure 4A). Next, we examined the expression of cleaved microtubule-associated protein 1 light chain 3 (LC3-II), which is required for autophagosome transport and maturation as well as a well-accepted monitor of autophagy<sup>[2,9]</sup>. After 24 h of FLX treatment, only SUM149PT showed increased level of LC3-II (data not shown). By 48 h, the LC3-II level in SUM149PT was significantly elevated compared to Late MCF10A (Figure 4A). Meanwhile, autophagy was not induced in Early MCF10A.

Induced cellular stress results in orchestration of several processes that dictate whether cells live or die. These processes include autophagy, ER stress, and apoptosis. The link between these processes has been elucidated only in the past 9 years. An important regulator and sensor of ER stress is BiP, which maintains proper protein folding and helps restore misfolded proteins<sup>[34]</sup>. In our study, only SUM149PT showed an apparent increase in BiP following exposure to FLX (Figure 4B). But UPR was induced in both FLX-treated SUM149PT and Late MCF10A, as indicated by an increase in PERK-mediated activation of translation

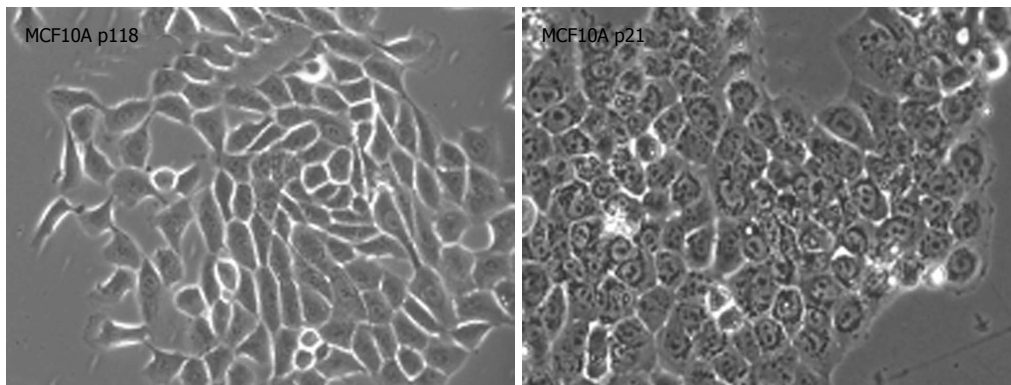
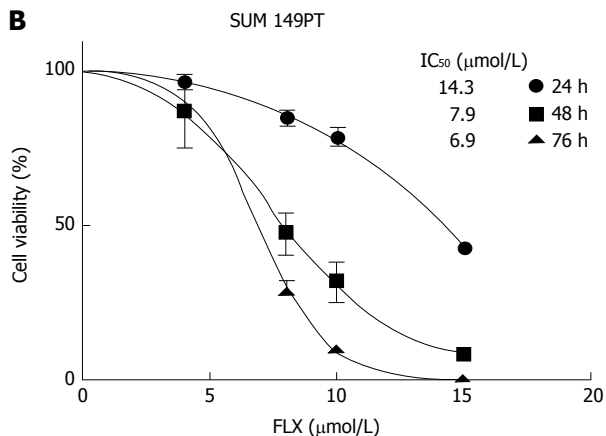
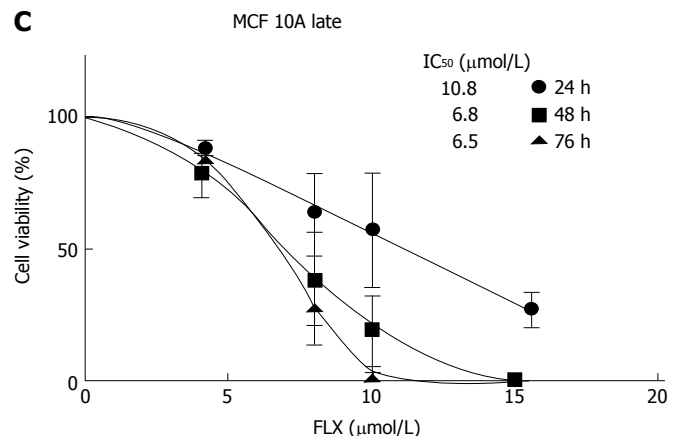
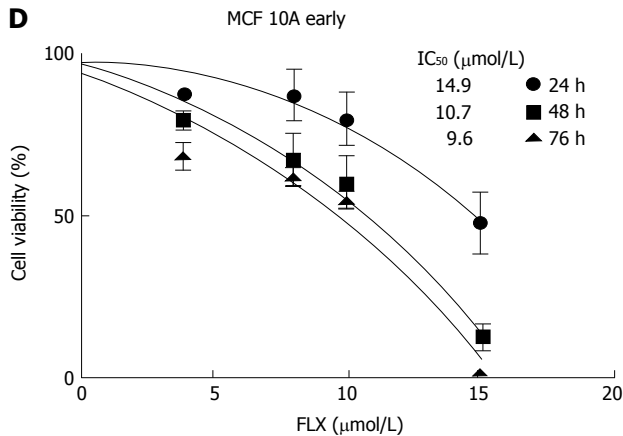
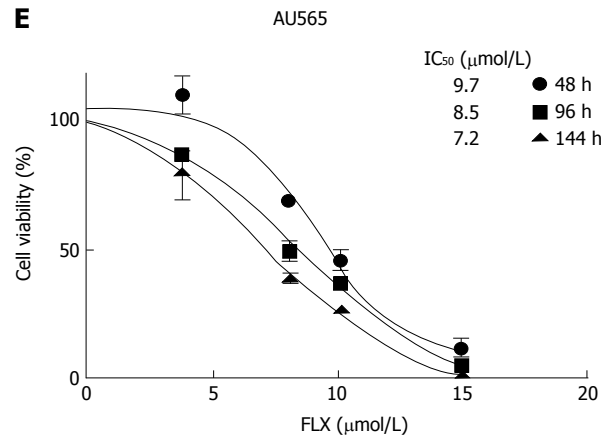
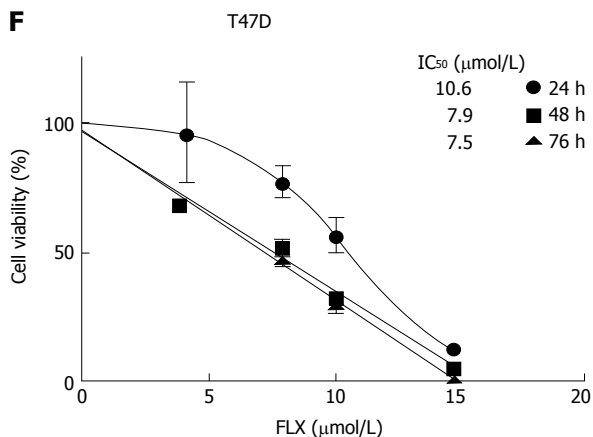


**Figure 1** Expression levels of proteins along the phosphoinositide 3-kinase/Akt/mammalian target of rapamycin, mitogen-activated protein kinase/extracellular signal-regulated kinase, and apoptosis pathways vary by cell lines. Statistically significant and differentially expressed proteins, following (A) 24 h and (B) 48 h treatment of 10  $\mu$ mol/L FLX, were indicated with stars; (C) Expression of few selected proteins across cell lines after 48 h of 10  $\mu$ mol/L fluoxetine was confirmed by Western blotting. For detection of p70 S6K and ERK1/2 activation, cell extracts were loaded at 100  $\mu$ g and 30  $\mu$ g per lane, respectively. MEK: Mitogen-activated protein kinase; ERK: Extracellular signal-regulated kinase; FLX: Fluoxetine; p70 S6K: p70 S6 Kinase; GSK3: Glycogen synthase kinase 3; eIF4G: Eukaryotic translation initiation factor 4G.

initiation factor eIF2 $\alpha$  at S51. Activation of eIF2 $\alpha$  has been shown to increase NF $\kappa$ B activity as well as inhibit protein synthesis<sup>[35,36]</sup>. Both RPPM and Western blot data indicated an increase in NF $\kappa$ B activity in SUM149PT and Late MCF10A after 48 h of FLX treatment (Figure 1B and 4B). The increased eIF2 $\alpha$  S51 levels in both cell lines were also consistent with the inhibition of eIF4G-mediated protein synthesis (Figure 1B). Meanwhile, the Early MCF10A did not show further increase in BiP, PERK, eIF2 $\alpha$  S51, and NF $\kappa$ B p65 S536 (Figure 4C),

suggesting no appreciable UPR in these normal cells with FLX treatment.

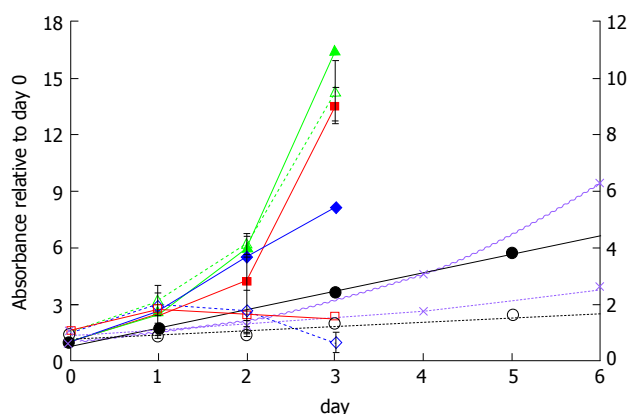
Although previous study has linked the activation of NF $\kappa$ B to autophagy through modulation of essential autophagy gene *Beclin-1*<sup>[37]</sup>, our study did not show a change in basal Beclin-1 protein levels with FLX treatment (data not shown). This suggests that Beclin-1 expression in mammary epithelial cells is not dependent on NF $\kappa$ B activation. However, a plausible link between UPR and autophagy induction in FLX-treated SUM149PT

**A****B****C****D****E****F**

**Figure 2 Time-dependent and concentration-dependent inhibition of cell growth induced by fluoxetine.** A: Bright field images of late and early passage of MCF10A cells were shown in 20 × magnification; B-F: The IC<sub>50</sub> values for the dose-response curves for each cell line were also indicated; cell viability for each cell line was measured spectrophotometrically by means of MTT assay. Data represented the average of optical densities normalized to untreated samples ± SEM of 6 measurements for each concentration. MTT: 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; FLX: Fluoxetine.

is BiP, which has been shown to be necessary in

the maturation step of autophagic vesicle (Figure



**Figure 3** Effect of incubation time on microwave theory and techniques reduction by cell lines in the absence (solid lines) and presence (dotted lines) of 10  $\mu\text{mol/L}$  fluoxetine. Reduction of MTT by viable Late MCF10A (diamonds), SUM149PT (squares), Early MCF10A (triangles), T47D (circles), and Au565 cells (crosses) was monitored at absorbance of 570 nm and normalized to day 0. The ordinate axis on the right represents the absorbance values for T47D and Au565 cells. MTT: 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide.

5), downstream of Beclin-mediated membrane nucleation<sup>[9,10]</sup>.

Cells undergoing prolonged autophagy and ER stress eventually succumb to death. Our RPPM data indicated an increase in pro-apoptotic Bax in Late MCF10A after 24 h of FLX (Figure 1A) as well as activated caspase-7 at 48 h in both SUM149PT and Late MCF10A (Figure 1B), suggesting that both cell lines undergo apoptosis. We confirmed caspase-7 activity by monitoring PARP cleavage, which was significant only in SUM149PT after 48 h of FLX treatment (Figure 4C). The lack of cleaved PARP in treated Late MCF10A does not necessarily correlate with inactive caspase-7. Rather, PARP cleavage site is either modified in this particular cell line or inaccessible by the antibody used in the Western blot analysis. Meanwhile, FLX did not induce apoptosis in Early MCF10A.

During ER stress, calcium released into the cytoplasm may enter the mitochondria to induce the intrinsic pathway to apoptosis<sup>[38]</sup>. Few members of the caspase family have been implicated in ER stress conditions. ER-resident procaspase-12 is cleaved or activated by protease calpain in response to calcium release<sup>[39]</sup>. Another study suggested that translocation of caspase-7 from the cytoplasm to the ER can cleave caspase-12 and mediate cell death<sup>[40]</sup>. In our study, there were no significant changes in calpain and cleaved caspase-12 levels in any of the cell lines with FLX treatment (Figure 4C). This data suggests that the observed FLX-induced cell death in SUM149PT and Late MCF10A is mediated through the mitochondrial apoptotic pathway.

### Persistent UPR and autophagy in SUM149PT promotes apoptosis

To closely determine the effects of FLX treatment in the aggressive TNBC line SUM149PT, the protein levels of the aforementioned regulators of ER stress, UPR, autophagy, and apoptosis were monitored at different

times by Western blots. As Figure 4D indicated, the metabolic sensor AMPK was activated as early as 2 h of FLX treatment. Modest but increased expression levels of BiP, eIF2 $\alpha$  S51, and LC3-II at 24 h indicated concurrent induction of UPR and autophagy, which were sustained up to 48 h. As a consequence of excessive ER stress, cell death mediated by mitochondrial apoptosis ensues. The increase in caspase-7 activation, as monitored by PARP cleavage (Figure 4C), coincided with a decrease in anti-apoptotic Bcl-2 level (Figure 4D). In FLX-treated SUM149PT, we would expect that the negative regulation of Beclin by Bcl-2 at the ER surface would be negligible and further support autophagy induction, given the role of Beclin in membrane nucleation<sup>[38]</sup>.

Although FLX-treated SUM149PT showed UPR at 48 h (Figure 4D), we could not detect levels of CHOP and GADD34 that are known to promote apoptosis as a result of prolonged ER stress (data not shown). The absence of these proteins suggests instability, as previously shown in mouse embryonic fibroblasts (MEFs) that have been treated with classical inducers of ER stress, such as thapsigargin (TG) and tunicamycin (TM)<sup>[41]</sup>. In this study, Rutkowski *et al.*<sup>[41]</sup> demonstrated that CHOP was rapidly degraded with a half-life of 4 h or less, while BiP expression was robust with a half-life of about 48 h. Lack of CHOP expression in the time points tested in our study was not surprising. Given the high dose of FLX (10  $\mu\text{mol/L}$ ) used in SUM149PT compared to the low dose of TG (2.5 nmol/L) or TM (30 nmol/L) in MEFs suggests that FLX is a less potent inducer of UPR than either TG or TM. Comparison of CHOP and GADD34 protein levels in an *in vivo* model treated with either classical UPR inducers or FLX will be an important future direction of this study.

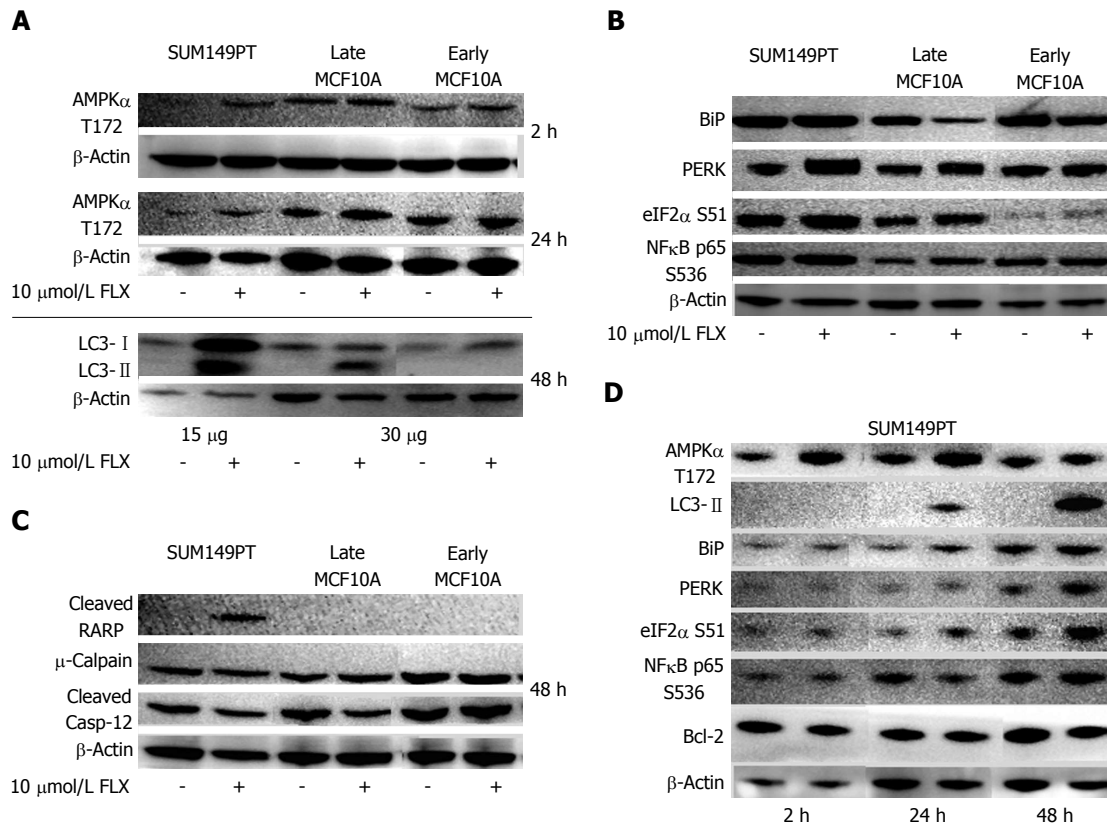
### Fluoxetine affects cell cycle progression

Cells that undergo UPR are subjected to global translation repression and cell cycle arrest<sup>[42]</sup>. To determine which phase of the cell cycle is modulated by 10  $\mu\text{mol/L}$  FLX treatment, we performed FACS analysis on cells that undergo significant UPR and apoptosis. T47D cells were used for comparison, given the cytostatic (vs cytotoxic) effect of FLX in this cell line (Figure 3). As Table 1 indicates, the proportion of SUM149PT and Late MCF10A cells entering the DNA synthesis (S) and mitosis (G2/M) phase decreased significantly with time, along with an increase in the growth (G1) phase. Meanwhile, FLX treatment in T47D did not change any phase of the cell cycle. Together, these results suggest that FLX-induced UPR in SUM149PT and Late MCF10A is associated with G1 arrest, which is consistent with previous studies that described the effect of FLX in colon (HT29), breast (MDA-MB-231), and cervical (SiHa) cancer cell lines<sup>[20,22]</sup>.

## DISCUSSION

In the present study, we showed that the treatment of TNBC line SUM149PT with antidepressant fluoxetine induces autophagy (Figure 4A) with concomitant decrease in cell growth (Figures 2 and 3). The activation





**Figure 4** Key proteins along the autophagy, unfolded protein response, and apoptosis pathways were examined across cell lines after 10 μmol/L fluoxetine treatment at various times. A, D: Critical effector of autophagy, AMPK, was activated after 2 h and 24 h of treatment in SUM149PT. Autophagy in cell lines was detected by the presence of cleaved LC3 (LC3- II) bands; B, C: The balance, level, and duration of ER stress sensors (B) and effectors of UPR (C) may ultimately dictate whether a cell lives or dies; D: Protein levels were monitored for up to 48 h in SUM149PT after fluoxetine treatment, showing concurrent induction of UPR and autophagy by 24 h. Blots shown were representative of at least 3 different experiments. The amounts of total cell extracts loaded for each cell line were indicated in the bottom panels in (A) for cleaved LC3 detection, while total loading in all cell lines for AMPKα detection was 100 μg. In panels B, C, and D, the amount of cell extracts loaded in each lane was 30 μg, 100 μg and 50 μg, respectively. Since the Abcam antibody for cleaved LC3 detection was very sensitive in panel A, we chose another manufacturer (cell signaling technology) to detect the same cleaved protein in panel D without the problem of overexposure during chemiluminescence. UPR: Unfolded protein response; ER: Endoplasmic reticulum; AMPK: Adenosine monophosphate kinase.

of AMPK, but a decrease in Akt and ERK activation, are likely to contribute to FLX-mediated cytotoxic autophagy in this cell line as early as 24 h (Figures 1, 2, and 4A). In contrast, the autophagy in Late MCF10A after 48 h of treatment may be dependent on ERK activation as previously reported for breast cell lines<sup>[43,44]</sup>. However, unlike those cells with survival advantage, Late MCF10A growth was significantly inhibited. Whether or not this growth inhibition is due to nuclear translocation of ERK to promote p53 upregulation and subsequent apoptosis, as has been suggested in some models<sup>[45]</sup>, remains to be determined.

Both SUM149PT and Late MCF10A are rapidly dividing cells (Figure 3) and have increased metabolic demands. The FLX-induced decrease in protein synthesis mediated by p70 S6K or eIF4G is likely to contribute to metabolic stress, thereby promoting autophagy in these cells. Given that the spindle-shaped Late MCF10A used in our study may have undergone some biochemical and/or genetic changes due to continual passaging, we acquired a different MCF10A line that has not been passaged extensively and shows normal cobblestone morphology (*i.e.*, Early MCF10A). The 48 h FLX treatment

did not induce autophagy (Figure 4A) but reduced cell growth in Early MCF10A (Figure 2) at a smaller proportion (40.1%) compared to Late MCF10A (81.3%) and SUM149PT (68.2%). This result is consistent with chemical-induced autophagy that is selective for only transformed mammary epithelial cells<sup>[44]</sup>. In preliminary testing of another TNBC line, MDA-MB-231, we found that the FLX-induced cytotoxicity in these cells was also associated with autophagy induction (data not shown). To our knowledge, this study is the first report of FLX-induced autophagy that results in significant growth inhibition of aggressive TNBC line.

In addition, the observed FLX-induced autophagy may be the result of ER stress and subsequent induction of UPR, consisting of PERK-dependent phosphorylation of eIF2α (Figure 4B), which can lead to translation inhibition of IκBα and subsequently NFκB activation<sup>[35,36]</sup>. Our RPPM data indicated residual IKK activity, as measured by IκBα S32/S36, even after 48 h of FLX treatment (data not shown), which promotes proteasomal-mediated degradation of IκBα, followed by NFκB translocation to the nucleus and subsequent activation. The FLX-induced NFκB activation in SUM149PT (Figure 1B),



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of apoptosis (Figure 4). Taken together, our study suggests that the FLX-induced UPR and autophagy in rapidly dividing SUM149PT and Late MCF10A promotes apoptosis, thus showing the intricate crosstalk between ER and mitochondria. Despite the network complexity, observations reported by us and others provide further evidence how individual components of UPR, autophagy, and apoptosis coordinately regulate cell fate (Figure 5). In regards to FLX-induced UPR in SUM149PT after 48 h, we hypothesized that activation of NFκB may promote transcription of apoptotic genes (*i.e.*, Bax) over cell survival genes (*i.e.*, Bcl-2), thereby favoring cell death through the mitochondrial pathway.

There are several limitations in the present study that precluded us from making general conclusions about the effects of FLX in molecular subtypes of breast cancer. First, each cell type has innate adaptive response to ER and metabolic stress. The extent to which cells will either adapt to stress or die will depend on (1) the genetic/biochemical makeup that dictates proper cellular response, (2) time of exposure to external and/or internal stress stimuli, and (3) the delicate balance between cell survival or death promoting genes. Second, the extent of cytotoxic response in basal-like SUM149PT may not be similar to other subtypes of TNBC that have been recently described<sup>[47]</sup>. Third, comparison of cytotoxic profiles between FLX and classical inducers of ER stress was

**Table 1** Proportion of cells in each cycle phase in the absence and presence of fluoxetine

	SUM149PT Control	SUM149PT 10 µmol/L FLX	Late MCF10A Control	Late MCF10A 10 µmol/L FLX	T47D Control	T47D 10 µmol/L FLX
24 h						
Sub G	1.1 (0.34) <sup>1</sup>	1.72 (0.55)	0.66 (0.13)	1.77 <sup>2</sup> (0.2)	1.86 (0.26)	1.63 (0.18)
G1	39.6 (0.74)	62.41 <sup>2</sup> (1.02)	60.26 (1.59)	79.89 <sup>2</sup> (0.71)	56.03 (2.05)	59.21 (0.55)
S	34.64 (1.28)	20.18 <sup>2</sup> (1.11)	23.78 (1.16)	10.75 <sup>2</sup> (0.43)	21.14 (1.12)	18.26 (1.45)
G2/M	25.26 (0.79)	16.12 <sup>2</sup> (0.3)	15.8 (0.65)	7.86 <sup>2</sup> (0.13)	21.44 (0.98)	21.39 (1.32)
48 h						
Sub G	1.91 (0.42)	6.67 (2.47)	0.41 (0.05)	10.26 <sup>3</sup> (0.52)	1.7 (0.32)	2.66 (0.39)
G1	46.79 (1.94)	67.73 <sup>3</sup> (1.16)	65.48 (0.93)	79.77 <sup>3</sup> (1.3)	57.52 (2.01)	62.35 (1.31)
S	29.69 (2.79)	14.01 <sup>2</sup> (0.84)	22.24 (0.6)	6.59 <sup>3</sup> (0.72)	22.33 (1.54)	18.07 (1.19)
G2/M	22.24 (1.08)	11.90 <sup>2</sup> (2.64)	12.31 (0.43)	3.58 <sup>3</sup> (0.13)	18.95 (0.24)	17.32 (0.29)

<sup>1</sup>Numbers in parentheses represent standard errors; <sup>2</sup>P-value < 0.05 compared to matching control; <sup>3</sup>P-value < 0.001 compared to matching control; FLX: Fluoxetine.

not performed in the present study. Xenograft models of SUM149PT and another TNBC subtype will be an important follow-up *in vivo* study to obtain important insights to the mechanism of action of FLX and potent ER stress inducer, thapsigargin.

In summary, our study demonstrated the complex actions of FDA-approved drug in malignant mammary epithelial TNBC cells that go beyond the inhibition of selective serotonin re-uptake. In addition to its utility for treating clinical depression, FLX has been used to improve quality of life in cancer patients<sup>[48]</sup>. Recently, FLX has been shown to reverse the multidrug resistance in cancer cells, enhancing the apoptotic effects of chemotherapeutics<sup>[23]</sup>. Here, we employed high-throughput RPPM to monitor FLX-induced changes in the expression of proteins encompassing the RTK/Akt/mTOR, MEK/ERK, and apoptotic pathways to complement our functional data. Our data analysis pointed to few proteins that play a role in cellular homeostasis and stress response. These proteins are components of the highly integrated autophagy, UPR, and apoptosis in response to ER and metabolic stress. The apparent sensitivity of TNBC SUM149PT to stress-mediated apoptosis has important clinical implications, given the aggressive biology of the inflammatory breast tumor that the cell line was originally derived and frequency of therapeutic resistance. Currently, a multi-modal approach (systemic chemotherapy, surgery, and radiation) is used to treat inflammatory breast cancer. Given the safety profile<sup>[49]</sup>, potential as a chemosensitizer<sup>[23]</sup>, and induction of ER stress-mediated apoptosis (Figure 5), FLX may provide additional benefit to current treatment modality for inflammatory TNBC. The anti-proliferative effect of FLX alone and in combination with chemotherapeutic will have to be tested in an *in vivo* model of TNBC in the near future.

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## COMMENTS

### Background

The ability of widely prescribed antidepressant fluoxetine to induce cell death or chemosensitize cancer cells has been described previously, but the mechanism of action is not well understood and appears to be cell type-dependent. While the inhibition of extracellular signal regulated kinase pathway and cell cycle progression has been proposed in two different breast cancer cell lines, the comparative studies did not employ non-transformed breast epithelial cells as additional control. Thus, information regarding the selectivity of fluoxetine-induced growth inhibition in molecular subtypes of breast cancer and normal breast cells is lacking.

### Research frontiers

Given the heterogeneity of breast cancers, including the aggressive triple negative breast cancer subtypes, efforts to identify targets of therapeutic intervention are greatly needed to improve clinical outcome and survival of women diagnosed with such phenotype.

### Innovations and breakthroughs

The authors' study is the first to describe the selective cytotoxicity of fluoxetine for basal-like inflammatory triple negative breast cancer (TNBC) cells over non-transformed mammary epithelial cells that involves the unfolded protein response and autophagy pathways.

### Applications

Inflammatory breast cancers have a high likelihood of residual disease and recurrence. The ability of fluoxetine to promote unfolded protein response, autophagy, and subsequent death in our preclinical model of inflammatory breast cancer may not only alleviate psychological stress but also potentially reverse therapeutic resistance. The utility of FLX as a potential adjuvant to treatment regimen of inflammatory breast cancer will have to be evaluated in xenograft models of TNBC.

## Terminology

Unfolded protein response (UPR) and autophagy are both physiological responses to oxidative and metabolic stress with the goal of restoring balance in correctly folded proteins and energy sources, respectively. However, prolonged cellular stress can lead to apoptosis. Because UPR and autophagy promote survival and death outcomes, mechanistic insights to their inter-dependent functions may lead to development of new treatment strategies against many diseases where both processes have been implicated.

## Peer-review

The paper is good, it has accomplished with many different cell lines.

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