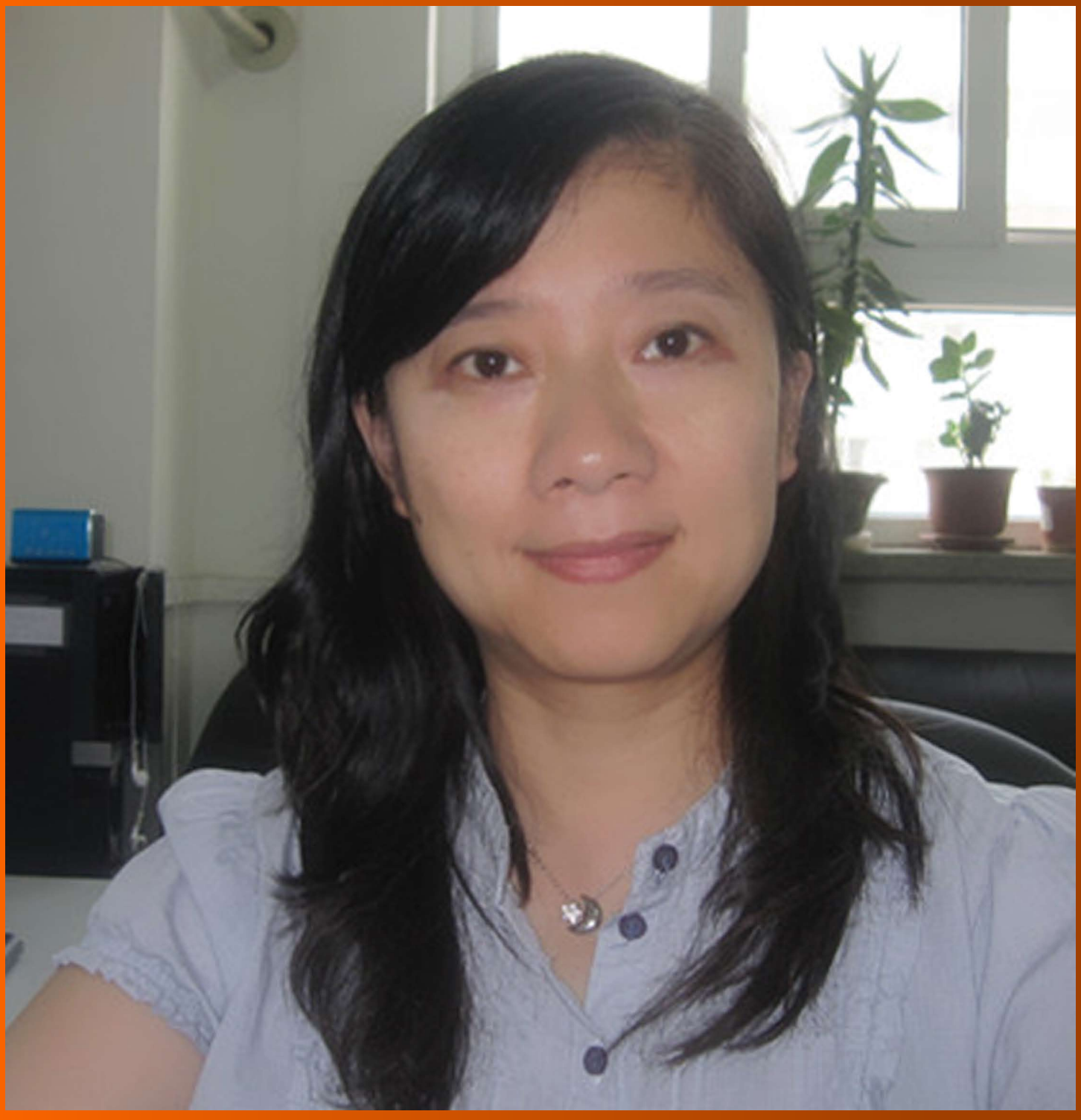
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EDITORIAL

Neoadjuvant treatment for resectable pancreatic adenocarcinoma

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Abstract

Pancreatic adenocarcinoma is the fourth leading cause of cancer mortality in the United States in both men and women, with a 5-year survival rate of less than 5%. Surgical resection remains the only curative treatment, but most patients develop systemic recurrence within 2 years of surgery. Adjuvant treatment with chemotherapy or chemoradiotherapy has been shown to improve overall survival, but the delivery of treatment remains problematic with up to 50% of patients not receiving postoperative treatment. Neoadjuvant therapy can provide benefits of eradication of micrometastasis and improved delivery of intended treatment. We have reviewed the findings from completed neoadjuvant clinical trials, and discussed the ongoing studies. Combinational cytotoxic chemotherapy such as fluorouracil, leucovorin, irinotecan, and oxaliplatin and gemcitabine plus nanoparticle albumin-bound (nab)-paclitaxel, active in the metastatic setting, are being studied in the neoadjuvant setting. In addition, novel targeted agents such as inhibitor of immune checkpoint are incorporated with cytotoxic chemotherapy in early-phase clinical trial. Furthermore we have explored the utility of biomarkers which can personalize treatment and select patients for target-driven therapy to improve treatment outcome. The treatment of resectable pancreatic adenocarcinoma requires multidisciplinary approach and novel strategies including innovative trials to make progress.

Key words: Pancreatic cancer; Resectable pancreatic adenocarcinoma; Neoadjuvant treatment; Biomarkers; Chemotherapy; Surgery

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Core tip: The treatment of resectable pancreatic adenocarcinoma requires multidisciplinary approach and novel strategies including innovative trials to make progress. Data from completed neoadjuvant clinical trials are reviewed, and important ongoing studies are presented. Biomarkers for patient selection and personalized medicine are discussed.

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INTRODUCTION

Pancreatic cancer can arise from either the exocrine or endocrine cells. Cancer of the endocrine pancreas, also known as pancreatic neuroendocrine tumor, is uncommon and has a relatively better prognosis than pancreatic adenocarcinoma (cancer of the exocrine pancreas). In the United States, approximately 48960 people are diagnosed with cancer of the exocrine pancreas each year, and an estimated 40560 people will die from their disease; it is the fourth leading cause of cancer mortality in men and women[1]. Globally, it is the seventh leading cause of cancer mortality in men and women, causing more than 300000 deaths annually[2]. Most patients with pancreatic adenocarcinoma will die within two years of diagnosis, and the 5-year survival rate is less than 5%[3]. The lack of a low cost screening test with high sensitivity and specificity contributes to most cases being diagnosed at an advanced stage.

Staging of pancreatic adenocarcinoma is usually done with tri-phasic pancreatic-protocol computed tomography scan of abdomen and pelvis and chest imaging. Based upon imaging, the tumor is classified as resectable, borderline resectable, locally advanced, or metastatic. Approximately 15% to 20% of patients are diagnosed with resectable disease and 45%-55% of patients are diagnosed with metastatic disease[4]. Appropriate staging allows the selection of patients who will have the best chance for curative intent resection (R0). Patients with borderline resectable disease are often given neoadjuvant treatment for tumor down-staging to render resection afterwards. Up to about one-third of patients with borderline-resectable tumors could have resectable disease after neoadjuvant treatment[5]. However, the role of neoadjuvant treatment for resectable pancreatic cancer remains unclear.

Surgical resection remains the only curative treatment for pancreatic cancer. A tumor is considered resectable if there is no arterial tumor contact [celiac axis (CA), superior mesenteric artery (SMA), common hepatic artery] and there is no tumor contact with the superior mesenteric vein (SMV) or portal vein (PV) or ≤ 180-degrees contact without vein contour irregularity on imaging study[6]. A tumor is considered unresectable if there is distant metastasis or unreconstructible SMV/PV due to tumor; if the tumor involves the pancreatic head it is unresectable if there is more than 180-degree encasement of the SMA or CA; a tumor of the pancreatic body or tail is unresectable if there is more than 180-degree encasement of the SMA or CA or tumor contact with the CA and aortic involvement[6].

Surgical management may include pancreaticoduodenectomy or pancreatectomy. It has been shown that pancreatic cancer patients undergoing surgery have better outcomes at high-volume hospitals, and the multidisciplinary approach and experienced surgeon seem to contribute most to the outcome of patients receiving pancreatic surgery[7]. The incomplete resection with positive surgical margins is frequent, reported 40% to 50% in most series[8]. The survival rate for patients with positive surgical margins is similar to that of patients who have locally-advanced disease[9]. The long-term survival after surgery remains low due to high rate of systemic recurrence: About 10% for node-positive disease and about 25% to 30% for node-negative disease[10-13]. Adjuvant treatment has been shown to improve survival as demonstrated in studies such as ESPAC-1, CONKO-001, ESPAC-3, RTOG 9704, and GITSG[14]. However, the delivery of postoperative treatment can be problematic with up to 50% of patients not receiving the intended treatment due to post-operative complications[15,16]. About 15% of patients may develop overt metastatic disease during postoperative recovery period, therefore early initiation of adjuvant chemotherapy within 20 d after surgery has been shown to improve disease-free and overall survival[8,17].

COMPLETED NEOADJUVANT TREATMENT STUDIES FOR RESECTABLE PANCREATIC CANCER

Due to the high rate of micrometastasis with systemic recurrence and difficulty in achieving R0 resection and delivering adjuvant therapy in time, neoadjuvant therapy has been studied to seek improvement in resection and survival. Potential benefits include early treatment of micrometastatic disease, tumor shrinkage for complete resection, and better tumor oxygenation plus drug delivery during chemoradiotherapy. It can also facilitate patient selection for surgery since those patients with disease progression at restaging would likely not benefit from resection.

Currently there is no data that clearly demonstrates improved resectability or survival with neoadjuvant treatment compared with initial surgery followed by adjuvant therapy. The National Comprehensive Cancer Network suggests not administering neoadjuvant therapy outside of a clinical trial. There have been several meta-analyses that have examined the benefit of neoadjuvant therapy. One analysis of 4394 patients in 111 phase Ⅰ/Ⅱ studies with about 1120 patients with resectable disease in 35 studies found no difference in overall survival between neoadjuvant and adjuvant treatment[5]. A meta-analysis of 19 studies in neoadjuvant chemoradiation showed that patients receiving neoadjuvant chemoradiotherapy were less likely to have a positive resection margin but they had an increased risk of perioperative death[18]. Another study of 536 patients in 14 phase Ⅱ studies concluded that patients with locally advanced

Table 1 Selected published neoadjuvant phase Ⅱ trials in resectable pancreatic adenocarcinoma between 2006-2015

Trial/reference published year	Trial phase/patient number	Treatment regimen	Primary endpoint	Result
NCT00335543/2015[63]	Ⅱ, randomized/66 (254 planned)	Upfront surgery *vs* chemoradiation with gemcitabine/cisplatin and radiotherapy of 55.8 Gy	Median survival	Median survival: 14.4 mo *vs* 17.4 mo (P = 0.96). Overall R0: 48% *vs* 52% (P = 0.81)
NCT00536874/2014[64]	Ⅱ/38	Gemcitabine and oxaliplatin	18-mo overall survival	18-mo overall survival: 63%. Median overall survival: 27.2 mo. Resection rate was 71%, and 74% of resection was R0
NCT00490360/2008[20,65]	Ⅱ/28	Gemcitabine and cisplatin	Resectability rate ⩾ 70%	Resection rate was 89%, and 80% of resection was R0. Overall survival was 26.5 mo
Evans *et al*[21]	Ⅱ/86	Chemoradiation with gemcitabine and radiotherapy of 30 Gy (in 10 fractions) for pancreatic head cancer	Clinical outcome	Overall R0: 74%. Median overall survival was 22.7 mo with a 5-yr survival of 27% (36% in R0)
Varadhachary[22]	Ⅱ/90	Gemcitabine and cisplatin followed by chemoradiation with gemcitabine and radiotherapy of 30 Gy for pancreatic head cancer	Clinical outcome	Overall R0 was 58%. Additional chemotherapy did not improve clinical outcome
Palmer *et al*[66]	Ⅱ, randomized/50	Gemcitabine *vs* gemcitabine and cisplatin	Resection rate	Resection rate was 54%: 9 (38%) in the gemcitabine arm and 18 (70%) in the combination arm

pancreatic cancer would benefit from neoadjuvant treatment[19]. Of note, some trials included in these analyses used older generation of chemotherapy and chemoradiation regimens such as 5-fluorouracil, cisplatin and mitomycin-C. Additionally, not only perspective studies, but retrospective cohort studies and case reports were also included in these meta-analyses, which are subjective to confounding and bias errors. Therefore, we have summarized the 6 published phase Ⅱ trials with gemcitabine-based regimen in the last 10 years (Table 1). Most of these trials have shown overall R0 rate around 50%. Two studies, neoadjuvant chemotherapy with gemcitabine and cisplatin reported by Heinrich *et al*[20], and neoadjuvant chemoradiation with gemcitabine reported by Evans *et al*[21], have demonstrated overall R0 rate of more than 70%. However, adding chemotherapy with gemcitabine and cisplatin before gemcitabine-based chemoradiation did not improve clinical outcome including overall R0 and survival rate[22].

Barbour *et al*[23] reported the result of GAP study: Phase Ⅱ study of gemcitabine and nab-paclitaxel for resectable pancreas cancer, a muticenter study conducted in Australia in 2015 ASCO Gastrointestinal Cancers Symposium. Patients in this study received 2 mo of pre-operative chemotherapy with gemcitabine and nab-paclitaxel, then underwent surgical resection. Patients received post-operative treatment based on their resection status (R0 *vs* R1). The primary endpoint was to examine the rate of R0 resection with all margins microscopically clear (minimum distance from tumor to resection margin ⩾ 1.0 mm), with a planned enrollment of 50 patients to aim for R0 rate of 85% or greater. However, this study was stopped after enrolling 42 patients due to a review by Independent Data and Safety Monitoring Committee showing the primary endpoint could not be met.

The ACOSOG Z5041 (NCT00733746) is a phase Ⅱ study in United States investigating overall survival at 2 years in patients receiving perioperative gemcitabine and erlotinib[24]. Erlotinib, an epidermal growth factor receptor (EGFR) tyrosine kinase inhibitor, has been shown to deliver statistically significant but marginal benefit in overall survival when adding to gemcitabine compared to gemcitabine alone as first-line treatment in patients with advanced pancreatic cancer[25]. The ACOSOG Z5041 met the accrual goal of 123 patients at end of 2013, and the result of the study is highly anticipated. The ACOSOG Z5041 will address the benefit of erlotinib as an adjunct to gemcitabine given perioperatively in resectable setting[26]. Additionally, this study will explore the biomarkers for response to erlotinib, such as E-cadherin whose expression is lost during epithelial-mesenchymal transition (EMT) contributing to cellular insensitivity to EGFR inhibition[27]. The NEOPAC (NCT01521702) is a phase Ⅲ randomized study in Europe comparing adjuvant gemcitabine *vs* neoadjuvant gemcitabine and oxaliplatin plus adjuvant gemcitabine[28]. The primary endpoint is progression-free survival, and the study has been terminated after enrolling about 25% of planned accrual.

ONGOING NEOADJUVANT TREATMENT STUDIES FOR RESECTABLE PANCREATIC CANCER

The landscape of systemic treatment in metastatic pancreatic cancer has changed significantly since 2011. Conroy *et al*[29] have shown FOLFIRINOX (5-fluorouracil plus leucovorin, irinotecan and oxaliplatin) delivered significant improvement of median overall survival when compared to gemcitabine alone in a randomized phase Ⅲ study enrolling 342 patients [11.1 mo *vs* 6.8 mo (P < 0.001)]. Of note, this study excluded patients

Table 2 Selected ongoing neoadjuvant trials for resectable pancreatic cancer

Trial	Trial phase	Treatment regimen	Primary endpoint	Planned accrual (patients)
NCT01900327 (NEOPA)	Ⅲ	Chemoradiation with gemcitabine and radiotherapy of 50.4 Gy *vs* upfront surgery	3 yr survival rate	410
NCT02172976	Ⅱ/Ⅲ	Perioperative FOLFIRINOX *vs* adjuvant gemcitabine	Median overall survival	126
NCT02047513 (NEONAX)	Ⅱ	Perioperative nab-paclitaxel/gemcitabine *vs* adjuvant nab-paclitaxel/gemcitabine	Disease free survival	166
NCT02305186	Ⅰ/Ⅱ	Neoadjuvant pembrolizumab plus chemoradiation with capecitabine and radiotherapy of 50.4 Gy *vs* neoadjuvant chemoradiation	Dose limiting toxicities; # of tumor infiltrating lymphocytes per high power field in resected tissue	56

with suboptimal performance status (ECOG 2 and beyond) or ages older than 76 years old. Von Hoff and colleagues[30] reported increased median overall survival with nab-paclitaxel plus gemcitabine compared to gemcitabine alone in a randomized phase Ⅲ study with 861 patients [8.5 mo *vs* 6.7 mo ($P < 0.001$)]. Therefore both FOLFIRINOX and nab-paclitaxel/gemcitabine have become preferred regimens in advanced pancreatic cancer, and are currently explored in the neoadjuvant setting (Table 2).

The NEPAFOX is a phase Ⅱ/Ⅲ multicenter study (NCT02172976) conducted in Germany with primary endpoint being median overall survival that has started recruiting patients with resectable or borderline resectable pancreatic cancer since November 2014. The phase Ⅱ study will randomize 126 patients to either surgery followed by 6 mo of gemcitabine or perioperative FOLFIRINOX (3 mo before surgery and 3 mo after surgery). After an interims analysis, the trial can be continued as phase Ⅲ to enroll 310 patients[31].

The NEONAX study (AIO-PAK-0313, NCT02047513) is a phase Ⅱ study conducted in Germany that has started recruiting patients with resectable pancreatic cancer since April 2015. This trial will randomize 166 patients to either perioperative treatment with nab-paclitaxel and gemcitabine (2 mo before surgery and 4 mo after surgery) or adjuvant treatment with nab-paclitaxel and gemcitabine. The primary outcome measure is disease-free survival, and aims to improve the disease-free survival rate at 18 mo in at least one arm to ⩾ 55%. This study will conduct biomarker study by collecting tumor tissue for exome sequencing, and circulating tumor DNA for biocorrelate and pharmacogenomic study[32,33].

The NEOPA study (NCT01900327; Neoadjuvant Treatment in Resectable Pancreatic Cancer) is an ongoing phase Ⅲ study in Germany that will randomize 410 patients to neoadjuvant gemcitabine-based chemoradiation *vs* upfront surgery[34]. Both groups will receive post-operative adjuvant treatment with gemcitabine. The primary endpoint is 3-year survival rate. This study is to examine the hypothesis that neoadjuvant gemcitabine-based chemoradiation increases the three-year overall survival by 12% compared to upfront surgery for resectable pancreatic cancer.

The Prep-02/JSAP05 study is a prospective randomized phase Ⅱ/Ⅲ trial conducted in Japan since January 2013 (clinical trial information: UMIN000009634)[35]. This study plans to enroll 360 patients with resectable pancreatic cancer, and randomizes them to either surgery followed by adjuvant chemotherapy with S1 for 6 mo or 2 mo of neoadjuvant chemotherapy with gemcitabine and S1 followed by surgery then 6 mo of adjuvant chemotherapy with S1. The primary study endpoint is resection rate for phase Ⅱ and overall survival for phase Ⅲ. This study plans to have 40 patients in each arm of the phase Ⅱ part, and moves on to phase Ⅲ if there are no more than 14 cases of non-resection in each arm of phase Ⅱ study. S-1 is an oral fluorinated pyrimidine, containing tegafur, 5-chloro-2,4-dihydroxypyridine and potassium oxonate at a molar ratio of 1:0.4:1[36]. Tegafur is a pro-drug of 5-fluorouracil, and S1 has been shown to deliver higher 5-fluorouracil levels in the plasma and the tumor tissue. The safety and efficacy of combination chemotherapy with gemcitabine and S1 for resectable pancreatic cancer have been reported in pilot study[37].

The UVA-PC-PD101 study (NCT02305186; Safety and Immunological Effect of Pembrolizumab in Resectable or Borderline Resectable Pancreatic Cancer) is a phase Ⅰb/Ⅱ multicenter study in patients with resectable or borderline resectable pancreatic cancer. This study will randomize 56 subjects in 2:1 to the experimental arm with pembrolizumab given concurrently with chemoradiation or control arm receiving chemoradiation only. Patients in both arms will receive surgery and adjuvant chemotherapy with gemcitabine. The primary outcome measures are to determine the safety of neoadjuvant chemoradiation with capecitabine in combination with pembrolizumab, and to examine and compare the difference in the number of tumor infiltrating lymphocytes (TILs) in resected pancreatic tissue between experimental and control arms[38]. The investigators hypothesize chemoradiation recruits TILs to the microenvironment of pancreatic cancer causing overexpression of programmed death-ligand 1 (PD-L1). PD-L1 binds to PD-1 on T-cells, and suppress cytotoxic T-cells. Pembrolizumab is a monoclonal antibody that targets the PD-1, and release the inhibition on cytotoxic T-cells. Therefore, it is expected that there are more immune effects at tumor tissues in the experimental arm than control arm. It will be interesting to see if this will

translate into improved clinical outcome.

BIOMARKERS IN RESECTABLE PANCREATIC CANCER

At this time there are no validated biomarkers for early pancreatic cancer. Carbohydrate antigen 19-9 (CA-19-9) is currently used as a marker for following patients during treatment for pancreatic cancer but it is non-specific and can be positive in other conditions such as cirrhosis of the liver, pancreatitis, cholangitis, and other GI cancers. Presence of circulating tumor cells in the peripheral blood has been found to be a negative prognostic factor in pancreatic cancer and potentially may have a role in patient selection for neoadjuvant treatment[39].

Whole exome sequencing in pancreatic cancer demonstrated four frequently mutated genes: *KRAS*, *TP53*, *CDKN2a/p16*, and *SMAD4/DPC4*. KRAS was found to be mutated in virtually all pancreatic cancer patients but genetic alterations in the other three genes were found to be associated with malignant behavior and may be a prognostic tool[40]. The inflammatory markers ferritin and C-reactive protein (CRP) have also been studied for prognostic and predictive value in advanced pancreatic cancer. The study demonstrated that patients with elevation in both biomarkers had a notable decrease in overall survival, and can possibly be a clinically useful tool[41].

The expression of E-cadherin, a calcium-dependent adhesion molecule, is frequently suppressed or lost during EMT of solid tumor malignancy including non-small cell lung and pancreatic cancers, which renders invasiveness and drug resistance[42,43]. Several retrospective studies in pancreatic cancer have shown poorer clinical outcome with decreased expression of E-cadherin[44-46]. Furthermore, E-cadherin interacts with EGFR, and down-regulation of E-cadherin contributes to decreased response and survival in patients receiving EGFR inhibitors for non-small cell lung cancer[47,48]. Ko *et al*[49] have recently reported the result of a phase Ⅱ study with combined inhibitors of EGFR, and MEK which is a key downstream effector of EGFR signaling, in advanced pancreatic cancer. They have found patients with tumors exhibiting an epithelial phenotype (demonstrated by high level of E-cadherin expression) were more likely to be sensitive to study treatment. The planned correlative investigation of ACOSOG Z5041 (NCT00733746), a completed perioperative phase Ⅱ study of gemcitabine and erlotinib for resectable pancreatic cancer, will provide further information on the interaction between EMT marker status and overall/progression-free survival after treatment.

Gemcitabine is a prodrug that is taken into cells *via* a nucleoside transporter[50]. The human equilibrative nucleoside transporter 1 (hENT1) has been studied as a predictive marker for treatment response, and there is data that hENT1 expression may correlate with response to gemcitabine[51-53]. These findings were not able to be validated in the metastatic setting in the LEAP trial[54].

The PD-1 is encoded by the *PDCD1* gene. It is primarily expressed by activated T-cells as negative co-stimulatory receptor; binding of PD-1 to its ligands, PD-L1 and PD-L2, downregulates T-cells and the immune system[55,56]. Many tumor cells express PD-L1 and PD-L2 which is a mechanism which allows escape from immune destruction of the tumor cells. Pembrolizumab is an anti-PD-1 antibody that is approved for use in metastatic melanoma and metastatic non-small cell lung cancer and is currently under study for other malignancies, and PD-L1 expression may be a potential marker for efficacy of anti-PD-1 studies for pancreatic cancer.

Elevated CRP levels in the plasma, a well-established marker of inflammation, at diagnosis correlate with higher tumor stage and grading and poorer clinical outcome in pancreatic cancer[57]. Patients with CRP greater than 13 mg/L had improved survival with ruxolitinib and capecitabine compared to capecitabine and placebo in a randomized phase Ⅱ study also known as the RECAP study enrolling 127 patients with metastatic pancreatic cancer (median overall survival of 83 d *vs* 55 d, $P = 0.01$)[58]. The CRP level could be a useful marker for patient stratification in the management of pancreatic cancer, and the JAK inhibitor ruxolitinib may improve clinical outcome in patients with elevated CRP. An ongoing phase Ⅲ study, known as JANUS 2, is examining these promising leads as a second-line setting in patients with advanced pancreatic cancer[59].

There is high prevalence of BRCA1/2 mutations in Ashkenazi Jewish with pancreatic cancer[60]. The *BRCA1* and *BRCA2* gene encodes large proteins that coordinate the homologous recombination repair double strand breaks (DSBs) pathway. Poly ADP-ribose polymerases (PARP) are a family of nuclear enzymes that regulates the repair of DNA single-strand breaks through the base-excision repair (BER) pathway. Since BRCA1/2-mutated tumors cannot utilize homologous recombination to repair DSBs, exposing these cells to PARP inhibitor, which shuts down BER rescue pathway, will lead to accumulation of DNA damage, genomic instability and cell death, also known as synthetic lethality[61]. Investigators from Memorial Sloan-Kettering Cancer Center have reported high response rate with combination of gemcitabine, cisplatin and veliparib, a PARP inhibitor, as first-line treatment in patients with andvanced pancreatic cancer and mutant BRCA[62]. Ongoing phase Ⅱ randomized study comparing gemcitabine and cisplatin with and without veliparib is currently underway (NCT01585805). This study will most likely provide us the information on using BRAC mutation as a biomarker for personalized treatment.

CONCLUSION

The need for more effective treatment regimens for resectable pancreatic cancer is highlighted by the

continued relatively low survival even in patients who receive surgical resection. Several studies utilizing more active chemotherapy regimens are pending results, and additional studies are ongoing. There also remains the need for accurate and cost-effective biomarkers to aid in the management of pancreatic cancer.

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EDITORIAL

Current treatment options for patients with initially unresectable isolated colorectal liver metastases

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Abstract

The development of liver metastases is a common clinical entity in the clinical course of colorectal cancer. For patients with isolated liver involvement, surgical resection is the only treatment that can provide a chance of prolonged survival and cure. However, most of these patients are not initially eligible for the surgery. Selected patients with initially considered to have unresectable disease may become resectable after systemic (chemotherapy ± biological therapy) and loco-regional treatment modalities including hepatic arterial infusion. Patients who have colorectal liver metastases ideally should be referred to a multidisciplinary cancer care team in order to identify the most optimal management approach.

Key words: Colorectal cancer; Conversion therapy; Liver metastases; Targeted therapy; Hepatic arterial infusion

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Core tip: A subset of patients presenting with unresectable colorectal liver metastases (CLM) patients may become eligible for resection following systemic (chemotherapy ± biological therapy) and loco-regional treatments, including hepatic arterial infusion. After successful complete (R0) resection of liver lesions, these patients can achieve long-term survival. Therefore, all patients with CLM should be discussed in a multidisciplinary team meeting to identify appropriate treatment options.

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INTRODUCTION

The liver is the one of the most attractive site for colorectal cancer (CRC) metastases. Up to 50% of CRC patients will experience liver metastases at some point during their clinical follow-up, and approximately 20%-30% of these patients will have isolated liver metastases. Complete surgical removal of all liver meta-

stases is the only treatment option providing the best opportunity for long-term survival in these patients[1-4].

However, most patients with isolated colorectal liver metastases (CLM) are not eligible for surgical resection due to the size, location or number of the lesions and anatomical constraints[5]. On the other hand, a subset of patients initially considered to have unresectable CLM may become eligible for resection following effective systemic chemotherapy. When the chemotherapy is used for that purpose, it is called conversion chemotherapy. Patients with resected CLM following conversion chemotherapy can achieve similar survival to those with initially resectable tumors[4]. In a large series reported by Adam *et al*[6], 1104 patients with unresectable CLM received chronomodulated chemotherapy regimens combining 5-fluorouracil (5-FU), leucovorin (LV), and oxaliplatin or irinotecan. After an average of 10 cycles of chemotherapy, tumor response that allowed secondary curative hepatic resection was seen in 138 (12.5%) of these patients. The 5- and 10-year overall survival (OS) rates after resection were 33% and 23%, respectively, which are better than those in patients who did not undergo resection, but less than those observed in patients who underwent initial complete metastasectomy at the same institution during the same period of time (48% and 30%, respectively, $P = 0.01$).

According to the new systematic review that looked at ten observational studies, the use of modern chemotherapy regimens including irinotecan and oxaliplatin with or without biological agents permitted secondary curative (R0) resection of CLM in 436 of 1886 patients (23.1%). The median OS following surgery was 45 (range, 36-60) mo and recurrence-free survival rate was 19%[7].

IS THERE AN OPTIMAL CHEMOTHERAPY REGIMEN FOR CONVERSION THERAPY?

It is clear that the regimens that can produce high response rates with an acceptable toxicity will lead to a higher rate of R0 resections of liver metastases and improved survival rates[8]. Folprecht *et al*[9] examined all published or presented trials as well as retrospective studies that report the rate of objective response and the rate of secondary resection following systemic chemotherapy in patients with initially unresectable CLM. They demonstrated a strong relationship between response rates to the regimen used and the liver resection rates in patients who have isolated liver involvement, and also speculated that highly active schedules can provide response rates as high as 70% and complete surgical resection rates as high as 50% in selected cases.

The standard doublet combinations of fluoropyrimidines plus either oxaliplatin (XELOX, FOLFOX) or irinotecan (FOLFIRI) offer conversion rates of between 9 and 33%, and their use remains a reasonable option for patients with unresectable CLM[8,10-12]. On the other hand, the administration of intensified triplet chemotherapy regimen of 5-FU, oxaliplatin and irinotecan (FOLFOXIRI) is an attractive strategy that may potentially increase response and resectability rates[13]. The Gruppo Oncologico Nord Ovest (GONO) performed a phase Ⅲ clinical study comparing FOLFOXIRI with FOLFIRI in front-line chemotherapy for patients with initially unresectable CRC[14]. In this trial, response rate (60% *vs* 34%, $P < 0.0001$) and R0 secondary resection rate was significantly greater in patients treated with FOLFOXIRI (15% *vs* 6%; $P = 0.033$, among all participants; and 36% *vs* 12%; $P = 0.017$ among patients with liver-only metastases). Furthermore, patients who received FOLFOXIRI had significantly improved progression-free survival (PFS) and OS than those who received FOLFIRI (median PFS, 6.9 mo *vs* 9.8 mo, $P = 0.0006$; median OS, 16.7 mo *vs* 22.6 mo, $P = 0.032$). As expected, FOLFOXIRI was found to be more toxic with regard to peripheral neurotoxicity and neutropenia, but they were manageable.

Masi *et al*[15] recently performed a retrospective analysis of pooled clinical data from 196 patients who received FOLFOXIRI because of initially unresectable metastatic CRC in phase Ⅰ-Ⅲ GONO studies. The primary aim of the investigators was to determine the long-term clinical results of patients undergoing a secondary complete resection and the effects of this regimen on perioperative surgical morbidity and mortality. They demonstrated that administration of this intensified regimen was associated with a high response rate of 70.4%, and a secondary complete (R0) resection was possible in 37 of 196 patients (19%) after a median of 5.5 mo of chemotherapy. In addition, four patients achieved a complete pathologic response. No perioperative mortality was recorded. Although 27% of patients developed perioperative complications, all of them resolved without sequelae. After a median follow-up period of 67 mo, the estimated OS rate at 5 and 8 years were 42% and 33%, respectively for the total patients population. For patients who had liver-only metastatic disease ($n = 25$), however, the median survival was 65 mo, with the estimated 5-year and 8-year survival rate was 43%. In the histopathological examination of chemotherapy-induced hepatic injury, a major cause for concern when treating patients with CLM, neither grade 3 vascular toxicity nor grade 4 steatosis, was detected.

The addition of targeted agents to chemotherapy backbones may further improve resectability rates of CLM[16-18]. According to the results of an initial phase 3 trial, bevacizumab, a monoclonal antibody directed against the vascular endothelial growth factor, only moderately improved resectability rates when added to oxaliplatin-based chemotherapy (8.4% *vs* 6.1%) in an unselected patient population with metastatic CRC[19]. Further data on the effects of bevacizumab on resection rates of CLM came from the Bevacizumab Expanded Access Trial investigating the safety of bevacizumab with fluoropyrimidine-based chemotherapy in the

first- line treatment of 1914 patients with metastatic CRC[20]. In 704 patients with liver-only metastases, 107 patients (15.2%) underwent hepatectomy, which was R0 resection in 85 out of 107 patients (79.4%). The 2-year survival rate was 89% in patients who underwent resection with curative intent and 94% in those who achieved complete R0 resection.

The BOXER (bevacizumab, oxaliplatin, xeloda in unresectable liver metastases) study investigated the efficacy of perioperative chemotherapy with bevacizumab plus capecitabine and oxaliplatin in patients with CLM who were considered ineligible for upfront resection due to following poor-risk features: The presence of more than four metastatic lesions, metastasis diameter > 5 cm, unlikely R0 resection, inadequate viable liver function if undergoing upfront surgical resection, inability to maintain adequate liver vascular perfusion, or the presence of synchronous metastases. After a median number of four cycles (range, 3-9) preoperative chemotherapy, objective tumor response was observed in 78% of patients and 40% of patients were converted from unresectable to resectable disease. Of these patients, 20% achieved an R0 resection[21].

In a phase 2 study, Masi *et al*[22] assessed the feasibility of FOLFOXIRI and bevacizumab combination in 57 patients with metastatic CRC. Among the 30 patients with liver-only metastatic disease, this regimen yielded an 80% objective response rate and 40% of these patients could undergo a curative (R0) resection. No perioperative mortality was recorded. Subsequently, the GONO reported the results of the phase 3 TRIBE (combination chemotherapy and bevacizumab as first-line therapy in treating patients with metastatic CRC) study comparing FOLFOXIRI plus bevacizumab with FOLFIRI plus bevacizumab in metastatic CRC patients[23]. FOLFOXIRI plus bevacizumab provided a significant increase in response rates (65% *vs* 53%) and PFS (median 12.1 mo *vs* 9.7 mo) compared with FOLFIRI plus bevacizumab. However, FOLFOXIRI plus bevacizumab did not improve the secondary curative R0 resection rate in the liver-only patient subgroup (28% *vs* 32%, $P = 0.823$).

In the OLIVIA randomized phase Ⅱ trial, 80 patients with initially unresectable CLM were randomized to receive bevacizumab plus modified FOLFOX6 or bevacizumab plus FOLFOXIRI[24]. The results showed that the combination of bevacizumab plus FOLFOXIRI improved overall resection rate (61% *vs* 49%) and R0 resection rate (49% *vs* 23%), and PFS (18.6 mo *vs* 11.5 mo) compared with bevacizumab plus FOLFOX6.

The results of phase Ⅲ CRYSTAL (cetuximab combined with irinotecan in first-line therapy for metastatic colorectal cancer) trial and phase Ⅱ OPUS (oxaliplatin and cetuximab in first-line treatment of metastatic colorectal cancer) trial have showed that the integration of cetuximab, a chimeric immunoglobulin G1 anti-epidermal growth factor receptor (EGFR) monoclonal antibody, to irinotecan or oxaliplatin-based first-line chemotherapy significantly improved response rates, R0 resection rates, PFS, and OS compared with chemotherapy alone in patients with metastatic CRC whose tumors did not harbor a KRAS mutation[25,26]. In the CRYSTAL trial, combined administration cetuximab and FOLFIRI resulted in an increase in the resection rate from 4.5% to 9.8% in the subgroup of patients with disease confined to the liver at presentation[25]. Similarly, in the OPUS study, the R0 resection rate for hepatic metastases doubled from 2.4% to 4.7% when cetuximab was added to FOLFOX4 regimen[26].

The CELIM randomized phase Ⅱ study was designed to assess the effect of cetuximab combined with chemotherapy (FOLFOX6 or FOLFIRI) on tumor response and secondary resectability of CLM[27]. A retrospective analysis of the study revealed that 70% of patients with KRAS wild-type disease achieved either a complete or partial or complete response after chemotherapy-biologic therapy, and the resectability rates increased from 32% (at baseline) to 60% (after treatment).

In the study by Ye *et al*[28], patients with KRAS wild-type unresectable colorectal liver-limited metastases were randomly assigned to receive chemotherapy (FOLFIRI or FOLFOX6) plus cetuximab or chemotherapy alone. Patients who received cetuximab plus chemotherapy had improved objective response rates (57.1% *vs* 29.4%; $P < 0.01$), and the R0 hepatic resection rates (25.7% *vs* 7.4%, $P < 0.01$) compared to patients who received chemotherapy alone.

Preliminary reports have suggested that response rates can be increased further by combining cetuximab with FOLFOXIRI regimen. The POCHER study investigated secondary liver resection rates following neoadjuvant treatment with cetuximab plus chronomodulated FOLFOXIRI in patients who were considered unsuitable for resection of their CLM at presentation[29]. After a median of six cycles of chemotherapy, a partial response was obtained in 79% of patients and R0 liver resection was possible in 60% of patients.

The MetaPan study evaluated the activity of adding panitumumab, a fully human monoclonal anti-EGFR antibody, to the capecitabine plus oxaliplatin (XELOX) combination as perioperative conversion treatment in CRC patients with unresectable liver-only metastases[30]. After conversion therapy, the overall response rate in the unselected patient population was 54%. However, in 35 patients with KRAS wild-type, response rate reached to 65%, which allowed for liver resection in 15 of these patients.

Petrelli *et al*[31] have performed a literature-based meta-analysis to determine the effects of cetuximab and panitumumab on objective response rate, the conversion rate, and survival outcome in patients with KRAS wild-type unresectable colorectal liver-limited metastases. They found that compared to chemotherapy alone, the addition of anti-EGFR agents significantly increased the response rate of liver metastases from 43% to 72% ($P = 0.0001$), and the curative (R0) resection rate of liver metastases from 11% to 18% ($P = 0.04$). Although anti-EGFR agents significantly reduced the risk of progression

by 32% (P = 0.002), they did not show any significant favorable effect on OS (P = 0.42).

Neither study found a significant increase in surgical complications with the use of biological agents in this setting[16-18]. There are currently no specific recommendations regarding the use of anti-EGFR antibodies in the preoperative period[16,17]. However, bevacizumab should be stopped at least 6-8 wk before surgery to reduce the risk of specific postoperative complications, including wound healing or bleeding problems[18,32].

HEPATIC ARTERIAL INFUSION

Since CLMs predominantly receive their blood supply from the hepatic artery, infusion of chemotherapeutic agents that have a high hepatic extraction rate [such as 5-fluorouridine (FUDR)] *via* the hepatic artery results higher drug concentrations in liver metastases compared with tumor-free liver parenchyma, which is supplied mainly by portals vein[33]. This can be achieved thorough a biocompatible pump that is implanted under the subcutaneous tissue of the abdomen and attached to a catheter placed in the hepatic artery, which delivers the chemotherapeutic drugs at a slow fixed rate.

Currently hepatic arterial infusion (HAI) chemotherapy is primarily recommended for the treatment of patients with unresectable liver confined metastatic CRC who had disease progression after first-line systemic chemotherapy[34]. However, available data suggest that HAI in combination with systemic chemotherapy or chemo-biologic therapy offer a chance for curative rescue resection to a substantial proportion of patients presenting with liver-limited metastatic CRC[34,35]. With this approach, down-staging to resectability occurs in 25%-50% of patients; the percentage can reach up to 57% in chemotherapy-naïve patients[10,11,35,36]. Importantly, the long-term overall survival can be obtained[10,11,35-37]. In the series reported by Goere *et al*[37], 87 patients with isolated unresectable CLM were treated with HAI of oxaliplatin with systemic 5-FU and LV. Seventy-nine percent of patients had previously received systemic chemotherapy. After the treatment, a curative resection was possible in 26% of patients, and the 5-year survival for these patients was 56% *vs* 0% for non-resected patients.

Ammori *et al*[38] reported the largest institutional series of 373 patients with unresectable CLM who were treated with HAI FUDR and systemic chemotherapy. Two hundred and ninety-six patients (79%) had been treated with systemic chemotherapy before HAI, and 43 of these patients received multiple lines of chemotherapy. Sixty patients (16%) had also extrahepatic disease at the time of HAI pump placement. Despite these unfavorable features, 25% of patients responded sufficiently to treatment and subsequently underwent complete resection and/or radiofrequency/microwave ablation. The median and the estimated 5-year survival for this conversion group were 59 mo and 47%, respectively, which were comparable to reports in the literature of patients initially presenting with resectable disease.

HAI can be associated with technical and liver-related complications. Technical complications including arterial thrombosis, catheter occlusion or dislodgement, extrahepatic perfusion, pump pocket infections or hematoma, have been reported up to 22% of patients[39]. However, most of these complications are manageable and overall rate of pump failure is around 9% at 1 year[39]. The most limiting hepatic toxicity related to HAI is biliary sclerosis, which has been reported in 4.6% of patients receiving HAI FUDR for unresectable CLM, and it can often be effectively managed, if detected early[40].

CONCLUSION

Surgical resection is currently the only curative approach for patients with isolated CRC liver metastases. Conversion chemotherapy may offer a chance for secondary resection in about one-third of these patients. Although, the optimal regimen for this is still unclear, a doublet combination of 5-FU plus either oxaliplatin or irinotecan remain the standard first-line option. The FOLFOXIRI triplet is a very attractive treatment option especially for patients who can tolerate this regimen. Despite lacking specifically designed randomized trials, available data suggest that the integration of targeted biological agents into chemotherapy may further improve tumor response and resectability. HAI should be considered in patients with extensive liver tumor burden and chemotherapy-refractory disease.

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REVIEW

Advanced oropharyngeal squamous cell carcinoma: Pathogenesis, treatment, and novel therapeutic approaches

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Abstract

Oropharyngeal cancer accounts for approximately 2.8% of newly cancer cases. Although classically a tobacco related disease, most cases today are related to infection with human papilloma virus (HPV) and present with locally advanced tumors. HPV related tumors have been recognized as a molecularly distinct entity with higher response rates to therapy, lower rates of relapse, and improved overall survival. Treatment of oropharyngeal cancer entails a multi-disciplinary approach with concomitant chemoradiation. The role of induction chemotherapy in locally advanced tumors continues to be controversial however large studies have demonstrated no difference in survival or time to treatment failure. Surgical approaches may be employed with low volume oropharyngeal cancers and with development new endoscopic tools, more tumors are able to be resected *via* an endoscopic approach. Given advances in the understanding of HPV related oropharyngeal cancer, ongoing research is looking at ways to minimize toxicities *via* de-intensification of therapy. Unfortunately, some patients develop recurrent or metastatic disease. Novel therapeutics are currently being investigated for this patient population including immunotherapeutics. This review discusses the current understanding of the pathogenesis of oropharyngeal cancer and treatment. We also discuss emerging areas of research as it pertains to de-intensification as well novel therapeutics for the management of metastatic disease.

Key words: Oropharyngeal cancer; Human papilloma virus; Transoral robotic surgery; Immunotherapy; Metastatic head and neck squamous cell carcinoma

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Core tip: Treatment of oropharyngeal cancer entails a

multi-disciplinary approach with concomitant chemoradiation. Given advances in the understanding of human papilloma virus related oropharyngeal cancer, ongoing research is looking at ways to minimize toxicities *via* de-intensification of therapy. Unfortunately, some patients develop recurrent or metastatic disease. This review discusses the current understanding of the pathogenesis of oropharyngeal cancer and treatment. We also discuss emerging areas of research as it pertains to de-intensification as well novel therapeutics for the management of metastatic disease.

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INTRODUCTION

Oropharyngeal cancer accounts for approximately 2.8% of newly diagnosed cancer cases and, in 2015, will result in 8650 estimated deaths[1]. Today, most cases are related to human papilloma virus (HPV) infections and many are curable with definitive combinations of surgery and radiation or chemoradiotherapy. Hence, HPV is a prognostic biomarker, but not yet predictive. As the field of clinical research continues to advance, methods for de-intensifying treatment for such patients are becoming more important. Here, we will review the epidemiology of oropharyngeal cancer as well as treatment strategies and areas of developing research for those afflicted with this disease.

EPIDEMIOLOGY, PATHOGENESIS, AND RISK STRATIFICATION

Classically, use of tobacco products has been the leading factor for development of oropharyngeal cancer, although this has been shifting with changes in societal trends in tobacco usage[2-4]. This increased risk pertains to use of cigarettes, cigars, and pipes and increases with the number of years an individual has smoked[5]. Smoking cessation resulted in a normalization of risk in casual smokers after approximately 15 years[6,7]. Additionally, tobacco usage during definitive therapy for head and neck cancer is associated with an increased rate of disease progression and death, particularly in those whose cancers are not related to HPV or are p16 negative[8]. Similarly, alcohol intake increases the risk of head and neck cancers in a dose dependent manner[7,9-11].

HPV, most notably genotype 16, has been identified as an increasing causative factor for oropharyngeal cancer and is chiefly seen in patients with minimal tobacco and alcohol use. This is especially important since the pathogenesis, presentation, and prognosis differ in HPV(+) *vs* HPV(-) oropharyngeal carcinomas. The molecular carcinogenesis of HPV associated oropharyngeal cancer has been explored in detail and is separate from that seen in HPV(-) cancer and relates to loss of cell cycle checkpoints[12,13]. In a subset of patients with chronic HPV infections, the viral oncoproteins E6 and E7 bind p53 and pRb/p21, respectively. The resultant effect is that E6 binding causes p53 degradation whereas E7 binding to pRb and p21 leads to an activation of transcription factors. These transcription factors cause malignant cells to progress into the G1 cell cycle phase which is unopposed due to the loss of p53. The latency from time of primary infection to development of malignancy is approximately 15-20 years. Over the last 20 years there has been an steady rise in the number of newly diagnosed HPV(+) oropharyngeal cancers, increasing from 16.3% to 71.7%, accompanied by a corresponding 50% decline in the incidence HPV(-) oropharyngeal carcinomas[3,14-16].

Clinically, HPV+ cancers are more likely to present in younger patients and involve the base of the tongue or tonsils[3,17,18]. Additionally, patients with HPV+ oropharyngeal cancers are much more likely to respond to therapy, have lower rates of disease relapse, and enjoy improved overall survivals. Furthermore, such tumors are less likely to develop second malignancies compared to matched HPV(-) patients[3,14-16,19]. Based on these studies, a model for risk stratification has been generated based on HPV status, smoking history, tumor stage, and nodal involvement. A classification of low, intermediate, or high risk disease has been generated, predicting 3 year overall survivals of 93%, 70.8%, and 46.2%, respectively[15]. Interestingly, a single center study analyzing survival and TNM staging in oropharyngeal cancers found that survival based on TNM status did not correlate with survival in those patients with HPV(+) disease, but it did correlate with survival in those with HPV(-) disease. A retrospective, multivariate analysis of the HPV+ patients, however, was able to generate an accurate prognostic model by including tumor stage, smoking status, and age by recursive partitioning analysis (RPA). Thus, the authors propose an RPA-based staging system in HPV-related oropharynx cancers, whereby stage Ⅰ cancers would be classified by T1-3, N0-N2b tumors, stage Ⅱ by T1-3, N2c, and stage III by T4 or N3 disease[20].

TREATMENT STRATEGIES

Surgical approaches

Surgical approaches are currently one of the primary modalities in the treatment of low volume oropharyngeal cancers. Early stage squamous cell carcinomas of the oropharynx can be managed with either surgery or radiation therapy. Given the significant acute and long term side effects of radiation therapy, minimally invasive surgical approaches [including transoral robotic surgery (TORS) and transoral laser microsurgery (TLM)] have

been increasingly employed for the management of early stage tumors. This increased utilization has been further driven by development of new endoscopic tools including the da Vinci Robot, enabling better visualization and surgical manipulation in the oropharynx. These technologies have allowed tumors only previously resectable *via* external and highly morbid approaches (mandibular split and pharyngotomy approaches) to now be treatable *via* the transoral route with significantly less morbidity. One report of TLM demonstrated the promise of this modality in patients with early stage oropharyngeal cancer (T1-4a, N0). In this study, sixty-nine patients in two centers underwent TLM and neck dissection, of which no patients were treated with adjuvant radiation. Excellent patient outcomes were reported, including a five year overall survival of 86%. Similarly, locoregional recurrences were quite low, with a 90% locoregional control rate in patients with T1 disease, and a 94% control rate in patients with T2 disease[21].

Although treatments with TORS and TLM are increasingly becoming employed in early stage oropharyngeal carcinomas, the bulk of the evidence supporting their use stems from the surgical management of patients with locally advanced (stage Ⅲ/Ⅳ) disease. The utilization of TORS was first reported in 2005[22], and since then has been described in numerous publications as an effective treatment for oropharyngeal cancers[23-25]. In one large case series of patients with locally advanced oropharyngeal cancers (T2-4a, N0-2c), treatment with TORS and selective neck dissections resulted in excellent outcomes, notably with a 98% 1-year disease specific survival. Regarding the need for further multimodality therapy, only 39% required radiation and 39% received chemoradiation. Based on these results, the use of TORS accompanied by selective neck dissection may be a method to de-intensify therapy, sparing patients from the toxic effects of adjuvant chemotherapy, and in some select cases, adjuvant radiation as well[26]. Further matched retrospective patient studies, directly comparing TORS to chemoradiation, have demonstrated that patients treated with TORS have less acute toxicities and a higher rate of recovery to baseline swallowing function at 12 mo[27]. Although these studies support the use of transoral surgery in select patient populations for both early and locally advanced, low volume oropharyngeal cancers, further multi-center, randomized studies comparing transoral surgery-based approaches to definitive chemoradiotherapy are needed in order to establish the role of primary surgery in standard of care practice.

Chemoradiotherapy

The management of locoregionally advanced oropharyngeal cancer (stage Ⅲ-ⅣB) is complex and emphasizes the need for a multidisciplinary approach as treatment for each patient is individualized based on the clinical setting. Currently, the treatment of locally advanced disease focuses around definitive chemoradiotherapy.

Organ preservation with chemoradiation has been studied exhaustively over the last 20 years. The relative benefit of concomitant chemotherapy and radiation has been established through numerous trials; however, the MACH-NC meta-analysis, which combined 93 randomized trials and more than 17000 patients, offers the most comprehensive perspective to date. In this study, concomitant chemotherapy and radiation was found to offer a significant improvement in 5-year overall survival compared to radiation therapy alone (33.7% *vs* 27.2%, absolute difference of 6.5% ± 1%). In an exploratory multivariate analysis, the observed effect of chemotherapy on improved survival decreased as a function of age; in the group of patients 70 and older, no improvement in survival was observed[28]. A similar analysis, presented at the 2015 American Society of Clinical Oncology (ASCO) annual meeting in Chicago, also noted lower survival rates in patients 70 years or older collectively from three previously published Radiation Thoracic Oncology Group (RTOG) studies[29]. A subsequent analysis, based on tumor site, also noted improvement of the 5-year overall survival rate in patients with oropharyngeal cancers, whereby the absolute benefit in 5-year overall survival was 8.1%[30].

A number of chemotherapeutic agents have been utilized as radiation sensitizers during concomitant therapy. However, the most commonly used regimens include high-dose cisplatin (100 mg/m^2 every 21 d for two or three doses), weekly cisplatin (30-40 mg/m^2), weekly carboplatin (AUC = 2) plus paclitaxel (45 mg/m^2), and weekly cetuximab. Landmark studies defining non-surgical approaches established high-dose bolus cisplatin as the original, standard concomitant agent[31-33]. Given the proven efficacy of bolus cisplatin, several phase Ⅱ studies and retrospective case series have sought to establish if weekly cisplatin is an effective and well-tolerated alternative[34,35]. Sharma *et al*[34] demonstrated that the addition of weekly cisplatin (40 mg/m^2) to radiotherapy improved overall survival when compared to radiation alone, though 40% of patients experienced Grade 3 or 4 toxicities in the concomitant arm as compared to 20% treated with radiation alone. Similarly, 29% of patients receiving cisplatin required treatment interruptions, compared to 9% in the radiation alone arm[34]. One meta-analysis found that increased cumulative cisplatin dose, regardless of schedule (bolus *vs* weekly), was associated with improvement in survival[36]. To date, there still are still no prospective, randomized published trials comparing weekly cisplatin and radiation with bolus cisplatin and radiation. Several retrospective reviews presented as abstracts suggest that survival may not be compromised with weekly platinum *vs* high-dose platinum-radiation regimens. Furthermore, patients with low risk disease (*i.e.*, p16+, low tumor volume, < 10 pack smoking histories) will inherently enjoy longer survival times regardless of the chemoradiotherapy regimen administered. Patients with poor prognosis tumors (T4, N2c, N3 tumors, > 10 pack year smoking histories), on the other hand, may benefit from high-dose cisplatin combined with radiation[37].

Given the persistent toxicities with weekly cisplatin and issues with renal failure, carboplatin has been explored alone or in combination with 5-fluorouracil or paclitaxel for use with radiation therapy[38,39]. In a pilot study of 60 patients, the combination of carboplatin and paclitaxel given concomitantly with radiation was well tolerated. Eighty-two percent of patients achieved a complete response and the 2 year overall survival rate was 62%. Fifty nine of the patients completed treatment, with the most common grade 3 toxicities being mucositis, dysphagia, leukopenia, and skin desquamation[38]. In another multicenter phase Ⅲ study, weekly carboplatin and 5-flurouracil given with radiation was compared to radiation alone in patients with locally advanced oropharyngeal carcinomas. Although this study demonstrated increased rates of grade 3 or 4 toxicities in patients receiving chemoradiation *vs* radiation alone (71% *vs* 29%), the three year overall survival rates favoring the chemoradiotherapy arm were impressive (51% *vs* 31%)[39].

Randomized, prospective studies comparing weekly platinum regimens to high-dose cisplatin with radiation have yet to be conducted. Investigators at the University of Michigan compared their institutional studies, utilizing weekly carboplatin and paclitaxel with intense modulated radiation therapy (IMRT) and bolus cisplatin with IMRT, in stage Ⅲ/Ⅳ oropharyngeal cancer patients *via* a matched, paired, retrospective analysis. This evaluation demonstrated that patients treated with high dose cisplatin had higher numbers of grade 3 or 4 toxicities (54% *vs* 40%). After accounting for HPV status, there was no significant difference noted in overall or progression-free survival between the two treatment arms[40].

The anti-EGFR monoclonal IgG1 antibody Cetuximab has been established as an effective agent for use with radiation therapy. In a large Phase Ⅲ trial, the median overall survival and 5-year overall survivals were both significantly improved with the addition of Cetuximab to radiation therapy over radiotherapy alone (49 mo *vs* 29.3 mo and 45.6% *vs* 36.4%, respectively). Of note, on exploratory multivariate analysis it was noted that the greatest benefit was seen in patients with oropharyngeal cancers but a benefit was not seen in those > 65 years old. In addition, it was noted that the development of a prominent acneiform rash (grade 2 or greater) was associated with a significantly improved overall survival[41,42]. Analysis of the effect of cetuximab on overall survival based on pre-treatment characteristics demonstrated that the addition was most beneficial in non-elderly men with oropharyngeal tumors, grade 1-3 tumors, node positive (N1-3), with good performance status[42]. A biomarker analysis evaluating outcomes related to HPV status was recently conducted on this study, and the results were presented at the 2014 ASCO annual meeting in Chicago. This investigation demonstrated improvement in OS with the addition of cetuximab to radiation in both HPV+ *vs* HPV- tumors, though a greater degree of improvement was seen in those tumors which were p16+. This study was exploratory in nature and not powered to make definitive conclusions; however, it does confirm that HPV is a prognostic biomarker, not yet predictive[43].

Given the improvement in clinical outcomes seen with cetuximab, several large trials have sought to answer whether the addition of anti-EGFR monoclonal antibodies (cetuximab or panitumumab) to conventional platinum based chemoradiation results in clinical improvement. Each of these studies has failed to demonstrate improvement in clinical outcomes with the addition of EGFR inhibition[44,45]. One of these studies did demonstrate that although EGFR expression did not distinguish outcome in patients treated with cetuximab, patients with p16 positive oropharyngeal carcinomas had a better 3 year progression free survival (72.8% *vs* 49.2%) and overall survival (85% *vs* 60.1%)[44]. Unplanned post-hoc analysis of RTOG 0522 (reviewing the role of cisplatin based chemoradiotherapy plus cetuximab) demonstrated that patients with high baseline metabolic tumor volumes on PET/CT had an inferior response to chemoradiotherapy in terms of progression-free survival and locoregional control. Interestingly, this remained an independent prognostic factor on multivariate analysis even after factoring for T stage[46].

Based on the evidence of efficacy with the use of Cetuximab as a radio-sensitizing agent, the question has arisen regarding the comparative efficacy *vs* a platinum based regimen. A published single center retrospective study was recently published describing the outcomes of patients with locally advanced head and neck squamous cell carcinoma treated with concurrent chemoradiation stratified by chemotherapeutic agent. It was noted that patients treated with platinum based chemotherapy had significantly superior relapse free and overall survival compared to those treated with cetuximab monotherapy or in combination with chemotherapy[47]. One meta-analysis including 15 trials and 1808 patients which was presented in a preliminary form demonstrates that studies to date support a greater improvement in both locoregional recurrence and overall survival with the use of cisplatin. However, this study had significant heterogeneity and did not account for p16 status[48]. Other studies comparing panitumumab and radiation with cisplatin and radiation have also failed to demonstrate the improvements of this fully human monoclonal antibody against EGFR to the standard of care[49,50]. Ongoing studies are still seeking to answer this question in select populations, including RTOG 1016.

The role of induction chemotherapy in oropharyngeal cancer has been debated extensively and there continues to be some controversy regarding its role. In general, the use of induction chemotherapy has been intended to decrease the rate of distant metastases, to cause rapid cytoreduction, to offer high doses of chemotherapy to tumor prior to disruption of vasculature by radiation, and to decrease tissue volume requiring

exposure to radiation[51]. Three large, randomized phase Ⅲ studies have been performed to date evaluating the role of induction *vs* concurrent chemoradiation, all of which demonstrated no difference in survival or time to treatment failure[52-54]. In the recently published DeCIDE trial, evaluating induction chemotherapy primarily in oropharyngeal cancer, enrollment was difficult and the study was closed after enrollment of 285 of the planned 400 patients. Although overall survival was no different between the arms at three years, one should note that (albeit not statistically significant) the difference in the rate of distant failure was 10% in the induction chemotherapy group *vs* 19% in the concurrent chemoradiation group. HPV status was available for only 49 patients and on subgroup analysis is was noted that there was no statistically significant difference in overall survival between HPV(+) and HPV(-) patients[54]. Early results of a phase Ⅲ trial from Italy, comparing induction chemotherapy followed by definitive chemoradiotherapy *vs* concomitant chemoradiation with cetuximab *vs* cisplatin and 5-flourouracil (5-FU) *via* 2 × 2 factorial design, were presented at the 2014 ASCO annual meeting in Chicago. This trial had a primary endpoint of 3 year overall survival between the induction *vs* no induction groups. Preliminary results demonstrated a statistically significant improvement with induction chemotherapy in both median progression-free (29.7 mo *vs* 18.5 mo, $P = 0.12$) and overall survival (57.6 mo *vs* 45.7 mo, $P = 0.03$). On unplanned subgroup analysis, these improvements were not seen in patients with oropharyngeal cancers. Additionally, when compared with similar previously published trials as historical controls[41,44,54], both progression-free survival and overall survival appear to be lower across the board, for which the etiology is unclear. Reporting of HPV status amongst the treatment groups is pending and will be important in fully interpreting the results of this study[55].

Investigators at the University of Michigan have studied the use of induction chemotherapy as a means of chemoselection, whereby patients with oropharyngeal cancers who had a response to one cycle of induction chemotherapy were treated with definitive chemoradiation, whereas those patients without evidence of response proceeded to salvage surgery. In this study, induction therapy failed to successfully select patients for surgical salvage, but a subgroup analysis demonstrated that higher HPV titers were associated with a significant reduction in tumor burden following the administration of a single cycle of chemotherapy, demonstrating the robust response of p16 positive oropharyngeal tumors to cytotoxic agents[56]. In the companion paper published with this article, correlative analysis noted that EGFR expression was inversely associated with response to chemoselection as well as patient outcomes including disease specific survival and overall survival. Moreover, when biomarkers were combined low EGFR and high p16 expression were associated with a good response to chemoselection however the combination of high EGFR expression, low p53 expression, and high Bcl-xL expression was associate with a poor response to chemoselection and overall survival[57].

DE-INTENSIFICATION OF THERAPY

Although chemoradiotherapy has improved survival outcomes in patients with loco-regionally advanced oropharyngeal cancers, this has come at the expense of both acute and late treatment related toxicities. These toxicities substantially impair patients' quality of life, potentially for the remainder of their lives, and include long-term swallowing dysfunction as a result of radiation. HPV+ oropharyngeal cancer is now being increasingly recognized as a biologically distinct malignancy with a distinct disease course and response to therapy. Moreover, HPV+ tumors have higher response rates to multimodality therapies, lower rates of disease relapse, and improved overall survivals compared with HPV- tumors. In an attempt to mitigate acute and late toxicities, an area of research looking to define patients with low risk oropharyngeal cancer who may be candidates for de-intensification of therapy is actively underway. Proposed methods of de-intensification include decreasing doses of radiation (so called de-escalation) or switching from cisplatin based radio-sensitization to targeted therapy with cetuximab.

To date, few published trials provide insight into this matter, and hopefully with the maturity of several ongoing prospective trials, there will be a body of literature as to guide the field. One retrospective study sought to define the pattern of recurrence in HPV + low risk patients (< 10 pack-year smoking and T1-T3 disease) based on treatment with radiation alone *vs* concomitant chemoradiation. It was shown that low risk patients, those with N0-N2a nodal involvement, had no difference in disease control rates with the introduction of chemo-sensitization as compared to those receiving only radiotherapy[58]. Given the retrospective nature of this study and the fact that the majority of patients not receiving chemotherapy were those with advanced age or restricting medical co-morbidities, it is difficult to draw definitive conclusions. However, this research certainly supports the consideration for de-escalation of therapy in a subset of low risk patients. Currently, RTOG 1333 is assessing such an approach with the primary endpoint of 2 year progression free survival. In this study low, risk patients (HPV+ with a ⩽ 10 pack-year smoking history) with oropharyngeal cancer are being randomized to either radiation (60 Gy, 2.0 Gy/fraction in 6 wk) with concurrent weekly cisplatin (40 mg/m^2 × 6 doses) or radiation alone (60 Gy of radiation, 2.0 Gy/fraction over 5 wk)[59]. As a chief aim of de-escalation is improving treatment related toxicities, one of the main secondary endpoints being followed in this trial includes quality life, most notably swallowing function. ECOG 3311 is an ongoing risk stratified randomized phase Ⅱ

study evaluating an approach of TORS followed by a risk adapted approach in patients with HPV(+) stage Ⅲ/Ⅳ oropharyngeal carcinoma. In this study, based on post-operative findings low risk patients will be observed, intermediate risk patients will be treated with radiation alone, and high risk patients will be treated with chemoradiation.

ECOG 1308 is a prospective, phase Ⅱ study that also examined the role of de-escalation. In this trial, patients were treated with 3 cycles of induction chemotherapy, and if they were found to have a complete response, they were treated with weekly cetuximab and low dose intensity IMRT (54 Gy/27 fractions). If, on the other hand, patients had less than complete response, they received weekly cetuximab with full dose IMRT (68.3 Gy/33 fractions). Preliminary analyses demonstrated that patients with complete responses, treated with low dose IMRT, had an improved 2 years progression free and overall survival compared to those patients in the standard-dose IMRT arm. Additional insights from the analysis of the patient cohort receiving low dose radiotherapy demonstrate that progression-free survival and overall survival were better in patients with a ⩽ 10 pack-year smoking histories and low volume (< T4, T1-N2b) disease. This favorable risk cohort had a significantly improved 2 year progression-free survival compared to other enrolled patients (96% *vs* 64%)[60]. Although this data yields valuable insights into the potential for reducing intensity of treatment for a select population of oropharyngeal cancer patients, a larger, multi-center phase Ⅲ is needed study to verify the results of this de-escalation trial, comparing this concept to standard cisplatin and radiotherapy.

Finally, RTOG 1016 is an ongoing non-inferiority phase Ⅲ trial that is seeking to identify the role of substituting Cetuximab for high dose bolus Cisplatin (100 mg/m^2 q 21 d × 2 doses) in combination with accelerated IMRT. This protocol exclusively enrolled 1000 patients with p16+ locoregionally advanced oropharyngeal cancer (clinical stage T1-2 N2a-N3 or T3-4 any N) with any smoking status. In addition to defining whether the substitution of cisplatin is non-inferior to standard therapy, this study will assess the effect of tobacco exposure and molecular profiles on patient outcomes. This study is now closed to accrual and the results are eagerly awaited.

LOCALLY RECURRENT AND METASTATIC DISEASE

Despite increased understanding of oropharyngeal cancer and advances in treatment of both early stage and loco-regionally advanced disease, a number of patients still develop locally recurrent and metastatic disease. Evidence now supports that HPV(+) oropharyngeal cancer patients who develop progression have a better median overall survival than those cancers which are HPV(-) (2.6 years *vs* 0.8 years). Fakhry *et al*[61] noted a worse survival upon progression in patients with distant metastases or those who initially presented with T4 lesions. Patterns of recurrence are also related to HPV status in oropharyngeal cancers. HPV(+) status markedly reduces the risk for loco-regional recurrence (HR = 0.09, P = 0.03)[62] and in one study was associated with a longer time to distant failure (16.4 mo *vs* 7.2 mo)[63].

The goal of therapy in patients with locally recurrent or metastatic oropharyngeal cancer who are treated with chemotherapy is palliative. As prognosis is poor and effective treatment options are limited, enrollment onto clinical trials offers the best possible care, especially for those who have failed a front-line platinum containing regimens. If trial involvement is not possible, numerous treatment modalities with standard agents may be considered.

Surgical salvage should be entertained in select situations as a treatment for locally recurrent or metastatic oropharyngeal cancer. Recent studies have demonstrated that surgery is an effective treatment option, often improving survival. One large study of 181 patients demonstrated that even when factoring in T/N stage, progression type (distant *vs* locoregional), smoking history, and p16 status to a multivariate analysis, salvage surgery still remained a significant predictor of overall survival (HR = 0.56, P = 0.02)[61]. Another similar retrospective study attempted to gain similar insight; however, this evaluation also considered whether salvage treatment with nonsurgical methods or with surgical methods offered superior overall survival. The investigators found that surgical salvage offered an improvement in overall survival compared to those treated with salvage radiation or chemotherapy. Similar to previous studies, this finding remained significant even on multivariate analysis when p16 status, T/N stage, smoking history, site of disease recurrence, and number of sites with disease recurrence were factored in[64].

If surgical salvage is not an option, there are numerous classes of cytotoxic chemotherapy drugs including platinum agents, taxanes, methotrexate, 5-FU as well as the anti-EGFR targeted therapy, cetuximab, which have proven efficacy in metastatic head and neck cancer. Response rates to chemotherapy range between 10%-30% with single agent regimens and 20%-40% for multi-drug regimens[65-67]. It is important to appreciate that although conventional cytotoxic agents may be combined as doublet therapies (traditionally platinum based), these combinations increase response rates but not overall survival, and they have notable increases in toxicities[66]. There have been no studies showing superiority of one cytotoxic regimen over the other, median overall survivals ranging from 6.6-8.7 mo[65-68]. Incorporation of cetuximab into a 5-FU and platinum containing regimens is associated with an increased objective response rate (36% *vs* 20%), progression free survival (5.6 mo *vs* 3.3 mo), and overall survival (10.1 mo

vs 7.4 mo) relative to platinum-5 FU doublet therapy in patients with metastatic head and neck cancer[69]. Although underpowered to draw conclusions, a post-hoc analysis of p16+ oropharyngeal cancers seemed to have a greater degree of benefit with the incorporation of cetuximab compared to those that were p16-[70].

FUTURE DIRECTIONS

There are currently numerous ongoing trials involving the treatment of oropharyngeal cancer. Among the current research avenues are novel predictive factors for recurrence and the development of immunotherapeutics. Although the prognosis of HPV+ advanced oropharyngeal cancer is impressive with 3 year survival rates of 62%-83%[71,72], there is an increasing rate of distant treatment failure, not accounting for 45% of long term deaths in the population[15,73]. Numerous prognostic factors have been explored as methods to better tailor therapy for those at increased risk, including micro-RNA, advanced T and N classification, and smoking status[58,74,75]. One novel finding, identified as prognostic as well as predictive, is the presence of matted nodes on pre-treatment imaging (CT or PET/CT). Matted nodes are defined as the presence of three lymph nodes abutting one another with loss of the intervening fat plane which is thought to represent radiologic evidence of extracapsular spread. Matted nodes have been identified in 20% of patients presenting with advanced oropharyngeal cancer. In one analysis, patients presenting with matted nodes had a three year disease specific survival of 58% *vs* 97% in those without. This bore out as a predictive marker on a further analysis and on a multivariate analysis whereby the presence of matted nodes remained an independent predictor of poor prognosis even when controlling for age, tumor classification, HPV status, and smoking status[76,77].

There has also been interest in searching for novel biomarkers as to guide patients at risk for reoccurrence. Retrospective analysis of patients with locally advanced HPV+ oropharyngeal cancer has demonstrated that patients who recurred were noted to have a significantly lower rate of E7 antibody clearance[78]. Prospective analyses are needed to determine the utility of E6 and E7 antibody clearance perhaps in combination with plasma HPV DNA levels. Two abstracts presented at the 2015 ASCO annual meeting may also aid in identifying patients at high risk for recurrence. In one study, loss of function tumor suppressor gene mutations appears to decrease the efficacy of treatments for locally advanced squamous cell carcinomas of the head and neck. Activating driver gene mutations, on the other hand, may define poor risk patients, in particular those with HPV(+) oropharyngeal carcinomas[79]. A second study evaluated the implication of persistent HPV-16 DNA detection in oral rinses in patients with p16 positive oropharyngeal carcinomas, treated for locally advanced disease. Data from this evaluation suggests that persistent oral HPV DNA in post-treatment rinses is strongly associated with poorer outcomes[80]. These findings may help to tailor intensification of therapy in high risk populations as to improve patient outcomes.

Immunotherapy [namely Programmed Death-1 (PD-1) inhibition] is currently one of the most exciting and rapidly changing areas of oncology with impressive response rates and improvements in overall survival seen in melanoma and lung cancer[81-83]. PD-1 targeting in head and neck cancer has been of interest as these malignancies [especially HPV(+) tumors] are thought to be quite antigenic[84]. In addition, pathologic samples in both HPV(+) and negative tumors have demonstrated a high frequency of PD-1 and PD-L1 expression, suggestive of a potential role for checkpoint inhibitors[85,86]. Preliminary results of the KEYNOTE-012 study, a phase 1b multisite study evaluating the activity of Pembrolizumab in patients with recurrent or metastatic HNSCC regardless of PD-L1 or HPV status, were reported at the ASCO Annual Meeting in 2015. An overall response rate of 24.8% and stable disease rate of 24.8% was reported with activity observed in both HPV(+) and HPV(-) patients. Although follow up was limited as only preliminary results were available, it was intriguing that the median duration of response was not reached[87]. An accompanying study analyzed this population as to try and identify predictors of response as both HPV and PD-L1 status have been non-discriminatory. It was demonstrated that an inflamed-phenotype gene expression, chiefly interferon gamma, was able to predict 6 mo progression free survival with a 95% negative predictive value and 40% positive predictive value[88]. Similar findings have been reported in melanoma where inflamed-phenotype gene expression signatures appear to predict benefit from pembrolizumab[89]. There are multiple ongoing phase Ⅱ/Ⅲ clinical trials investigating the role for Pembolizumab and Nivolumab in the setting of metastatic disease for head and neck cancer, which include the evaluation of markers to potentially identify responders[87]. Results of these studies will offer new insights and may drastically alter the treatment of metastatic oropharyngeal cancer.

CONCLUSION

The management of oropharyngeal cancer is complex and depends on a multidisciplinary team including otolaryngologists, medical oncologists, and radiation oncologists. Although great strides have been made in the last 20 years in approaches to organ preservation and risk stratification, improvements are needed in delineating the role of treatment de-intensification and development of novel therapeutics for the treatment of metastatic disease. We eagerly await final publications of the data from the recent ASCO annual meetings to further validate the use of several novel agents and treatment approaches.

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REVIEW

Current therapeutic strategies for advanced pancreatic cancer: A review for clinicians

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Abstract

Pancreatic cancer (PC) would become the second leading cause of cancer death in the near future, despite representing only 3% of new cancer diagnosis. Survival improvement will come from a better knowledge of risk factors, earlier diagnosis, better integration of locoregional and systemic therapies, as well as the development of more efficacious drugs rising from a deeper understanding of disease biology. For patients with unresectable, non-metastatic disease, combined strategies encompassing primary chemotherapy and radiation seems to be promising. In fit patients, new polychemotherapy regimens can lead to better outcomes in terms of slight but significant survival improvement associated with a positive impact on quality of life. The upfront use of these regimes can also increase the rate of radical resections in borderline resectable and locally advanced PC. Second line treatments showed to positively affect both overall survival and quality of life in fit patients affected by metastatic disease. At present, oxaliplatin-based regimens are the most extensively studied. Nonetheless, other promising drugs are currently under evaluation. Presently, in addition to surgery and conventional radiation therapy, new locoregional treatment techniques are emerging as alternative options in the multimodal approach to patients or diseases not suitable for radical surgery. As of today, in contrast with other types of cancer, targeted therapies failed to show relevant activity either alone or in combination with chemotherapy and, thus, current clinical practice does not include them. Up to now, despite the fact of extremely promising results in different tumors, also immunotherapy is not in the actual therapeutic armamentarium for PC. In the present paper, we provide a comprehensive review of the current state of the art of clinical practice and research in PC aiming to offer a guide for clinicians on the most relevant topics in the management of this disease.

Key words: Pancreatic cancer; Chemotherapy; Radiofrequency; Stereotactic radiotherapy; Irreversible electroporation

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Core tip: This review focuses on the current clinical practice in the treatment of pancreatic cancer (PC), and outlines research topics. PC is still a highly lethal disease, for a usual presentation stage not manageable with curative surgery. Up to now, new targeted therapies have not shown any positive impact on its dismal prognosis. Only slight improvements ensued from the availability of more active polychemotherapy regimens. From the point of view of a multimodal approach, in addition to surgery, new locoregional techniques are nowadays available, suitable for combination with systemic treatments, to increase disease control and survival.

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INTRODUCTION

In contrast to the general trend of increase in cancer survival, advances have been slow for pancreatic cancer (PC). Therefore PC is actually the fourth cause of cancer death, and it is expected it will be the second cause of cancer death by 2030 in Western countries. The American Cancer Society estimated that there will be 48960 new cases of PC in the United States in 2015, with 40560 deaths[1,2]. Despite surgery, locoregional therapy, chemotherapy and molecular therapies, the overall median survival is less than 1 year from diagnosis, highlighting the need for better therapeutic options. In fact, PC is frequently undiagnosed until the sudden appearance of prominent clinical symptoms and signs for advanced disease. Only in 10%-20% of cases the disease is resectable or borderline resectable, therefore suitable to surgery associated with neoadjuvant or adjuvant treatment, with curative purposes. In the last years different ablative techniques such as irreversible electroporation (IRE), radiofrequency ablation (RFA) and stereotactic body radiation therapy (SBRT) caught the attention of the scientific community. Such techniques may be an alternative to surgery in patients with a locally advanced disease, poor response to systemic therapy, and with a locoregional rather than metastatic growth pattern.

This review aims to explore the major questions still open regarding the management of the disease. We identified studies and systematic reviews by searching PubMed, ClinicalTrials.gov, and the Cochrane database from database inception to April 2015.

ARE THERE PROVEN RISK FACTORS IN PC? CAN WE PREVENT IT?

Current knowledge and unmet needs

Facing such a dismal prognosis cancer, a frequent question from patients is "Why? Why to me". PC has a multifactor etiology, whose better knowledge could be helpful to identify groups of people worthy of surveillance trials. A study on 117 meta-analytical and pooled reports estimated risk factors and the fraction of PCs attributable to them[3]. There is a moderately sized association between a family history of PC in first degree relatives, with multivariate-adjusted odds ratios (OR) of 1.8[4], justifying 5%-10% of cases. There is a significant association between PC risk and AB0 phenotypes (OR = 1.4 in non 0 blood type), and up to 19.5% of all cases of PC in populations with European ancestry could be attributable to a non-0 blood group[5]. Moreover, a multistage genome-wide association study[6] identified multiple susceptibility alleles to be further evaluated. A study of Maisonneuve and Lowenfels[7] however suggests that nearly two thirds of PC are due to potentially avoidable causes. The strongest associations are with tobacco smoking, that is the greatest behavioral risk factor for PC, and *Helicobacter pylori* infection, with estimated population attributable fractions of 11%-32% and 4%-25% respectively. Besides carcinogens, smoking also generates agents perpetuating inflammatory response, and heightens the risk of chronic pancreatitis. A higher risk of PC indeed is associated with chronic pancreatitis. In this perspective, Helicobacter pylori infection could have a role in pancreas carcinogenesis, through the induction of autoimmune pancreatitis[8]. Heavy alcohol intake, defined as a daily consumption of over 30 g, has a strong association with PC, with an attributable increased risk of 20%-30%. All or many of these risk factors could concur through complex interactions involving different pathways[9]. Diabetes, obesity and reduced adiponectin level are all related to insulin resistance, and probably share common pathways, which can be responsible for attributable fraction up to 16%-19%, with the opposite postulated protective effect of higher physical activity[10].

The strongest evidence for a protective effect is for atopic allergy, especially hay fever or allergy to animals, that could reduce PC risk up to 20%-30%[11]. A number of other postulated risk factors or protective factors like meat and fruit intake, or vitamin D circulating levels have a lower level of evidence and deserve further studies.

Cystic lesions occasionally detected with non-invasive abdominal imaging, prescribed for unrelated indications, deserve a separate discussion. Prevalence range of incidental pancreatic cysts in the adult population is from 2.2%-5.9% (depending on imaging technique)[12]. Their correct management is crucial for preventing and early treating of the disease. Especially intraductal papillary mucinous neoplasms can have a progression model similar to that of colonic polyps, with the risk of transforming into invasive cancer, more likely in cases with involvement of main pancreatic duct or with multiple lesions. But only a few of them actually progress to malignancy. Their optimal management is still controversial, based more on experts' opinions than on evidence from randomised studies. This uncertainty

about the prediction of future behavior is due to a lack of accurate diagnostic tools and prognostic factors. It exposes patients to a risk of overtreatment with unnecessary high-risk surgery, undertreatment or expensive long term imaging follow-up[13].

IS INTEGRATED APPROACH (SURGERY, RADIOTHERAPY AND CHEMOTHERAPY) THE GOLD STANDARD IN LOCALLY ADVANCED PC? WHAT IS TODAY THE ROLE OF SURGERY?

Current knowledge

Nearly 30%-40% patients at diagnosis have a borderline-resectable (BRPC) or locally advanced PC (LAPC)[14]. But despite the absence of distant metastasis, the overall survival (OS) of these patients is absolutely poor[15,16], and only radical surgery can give a chance for a cure[17]. Selected patients can have an improved outcome with a multimodal approach, combining chemotherapy with radiation therapy or surgery. The selection of a population of patients suitable to multimodal approach, however, needs an accurate identification of LAPC and BRPC. LAPC refers to cases with an extended involvement of adjacent structures[18]; whereas BRPC comprises a subset of patients eligible to an upfront resection, but with a high risk of residual microscopic disease (R1, according to the International Union Against Cancer Classification) caused by an involvement of nearby structures, such as superior mesenteric artery or celiac artery, not allowing a removal of the tumour without an arterial resection, thus greatly increasing the risk of R1 or R2 surgery. R0 resection only can cure PC. Unfortunately, there is a wide heterogeneity in the literature regarding the definition of resectability criteria. Moreover, BRPC patients are an ill-represented population in the majority of chemotherapy clinical trials.

In this context, upfront resection has been rated as a 2B recommendation in the National Comprehensive Cancer Network Guideline[18-21].

Although lacking high level evidence, there is a general consensus for neo-adjuvant chemotherapy, estimated able to convert to R0 resection 33% of LAPC/BRPC patients[22,23].

This therapeutic strategy has been historically based on fluoropyrimidines, 5-fluorouracil (5-FU) or capecitabine, combined with radiation and recently on gemcitabine induction followed by concomitant chemo-radiation with either gemcitabine or fluoropyrimidines[24,25]. At present, there are no data about the better neo-adjuvant chemotherapy regimen. But based on the observed results in the metastatic settings, FOLFIRINOX or gemcitabine plus nab - paclitaxel with or without subsequent chemoradiation might represent promising options. However, especially FOLFIRINOX suits only to fit patients, for high rate of G3-G4 toxicities[26-29]. The results of ongoing Alliance A021101 pilot trial (NCT01821612) could help clarify the role of a multimodal strategy of neoadjuvant FOLFIRINOX, followed by chemoradiation [50.4 Gy external beam radiation therapy (EBRT) with concomitant capecitabine], definitive surgery and postoperative adjuvant gemcitabine in BRPC patients.

For LAPC affected patients as well, a combined approach in LAPC could allow radical resection also in cases not eligible to upfront surgery. In several studies[30,31], and a meta-analysis by Gillen *et al*[23], gemcitabine-based combination regimens allowed a higher resection rate than single agent chemotherapy (33% *vs* 27%). In this meta-analysis OS was almost doubled in patients who finally underwent surgical resection of their tumour (20.5 mo *vs* 10.2 mo). Moreover, three meta-analyses have suggested a survival advantage in patients treated with gemcitabine-based chemo-radiation (CRT)[32-34].

Nevertheless, the role of chemoradiation in LAPC is still unclear, for conflicting results of clinical trials. Indeed, two studies reported improved OS with CRT (Gastrointestinal Tumor Study Group 9283[35] and ECOG 4201[36]) and Huguet *et al*[37] reviewed two perspective trials finding a survival advantage in patients treated with chemotherapy and chemoradiation *vs* patients treated with chemotherapy alone. Other interesting results were recently reported by Sherman *et al*[38] using docetaxel and capecitabine followed by gemcitabine and capecitabine combined with radiation therapy and surgery[38]. In this phase 2 trial, 20 out of 45 treated patients (44%) had R0 resection.

On the opposite, Chauffert *et al*[39] reported no advantage in OS and more toxicity with the addition of CRT to chemotherapy, and preliminary results of the international phase 3 GERCOR LAP-07 study demonstrated improved local control with the addition of chemoradiation to chemotherapy, but no difference in OS[40].

Waiting for definitive evidence about the usefulness of CRT, at present, the most widespread approach in fit patients is to start with induction chemotherapy followed by chemoradiation in absence of disease progression at the time of first radiological evaluation. This approach has two advantages: It avoids unnecessary radiotherapy in the nearly 30% of patients who undergo widespread disease progression during initial treatment, and it permits to test patient's tolerance to chemotherapy alone, before adding the relevant toxicities of a radiation concomitant to chemotherapy. Radiotherapy[41].

Standard dose radiation therapy is usually 50.4 Gy in 1.8-Gy fractions, although some trials reported the use of a 30-36 Gy in 3-Gy fractions schedule[42]. Better outcomes could come from the use of newer radiotherapy techniques like intensity-modulated radiation therapy (IMRT) and SBRT, suited to deliver higher biological dose[42]. Indeed, in a phase 2 multi-institutional trial, SBRT was feasible without unexpected toxicities and obtained a 1-year local progression-free survival (PFS) of 78%[43].

Unmet needs

An agreement about an unambiguous, rigorous definition of the BRPC could help to reach a homogeneous approach to borderline resectable disease, thus allowing comparison among different trials results. Despite multimodal treatments, not all BRPC and LAPC will become resectable up to R0, missing their chance for a cure. Deeper exploration of combination regimens is necessary to improve this outcome, especially through the identification of prognostic factors and biomarkers to predict the response or the resistance to the different treatments. At present, little evidence is available. As an example, SMAD4-deleted tumours are associated with widespread disease, whereas SMAD4-proficient tumours are associated with a more locally aggressive disease[44]. Nevertheless, the impact of SMAD4 on treatment outcome is far to be defined. In any case, a multidisciplinary management in high-expertise centers can increase the chance of cure for all patients with PC, and even more for those with BRPC and LAPC.

WHAT IS THE BEST FIRST LINE CHEMOTHERAPY IN INOPERABLE PC?

Current knowledge

Despite recent advances in our understanding of the molecular biology of PC, there has been limited progress in therapeutic options for metastatic disease, and traditional chemotherapy outcomes, even though improved, are still disappointing. The overall median survival from diagnosis is still less than 1 year, underscoring the need for the development of newer therapeutic options. The goals of chemotherapy are: The improvement of survival, the control of symptoms and the need to ensure a good quality of life for the patient. In the past, several studies have demonstrated the superiority of chemotherapy compared to best supportive care alone (BSC) and fluorouracil (5-FU), in different doses, schedules, and combination regimens, has been considered the cornerstone in the palliative treatment of metastatic PC[45]. Since 1997, gemcitabine monotherapy has represented the standard of care for patients with metastatic PC, when Burris *et al*[46] demonstrated that it was superior to 5-FU in terms of clinical benefit/efficacy, outcome measures and safety profile in patients with a baseline Karnofsky performance status (PS) $\geqslant$ 50. Gemcitabine subsequently represented a backbone in chemotherapy, in clinical trials investigating more intensive combination regimens. Due to its good tolerability and demonstrated efficacy, from 1997 to 2010 several studies had combined it with many other active cytotoxic agents, including fluorouracil[39], capecitabine[47], cisplatin[48], epirubicin[49], docetaxel[50-52], oxaliplatin[31], irinotecan[53,54], and pemetrexed[55]; but up to now, no conclusive results about an effective impact on survival. In contrast to other tumour types, with the exception of the negligible benefit showed by erlotinib[56], tested targeted therapies as cetuximab[57], bevacizumab[58], axitinib[59], tipirarnib[60], oftrametinib[61], trastuzumab[62], have largely failed to show any significant benefit when added to standard chemotherapy in metastatic PC.

In 2011 a combination regimen of leucovorin, fluorouracil, irinotecan, and oxaliplatin (FOLFIRINOX) obtained a meaningful survival benefit over single agent gemcitabine. FOLFIRINOX, providing a significant survival improvement of 4.3 mo in comparison to gemcitabine alone[63]. The median OS, PFS, and objective response rate (ORR) were significantly higher with FOLFIRINOX compared with gemcitabine alone (median OS, 11.1 mo *vs* 6.8 mo; PFS, 6.4 mo *vs* 3.3 mo; ORR, 32% *vs* 9%). FOLFIRINOX, however, showed an unfavourable toxicity profile compared to gemcitabine alone, including grade 3/4 neutropenia (46% *vs* 21%), febrile neutropenia (5.4% *vs* 1.2%), thrombocytopenia (9.1% *vs* 3.6%), sensory neuropathy (9% *vs* 0%), vomiting (15% *vs* 8%), fatigue (23% *vs* 18%), and diarrhea (13% *vs* 2%). Only well-selected patients with metastatic PC can therefore bear such a treatment without heavy side effects.

In 2013, nab-paclitaxel in combination with gemcitabine showed an improved median survival of almost two months (1.8), compared to gemcitabine alone[64]. It also increased OS at 1 and 2 years, with a tolerable toxicity profile. Grade 3/4 adverse events occurred, as expected, more often with the combination therapy and included neutropenia (38% *vs* 27%), febrile neutropenia (3% *vs* 1%), fatigue (17% *vs* 7%), diarrhea (6% *vs* 1%), and neuropathy (17% *vs* 1%). In September 2013, nab-paclitaxel in combination with gemcitabine was approved by the FDA for first-line treatment of metastatic PC of the pancreas.

Unmet needs and proposals

Clinical trials results suggest that combination chemotherapy with regimens FOLFIRINOX or gemcitabine plus nab-paclitaxel are an acceptable option for patients with good PS, good pain management, and adequate nutritional intake. It is still not clear which is the best: FOLFIRINOX or gemcitabine and nab-paclitaxel? The median OS obtained in the two different trials was 11.1 mo with FOLFIRINOX and 8.5 mo with gemcitabine plus nab-paclitaxel. A direct comparison of the results of the two trials, conducted on different populations, is impossible.

In our opinion, both FOLFIRINOX and gemcitabine plus nab-paclitaxel are reasonable choices for first-line therapy in patients with Eastern Cooperative Oncology Group (ECOG) PS 0 or 1. For a better tolerability, the combination of gemcitabine and nab-paclitaxel could be an option also for patients with a slight worse PS, who cannot tolerate a FOLFIRINOX regimen, or in patients who have received FOLFIRINOX as neoadjuvant treatment. However, in common clinical practice only a small number of patients with metastatic PC presents with good PS. For the other patients gemcitabine

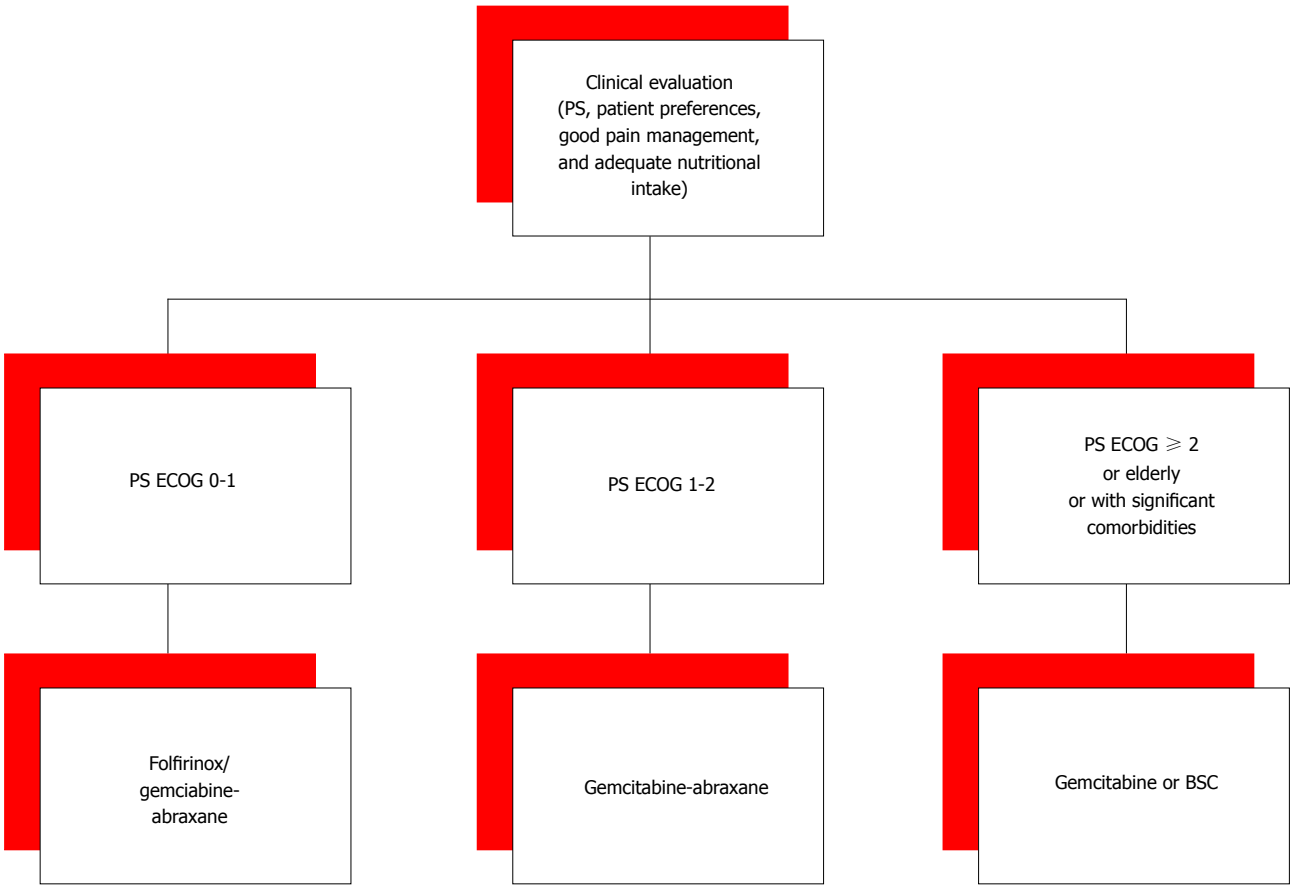


Figure 1 Proposal for the choice of the first line. PS ECOG: The eastern cooperative oncology group score of performance status; BSC: Best supportive care.

monotherapy is still the only therapeutic option (Figure 1).

Additional therapeutic advances are expected from studies evaluating strategies for depletion stromal, inhibition pathways of cancer (*i.e.,* Hedgehog, RAS-RAF-MAPK and PI3K-AKT), new chemotherapeutic drugs (*i.e.,* MM-398 irinotecan encapsulated into liposomal-based nano particles)[65,66], or the new era of immunotherapy. The identification of biomarkers continues to be clinically challenging but essential in order to tailor therapy to specific patients' subgroups in which the maximal antitumour effect from novel agents can be obtained.

WHAT IS THE ROLE OF THE LINES OF CHEMOTHERAPY SUBSEQUENT TO THE FIRST?

Current knowledge

Outside the context of clinical trials[67], median OS in patients with metastatic PC is 2.8-5.7 mo. However, despite the aggressiveness of this disease, in recent years the better results obtained with first-line chemotherapy have allowed a wider use of second-line treatments. In a retrospective study, the French and British oncologists analysed data of 400 patients treated for metastatic PC between 2009 and 2012. They collected patients' information about sex, age, PS, comorbidities, cancer-directed treatment, supportive care, adverse events and complications. The most common used first-line chemotherapy regimens were gemcitabine alone (46%), FOLFIRINOX (20.1%), gemcitabine/capecitabine (10.8%), and gemcitabine/oxaliplatin (9.5%). Approximately 40% of patients received second-line systemic therapy, whereas less than 20% received third-line systemic therapy[68]. About 45% of patients in phase Ⅱ-Ⅲ trial PRODIGE 4-ACCORD 11 received second-line therapy. FOLFIRINOX, despite significantly higher chemotherapy-related adverse events, allowed a better Quality of Life (QoL) than gemcitabine[69]. Since the QoL of patients with metastatic PC is more influenced by disease symptoms than by chemotherapy-related toxicity, the second-line chemotherapy could be a good option for selected patients. In a phase Ⅱ study, oxaliplatin-based regimen showed some activity in metastatic PC patients after failure of first-line chemotherapy with gemcitabine[70]. The CONKO-01 randomised phase Ⅲ multicenter study compared OFF (oxaliplatin, folinic acid and 5-FU 24 h) to BSC in patients with PC progressing while on gemcitabine therapy. Stratification included duration of first-line therapy, PS, and tumour stage. Trial terminated prematurely, after the accrual of 46 patients instead of 165 planned, probably for patients and physicians unwillingness to a randomisation in a BSC arm. Median second-line survival was 4.82 mo with OFF treatment,

compared to 2.30 mo with BSC. Median OS for the sequence GEM-OFF and for GEM-BSC was 9.09 and 7.9 mo, respectively. The OFF regimen was well tolerated with 13% of grade Ⅱ/Ⅲ gastrointestinal toxicities. This randomised trial has supported the hypothesis of the benefit of second-line chemotherapy in comparison to BSC alone, for patients with PC[71]. A further phase Ⅲ trial, CONKO-003, evaluated the effect on survival of oxaliplatin added to 5-FU, on second-line therapy. This trial randomised 168 patients with disease progression during first-line gemcitabine therapy, to folinic acid and 5-FU or oxaliplatin and 5-FU (OFF). In the OFF arm, the median OS and TTP were significantly extended in comparison to the 5-FU arm. The toxicities were similar between the two groups except for neurotoxicity, in 38.2% of OFF group patients[72]. However, oxaliplatin-based regimens for second-line chemotherapy have not given only positive outcomes. The PANCREOX trial randomised 108 patients after first-line gemcitabine, to mFOLFOX6 *vs* infusional 5-FU and folinic acid (5-FU/LV). The study showed no difference in median PFS (3.1 mo *vs* 2.9 mo, $P = 0.99$). Moreover mFOLFOX6 arm had a shorter OS and a higher patients number in mFOLFOX6 group withdrew for adverse events, thus the conclusion could be that this regimen is too toxic for this patients[73]. Irinotecan, alone or in combination with other drugs, could be another promising option for second-line therapy, in patients with metastatic PC after failure of gemcitabine. Also FOLFIRI has been proved, by some phase Ⅱ trials, to be a safe and potentially active regimen in this setting[74,75]. But a more interesting aspect is the availability of a new irinotecan formulation, encapsulated into liposome-based nanoparticles, potentially increasing drug stability and sustaining drug release in the tumour area. The NAPOLI-1 trial, a multicentre, open- label, three-arm, randomised phase Ⅲ trial, randomised 417 patients affected with metastatic PC, after prior gemcitabine-based therapy, to nano-liposomal irinotecan (MM-398) alone, or combined with 5-FU/LV, in comparison to 5-FU/LV. The combination of MM-398 + 5-FU/LV significantly improved OS, PFS, TTF, and ORR in comparison to 5-FU/LV. Median OS was 6.1 and 4.2 mo respectively. And median PFS 3.1 and 1.5 mo. MM-398 alone did not demonstrate any statistical improvement in efficacy. Many phase Ⅱ trials have investigated other therapeutic options as taxanes[76,77], capecitabine[78], S1[79], FOLFIRI and FOLFOX[80], FOLFIRINOX[81,82], nab-paclitaxel[83] for the treatment of chemorefractory patients, but more confirmation studies are needed.

Unmet needs and proposals

Given the evidence of some benefit from second-line therapy, questions still remain about which optimal drugs and regimens and for which patients. Moreover, available second-lines therapies further questions concern the optimal treatment sequences. For patients who received FOLFIRINOX in the first-line setting, the second-line option is often a gemcitabine-based therapy. The association nab-paclitaxel with gemcitabine proved to be effective in the front-line setting, but lack efficacy data in second-line setting. While, for the patients who received nab-paclitaxel and gemcitabine in the first-line setting, an oxaliplatin-based treatment may be considered in the second-line (Figure 2). Choosing second-lines options very aggressive behaviour of PC and the relatively rapid QoL deterioration have not to be forgotten. The choice of second-line treatment should always be done with close attention to PS, patient's age, the presence of comorbidities, and patient preferences.

ARE LOCOREGIONAL TREATMENTS ACHIEVABLE ALTERNATIVES TO SURGERY? ARE THEY USEFUL IN LAPC OR METASTATIC PC?

Current knowledge

Roughly 40% of PC diagnosis are of LAPC, because non-metastatic but unresectable disease, not suited to surgery with radical intent. So far, in this setting, sole palliative chemotherapy can only give slight survival improvement. But there are further options of several innovative local ablative therapies, including RFA, IRE, SBRT, high-intensity focused ultrasound (HIFU), microwave ablation (MWA), photodynamic therapy (PDT), and cryoablation (Table 1). Ablative therapies based on thermal tumour damage include RFA, HIFU, cryoablation and MWA while IRE, PDT and SBRT are non-thermal ablative methods. Actually, despite their proven safety, feasibility and reproducibility, novel ablative methods in LAPC or metastatic PC have still to demonstrate a benefit on survival in large prospective randomised studies[84].

Stereotactic body radiotherapy: In the last few years, SBRT gained increasing interest for its better and longer lasting outcomes, as well less toxicity than conventional EBRT. The SBRT can selectively deliver a higher dose of radiation to a target lesion, in single or multiple sessions. When using SBRT it is of paramount importance the precise delineation of the therapeutic target and the correct evaluation of possible target motion, in particular for pancreas, in a site affected from breathing movements. For this reason, the treatment planning uses four-dimensional diagnostic imaging. SBRT may be delivered using non-isocentric technique, IMRT, or volumetric-modulated arc therapy[85]. Despite the above mentioned characteristics which seem to improve some of the major limits of EBRT, the role of SBRT in LAPC and BRPC is not clearly defined yet, though some interesting preliminary evidence of its activity has been recently reported. As an example, in a single centre institution experience, the authors reported a median OS of 18.4 mo and median PFS of 9.8 mo in 88 patients affected by LAPC and BRPC treated with SBRT (2-30 Gy in five fractions on the planning target volume) with an acceptable toxicity profile (3.4% of

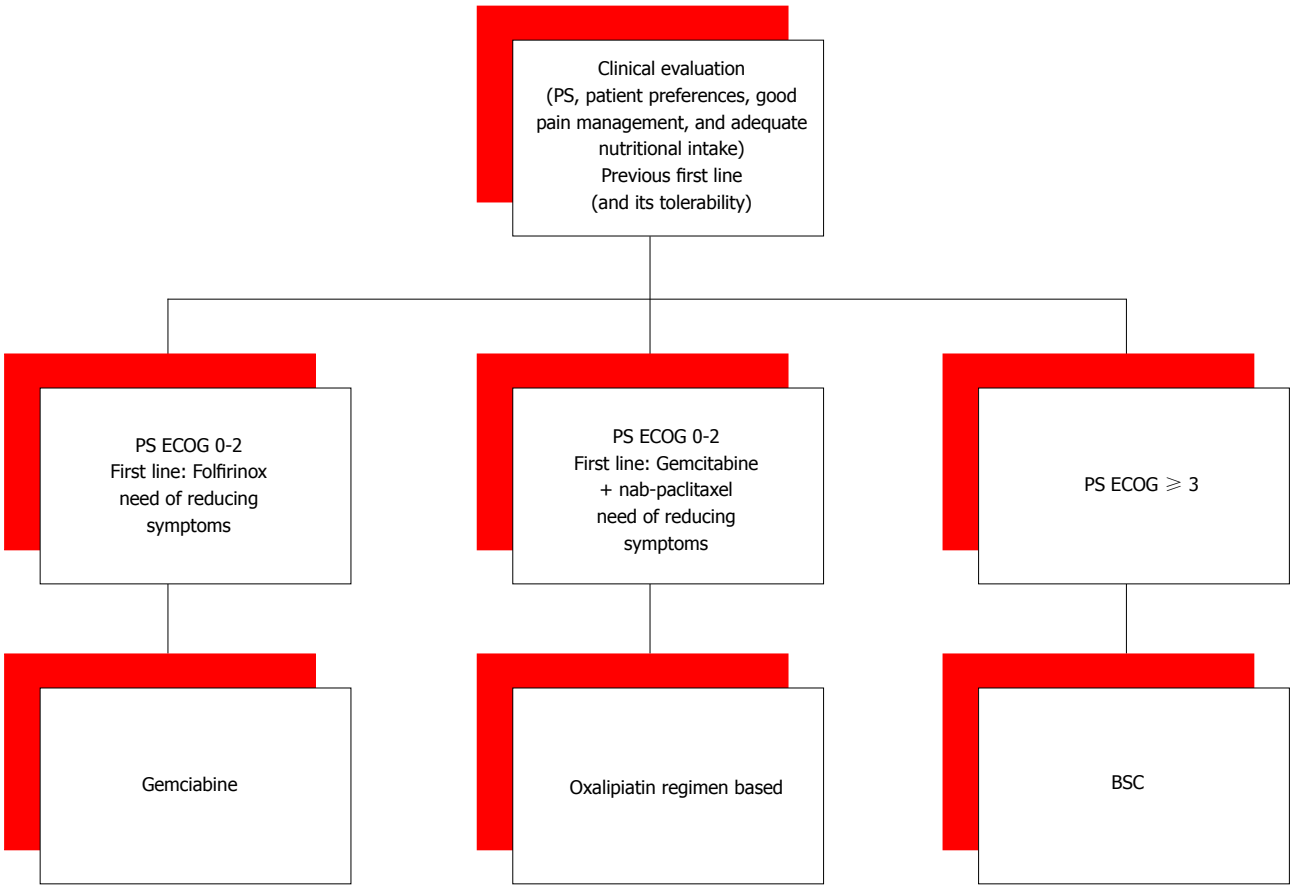


Figure 2 Proposal for the choice of the second line. PS ECOG: The eastern cooperative oncology group score of performance status; BSC: Best supportive care.

Table 1 Ongoing clinical trials about locoregional treatments in locally advanced pancreatic cancer

Combination	Inclusion criteria	Start	Clinical trial identifier[1]	Expected end of accrual
FOLFIRINOX + SBRT	T ⩽ 7 cm, non-metastatic	November 14	NCT02292745	November 20
RFA	Unresectable, also metastatic	June 14	NCT02166190	June 16
Cryoablation	Borderline resectable/locally advanced	November 14	NCT02336672	November 16
Radioembolization	unresectable/failure of celiac alcholization	Not available	NCT01786850	Not available
Irreversible electroporation (PAN.FIRE)	T < 5 cm, non-metastatic	September 13	NCT01939665	June 16

RFA: Radiofrequency ablation; SBRT: Stereotactic body radiation therapy; [1]Data Available from: URL: http// www.clinicaltrials.gov (last access 2015 May 24).

> G3 gastrointestinal toxicity)[86]. Furthermore, SBRT led to improved pain control in five out of six studies in which this outcome has been evaluated[87]. Moreover, as previously mentioned, SBRT can be delivered in association with chemotherapy with interesting preliminary evidence of activity (*e.g.*, gemcitabine). Some trials are currently ongoing trying to better clarify the role of SBRT in PC and its activity in association with more recent combination regimens (*e.g.*, SBRT with FOLFIRINOX, NCT 02292745).

RFA is the commonest thermal ablative technique used to treat tumours. It causes both direct thermal destructive effect and stimulation of antitumour immunity, through the expression of heat shock protein. RFA appears to be an attractive treatment for LAPC. According to the experience of Spiliotis *et al*[88] it should not be offered as an option for resectable PC, but it has shown to improve survival in 25 consecutive patients with inoperable LAPC who underwent palliative therapy with or without RFA. Median OS was 13 mo in patients receiving palliative therapy alone, compared to 33 mo in those who received RFA too (P = 0.0048). Moreover, RFA could be an option for patients with liver only metastasis in locally controlled PC. In a retrospective review by Park *et al*[89], RFA of liver metastasis was performed on 34 patients with PC, after the pancreatectomy or at the same time of the pancreatectomy. Median OS after liver metastasis treatment was 14 mo. In multivariate analysis, a single < 2 cm diameter liver metastasis and good or moderate differentiation were independent predictors for longer patient survival (P = 0.27, P = 0.16)[90].

Pancreatic Cryo Ablation (PCA) is a technique that uses single (or multiple) argon based probe in order to freeze the tumour. In most cases two cycles of freezing are used. It is currently used in several centers in the Far East for unresectable and often metastatic pancreatic ductal adenocarcinoma. Reported complications include acute pancreatitis, bleeding, leakage of bile, and delayed gastric emptying. No randomised trials have evaluated the efficacy of cryoablation, but Niu *et al*[91] retrospectively assessed the effect of comprehensive cryosurgery (ablation of intrapancreatic and extrapancreatic tumours) plus immunotherapy in 106 metastatic PC patients (cryoimmunotherapy: 31 patients, cryotherapy: 36 patients, immunotherapy: 17 patients and chemotherapy: 22 patients). Median OS was higher in the cryoimmunotherapy (13 mo) and cryotherapy groups (7 mo) than in the chemotherapy group (3.5 mo; both $P < 0.001$) and was higher in the cryoimmunotherapy group than in the cryotherapy ($P < 0.05$) and immunotherapy groups (5 mo; $P < 0.001$). In both the cryoimmunotherapy and cryotherapy groups, median OS was higher after multiple cryoablations than after a single cryoablation ($P = 0.0048$ and 0.041, respectively). A single institution retrospective review suggested effectiveness of PCA in palliation of cancer pain, on 62 patients, in combination with celiac plexus block. Some slight adverse effects (*e.g.*, increased serum amylase, abdominal distension and nausea, abdominal bleeding) had disappeared by 3 wk, spontaneously or after symptomatic treatment. A significant difference was found between pretreatment and post-treatment pain frequency ($P = 0.0019$), regardless of the presence of advanced ($P = 0.0096$) or metastatic ($P = 0.0072$) cancer, and pain control was reported to last for more than 8 wk, without severe side effects[92].

Radio Embolization (RE) is a form of brachytherapy, which involves the direct intra-arterial delivery of radioactive isotopes close to or into a tumour. RE with intravascular yttrium-90 microspheres has been shown to be a safe and efficacious treatment of unresectable primary and metastatic hepatic tumours. RE is well tolerated with minimal toxicity. Patients may experience a short lasting post embolization syndrome, characterized by fatigue, nausea, abdominal pain, and/or a transient rise in liver function tests. RE for the treatment of liver metastasis from PC is investigational[93].

MWA is an emerging modality, performed either under percutaneous ultrasound guidance or through a laparotomy. Although operative temperatures may be higher with MW than with RFA, heat sink effects are less prominent, with less procedure related pain. Multiple probes can be used at the same time, reducing operative time. In a retrospective series[94], 10 patients with unresectable LAPC were treated with MW and palliative bypass surgery. In 5 of them MW was administered percutaneously, while in the other 5 it was delivered during laparotomy. One late major complication occurred, without any visceral injury being detected. No patient underwent further surgery. All patients had an improvement in QOL. In conclusion, MW ablation is a feasible approach in the palliative treatment of PC, but further studies are necessary.

Trans Artherial Chemo Embolization it is an interventional radiology procedure, of intra-arterial catheter-based chemotherapy. The selective administration of small drug-coated particles allow high doses directly to the tumour bed while sparing the surrounding liver tissue. For reported very limited experience, regarding liver metastasis from PC, its use is purely investigational[95].

PDT is a minimally invasive and safe method of treating cancer using an intravenous adinistered photosensitizer, activated by a specific wavelength of light, to kill tumour cells. The activated photosensitizer, produces singlet oxygen from molecular oxygen, which in turn causes tumour necrosis. There is also indirect cell death caused by induced hypoxia through tumour vasculature damage, without significant damage to connective tissues. The VERTPAC-01 trial investigated the safety and efficacy of PDT in 15 patients with LAPC using Verteroporphin. In 11 of 13 assessable patients, tumour size was stable at 1 mo, and in 6 of them stability was maintained at 3 mo. The technique proved to be feasible and safe and the authors concluded that it warrants further studies and may have a role in the multimodal treatment of PC[96].

HIFU The intention of a HIFU treatment is to deliver ultrasound energy to a well-defined targeted volume at depth, and to induce complete coagulation necrosis of the tumour. It can be administered with continuous or pulsed modality. HIFU doesn't need the placement of a needle and it is characterized by a low rate of adverse events[87]. In a recent trial of HIFU, administered in addition chemotherapy or chemoradiotherapy to 30 patients with stage Ⅲ/Ⅳ PC, the rate of symptom relief effect was 66.7% and the disease control-rate was 86% (mainly stable disease). The procedure was well tolerated, with moderate adverse events occurred in 10% of cases, mainly pseudocyst formation and mild pancreatitis[97,98].

IRE is a nonthermal ablative technique that uses ultrashort pulsed but very strong electrical fields. Formation of nanopores and micropores in the lipid bilayer of cell membranes induces cancer cells apoptosis[99,100]. No results of randomised trial are currently available. In the largest prospective series in LAPC, 54 patients have undergone an open approach IRE for unresectable cancer. The outcomes were compared to those obtained in 85 matched stage Ⅲ patients, treated with chemotherapy and radiation therapy alone. The IRE procedure was given in addition to standard treatment: Chemotherapy or chemoradiation therapy in forty-nine (90%) patients and chemotherapy or chemoradiation after IRE in forty patients (73%). The 90 d mortality was 2%. IRE was associated with an increase in local progression-free survival (14 mo *vs* 6 mo; $P = 0.01$),

distant progression-free survival (15 *vs* mo 9 mo; P = 0.02), and OS (20 mo *vs* 13 mo; P = 0.03)[101]. In a percutaneous approach IRE study, in 14 patients IRE was performed. All patients had received chemotherapy or radiation previously. Two patients underwent surgical resection with margin-negative and both had long disease-free survival (11 and 14 mo). There were no procedure-related deaths[100]. IRE appears to be feasible and safe, but it doesn't improve OS compared with standard treatments, because of rapid progression of distant metastasis. IRE could be used as an additional treatment when surgical resection is possible but with hight risk of margin-positive (R1).

Regional intra-arterial chemotherapy: Intra-arterial chemotherapy aims both to increase drug concentrations in tumours tissues and to maintain low systemic drug levels. A recent meta-analysis and systematic review of randomised controlled trials included 155 patients receiving Regional Intra-Arterial Chemotherapy (RIAC) and 143 patients receiving systemic chemotherapy[102]. The RIAC efficacy seems to be evidenced by response rates of 58.06% with RIAC *vs* 29.37% with systemic treatment. Also, clinical benefit seems to be in favor of the RIAC (78.06% *vs* 29.37% respectively). The median survival time with RIAC (5-21 mo) was longer than for systemic chemotherapy (2.7-14 mo). Side effects were fewer in patients treated with RIAC (49.03%) than in those treated with systemic chemotherapy (71.33%), but the only statistically significant difference was for hematological side effects (60.87% *vs* 85.71% respectively). Despite these results, RIAC is not commonly used in clinical practice because it is invasive and requests hospitalization, with consequent risks of complications. A possible application of this technique, to further explore, could be in the neoadjuvant setting, in order to increase the resection rates and then probably OS with local advanced PC[103].

Unmet needs and proposals

Locoregional therapies alternative to surgery and radiation, for unresectable PC, are attractive and a number of studies demonstrated that they are feasible and reproducible. All of them should be considered as having a complementary role in the multimodal management care model. In metastatic setting, few data are available, most of them concerning RFA, these could be a safe and feasible strategy for extending survival in selected patients. Albeit several studies have anyway shown improved outcomes (changes in stage, diagnosis, or treatment plan), long-term survival data are lacking. Large prospective randomised studies are mandatory to assess the efficacy of these techniques and define their role/position in future treatment algorithms for the management of LAPC. Their main interest is in the context of a multidisciplinary-team patient evaluation, that is the best option to help patients cope with this challenging cancer[104-106].

IS MOLECULAR BIOLOGY THE NEW ROUTE IN DIAGNOSIS AND THERAPY?

Current knowledge

PC has a mean of 50 to 60 somatic mutations in protein-coding genes and at least 4 to 6 of them are driver mutation-driven in proto-oncogenes or tumour suppressor genes[106]. In addition, these somatic mutations are distributed in several key molecular pathways, probably ten or more[107], thus facilitating the acquisition of both intrinsic and secondary resistance to chemotherapy and targeted agents.

The commonest genome aberrations of PC are[108]: (1) the chromosomal rearrangements, widespread among the cancer genome and very common; (2) the KRAS oncogene mutated in nearly 90% of PC; (3) the tumour-suppressor genes TP53, SMAD4 e CDKN2A inactivated in more than 50% of cases.

Some key features of PC have been recently elucidated by the results of whole genome analyses of 100 cases of PC[109]. In particular, according to structural variations profiles and implicated molecular mechanisms underlying, PC can be classified into 4 subtypes defined as: (1) "stable", 20% of cases, with low (< 50) structural variation events and frequent aneuploidy, suggesting defects in cell cycle/mitosis mechanisms; (2) "locally rearranged", 30% of all samples, exhibiting a significant focal event in 1 or 2 chromosomes. In nearly one-third of cases it was present a gain of known oncogenes, mainly KRAS, SOX9 and GATA6, but also therapeutic target genes as ERBB2, MET, CDK6, PIK3CA, but with a low individual prevalence; (3) "scattered", 36% of samples, exhibiting a moderate range of non-random chromosomal damage and less than 200 structural variation events; (4) "unstable", 14% of cases, with a large (> 200) number of structural variation events suggesting defects in DNA maintenance including both mutations in BRCA pathway and mutations in other pathways involved in genomic instability, with a possible association with sensitivity to platinum agents and PARP inhibitors.

Moreover, the techniques of circulating cell-free tumour DNA (cfct-DNA) or circulating tumour cells, even if there are still very limited data in PC, seem a very interesting and promising way to study dynamically the global amount of cancer mutation. The cfct-DNA can be detected in respectively > 75% and 48% of patients with advanced or localized PC[110].

Unfortunately, up to now no single targeted agent, in preliminary clinical and preclinical data, has demonstrated to have a relevant impact on the natural history of metastatic PC. Strategies employed in these trials have involved mainly the inhibition of EGFR-MEK pathway and farnesyl-transferase. Targeted agents have been studied in PC mainly in combination with standard chemotherapy, in most cases gemcitabine. The association of chemotherapy with targeted agents blocking a single pathway in a molecularly unsele-

Table 2 Available clinical results about multitarget inhibition in pancreatic adenocarcinoma

Combination	Molecular targets	Frequence of mutation[1]	Setting/combination	Results
Everolimus + Erlotinib (Javle 2010)	mTOR + EGFR	+, +	Phase Ⅱ, 16 patients, chemo-refractory	No responses
Bevacizumab + Erlotinib (Van Cutsem 2009)	VEGF + EGFR	+, +	Phase Ⅲ, 301 patients, plus GEM + ERLO	No increase in OS respect GEM+ ERLO
Cixutumumab + Erlotinib (Philip 2014)	IGF-1R + EGFR	+, +	Phase Ⅰb/Ⅱ, 126 patients, plus GEM	No increase in PFS and OS respect GEM + ERLO
Sunitinb (Bergmann 2015)	VEGFR + PDGFR	+, +	Phase Ⅱ, 106 patients, 1st line, plus GEM	No increase in TTP and OS respect GEM

GEM: Gemcitabine; ERLO: Erlotinib; Nab-P: Nab-paclitaxel; OS: Overall survival; PFS: Progression-free survival; mTOR: Mammalian target of rapamycin; EGFR: Epidermal growth factor receptor; IGF-1R: Insulin-like growth factor 1 receptor; VEGFR: Vascular endothelial growth factor receptor; PDGFR: Platelet-derived growth factor receptor; TTP: Time to progression. [1]To obtain this parameter, a mean between the frequency of somatic mutations in the target was calculated from the paper by Biankin *et al*[108] and Waddel *et al*[109], Figure 1. Three parameter were possible: +++ ⩾ 75%, ++ > 50%, + ⩽ 50%.

Table 3 Ongoing clinical trials about multitarget inhibition in pancreatic adenocarcinoma

Combination	Target	Frequence of mutation[1]	Setting	Clinical trial identifier[2]	Expected end of accrual
Dovitinib	FGRFR + PDGFR + VEGFR	+, +, +	Phase Ⅱ, + GEM and CAPE	NCT01497392	Sep-16
Trastuzumab + Erlotinib	EGFR2 + EGFR	+, +	Phase Ⅱ, + GEM	NCT01204372	Apr-15
MEK162 + Ganitumab	MEK1 + IGF-1R	+, +	Phase Ⅱ, multi-disease, chemorefractory	NCT01562899	Apr-15

GEM: Gemcitabine; MEK 1: Mitogen-activated extracellular signal regulated kinase 1; CAPE: Capecitabine. [1]To obtain this parameter, a mean between the frequency of somatic mutations in the target was calculated from the paper by Biankin *et al*[108] and Waddel *et al*[109], Figure 1. Three parameter were possible: +++ ⩾ 75%, ++ > 50%, + ⩽ 50%; [2]Data Available from: URL: http// www.clinicaltrials.gov (last access 2015 May 24).

Table 4 Available and ongoing clinical results about drugs targeting mainly stroma in pancreatic adenocarcinoma

Combination	Target (s)	Setting	Clinical trial identifier[1]	Expected end of accrual
Demcizumab (Gracian 2014)	Cancer stem cells by DLL4 inhibition	Phase Ⅰb, plus GEM +/- Nab-P	NCT01189929	Concluded. presented at ASCO 2014: Increase in ORR, cardiovascular toxicity
Ruxolitinib (Hurwitz 2014)	Inflammation by JAK/STAT inhibition	Phase Ⅱ, 2nd line, plus CAPE	NCT01423604	Concluded. presented at ASCO 2014: Benefit in patients with elevated CRP
PEGPH20 (Hingorani 2013)S	HA by Pegylated-hyaluronidase	Phase Ⅱ, 1st line, plus GEM	NCT01453153	Concluded. presented at ASCO 2013: ORR 33%, especially in patients with high HA expression
″	″	Phase Ⅱ, plus GEM + Nab-P	NCT01839487	July 16

CAPE: Capecitabine; CRP: C-reactive protein; GEM: Gemcitabine; HA: Hyaluronic acid; ORR: Objective response rate; DLL4: Delta like ligand 4; ASCO: American society of clinical oncology; JAK/STAT: Janus kinase/signal transducer and activator of transcription; Nab-P: Nab-paclitaxel. [1]Data Available from: URL: http// www.clinicaltrials.gov (last access 2015 May 24).

cted PC population has not led to relevant increase of treatment outcomes as, for example, in the case of erlotinib, tipifarnib, anti MEK-drugs like selumetinib and trametinib, trastuzumab and bevacizumab. Strategies involving a multiple blockade seem more promising due to the complexity of PC genome: Available clinical data and ongoing trials in this setting are described in Tables 2 and 3[111-115]. Data about multiple pathway inhibition strategies are available only in preclinical models[116].

A very peculiar feature of PC is its ability to promote the growth of a complex peritumoural stroma, with desmoplasia and altered vascularization. This surrounding environment greatly hinders antitumour drugs to reach active concentration into the tumour[117]. As far as inhibition of stroma is concerned, some recent preclinical data showed a possible benefit from hyaluronidase, an enzyme able to dissolve extracellular matrix[118], which is being tested in association with chemotherapy. Moreover, in a preclinical model, the concurrent administration of gemcitabine plus saridegib, a multiple Hedgehog signalling pathway inhibitor, increased vascular density and intratumoural concentration of gemcitabine. Clinical data about these approaches are resumed in Table 4[119-122]. Results from phase Ⅱ and phase Ⅲ trials exploring other treatment targets, as Hedgehog pathway, angiogenesis and immune regulation are expected in the next years[111]. Both the genomic instability and the complex tumour-stroma interactions promote the development of a relevant spatial and temporal molecular heterogeneity[123].

PC stem cells (PCSCs) are believed to promote tumour growth and progression through a number of mechanisms, including differentiation into bulk tumour cells, metastasis, alteration of adjacent stromal cells, and

evasion of conventional therapies. Possible strategies to target PCSCs involve inhibiting specific proteins and pathways, such as c-Met, Alk-4, Notch pathway and gamma-secretase. These approaches are in a preclinical stage of development[124] (Table 3).

Regarding epigenetic modifications, key tumour suppressors genes, with a well-established role in PC, may be altered through hypermethylation. And permissive histone modifications may be the cause of oncogenes upregulation. Moreover, factors involved in tumour invasiveness can be aberrantly expressed through deregulated microRNA. In this perspective, a potential therapeutical target in order to modify epigenetics is the enzyme enhancer of zeste homolog 2 (codified by the gene EZH2) which, when overexpressed, contributes to PC growth. Only preclinical data are available[125].

Unmet needs and proposals

Although presenting a molecular landscape shared with other neoplasms (*e.g.,* colorectal and breast cancer) PC has a worse prognosis. As described above, the very complex genomic landscape with the simultaneous activation of multiple relevant pathways and the complexity of cancer microenvironment could be key factors in determining the disappointing results of targeted agents in PC. From a clinical point of view, due to the increasing availability of targeted agents, a deeper understanding of PC's biology is desirable and remains the mainstay of clinical research in PC.

The recent availability of next-generation sequencing techniques and the creation of joined multicenter working groups has greatly increased the knowledge of the mutational landscape of PC and raised the possibility to perform a personalized medicine even in such as "distressing" setting[126].

Starting from the current knowledges, possible research strategies to improve the results of targeted agents could be the simultaneous inhibition of multiple pathways, the combination of stroma targeting agents with other possibly effective drugs (*e.g.,* chemotherapy), targeting PCSCs, targeting epigenetic alterations.

Moreover, the heterogeneity of the disease has to be taken in account. A better knowledge of pathways and targets and of distinct genetic features can help in defining prognostic and predictive factors to select or stratify patients accrued in clinical trials. As an example, a significant proportion of subtype 2/locally arranged PC harbor mutations in "druggable" genes (as ERBB2 and MET) and many subtype 4/unstable PCs have defects in DNA repair mechanisms suggesting the hypothesis this group could have a particular sensitivity to platinum agents and PARP inhibitors, to prospectively test in further trials.

CONCLUSION

Despite new biomolecular knowledge and the efforts to define new therapeutic approaches in PC in all the setting of care, there are still many unresolved issues. In fact, starting from the definition of resectable disease to the evaluation of the best locoregional treatment in LAPC, everything today is constantly evolving in clinical practice and there is still no uniformity of view from center to center. Moreover in the era of cancer treatment based on specific molecular alterations and of immunotherapy rather than chemotherapy, PC seems to go against the grain. Disappointing results of targeted therapy studies have not allowed us to add new weapons to systemic treatments, and immunotherapy is still object of clinical trials. Furthermore, the high biological aggressiveness of PC and the incomplete knowledge of the biology of this disease have hampered the development of new more efficacious strategies of target selection and drug development. Hence, PC is still an undefeated enemy, with high and early mortality, high genetic complexity and lack of prognostic and predictive factors that can drive the clinical decision. Efforts to define and validate prognostic and predictive factors as well as the genetic and molecular basis that can help the oncologist in everyday clinical practice must be carried over. A multidisciplinary team is crucial in order to rapidly and effectively translate clinical and preclinical findings into valuable and applicable data for the clinical setting.

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REVIEW

Current status of ultrasound-guided surgery in the treatment of breast cancer

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Abstract

The primary goal of breast-conserving surgery (BCS) is to obtain tumour-free resection margins. Margins positive or focally positive for tumour cells are associated with a high risk of local recurrence, and in the case of tumour-positive margins, re-excision or even mastectomy are sometimes needed to achieve definite clear margins. Unfortunately, tumour-involved margins and re-excisions after lumpectomy are still reported in up to 40% of patients and additionally, unnecessary large excision volumes are described. A secondary goal of BCS is the cosmetic outcome and one of the main determinants of worse cosmetic outcome is a large excision volume. Up to 30% of unsatisfied cosmetic outcome is reported. Therefore, the search for better surgical techniques to improve margin status, excision volume and consequently, cosmetic outcome has continued. Nowadays, the most commonly used localization methods for BCS of non-palpable breast cancers are wire-guided localization (WGL) and radio-guided localization (RGL). WGL and RGL are invasive procedures that need to be performed pre-operatively with technical and scheduling difficulties. For palpable breast cancer, tumour excision is usually guided by tactile skills of the surgeon performing "blind" surgery. One of the surgical techniques pursuing the aims of radicality and small excision volumes includes intra-operative ultrasound (IOUS). The best evidence available demonstrates benefits of IOUS with a significantly high proportion of negative margins compared with other localization techniques in palpable and non-palpable breast cancer. Additionally, IOUS is non-invasive, easy to learn and can centralize the tumour in the excised specimen with low amount of healthy breast tissue

being excised. This could lead to better cosmetic results of BCS. Despite the advantages of IOUS, only a small amount of surgeons are performing this technique. This review aims to highlight the position of ultrasound-guided surgery for malignant breast tumours in the search for better oncological and cosmetic outcomes.

Key words: Breast neoplasms; Segmental; Surgery; Ultrasonography; Mastectomy; Cosmetics; Margins; Volume status; Wire localization; Radioguided surgery

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Core tip: Despite improved survival and local recurrence rates of breast cancer patients in the past years, there is still much to be gained in surgical treatment. Unacceptable rates of involved margins are described, with up to 25% of the patients undergoing re-excision after breast conserving surgery. The most frequently used excision methods are wire-guided and radioguided localization for non-palpable tumours and palpation-guided localization for palpable tumours. Although ultrasound-guided surgery is a simple and non-invasive technique, it is not frequently used. This review highlights the position of ultrasound-guided surgery for breast cancer in the search for better oncological and cosmetic outcomes.

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INTRODUCTION

Breast conserving therapy

Breast cancer is the most commonly diagnosed cancer worldwide, including low and middle-income countries and incidence is rising with an estimated 1.67 million new cancer cases diagnosed in 2012 (25% of all cancers). In the western world, approximately 1 in 8 women (13%) will develop breast cancer over the course of their lifetime[1].

Since disease-free and overall survival rates after breast conserving therapy (BCT) are known to be comparable with patients treated by mastectomy, BCT is established as the standard of care in women with early stage breast cancer[2-4]. BCT refers to a combination of breast conserving surgery (BCS) followed by whole breast irradiation to eradicate any microscopic residual disease. The widespread use of screening and the development of more effective treatment methods have been associated with improvement in terms of overall survival and recurrence rate, with 5 year survival rates for early stage (Ⅰ and Ⅱ) of more than 92%[5-8].

Primary goal: The primary goal of BCS is to remove the tumour with clear margins. Margins positive or focally positive for tumour cells are associated with a high risk of local recurrence and, in the case of tumour positive margins, re-excision or even mastectomy must be performed[9,10].

Incidences for tumour-involved margins in BCS have been reported up to 40%[11-15]. However, direct comparison of studies is difficult due to the use of varying definitions for positive margins, for instance a "close margin" is used for either a positive and negative margin. In the United Kingdom previous guidelines recommended a margin > 2 mm, however current guidelines do not encompass a clear definition on margin status and they recommend breast units to have local guidelines regarding acceptable margin width[16]. Danish National Guidelines recommend tumour-free margins $\geqslant$ 2 mm[17]. Other European countries such as Germany and France have BCS guidelines on margin status that indicate that patients with margins $\leqslant$ 1 mm should undergo additional surgery[18,19]. In the Netherlands and the United States guidelines for BCS are stating all specimens without tumour-cells at the inked margins are tumour free margins, and these specimens do not necessitate additional local treatment such as surgery or radiotherapy[10,20].

In the recent St. Gallen International Breast Cancer Conference 2015, the majority of the panelists agreed that the minimal acceptable surgical margin was "no ink on invasive tumor" in women undergoing BCS for invasive breast cancer and proceeding to standard radiation and adjuvant systemic therapy[21]. However, two recent surveys in the United States have reported that, against the national breast cancer guidelines, 85% of breast surgeons do not accept a tumour-free margin less than 1 mm[22,23].

In the United States, a striking number of approximately one-fourth patients who undergo initial BCS for breast cancer will have a subsequent operative intervention[24]. Evidently there remains an international controversy regarding the definition of tumour margins. It is, however, important to note that a tumour-free resection margin of > 1 mm is unrelated to local recurrence or overall survival, and the range of local recurrence rates is 2%-5%[5,25].

Secondary goals: Together with increasing breast cancer incidence, the improved outcome has resulted in a growing population of breast cancer survivors and there has been considerable interest in secondary goals such as cosmetic outcome and quality of life of (QOL)[26-33]. The achievement of tumour-free margins during BCS is of great importance for local recurrence but also for the cosmetic outcome. Positive resection margins result in additional treatment, such as higher radiotherapy dose, re-excisions and even mastectomy, these additional therapies will ensure oncological safety but negatively influencing the cosmetic outcome[30,31]. Besides young age, central inner quadrant localization, axillary dissection, re-excision and complications, larger

excision volumes and secondary radiotherapy (administration of boost and whole breast irradiation dose) are the two key determinants of cosmetic outcome[29,32,33].

Poor cosmetic outcomes are observed in up to 30% of patients after BCS[32-34]. In a large survey among 963 women treated with BCS for breast cancer, cosmetic results were scored as 3.4 on a 5-point scale with from 1 (very dissatisfied) to 5 (very satisfied)[35].

The importance of achieving optimal oncological control may lead to an unnecessarily large resection of breast tissue. Literature shows that cosmetic failure rates are significantly higher when a lump exceeds 50-100 cm^3[29,32,36-37]. However, these studies dated from the 90's and recent data on volume are scare. When the surgical accuracy of BCS is improved by a higher rate of margin clearance and smaller excision volume, this will improve not only oncological outcome, but also improves patient satisfaction and cosmetic outcome.

In many cases, an unnecessarily large volume of healthy breast tissue is excised along with the tumour, while clear margins are not assured[13,38,39]. High excision volumes are rarely related to the size of the tumour. Therefore, as a tool to define the amount of tumour and the excess healthy breast tissue in a surgical specimen, the calculated resection ratio (CRR) was introduced, indicating excess healthy tissue resection[13]. The CRR represents a comparison of the total resection volume to the optimal resection volume. This means that in an ideal situation, the specimen volume is equal or smaller than the optimal resection volume and the CRR $\leqslant$ 1. For example, in a retrospective study, 10.7% of 726 patients with T1-T2 tumours still had positive or focally positive margins when the CRR was > 4.0, meaning that the tumour is often located eccentrically in the surgical specimen[13].

Despite the ongoing development of techniques for diagnosing and treating breast cancer, the current techniques BCS in many cases do not meet primary and secondary goals and there is still much to be gained. This review aims to highlight the position of ultrasound-guided surgery for breast tumours in the search for better oncological and cosmetic outcomes.

Non-palpable breast cancer

Due to the development of imaging techniques and screening programs, the incidence of non-palpable breast cancer has increased with up one third of the diagnosed breast cancer being non-palpable. In this group, DCIS represents a challenging problem for breast-conserving surgery (BCS) given that it is typically non-palpable and non-contiguous. Different management procedures are used to remove the non-palpable tumour with optimal resection margins: Wire guided localization (WGL), radio guided localization (RGL) or intra-operative ultrasound-guided surgery (IOUS)[33-52]. Despite improved techniques intra-operatively, unfortunately no assessment can ensure clear lumpectomy margins during surgery.

Wire guided surgery: WGL has been the most commonly used technique for non-palpable breast cancer in the past years. Pre-operatively, a thin, hooked wire is placed into a non-palpable lesion under mammographic, sonographic, or CT guidance. When the lesion is visible on ultrasound, this is the easiest approach because the wire can directly be placed under ultrasound guidance. The mammographic approach is based on measurements of distances between the lesion and the nipple (or other reference points) performed on the two projections of the mammogram. Subsequent mammograms are then obtained to reposition the wire more accurately, and a confirmatory mammogram is finally obtained.

After WGL, positive margins are described in 10%-43% of patients with up to 40% re-excisions after initial surgery[38,42-44]. The reported CRR after WGL is 2.8-4.3[13,39]. Volumes and cosmetic outcome must be compared to other techniques in the same study groups and therefore are mentioned in the next paragraph.

Even though WGL has proven to be a useful localization tool, it is associated with several shortcomings. The wire tip gives no indication of the extent of the tumour and the amount of tissue to be excised is estimated by the surgeon intraoperatively. This could explain the high amount of incomplete tumour resections.

Additionally, the wire may migrate, become displaced or transected[45]. Also the extra pre-operative procedure is demanding for the patient pre-operatively with pain and discomfort caused by the wire.

Radio-guided surgery: Due to the technical and scheduling difficulties of WGL, radio-guided surgery (RGS) was developed, in the form of radio-guided occult lesion localization (ROLL) and radio-guided seed localization (RSL). ROLL uses the radiotracer which is injected intra-tumourally for the sentinel lymph node procures to guide surgical excision of the primary tumour. The gamma-detecting probe guides the localization of the lesion throughout the surgical procedure. In RSL, a radio-opaque titanium seed containing Iodine-125 is placed into the tumor under stereotactic or ultrasound guidance. The seed can be placed days to weeks preoperatively. Again, a handheld gamma probe is used to guide surgical resection of the tumor during surgery.

RGS has been prospectively compared to WGL. Overall tumour free margin rates of RGS range from 73% to 96% with a weighted average of 90%[42,47,48]. The range of re-excisions reported is 4.6%-27%[38,45].

Sajid *et al*[42] showed in a meta-analysis the risk of having positive resection margins following WGL was higher than ROLL. (OR = 0.47; 95%CI: 0.22-0.99; z = 1.99; P < 0.05). A systematic review by Lovrics *et al*[40] demonstrates that WGL produces higher positive margins rates and more re-operations. However, Postma in their randomized controlled trial, showed WGL is comparable to ROLL in terms of complete tumour excision and re-excision rates and ROLL was leading to larger excision volumes (71 cm^3 *vs* 64 cm^3). The average excision volume of five studies including 1077 patients with DCIS or invasive breast cancer was 86 cm^3[47].

Further evaluation about the effect of the specimen volume on margins status demonstrated no correlation and was difficult to interpret because of varying patient populations and the fact that excision volumes were missing in most studies.

One retrospective study mentioned CRR with a significant, and clinically relevant difference for ROLL (CRR = 3.8) compared to WGL (CRR = 2.8, $P = 0.043$)[15]. However, in both techniques CRR is more than one, meaning a large amount of healthy tissue is resected. Cosmetic outcomes assessed were similar after WGL and RGS in a prospective, randomized trial. Most patients rated their overall cosmesis as "excellent" or "good" (76% WGL, 80% RSL). This comparable outcome may reflect the similar reoperation rates and volumes of excision between groups[34]. An advantage of RGS compared to WGL is the fact that it can be done weeks pre-operatively. However, it still requires radioactive material being transported and attendance to the radiology department prior to surgery. Therefore, it does not overcome the scheduling conflicts between radiology or nuclear department and the surgery department. Additionally, with RGS, the borders of the tumour are not visible during surgery, there is only a diffusion zone guided by the gamma probe. The risk of seed migration and failure of seed placement ranged from 0%-0.6% and 0%-7.2% respectively[45]. Unfortunately RGS remains having an invasive component with discomfort for the patient.

Intra operative ultrasound: Since high-frequency real-time ultrasonography was introduced in the 1970s, technological advances have improved sensitivity and reduced the size of ultrasound scanners, making them practical and able to be used close to the bed-side or in the operating theatre. In the 1980s Schwartz *et al*[48] were the first to describe IOUS as an effective and accurate technique for localizing non-palpable breast masses, facilitating excision and diagnosis with a minimum of patient inconvenience and discomfort as well as utilizing hospital resources efficiently. The ideal localization procedure would be non-stressful for the patient and would allow accurate targeting and removal of the lesion central in the specimen, while removing as little tissue as possible with tumour-free margins. In the current series, IOUS met these objectives better than did WGL. The rate of successful intra-operative localization in, ultrasound visible, non-palpable tumours varies between 95%-100%[49-53]. In 2002, a randomized clinical trial from Rahusen *et al*[53] involving 49 patients with non-palpable breast cancer, demonstrated IOUS to be superior to WGL concerning margin clearance. In 2013, a systematic review and meta-analysis of patients with non-palpable breast cancer treated with IOUS *vs* WGL was performed. One RCT[50] and nine cohort studies with control WGL groups were identified, containing 739 patients[15,39,52-58]. The rate of involved surgical margins for IOUS varies between 0%-19%. In the effects model there was a statistically significant difference between IOUS and WGL in terms of tumour-free margins favoring IOUS. (OR = 0.52; 95%CI: 0.38-0.71)[49]. Another meta-analysis of Pan *et al*[59] also demonstrated a statistically significant increase in the incidence of pathologically negative margins with the use of IOUS, for both non-palpable and palpable breast cancers. A limitation of a meta-analysis by Ahmed *et al*[49] is the heterogeneity of *in situ* cancer (DCIS) amongst the small cohorts studies. The trend is towards higher percentages of *in situ* cancer in the WGL groups within the meta-analysis, but this trend does not reach a statistically significant value ($P = 0.65$). Because patients were not randomized to either cohort, more difficult cases with extensive DCIS may have been selectively approached with bracketed needle localization, whereas more "straightforward" cases of limited disease may have been chosen for ultrasound-guided excision.

Excision volumes for non-palpable breast cancer after IOUS and WGL are mostly mentioned in (retrospective) cohort-controlled studies. Different outcome of resection volume between groups were seen ranging from no difference to smaller volume in IOUS[39,58]. However, selection bias has occurred in the localization technique to assess large and clearly visible tumours without microcalcification; on average there was a larger tumour size in the IOUS group. This is indicating that, with IOUS more optimal resection volumes are obtained. The excess breast tissue resection therefore must be determined using the CRR. For example, Barentsz *et al*[39] showed total resection volumes was similar in both groups. (56.6 cm^3 *vs* 62.8 cm^3, $P = 0.66$) Because of the larger tumour size in the IOUS group, the CRR was smaller. (3.3 *vs* 4.3) in the IOUS group ($P = 0.018$). No study specifically measured cosmetic outcome after IOUS in non-palpable breast cancer compared with other techniques. However, as the volume of resection decreases and better margins are achieved, we expect cosmesis to be positively affected, as is patient satisfaction[38].

IOUS is accurate, simple and it carries a minimal risk of procedure-related complications[50,51,60]. It overcomes the issues associated with WGL an RGS because it does not require preoperative localization at the radiology or nuclear department and being a less invasive procedure. IOUS can also be used *ex vivo* to verify the presence of a tumour in a resected specimen. Downsides of IOUS include the need for or visibility of the breast lesion on ultrasound. IOUS is not very accurate for lesions presenting as clustered microcalcifications. Patients with DCIS and multifocal invasive cancer will be at increased risk for a positive margin and at increased risk for re-excision or mastectomy, also after IOUS. However, to overcome this problem, a marker which is visible on ultrasound could be placed intra-tumourally. Additionally, the availability of a surgeon with ultrasound experience or a preoperative assisting radiologist is mandatory in performing IOUS.

Palpable breast cancer

In palpable tumours, surgeons are performing blind surgery trusting on pre-operative imaging and their tactile skills, which can be problematic, especially in dense breasts[61]. A high incidence of positive margins after palpation guided surgery (PGS) up to 40% is described[13,61-63]. Moreover, it has been shown that many surgeons tend to overexcise volumes of healthy tissue in an effort to obtain adequate margins. Median excision volume of PGS is over two times too large[13].

Only a few reports have been published of the use of ultrasound-guided surgery in palpable breast cancer. In 2001, Moore and colleagues were prompted to prospectively evaluate IOUS in women with palpable breast cancer because of poor results obtained with PGS. They compared 27 patients undergoing IOUS with 24 undergoing PGS and their findings were striking. Only 3% positive tumour margins were noted in the ultrasound-guided surgery group compared with 29% in the palpation-guided surgery group (P < 005)[61]. After this, other retrospective studies showed IOUS of palpable breast cancers to be associated with markedly reduced rates of involved margins and re-excisions[59,61-64]. The COBALT-trial was the first multicenter randomized controlled trial for palpable cancer comparing IOUS with the PGS, in order to improve both oncological and cosmetic outcomes. The primary results of this trial showed a dramatic difference in margin involvement with 3% of tumour-involved margins for the invasive component in the IOUS-group compared to 17% in the PGS-group, and thus a significant decrease in additional treatment required in the IOUS group [2% re-excision and 9% boost in IOUS (total, 11%) *vs* 7% mastectomy, 4% re-excision and 16% boost in the PGS group (total, 27%)][65]. Moore *et al*[61] found that the volume of the lumpectomy specimen was smaller in the IOUS group (104 cm^3) *vs* their palpation-guided group (114 cm^3). In the COBALT study, IOUS results in significantly reduced excision volumes and CRR compared with PGS[65]. A CRR greater than 2.0 was seen in only three (5%) women in the ultrasound-guided surgery group *vs* 20 (29%) patients in the palpation-guided surgery arm (P < 0.0001). Minor lesions of additional ductal carcinoma *in situ* (DCIS) were found inside or within several millimeters of the invasive tumour by the pathologist in 73 (55%) of the 132 palpable tumours in the COBALT trial. Despite the fact that United States cannot always detect DCIS, the rate of tumour-free margins was high, even in cases with additional *in situ* carcinoma (11% in the IOUS group compared with 28% in the PGS group). It could be explained by the increased accuracy with IOUS in the localization of the central point of the tumour, which allowed complete resection of the additional DCIS. Earlier studies have mentioned IOUS to improve the cosmetic results and patient satisfaction in palpable breast cancer[61,63]. The COBALT-study clearly showed IOUS resulting in better cosmetic outcome than PGS; 21% of the overall responses were excellent and 6% were poor with IOUS, while 14% and 13% of responses were excellent and poor, respectively, with PGS. Consistently, IOUS had smaller odds of having worse cosmetic outcome than PGS (OR = 0.51, P = 0.045)[66].

Learning curve

Hands-on ultrasound education for surgeons and the ongoing improvements in imaging technology have made surgeon-performed breast ultrasound an effective method of identifying palpable breast lesions. With proper teaching, adequate practice, and close supervision leading to progressive independence, breast surgeons can acquire comprehensive skills that will enable them to successfully incorporate breast ultrasound and ultrasound-guided breast procedures into their clinical practice[50,51,67-72]. A weekly half-day training minimally invasive breast biopsy for breast fellows with hands-on, "live-patient" breast ultrasound training, showed proficiency in performing breast ultrasound by the 12th week[69]. In the study by Krekel *et al*[70] surgeons underwent an ultrasound-training program performing ten cases, under the strict supervision of a breast radiologist. The learning curve for surgeons to develop the adequate skills was short, after the first two supervised cases, resections reached optimal volumes. After eight procedures, surgeons acquire the expertise to perform IOUS.

Despite the good results of IOUS, the utilization of this technique amongst breast surgeons remains consistently low, with surveys of American and Australasian breast surgeons suggesting figures between 2.8%-17%, respectively[73,74]. The main reasons for the low amount of American surgeons performing IOUS were related to their radiology department with almost half stating that radiologists had prohibited them from scanning, the remainder being due to a combination of a lack of time, hospital restrictions, lack of confidence and reimbursement as well as medico-legal liability[71]. The lack of performing IOUS by breast surgeons gains more relevance with the increasing evidence of improving outcome of BCS in palpable and non-palpable breast cancer.

Cost-effectivity

With experienced surgeons, excision time is similar between IOUS and other guidance techniques, although there is extra time for the pre- and post-surgical use of the United States system which will account for 5-10 min[53,57].

In the ROLL-study, QOL effects between ROLL and WGL were similar (difference 0.00 QALYs 95%CI: -0.04-0.05). Total costs were also similar for ROLL and WGL[73]. In palpable breast cancer, a cost-benefit analysis applied to IOUS *vs* other localization techniques for invasive breast tumours evaluating costs in terms of reoperation and complication-related costs as well as the procedural costs themselves has been performed by Haloua *et al*[74] Although the cost of IOUS is more expensive, the overall cost of performing an IOUS procedure *vs* palpation only was €154 cheaper per patient

due to a reduced rate of tumour involved margins and thereby the avoidance of cost of additional treatments. Above 30 patients, use of the USS system leads to cost savings.

Future directions

Because most of the current operative techniques for BCS in palpable and non-palpable breast cancer result in a high rate of positive margins, re-excisions and resection volumes with impact on cosmetic results, surgeons have been proactive in searching for better surgical techniques of BCS in two ways.

Firstly, surgeons have continually used ways to decrease the amount of positive margins and rate of re-excisions such as cavity shaves and touch-prep or intra-operative frozen section assessment of the margins. There is still much debate about the usefulness of these methods and their influence on re-excisions and volume resected[75-80]. These methods are potentially useful as additional methods to decrease the overall volume of excised tissue and re-excisions. However, it is preferable to perform a small lumpectomy with adequate CRR and the tumour centrally in the specimen in the first place.

Another available method of intraoperative margin evaluation is the MarginProbe (Dune Medical Devices, PA, United States). This device allows for *ex vivo* evaluation of the resected specimen and is especially useful in detecting DCIS. Adjunctive use of the Margin Probe device during BCS improved surgeons' ability to identify and resect positive lumpectomy margins in the absence of intraoperative pathology assessment, reducing the number of patients requiring re-excision[81-82]. Due to the *ex vivo* use of the margin probe, we think this method could be used alongside IOUS but should not be seen as a replacement method.

Additionally, the search for improving ultrasound-guided surgery is ongoing. Technical aspects are improved, such as the development of a portable three-dimensional ultrasound systems[83]. Also, IOUS is combined with other techniques such as needle localization or intraoperative margin assessment[75,77,84]. Combined techniques are especially useful in those tumours who are non-palpable and not visible on ultrasound. Ivanovic *et al*[75] recently showed a technique of excising palpable and non-palpable breast cancer by intraoperative ultrasound with an especially constructed marking needle, being placed while the patient is anesthetized. Preliminary results showed this technique to be feasible with good oncological safety with only one patient with a positive resection margin (3%).

Secondly, the volume of normal breast tissue excised at the time of BCS must be minimized by centralizing the tumour in the surgical specimen and, in cases where a larger excision volume is necessary for oncological reasons, volume displacement and replacement techniques are utilized. This last mentioned approach is referred to as oncoplastic breast surgery (OPBS) and combines oncological resection with plastic surgery techniques in a single procedure. The term may refer to simple volume-displacement techniques or to more complex techniques of volume replacement by using local or regional flaps. The proposed benefit of OPBS is the ability to achieve wide surgical margins, with a higher chance of obtaining tumour-free resection margins than with standard BCS. A recent study showed 11.9% positive margins and a 91% breast conservation rate[85]. Additionally, it is surprising to notice in a systematic review on OPBS that tumour-free margins ranged in the included studies from 78% to 93% with OPBS, resulting in a conversion to mastectomy in 3% to 16% of all OPBS cases. However, most studies showed significant weaknesses including lack of robust design and important methodological shortcomings, negatively influencing generalizability[86]. Therefore there is a need for well-designed comparative studies to create high quality guidelines, ensuring uniform indications for OPBS in breast cancer patients.

Considering the outcomes of studies performing IOUS, the results of OPBS may also be improved with the use of ultrasound-guidance. In patients with large tumours, a high amount of volume must be resected, even with IOUS. In these cases, by performing IOUS a safe margin with minimal volume of healthy breast tissue will be excised while achieving a good cosmetic outcome. We do think that if the tumour volume to mammary volume ratio is low, IOUS could be sufficient to achieve both tumour-free surgical margins and a good cosmetic result without concomitant reconstruction techniques.

As mentioned earlier, IOUS is not applicable in every patient. However, when applicable, IOUS is an accurate, non-invasive and technically feasible method. Although we embrace new studies to improving surgical outcome and reducing the need for re-excision in BCS, it does not seems necessary to develop new and expensive techniques for patients already suitable for IOUS. Despite advances and usability of ultrasound, the main problem remains that the use of ultrasound by breast surgeons is consistently low, as is the presence of a radiologist in the operating theatre. Surgeons who wish to provide optimal, state-of the art care for patients with breast cancer should embrace IOUS. Adequate ultrasound training should be included in the surgical curriculum for breast surgical trainees.

CONCLUSION

The best evidence available demonstrates the benefits of IOUS in BCS with a high proportion of negative margins and optimum resection volumes compared with other localization techniques in palpable and non-palpable breast cancer visible on ultrasound. With intra-operative United States guidance, surgeons can more accurately delineate the tumour by direct feedback and thereby achieving a centrally localized tumour in the specimen. Next to this, IOUS is shown to be a method which can be learned easily by surgeons and being cost-effectiveness because of less additional therapy

applied as a result of negative margins. The oncological, cosmetic and logistical advantages of IOUS for patients with breast cancer have to be recognized by every surgeon performing BCS.

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REVIEW

Historical review of the causes of cancer

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Abstract

In the early 1900s, numerous seminal publications reported that high rates of cancer occurred in certain occupations. During this period, work with infectious agents produced only meager results which seemed irrelevant to humans. Then in the 1980s ground breaking evidence began to emerge that a variety of viruses also cause cancer in humans. There is now sufficient evidence of carcinogenicity in humans for human T-cell lymphotrophic virus, human immunodeficiency virus, hepatitis B virus, hepatitis C virus, human papillomavirus, Epstein-Barr virus, and human herpes virus 8 according to the International Agency for Research on Cancer (IARC). Many other causes of cancer have also been identified by the IARC, which include: Sunlight, tobacco, pharmaceuticals, hormones, alcohol, parasites, fungi, bacteria, salted fish, wood dust, and herbs. The World Cancer Research Fund and the American Institute for Cancer Research have determined additional causes of cancer, which include beta carotene, red meat, processed meats, low fibre diets, not breast feeding, obesity, increased adult height and sedentary lifestyles. In brief, a historical review of the discoveries of the causes of human cancer is presented with extended discussions of the difficulties encountered in identifying viral causes of cancer.

Key words: Infections; Causes; Cancer; Carcinogens; Historical; Etiology; International agency for research on cancer

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Core tip: The International Agency for Research on Cancer has worked for around 45 years evaluating the scientific literature, concerning the potential of around 1000 different agents to cause cancer. Those agents which were determined to definitively cause cancer in humans are reviewed from a historical perspective. It is reviewed how there were many complexities in identifying infectious agents as causes of cancer. The author incidentally discovered while writing this review that natural factors are an additional and relatively underappreciated cause of cancer.

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INTRODUCTION

The question of "what causes cancer" has intrigued people for generations. In 1950, the World Health Organization sponsored an international symposium, and the attendees were intrigued by the dramatic variations in the types of cancer found in different areas of the world[1]. It was learned that people who migrated to other countries, developed types of cancer common to their adopted countries, rather than their homelands. This implied that most cancers were caused by exposures in the environment, rather than inherited genetic factors. The symposium led to the creation of the International Agency for Research on Cancer (IARC) in 1965 which was instructed to conduct multidisciplinary investigations of the *causes* of human cancers[1,2]. The assessments of the IARC were initially based only on epidemiological evidence[3], and then later the criteria were extended to include experimental evidence[4].

There has been a widespread notion that synthetic agents are the cause of most cancers, so this review begins with a review of the discoveries of occupational and pharmaceutical agents which cause cancer, wherein it becomes evident how this opinion arose. The next section recounts how there has also been a strong suspicion that infectious agents cause cancer, and includes a description of the exhaustive search for viruses which cause cancer. This is followed by a section which discusses natural factors and non-viral infectious agents which have been demonstrated to cause cancer.

Numerous resources were frequently consulted, and influenced the selection of topics for discussion in this review. The historical treatise by Shimkin[5], the historic milestones outlined by Sirica[6], and the monograph by Ludwig Gross[7] were consulted many times. Most importantly, the monographs by the IARC were used to identify which agents have been determined to cause cancer in humans, and were frequently used to identify the earliest and most influential studies. "Food, Nutrition, Physical Activity and Prevention of Cancer: A Global Perspective" is an expert report published by the World Cancer Research Fund and the American Institute for Research on Cancer[8] which provided supplementary analyses for a variety of natural agents.

OCCUPATIONAL, PHARMACEUTICAL, AND TOBACCO

Early studies

Early epidemiological studies: The earliest carcinogens to be identified were generally associated with specific occupations. Bernardino Ramazzini[9] observed in 1713 that nuns suffered from high rates of breast cancer which he attributed to their celibate life. Percivol Pott[10] documented in 1775 that chimney sweeps frequently developed cancer of the scrotum which he deduced to be caused by their heavy exposure to soot. A century afterwards, reports emerged that a variety of other occupations were associated with increased rates of cancer. Richard von Volkmann[11] diagnosed three cases of scrotal cancer in 1875 among coal tar distillers in Germany, which was quickly followed by similar reports by other physicians[12]. Joseph Bell[13] described two cases of scrotal cancer among shale oil workers in Scotland in 1876, and commented that the cancer was quite common among shale oil workers. Harting and Hesse documented in 1879 that miners in the Black Forest regions of Schneeberg in Germany and Joachimsthal in Czechoslovakia suffered from a high mortality due to lung cancer[14,15]. Ludwig Rhen[16,17] reported in 1895 that long term dye workers in Germany frequently perished of bladder cancer. Wilhelm Conrad Röntgen[18] discovered X-rays in 1895, which were heralded as a phenomenal discovery, because they permitted the painless visualization of bones. The early radiologists routinely tested the performance of their equipment by exposing their hands. Then a few days after a prolonged exposure, an extremely painful skin condition termed radiodermatitis developed[19,20]. A decade after Röntgen's discovery of X-rays, case reports began emerging from many diverse areas of the world, that radiologists were succumbing to skin cancers[21,22].

A few non-occupational agents were also identified during this period. John Hill[23] reported in 1761 that immoderate use of tobacco snuff was associated with the occurrence of nasal cancers[24]. Sir Johnathan Hutchinson[25] observed in 1881 that patients who used a tonic which contained arsenic for extended durations frequently developed keratosis lesions which sometimes progressed to skin cancer.

Early experimental studies: In the late 1800s, there were three fundamental theories of the cause of cancer[26-28]. Virchow proposed that cancer was a product of chronic irritation[28,29]; Lobstein and Recamier, and later Cohnheim hypothesized that cancer was the result of displaced embryonal tissue[28,29]; others surmised that cancer was caused by an infectious (or parasitic) agent[27,28,30]. Numerous researchers attempted to induce cancer in experimental animals, based on one of these theories. However, experiments to produce tumors with irritating chemicals produced only benign growths[31]. Work to prove Cohnheim's theory by transplanting embryonal or fetal tissue into adult hosts similarly failed to induce malignant growths[32]. A broad range of microbes were identified in cancerous growths. However attempts to extract the microbes and produce cancers, could not induce cancers reproducibly[28]. Experimental induction of cancer was considered to be important, because this was expected facilitate the development of preventative measures and effective treatments[32].

In 1908, Ellermann and Bang[33,34] reported that a cell-free filtrate caused a leukemia in chickens, and Peyton Rous[35,36] reported that a cell-free filtrate produced

a sarcoma in chickens shortly afterwards. However, work with chickens seemed to be irrelevant to humans, so efforts to produce experimental cancer based on the other theories continued unabated.

Jean Clunet designed an experiment which simulated the procedures of early radiologists, who developed radiation burns after prolonged exposures to X-rays[37,38]. He administered X-rays to four rats, at dosages sufficient to induce epidermal ulcerations, then allowed the lesions to heal for a few days, and repeated the exposure[37,38]. Cancer developed in one of two surviving rats at the site of ulceration. However, the experiment was not widely accepted as a success for three reasons: Only one rat developed cancer, the tumor resembled spontaneous tumors of rats, and other experimenters had difficulty reproducing the experiment[39].

Katsusaburo Yamagiwa was a young associate professor, whom the Japanese government considered to have good potential. They sent him to Virchow's Institute in Germany, where he studied pathology from 1892-1894[26]. von Volkmann's study of skin cancers among coal tar workers had become very well known by the time of Yamagiwa's studies, with numerous other investigators reporting additional cases[40,41]. Yamagiwa was intrigued by these reports, so he devised to induce skin cancer in rabbits, by exposing them to conditions which resembled occupational exposure to coal tar. The ears of rabbit's were not known to be susceptible to spontaneous cancers, so he decided to apply tar to their ears. He reasoned that, since previous attempts to induce experimental tumors produced only benign lesions which regressed, then he should continue to apply tar when the benign lesions emerged. He surmised that the application of tar to benign lesions could promote further changes, which would progress to malignancy[40,41].

When Yamagiwa returned to Japan, he applied tar to the ears of 137 rabbits, and repeated the application every two or three days. Seven rabbits eventually developed cancerous lesions. The average cancer developed after five months of tarring; some cancers only emerged after a year of tar application[40,41]. Yamagiwa recorded the occurrence of metastasis in two of the rabbits, which confirmed the malignant nature of the tumors, and the experiment became widely regarded as the first successful experimental induction of cancer.

Numerous experimenters attempted to replicate Yamagiwa's experiment. Many endeavors to reproduce the experiment failed, which led investigators to decipher why Yamagiwa's experiment was efficacious. It became evident that many previous experimenters failed because they did not continue their treatments for a sufficient duration. Woglom colorfully reflected in 1926, that Yamagiwa and Ichikawa were possessed of "infinite patience", because they continued to apply tar for many months without evidence that cancers would develop[42]. Murray Shear[43] similarly conjectured that other investigators had terminated their experiments early, because they thought they were hopelessly "kicking a dead horse". Another reason for failure was because an insusceptible species had been chosen, since it was not appreciated that most carcinogens are species specific[42]. A further reason for Yamagiwa's success was that he chose to use many animals, because only seven of 137 rabbits developed cancer, and only two developed metastases.

1920-1950

Kennaway's experimental work: During 1920-1950, additional evidence emerged that synthetic agents were the cause of human cancer. Ernest Kennaway[44] studied many modifications of Yamagiwa's experiment. He was intrigued by observations that some fractions of coal tar induced cancer, while other fractions were ineffective. He suspected that this was due to an active component, which was present in only minute quantities, similar the vitamins in foods, or hormones in tissues which were first discovered during this period. An intense search was undertaken to identify "the cancer producing compound in coal-tar" under his direction[45,46]. Kennaway and Hieger[47] identified dibenz(a,h)anthracene in 1930 as the first pure chemical compound to induce cancer in experimental animals. This was followed by the isolation of benzo(a)pyrene as the major "cancer producing compound of coal-tar" in 1933[48]. Many other chemicals were identified that induced cancer in experimental animals during this period which are too numerous to describe, but were reviewed in 1947[49].

Occupational studies: A few additional reports of occupational carcinogens emerged during this period. During 1915-1929, young women in the United States were recruited to paint watch dials with a new florescent paint. The paint contained minute quantities of the newly discovered element named radium which illuminated the dials at night. The women ingested the isotope incidentally by pointing their paint brushes with their mouths. Shortly after they began the work, it was reported that many of them developed decreased levels of polymorphonuclear leukocytes and lymphocytes. A few years later, it was reported that numerous women were diagnosed with necrosis of the jaw bones which were frequently fatal[50]. After further follow-up, in 1929, it was recounted that the survivors commonly succumbed to osteosarcomas of the jaw bones[51,52]. X-rays, coal tar dyes and radium appeared to be astonishing discoveries when they were first introduced, but then horrendous diseases developed in those exposed to the new discoveries. The notion that synthetic agents were the cause of cancer was emerging.

During this period, the Report of the Chief Inspector of Factories and Workshops in England, described an increased incidence of nasal cancer among workers in a large nickel refining company in South Wales[53]. Machle

and Gregorius reported that men in the United States, whose employment involved industrial exposure to the fumes of chromate, developed lung cancer at a 25 fold higher rate compared to workers in other industries[54].

1950-1980

Numerous notable occupational studies: A strong groundswell of interest in cancer began to emerge in the 1950s and the disease received more systematic study. Some carcinogens were reported which caused very high rates of cancer. Robert Case worked to identify which of the 100s of chemicals used in the dyestuffs industry in England and Wales caused the high rates of bladder cancer among dye workers. Case analyzed the various combinations of chemicals had been used by persons who developed bladder cancer, and after a meticulous analysis, he deduced that 30%-50% of the workers who had long term exposure to β-naphthylamine developed bladder cancer[55]. He also estimated that about 10% of workers exposed to benzidine developed bladder cancer[55].

Another study reported that seventeen percent of a subset of workers involved in the production of 4-aminobiphenyl, (a chemical that was used as an antioxidant in the rubber industry), developed bladder cancer[56]. Six of eighteen (30%) of workers exposed to bis(chloromethyl)ether, (an important intermediate in the synthesis of organic compounds), were reported to have developed lung cancer after only six years of exposure[57,58]. A similar compound, chloromethyl methyl ether, induced lung cancer in 14 of 91 (15%) of exposed workers[59,60]. Thirty three cases of the extremely rare mesothelioma were reported among the residents of the asbestos mining area of North Western Cape Province in South Africa[61]. Karin is a small village in the Anatolian region of Turkey, which has high deposits of erionite, an asbestos-like mineral, which occurs naturally near the surface of the earth in the region. Erionite is easily cut into large blocks which have been traditionally used for construction of homes and multiple other purposes in the region[62]. Eighty-two of 179 (45.8%) deaths in the village of Karain have been reported to be due to mesothelioma[63,64].

There were discoveries of additional occupational exposures which cause cancer which were also significant, though less striking. Three cases of a very rare cancer, angiosarcoma of the liver, occurred among workers employed in the manufacture of vinyl chloride[65]. A case series of workers occupationally exposed to benzene, reported a high rate of hemocytoblastic leukemia[66]. The cause of the high rates of lung cancer among miners had been a mystery. Arsenic and cobalt were each been suspected, but during this period, it was deduced that radon emitted from uranium was the principal cause[67]. A follow-up of the British veterans of the 1914-1918 war was published. The report estimated that the veterans who were exposed to mustard gas had a twofold increase in death due to cancers of the lung and pleura over the expected number[68]. Follow-up of the survivors of the Hiroshima and Nagasaki atomic bomb explosions of 1945 was published, and it was reported that they had increased rates of leukemia, as well as other types of cancer[69].

The cigarette smoking and lung cancer controversy: Sophisticated methods for statistical analysis were developed to detect additional causes of cancer during this period. By the mid-1940s, it was evident that the rate of lung cancer was increasing at epidemic proportions, but the cause was unknown. Austin Bradford Hill sought to study medicine, but was unable when he contracted tuberculosis, so he completed a BSc in economics by correspondence while convalescing. Following this, Professor Major Greenwood mentored him in statistical methods[70,71]. Hill developed an interest in formulating mathematical methods to discern the health effects of exposure to chemicals introduced since 1900. He became involved in a committee, which designed a comprehensive epidemiological study to attempt to decipher the cause of the increasing rates of lung cancer[72]. Hill hired Richard Doll, a young physician who preferred to work with mathematics over patients[72]. A case-control study was designed which would investigate the effects of a wide variety of exposures that were new to the 1950s, which included automobile exhaust, road tars, atmospheric pollution, and cigarette smoking[72]. Doll and Hill[72,73] were surprised when their analysis showed that only cigarette smoking was correlated with the incidence of lung cancer. Doll himself was a cigarette smoker, as the harmful effects of smoking were generally unsuspected[74]. They decided to follow up with a prospective trial, in order to test for possible undetected flaws of the case-control study. After 29 mo of follow-up, thirty five lung cancer deaths occurred among 24, 389 men. The highest proportion of lung cancer cases occurred among the heaviest smokers in both the case-control and prospective studies which was interpreted as confirming that smoking was the cause[75]. Numerous other studies of smoking and lung cancer were published both before and after Doll and Hill's studies, which reported similar results. The studies were reviewed by the Royal College of Physicians of London in Great Britain in 1962[76], the United States Surgeon General in 1964[77] and more recently by Doll[78].

The studies of smoking and lung cancer were initially received with incredulity by the medical community. Firstly, because smoking was considered a benign activity, with some physicians recommending cigarette smoking because there were suggestions that smoking had various health benefits[74,79,80]. Secondly, the usefulness of mathematics to discern the cause of a disease, was a new discipline and not universally accepted[81-83]. The conventional approach to demonstrate disease causation was experimentation. The controversy concerning whether cigarette smoking causes lung cancer prompted extensive discussions of

the criteria to determine whether exposure to an agent causes a disease[81,84], and was the impetus for Hill to develop a seminal list of principles to discern whether epidemiological associations are causal[85]. The smoking and lung cancer controversy contributed strongly to the establishment of modern cancer epidemiology[1].

Pharmaceutical studies: Beginning in the late 1960s, pharmaceuticals began to be frequently identified as carcinogens. Users of high doses of analgesic mixtures containing phenacetin were reported to develop high rates of carcinoma of the renal pelvis[86]. Organ transplant recipients, who used the immunosuppressant drug azathioprine, were reported to develop high rates of lymphomas[87]. Studies of women who took diethylstilboestrol during pregnancy revealed that their female children had high rates of the extremely rare adenocarcinoma of the vagina in adulthood[88]. Post-menopausal women, who used estrogen replacement therapy, were reported to have a high risk of developing endometrial cancer[89]. Four of 5 patients who received high cumulative doses (200 g or more) of chlornaphazine, a chemical related to β-naphthylamine for treatment of Hodgkin's lymphoma, developed invasive bladder carcinomas[90,91].

Research concerning chemical warfare agents had shown that the sulphur and nitrogen mustards (β-chloroethyl sulphides and amines) exerted strong cytotoxic activity on rapidly proliferating tissue, especially lymphoid tissue, bone marrow and epithelium of the gastrointestinal tract[92]. Subsequently, numerous analogues were developed for use as therapeutic agents for the treatment of cancer. Melphalan[93], busulfan[94], and cyclophosphamide[95] were each shown to be associated with increased rates of acute nonlymphocytic leukemia (ANLL).

Medical radioisotopes were also found to be associated with high rates of cancer. Patients suffering polycythaemia vera were treated with radioactive phosphorus ($^{32}PO_4$) during this period. They developed high rates of leukemia[96]. Patients who were administered a contrast medium which contained radioactive thorium, for imaging purposes, developed high rates of the rare angiosarcoma of the liver[97,98]. The use of thorium was curiously preceded, by numerous cautions predicting its probable carcinogenic effects due to its radioactivity[99,100].

1980-present

Sophisticated occupational studies: The most obvious agents were already identified in previous periods, so many studies published after 1980 were frequently re-examinations of previously tested agents. Cogliano *et al*[101] described how the IARC relaxed the criteria which are required to classify agents as carcinogenic during this period. They recounted how agents which lacked epidemiologic evidence of carcinogenicity were permitted to be classified as group 1 carcinogens based only on mechanistic evidence. The author observed that epidemiological studies generally became larger, employed more sophisticated methods of analysis and frequently only reported modest increases.

Ortho-toluidine is an aromatic amine which belongs to the same class of chemicals as β-naphthylamine. It is used in the synthesis of dyes, herbicides, synthetic rubber and other chemicals[102]. Ward *et al*[103] reported that six of 73 workers exposed to ortho-toluidine for over ten years in a synthetic rubber manufacturing plant developed bladder cancer which yielded a standardized incidence ratio of 27.2.

A study of workers in Vermont granite manufacturing plants, reported that exposure to silica dust was associated with increased rates of lung cancer. A standardized mortality ratio (SMR) of 1.81 was reported for workers exposed to silica dust for over 30 years[104]. Exposure to diesel exhaust was also reported to be associated with a higher risk of lung cancer. A cohort of 54973 United States railway workers who were exposed to diesel exhaust for 24.8 years were estimated to have a relative risk of lung cancer of 1.40 based on 4351 lung cancer deaths[105].

Sulfuric acid has been used to remove oxides from the surfaces of steel in preparation for painting and other coating processes. Exposures to mists of sulfuric acid have been found to cause laryngeal cancer. A study of 879 steelworkers who were exposed to mists of sulfuric acid for a mean of 9.5 years reported a standardized incidence ratio of 2.30 for laryngeal cancer based on nine cases[106].

Exposure to formaldehyde was determined to cause nasopharyngeal carcinoma (NPC) during this period. Eight of 25619 workers succumbed to nasopharyngeal cancer after exposure to formaldehyde for a median of 35 years. A SMR of 2.10 was estimated from this study[107].

1,3-butadiene is a chemical used in the production of synthetic rubbers and polymers, which has been ascertained to cause non-Hodgkin's lymphoma. Four of three hundred and sixty-four men involved in 1,3-butadiene production died of non-Hodgkin's lymphomas for a SMR of 5.77[108,109].

2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD), is a large complex molecule which occurs as a by-product of some industrial chemical reactions, which has received much media attention as a probable carcinogen. Fingerhut *et al*[110] conducted a study of mortality among twelve plants in the United States, which produced chemicals contaminated with TCDD. They reported a slight increase in all cancers combined, though the increase was limited to workers with the heaviest exposures to TCDD[110].

Trichloroethylene is a solvent which has been used in the dry cleaning industry and for degreasing metal machine parts that has been determined to cause renal cell cancer. A case-control study from France reported an odds ratio of 1.96 for the development renal cell carcinoma among workers with high cumulative expo-

sure, after adjusting for various factors[111].

Ethylene oxide is used mainly as a disinfectant and sterilizing agent in medical facilities for the manufacture of sterile disposable items, which has been determined to cause lymphatic and haematopoietic cancers. The IARC examined the epidemiological evidence concerning exposure to ethylene oxide, and concluded that mortality from lymphatic and haematopoietic cancers were "only marginally elevated"[112].

Beryllium is a metal with a high strength and excellent electrical conductivity which is commonly used in electronics, which has been concluded to cause lung cancer. A study of 689 patients with beryllium lung disease estimated a SMR of 2.0 for the mortality due to lung cancer based on 28 deaths[113]. The IARC has concluded that beryllium is a group 1 carcinogen[114], though various investigators have challenged the conclusion[115].

4,4'-Methylenebis(2-chlorobenzenamine) (MOCA) is a chemical used in the synthesis of some polyurethane products which has been designated as a bladder carcinogen. Screening of 540 workers exposed to MOCA detected two noninvasive papillary tumors of the bladder[116]. The IARC designated MOCA as carcinogenic to humans based on mechanistic rationale[117].

Some of the early nuclear industry personnel in Russia were exposed to high levels of plutonium. However, the reports were originally published in Russian in "classified" reports or journals, which were unavailable to western scientists. The 1990s witnessed a relaxation of this secrecy, and it was reported that workers exposed to high levels of plutonium developed high rates of lung, liver and bone cancers[118,119]. The Chernobyl accident in 1986 resulted in release of iodine-131 into the atmosphere, which was followed by increased rates of childhood thyroid cancers in the nearby areas of Belarus, Russian Federation and the Ukraine[120].

Additional pharmaceutical studies: Additional pharmaceuticals were identified as carcinogens. Patients who had been treated with MOPP (nitrogen mustard, vincristine, procarbazine, and prednisone) for Hodgkin's disease, were reported to have an increase in ANLL[121]. A randomized trial of polycythemia vera patients reported a thirteen fold increase in ANLL among those receiving the nitrogen mustard drug chlorambucil, which was strongly related to the dosage[122]. A dose-response relationship was reported between the use of the nitrosourea drug semustine and the occurrence of ANLL[123,124]. Patients who were treated with cyclosporine immunosuppressive therapy, developed increased frequencies of lymphoma, Kaposi's sarcoma and skin cancer[125]. Patients who used tamoxifen were reported to have an increased risk of endometrial cancer[126], but to have a reduced risk of recurrence of breast cancer[127].

Etoposide is a semi-synthetic derivative of an extract of the roots and rhizomes of species of the genus Podophyllin[128]. Ratain *et al*[129] reported that 4 of 21 lung cancer patients who survived longer than one year, of a chemotherapy regimen which included etoposide, developed ANLL. Thiotepa and treosulfan are alkylating agents used in chemotherapy which have each been shown to increase the risk of various types of leukemia[130-132].

The use of combined (estrogen/progestin) oral contraceptives was reported to be associated with decreases in ovarian and endometrial cancers[133,134]. Epidemiological studies concerning the association between oral contraceptive use and breast cancer produced inconsistent results which were difficult to interpret. Some studies reported higher rates, others reported lower rates, and still others reported no effect. A reanalysis of 53297 women with breast cancer and 100239 controls from 54 epidemiological studies reported a relative risk of 1.24 (95%CI: 1.15-1.33) among current users, and a relative risk of 1.07 (95%CI: 1.02-1.3) among previous users who discontinued usage for 5-9 years[135].

Emerging studies of possible new synthetic carcinogens

Having considered the past and present advancements in our understanding of the causes of cancer, it is interesting to consider a few promising areas which are presently under investigation. The effect of prenatal exposures on the rates of cancers in both childhood and also in adulthood is generating interest. The concept of prenatal exposure has been well demonstrated in principle. Prenatal exposure to diethylstilboestrol has been demonstrated, to cause the rare adenocarcinoma of the vagina in the female offspring of mothers who used the drug during pregnancy[88]. A considerable amount of literature indicates that many chemicals can have biological effects at extremely low levels, at levels far below those currently recognized by government regulatory agencies such as the United States Environmental Protection Agency (EPA)[136-138]. Evidence is emerging that *in utero* exposure to xenoestrogens causes reproductive cancers. Bisphenol A (BPA) is a xenoestrogen which has been widely used in the manufacture of polycarbonate plastics, which are used as food storage containers, and epoxy resins which are used to line food and beverage cans[139,140]. BPA has been shown to leach into food products at very low levels[141,142], and to accumulate in the amniotic fluid of pregnant women[143]. When BPA was administered to pregnant nonhuman primates at levels similar to human exposure levels, the histology of the mammary glands of the newborn females was altered[144]. Rats which received *in utero* exposure of BPA, at 1/20000 the level currently estimated by the EPA as the lowest observable adverse effects level, developed mammary gland ductal hyperplasia and carcinoma *in situ*[145]. Male rat fetuses which were exposed to low levels of BPA displayed an increased propensity to develop prostatic intraepithelial neoplasias[146]. It will be interesting to follow the research concerning *in utero* exposure to BPA, as well

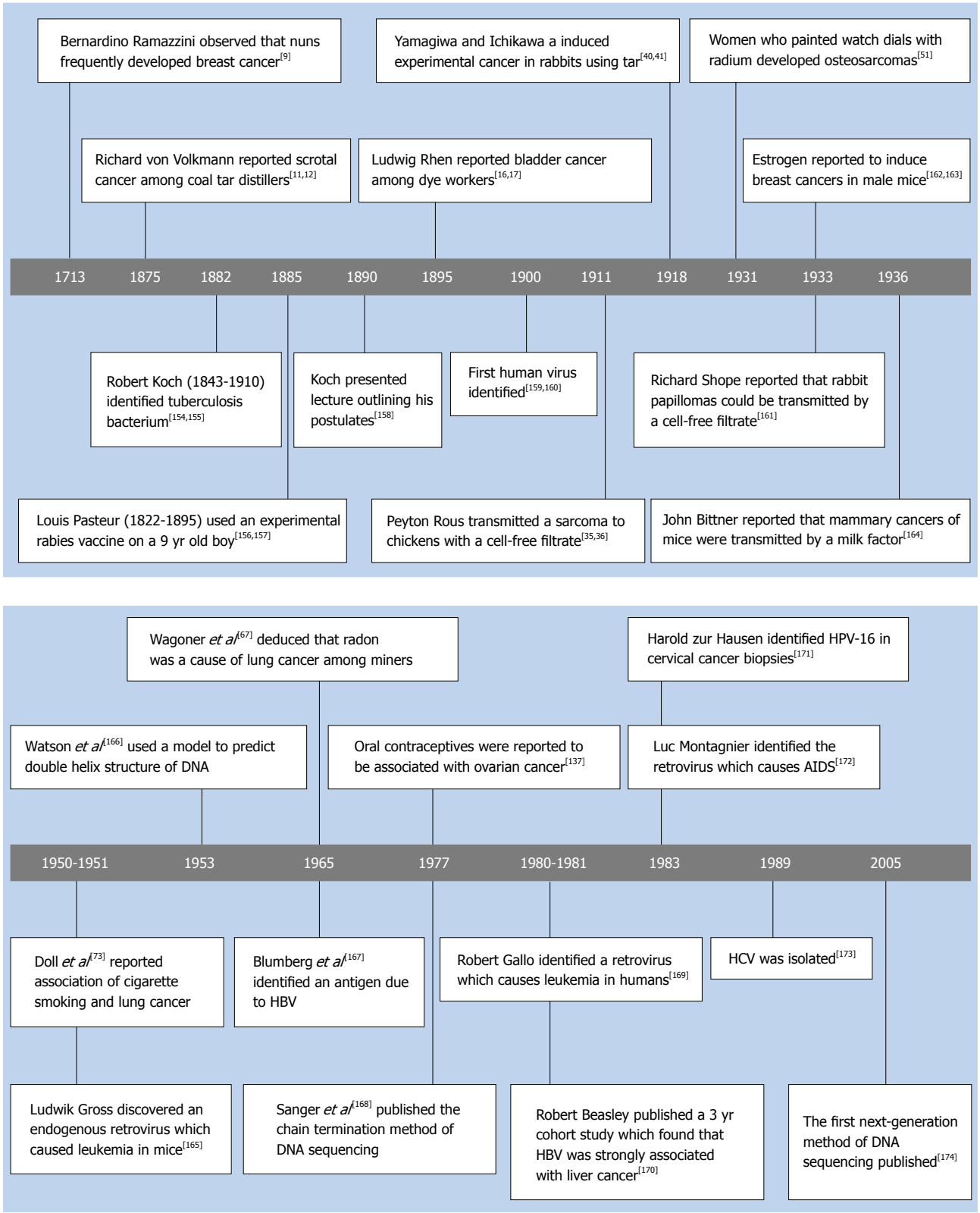


Figure 1 Some milestone publications concerning the causes of cancer, microbiology, and technology.

as other xenoestrogens as possible contributors to the high rates of breast and prostate cancers in developed countries[147-149].

A very recent review has highlighted the fact that most research has analyzed the effects of single agents, while human exposures involve complex combinations of agents, and that combinations of agents may have synergistic effects. They encourage researchers to investigate the effects of low doses of combinations of chemicals[150].

VIRAL

Early experimental studies

Belief in a contagious cause of cancer began in classical times, when it was hypothesized that a single infectious organism caused every kind of cancer. A tremendous amount of work was performed searching for a contagious cause, but nothing was found which could be confirmed[151-153]. In the late 1800s Pasteur and Koch demonstrated the contagious origin of many diseases (see Figure 1)[9,11,12,16,17,35,36,40,41,51,67,73,137,154-174] which prompted many more searches for a microbial cause of cancer[151-153]. In 1900, the first human virus was identified, with the isolation of the yellow fever virus[159,160]. Shortly afterwards, Borrel[175,176] hypothesized that cancer had a viral etiology. Following this, Vilhelm Ellermann and Oluf Bang[33,34,177] reported that a cell-free filtrate induced leukemia in chickens, and Peyton Rous[35,36] reported that another cell-free filtrate caused a sarcoma in chickens. However, these early reports kindled little interest, because many orthodox pathologists did not accept the notion that leukemia was a form of cancer. Furthermore, the chicken seemed to be too different from humans for the work to be relevant[153,178].

A well studied observer reflected in 1903[27] that the number of reports which claimed to have identified a microbial cause of cancer peaked in 1887, and then steadily decreased each year thereafter. Interest faded when none of the results could be confirmed[28,179]. The search for an infectious cause of cancer became so intense, and extended for so long, that it eventually "led to wide acceptance of the dogma that cancer did not, and could not have an infectious agent as its cause"[153,180]. An infectious cause came to be considered as ruled out by most well respected investigators when the first studies of chicken cancers were published[181-183]. Furthermore, numerous reports had emerged that a variety of industrial exposures, such as chimney soot, coal tar, dye chemicals, and X-rays caused cancer in humans when these studies emerged, which further eroded interest in the virus studies.

1920-1950

Additional experimental studies: Rous worked with the chicken virus for a decade, and then became discouraged so he abandoned the work[180]. Following this, William E Gye published an independent investigation of the virus, which incited fierce controversies[184-186]. He argued that the work was an embarrassment to both sides. Those in favour of the parasitic theory found little support in the work, because it was not evident how the agent could provide a unifying concept for the tremendous variety of tumors which occur in so many different species[184,186]. The virus only induced one type of cancer in one species. Those opposed to the parasitic theory argued that the Rous sarcoma virus (RSV) was purifiable by chemical methods which no living organism could survive[186]. Other opponents argued that the sarcoma was a disease peculiar to chickens, not really a cancer, and therefore not relevant[184,185].

Methods to successfully transplant cancerous tissue were developed in the late 1800s. Carol O Jensen and Leo Leob independently produced a considerable amount of research on the nature of transplanted cancers by 1903[187]. They each searched for evidence to prove/disprove the infectious cause of cancer theory. Jensen reported that cancers which were transplanted as whole cells frequently survived in a new host, but when cancer cells were crushed in a mortar, the inoculation was never able to produce cancers in the recipients[188,189]. This seemed to be definitive evidence that the transplanted cancers were not the product of transmission of an infection.

The first crude electron microscope (EM) was only developed in 1933[190], and the RSV was not visualized until 1947[191]. The EM only became consistently reliable for the examination of biological specimens in the 1950s[192]. Hesitation to accept the discoveries of viral induced cancers in chickens is understandable.

A few additional viruses were identified which induced cancer during this period. Shope demonstrated that rabbit papillomas could be transmitted with a cell-free filtrate in 1933[161]. Bittner reported that mammary carcinomas of mice could be induced by an infectious agent transmitted in the milk in 1936[164]. Lucke reported in 1938 that a virus caused renal adenocarcinoma in leopard frogs[193]. The reports of Shope and Bittner were the first evidence that viruses could induce tumors in mammals, but interest in synthetic industrial agents as causes of cancer had become overwhelmingly convincing.

1950-1980

A golden era for animal experimental studies: Endogenous viruses were discovered in the 1950s. Mice had been systematically bred for genetic inheritances of high and low frequencies of leukemia in the 1920s and 1930s[194]. In 1951 Gross[165] reported that a cell-free extract from the high frequency Ak strain, induced leukemia in the low frequency C3H strain. In related works, normal chicken embryos were reported to contain an antigen, which was indistinguishable from an antigen of a virus which caused leukemia in chickens in 1967[195]. There was an intriguing report, that either ionizing radiation or chemical carcinogens could induce the replication and spontaneous release of an avian leukemia virus from normal uninfected cells in 1971[196], and another report indicated that the murine leukemia virus could be similarly induced[197,198]. These curious reports became comprehensible when it was found in 1972, that retroviruses frequently integrate into the host cell DNA[199]. Selective breeding for high rates of cancer using the principles of genetics had unintentionally selected for latent endogenous retroviruses which were transmitted vertically[200].

In 1964 Jarrett *et al*[201,202] discovered a retrovirus which caused lymphomas in common domestic cats,

which has been estimated to account for as many as 70% of the lymphomas occurring in domestic cats[203]. During this period, virus particles were detected in cultured human Burkitt's lymphoma cells[204]. An antigen was discovered in a hemophiliac patient, which would later be found to be produced by the hepatitis B virus (HBV)[167]. Breast milk of women who were at high risk of developing breast cancer, was reported to contain particles which were morphologically similar to the MMTV, and to have reverse transcriptase activity[205].

The virus cancer program: The discovery of the mouse leukemia virus[165], along the chicken leukemia virus[33,34], and the feline lymphoma virus[201,202], made it seem reasonable that similar agents could induce leukemia in other species[206]. There were many other discoveries leading up to, and during this period, and additional reasons which fuelled interest in a possible role of viruses in human cancers[7,207]. Some were predicting that viruses would be found to have a universal role in all cancers, including those induced by radiation and chemicals. Others advocated a more bridled enthusiasm. They accepted that viruses were involved in some cancers, but they did not accept the hypothesis that viruses were involved in all cancers[207].

In 1968 the Congress of the United States considered the evidence that viruses induce cancers in animals to be overwhelming. They expected the viruses responsible for human cancers to be found soon, so they began investing ten million dollars a year in the virus cancer program at the National Cancer Institute[208,209]. Much work focussed on searching for human oncogenic retroviruses, analogous to the endogenous retroviruses which were found in animals. A decade was spent searching for the suspected viruses, but very few were found, and those which were found could not be grown in culture. Adding further to the disillusionment, some agents were eventually shown to be contaminants[200-211], while other agents could not withstand the increasingly sophisticated methods of analysis which were being developed[212,213]. The notion became widespread again that human cancers were not caused by viruses. The mood became analogous to the scepticism of the early 1900s[214]. Suspected cancer causing viruses had previously been frequently termed "tumor viruses". Naysayers became so confident that a virus would never be found which caused human cancer, that they mockingly called them "rumor viruses"[212,215].

1980-present

Major advances in the establishment of the roles of viruses in human cancer occurred during this period for numerous reasons. Laboratory methods were improved which enabled the analysis of much lower levels of viruses. The epidemiological methods which had been developed to evaluate the association of tobacco smoking with lung cancer were revised for analysis of infectious agents with other types of cancer. However each virus presented unique difficulties, as described below.

Human T-cell leukemia virus: Robert Gallo's only sibling was diagnosed with leukemia when she was only five years of age, and Robert was eleven. Influenced by his sister's physician, Gallo enrolled as a medical student in 1960, and studied the biochemistry of blood cells[216]. In 1970, it was discovered that RNA viruses replicate using the reverse transcriptase enzyme[217,218], and Gallo decided to work developing enzyme assays to detect viral reverse transcriptase, as a more sensitive method of detecting possible cancer causing viruses[219] (The EM had become the gold standard, but it was tedious and time consuming).

Gallo *et al*[219] were determined to search for a retroviral cause of human leukemia when other researchers were abandoning the search. They were provoked by the discoveries of a retrovirus which caused leukemia in nonhuman primates[220], and another retrovirus which caused leukemia in cattle[221]. The discovery of the bovine leukemia retrovirus intrigued them because it induced leukemia with only very low levels of replication. They reasoned that human leukemia could have similarly low levels of replication, and therefore be difficult to isolate[210].

They eventually found a retrovirus in a T-cell line established from a patient with a cutaneous T-cell lymphoma, which they termed the human T-cell leukemia retrovirus (HTLV-1) in 1980[169,210]. Hinuma *et al*[222] reported in 1982 that 140 of 142 patients with adult T-cell leukemia (ATL) were positive for the virus from certain areas of Japan. Southern blot analysis showed that all cases contained the virus, and that it was integrated into the host genome in monoclonal form, indicating that infection and integration occurred before clonal expansion of the tumor cells[223]. The discovery of HTLV-1 has been reviewed[210,224,225].

The virus causes only a particular type of leukemia, which is common in some areas of Japan, but it is rare in the United States. Parkin *et al*[226] estimated that the global fraction of leukemia due to HTLV-1 is only about 1%, but the principle that viruses could cause human cancer, was finally proven. Convincing evidence that a retrovirus causes common forms of leukemia has still not emerged, though evidence concerning other infectious agents continues to be intriguing[227].

The mechanism by which HTLV-1 causes leukemia is only partially understood. The virus integrates into the host genome as a component of its regular replication cycle. It is found as a single integrated provirus in around 80% of ATL patients, with the remaining ATL patients displaying either two integration sites or multiple clones[228]. The virus codes for a protein termed Tax, which has multiple effects which are predicted to induce transformation[229]. However, it is perplexing that about 60% of patients with ATL have lost expression of the *tax* gene[230], so investigators have been searching for another mechanism to account for how HTLV-1 causes ATL. It has been reported that the HTLV-1 basic leucine

zipper factor *(HBZ)* gene is transcribed in all cases of ATL[231]. *HBZ* RNA promotes T cell proliferation[231], and transgenic mice which express the *HBZ* gene in $CD4^+$ cells develop lymphomas[232]. For recent reviews of HTLV-1 replication and ATL see[233,234].

Human immunodeficiency virus: In the summer of 1981, the first reports of a mysterious new syndrome emerged. Young male homosexuals in New York City and California were reported to have phenomenally high rates of *Pneumocystis carinii* pneumonia[235], and unprecedented rates of Kaposi's sarcoma (KS)[236].

The etiology of the acquired immunodeficiency syndrome (AIDS) perplexed the medical community and many bizarre theories were suggested. Max Essex suggested to Gallo that some feline leukemia retrovirus variants caused immunosuppressive syndromes[224,237]. Gallo reasoned that HTLV sometimes caused immunosuppression, and that the geographic distribution of HTLV across Africa resembled the distribution of AIDS in Africa[238]. He wondered if AIDS could be caused by a retrovirus closely related to HTLV. He decided to abandon the search for retroviral causes of human leukemia/lymphoma, in order to investigate whether the cause of AIDS was a retrovirus, and possibly intercept an impending worldwide epidemic[215]. Luc Montagnier *et al*[239] at the Pasteur institute in France had also been searching for oncogenic human retroviruses, when the AIDS epidemic emerged. Inspired by Gallo's theory, they decided to redirect their resources to search for a retroviral cause of AIDS as well. Using a reverse transcriptase assay Luc Montagnier and Francois Barre-Sinoussi reported identification of a retrovirus in the lymph node biopsy of a patient in the early stages of AIDS in May of 1983[172].

Montagnier *et al*[240] reported detection of antibodies to the retrovirus in the serum of 60% of patients with the pre-AIDS lymphadenopathy syndrome, but in only 20% of patients with AIDS, so the significance of their discovery was initially uncertain. Following this, Sarngadharan *et al*[241] reported that they had developed an improved method to detect antibodies to the virus, and that they found around 90% of cases with AIDS were seropositive in May of 1984. This was followed a few months later by two independent reports of detection of antibodies to the virus in around 90% of patients with AIDS[242,243], which confirmed the virus as the cause of AIDS. Shaw *et al*[224] also developed a cell line capable of yielding sufficient quantity of the virus to be studied and they produced a commercial blood test for the virus in May of 1984[244]. Montagnier *et al*[245] reported development of a comparable method of production of the virus in July of 1984, and a reliable method to detect antibodies in Oct of 1984[246]. Of course the virus was later termed the human immunodeficiency virus (HIV).

Luc Montagnier and Francoise Barre-Sinoussi received the prestigious Nobel Prize for their identification of the virus in 2008 (http://nobelprize.org). A succinct review of the chronology of events concerning the discovery of HIV has been published[247]. Gallo and Montagnier have both written detailed accounts of their personal reflections of this period[248,249]. Robin Weiss has also written a concise informative review of the discovery of HIV[250].

Around 50% of patients diagnosed with AIDS, were also diagnosed with KS at the time of their AIDS diagnosis in the early 1980s[251,252], which would seem to be unequivocal proof that HIV causes KS. However, KS cells were found to be curiously not infected with HIV[253]. Large trials also demonstrated that non-Hodgkin's lymphoma occurred in around 4% of patients with AIDS at the time of their diagnosis, and is the second most common cancer among AIDS patients[254]. However, B cells of AIDS related lymphomas have also been reported to be devoid of the virus[255,256].

The mechanism of how HIV causes cancers is not straightforward. The virus infects T cells and macrophages which release an HIV encoded protein known as Tat, which is taken up by other cell types in the microenvironment. Tat has numerous effects which are also predicted to promote angiogenesis and carcinogenesis[257-259]. It appears that immunosuppression caused by HIV is a potent cofactor in KS and lymphomas, rather than a cause of these cancers, since they also occur at increased rates in transplant patients [Also see the discussion of human herpes virus 8 (HHV-8) below for additional discussion of KS].

The IARC concluded in 2012 that HIV causes not only Kaposi sarcoma and non-Hodgkin's lymphoma, but also Hodgkin's lymphoma, and cancers of the cervix, anus, and conjunctiva[260].

HBV: Baruch Blumberg was formally educated as a physician in the United States, and relates that his education left him "woefully ignorant about viruses"[261,262]. In the early 1960s he was searching for genetic polymorphisms which could account for differences among individuals in their susceptibility to diverse diseases. Harvey Alter was assisting him in this, when they found a precipitin in a serum which they were hoping was caused by a genetic polymorphism[167,263]. Following this, Blumberg became intensely interested in studying every detail about the precipitin. After many months of study, they noticed that a couple of patients who were negative for the antigen became positive, and that the patients coincidentally developed hepatitis[264-266]. Blumberg collaborated with some experienced electron microscopists and reported visualization of small particles in the serum of the patients which were later identified as the surface antigen of HBV[267].

It was not evident that HBV caused hepatocellular carcinoma (HCC) when it was discovered. The geographical correlation between the distributions of the rates of HBV infection and HCC became recognized a decade after the discovery of the antigen[268]. However, aflatoxin had been previously demonstrated to induce

liver cancers in rodents[269] and it was proven to cause liver cancers in primates as well[270], so aflatoxin seemed more likely to be the cause of HCC in humans. Furthermore, serum antigens of the virus were not reproducibly detectable at the time of diagnosis of HCC using the methods that were available in the 1970s[271,272]. Moreover, viral antigens were only present at much reduced levels in the tumor tissue compared to the surrounding normal liver tisue[273-275]. In spite of this evidence, Robert Beasley was determined to definitively test whether HBV caused HCC. He decided to study a healthy population without HCC, and to test them for serum HBV antigens using newly developed and highly sensitive radioimmunoassay techniques. He devised to follow subjects prospectively to determine which individuals developed HCC. It was difficult to get funding for a large study when the evidence seemed to indicate that aflatoxin was the cause of liver cancer. He recruited 22707 healthy male government employees in Taiwan, and tested them for a variety of indicators of HBV infection[170]. After three years of follow-up, forty one deaths due to HCC occurred. Only 15% of the employees were seropositive for hepatitis B surface antigen (HBsAg), but forty of the 41 men who developed HCC were seropositive for the antigen. The relative risk of HCC among men who were seropositive for HBsAg was calculated to be 223 (95%CI: 28-1479)[170]. This was evidence which could not be dismissed[276]. Vaccination of Taiwanese children has resulted in a pronounced decrease in the incidence of childhood HCC[277,278], which is confirmation that HBV causes HCC.

While epidemiological studies have provided convincing evidence that HBV causes HCC, investigations of the mechanisms of how it causes cancer have been less straightforward. A variety of mechanisms have been proposed, which can be divided into three general categories; viral proteins, inflammation and genetic instability. The virus produces oncogenic proteins; HBsAg and HBx have received the most intensive investigations. Mice which are transgenic for HBsAg or HBx have been reported to develop liver cancers[279,280]. The second general mechanism involves the immune response to the virus which results in a state of chronic inflammation, which produces cirrhosis that deteriorates into HCC[281,282]. The third general mechanism involves the integration of the virus into the host genome, which generates genetic instability. The normal replication cycle of HBV does not include integration into the host cell genome. However, the virus has been found integrated into the host cell genome in around 80% of HCC cases associated with HBV[283]. The integrated virus is fragmented and rearranged, so that it cannot produce infectious particles[284-286]. However, the integrated virus is attributed to induce chromosomal instability of the host cell[287]. Each of these mechanisms likely causes a few cases of HCC, with most cases involving two or all three of the above mechanisms. Cases of HCC which develop without cirrhosis are caused by viral integration and viral proteins. Cases which develop without viral integration are caused by inflammation/cirrhosis and viral proteins.

Human papillomavirus: Harold zur Hausen was intrigued by the fact that epidemiological evidence indicated that the risk of cervical cancer was correlated with sexual promiscuity, which suggested an infectious cause[288,289]. Herpes simplex virus type 2 seemed like a plausible agent, so he analyzed cervical cancer biopsies for the virus. However, the samples were found to be devoid of the virus, so he decided to investigate papillomaviruses[289]. The early methods of analysis for human papillomavirus (HPV) were crude and did not produce convincing evidence of a causal role. In the mid-1970s recombinant DNA technology was developed, and zur Hausen's group used the technology to sequence the virus. They discovered that the HPV exists as a variety of different types[290,291]. They identified HPV-16 and HPV-18 in cervical cancer biopsies[171,292]. Following this, the early epidemiological studies still did not consistently identify HPV DNA in cervical cancers, because the methods of analysis of HPV DNA varied substantially in sensitivity and specificity[293,294]. As the sensitivity of the techniques increased, HPV DNA became detectable in 97%-98% of cervical cancer biopsies[295,296].

HPV-16 and HPV-18 produce a number of proteins (E5, E6, and E7) which have various oncogenic activities[297,298]. E6 induces chromosomal instability by binding to the tumor suppressor protein p53, interfering with its normal function and inducing its degradation[299]. E7 also induces chromosomal instability by interfering with the normal functioning of the retinoblastoma family of proteins and inducing their degradation[300,301]. HPV replicates as an episome, in a cycle that does not involve integration into the host's cell genome[302]. However, the virus has been frequently found integrated into the host cell's genome. It is not known precisely how the virus becomes integrated, but it likely results when DNA breaks occur, which are likely promoted by episomal E6 and E7[303,304]. The integrated virus is found in a truncated form, though E6 and E7 usually remain intact, and continue to be transcribed. Numerous reports have found that E6 and E7 are transcribed at higher levels when it is integrated into the host genome[305], and the proteins produced by the integrated virus may have increased stability[306]. Studies which have analyzed the frequencies of integration in premalignant and invasive cancers have consistently reported higher rates of integration among invasive cancers[307,308]. It has also been consistently reported that the infectious episomal form of the virus is detected less frequently in invasive cancers than in premalignant lesions[308].

Recent prospective cohort studies have reported strong associations among women in whom HPV was detected on multiple occasions, which is consistent with cervical cancer developing from persistent infections. Analysis of ten years of follow-up of a cohort

in Copenhagen, reported that 13.6% (167/1229) of women who were positive for high risk HPV at enrolment developed high grade lesions (severe dysplasia or carcinoma *in situ*), while 20.0% (83/414) of women who tested positive at enrolment and tested positive again two years later developed high grade lesions[309].

Confirmation of the causative role of HPV in cervical cancer is expected to emerge, with the successful clinical trials of vaccines for HPV. Vaccine efficacy for a bivalent vaccine (HPV 16, 18) was tested in over 10000 HPV naïve women who were followed for 4 years. The efficacy was 64.9% (95%CI: 52.7-74.2) for protection against CIN2 irrespective of HPV DNA in the lesion, and 93.2% (95%CI: 78.9-98.7) for prevention of CIN3 irrespective of HPV DNA in the lesion. Seven cases of adenocarcinoma in situ occurred in the unvaccinated controls, whereas zero cases occurred in the vaccinated women[310]. Results of the efficacy of HPV vaccination against cervical cancer are expected to emerge in 5-10 years[311].

Initial studies with HPV focussed on identification of the cause of cervical cancer. Further studies have shown that mucosotrophic HPV types also cause cancers of the vulva, vagina, penis, oropharynx, oral cavity, and tonsil[312,313]. Zur Hausen was awarded the Nobel Prize for his work with HPV in 2008 (http://nobelprize.org). Robin Weiss has written a concise reflection of zur Hausen's work[250].

Hepatitis C virus: After the discoveries of HAV and HBV, it was found that most cases of transfusion associated hepatitis were caused by neither HAV nor HBV[314]. A non-A non-B (NANB) infectious agent was suspected of causing transfusion associated hepatitis. The NANB infectious agent was also suspected of causing HCC, based on case reports of transfusion recipients who developed chronic hepatitis, which progressed to cirrhosis, and then HCC[315,316]. However, the identification of hepatitis C virus (HCV) was excruciatingly difficult. The NANB hepatitis agent could be transmitted to chimpanzees; however efforts to isolate the virus using traditional methods, based on antigens and antibodies were without success for over fifteen years. Numerous tests were published, but none could be confirmed by independent laboratories[317,318]. The results were so perplexing and inconsistent, that it was speculated that the agent might only be present at very low levels[318], and/or the immune response could be very weak and therefore difficult to identify[318,319].

Finally, a massive systematic search was initiated by Michael Houghton *et al*[320] at the Chiron Corporation together with Daniel Bradley at the Centers for Disease Control. Their approach was to clone the agent before isolating it, using a "blind" immunoassay. The identification began in the early 1980s, and utilized the recombinant DNA techniques which had been recently developed. Michael Houghton *et al*[320] searched intensely for the elusive NANB hepatitis agent(s), using many different approaches to screen hundreds of millions of cDNA clones. After seven years, they identified the elusive agent, as a single stranded RNA molecule of about 10000 nucleotides, and termed it HCV[173]. Choo *et al*[321] have written a detailed review of the discovery of HCV.

After the identification of the clone, the development of an ELISA test for the detection of circulating antibodies was promptly published[322], and many reports of high rates of detection of antibodies to HCV in the serum of patients with HCC soon followed[323,324].

HCV is considered to cause HCC, though conclusive proof has been elusive. HCV induced HCC evolves through a progression of chronic hepatitis, to cirrhosis, to HCC which generally requires 20-30 years, or longer to develop, so prospective trials have been few. A meta-analysis of HCV positive cirrhotics reported that 17%-30% of patients developed HCC over five years of observation[325]. An eleven year prospective study followed 925 patients with antibodies to HCV. They reported a cumulative risk of HCC of 1.1% among those with undetectable HCV RNA levels and 14.7% among those with the high serum levels of HCV RNA[326].

Confirmation that HCV causes HCC has also been elusive. Treatment of HCV infection with α-interferon (or α-interferon plus ribavirin) results in decreased rates of HCC[327]. However, the demonstration that α-interferon reduces the incidence of HCC is complicated by the evidence that α-interferon has anti-carcinogenic effects independent of its antiviral effects[328-330]. A meta-analysis by Kimer *et al*[331] reported that the rate of HCC was reduced in both sustained virological responders [risk ratio (RR) = 0.15, 95%CI: 0.05-0.45] and also in non-responders (RR = 0.57, 95%CI: 0.37-0.85). The improved prognosis among non-responders is consistent with α-interferon having an anti-carcinogenic effect in addition to its anti-viral effect. Interferon-free treatment regimens are very recently becoming widely prescribed[332], so the long term effect of viral clearance by these treatments should provide a definitive confirmation of the causative role of HCV in HCC.

HCV has an interesting and complex life cycle. The virus replicates using an error prone polymerase which lacks proofreading activity. Consequently, HCV circulates in an individual as a heterogeneous population of sequences or quasispecies, which is very complex for the immune system to clear[333]. A systematic review estimated that 75% of individuals fail to eradicate the virus, because their immune response is only partially effective[334]. The principal mechanism by which HCV causes HCC is considered to be the chronic immune inflammatory response[335]. Around 40% of patients with chronic HCV develop cirrhosis after 30 years[336]. While HCC develops mostly among cirrhotics, it also develops at low rates among patients devoid of cirrhosis[337], which is interpreted as evidence that the virus may possess some directly carcinogenic effects. The virus replicates in the cytoplasm and does not integrate into the host genome as a component of its replication cycle or incidentally, unlike some of the viruses discussed above.

Epstein-barr virus

Burkitt's lymphoma: Dr Burkitt was an English missionary physician posted in Uganda, when he became impressed by the unusually high incidence of a very aggressive jaw and abdominal tumor among the local children. He published an account of it in 1958, then returned home to England in 1961, and presented public lectures on his recent studies[338]. A young Tony Epstein was performing electron microscopy studies of tumor viruses during this period, when he happened to decide to sit in on a lecture of Dr Burkitt's. Afterwards, Epstein requested Dr Burkitt to ship biopsy samples to him from Uganda[339], and a few years later Epstein *et al*[204] reported that they detected virus particles in cell cultures of the biopsy samples.

Henle *et al*[340] reported in 1969, that patients with BL had higher antibody titres to the Epstein-Barr virus (EBV) viral capsid antigen (VCA) than controls. This was followed by a seven year prospective study of 42000 Ugandan children, 14 of whom developed BL in 1978. This study found that high antibody titres to EBV VCA preceded the development of BL[341]. The virus was found to be present in monoclonal episomes in BL cells, which is consistent with the infection of a single normal cell occurring before the transformation and expansion of the tumor[342].

The above discussion has been limited to the endemic BL, which occurs principally in certain areas of Africa, and is strongly associated with malaria[343]. Malaria is discussed further in the natural factors section below. A sporadic form of the tumor occurs outside of Africa, in areas where malaria is rare, at much lower rates. The sporadic form of BL usually lacks detectable levels of EBV in the tumors. For detailed reviews of BL see the following references[344-346].

NPC: The incidence of NPC varies widely throughout the world; however EBV is consistently present in the undifferentiated form of NPC in various geographic regions[347,348]. High serum EBV antibodies have been found to precede the development of NPC[349], and to be informative in predicting the onset of NPC[350]. The virus is found in monoclonal episomes in every tumor cell, indicating that infection of the cells occurred prior to clonal expansion[342,351]. Administration of EBV-specific cytotoxic T-lymphocytes has induced remissions of cases of NPC which are refractory to conventional radiotherapy and chemotherapy[352,353].

Deciphering the mechanism of how EBV causes NPC has been complex. The virus has a dual tropism for B cells and epithelial cells. The life cycle is furthermore complicated by possessing a variety of different latency cycles as well as a lytic cycle[354]. The virus is present in NPC cells in a latent cycle. The virus produces a latent membrane protein 1 which has strong carcinogenic effects, but is only present at extremely low levels in most NPC biopsies[355]. NPC biopsies are characterized by high levels of untranslated EBV RNA which are termed EBER 1 and EBER 2[355]. NPC biopsies also have high levels of rightward transcripts derived from the *Bam*HI A region (BARTs) of the EBV genome[356]. The *Bam*HI A transcripts are multiple spliced which yield numerous microRNAs[357]. It is unclear precisely how EBERs and *Bam*HI A transcripts might induce malignant changes, but in recent years evidence has emerged that untranslated RNA, especially microRNAs, can have strong effects on cellular metabolism[358]. The mechanisms whereby EBV could induce malignancy, have been recently reviewed[355,359].

EBV has also been demonstrated to cause non-Hodgkin lymphomas among immunosuppressed patients, and some cases of Hodgkin lymphoma[360]. While EBV was discovered before HBV, HCV, HPV-16, HPV-18, and HIV, work to prevent cancers caused by EBV has dawdled behind achievements to prevent cancers caused by other viruses. A commercial vaccine for EBV is only in the early stages of development[361].

HHV-8: The initial identification of HHV-8 consisted of only a few epidemiological observations at the Centers for Disease Control, which were followed up by two or three scientists with very limited laboratory experience, who worked for only around three months[362]. Using polymerase chain reaction-based technology, the causative agent was identified as a new herpes virus, with homology to both EBV and herpesvirus saimiri[363]. However, definitive evidence that it causes KS has been challenging.

HHV-8 DNA is present in 91%-96% of KS biopsies[364]. Prospective studies which followed patients with AIDS (or transplant recipients) reported that 20%-50% of persons with detectable levels of HHV-8 in their peripheral blood develop KS[365-368]. Two studies reported that low levels of HHV-8 specific T-cell responses are associated with a high risk of developing KS among immunosuppressed patients[369,370].

Studies of antiherpes drugs usage for cytomegalovirus retinitis and other cytomegalovirus induced disease among HIV patients provide additional evidence that HHV-8 causes KS. Three large studies conducted before the widespread use of HAART, reported decreases in the number of cases of KS among HIV patients who were treated with the antiherpes drugs ganciclovir or foscarnet for cytomegalovirus infections (Reviewed by Casper *et al*[371]).

Not everyone has agreed that HHV-8 has been established as the cause of KS, or that KS is a form of cancer. It has been argued that KS is polyclonal reactive process, rather than a true malignancy. Furthermore, studies concerning the clonality of the virus indicate that some are monoclonal, but most are oligoclonal[372]. Many objections to whether KS is a true cancer, and whether HHV-8 is the cause are settled if it is considered that most KS lesions are preneoplastic with a propensity to progress to cancer[373]. HHV-8 is also attributed to cause a rare cancer termed primary effusion lymphoma[374]. For recent reviews see the following[374,375].

Emerging studies of possible new viral carcinogens

Tremendous progress has been made in understanding the causes of liver and cervical cancers since the 1980s. However, our understanding of breast, prostate, and colon cancers has made relatively little progress. It is striking that the rates of breast cancer are so high, and that few if any, agents have been identified which strongly increase or decrease the risk. The author hypothesizes that an initiating event occurs in all females during fetal development, which renders human breast tissue susceptible to the promoting effects of estrogen and a diet high in protein and calories.

It was decided during the early drafts of writing this review, that discussions of genetics would be beyond the scope of this review. However, it is ambiguous whether some elements of the human genome should be considered as infectious or genetic. The International Human Genome Sequencing Consortium has reported that only around 1.5% of the human genome consists of protein coding sequences. Around 50% of the human genome consists of repeat sequences which have been traditionally dismissed as uninteresting "junk". Most of the repeat regions are classified as transposable elements, which consist of; short interspersed elements, long interspersed elements (LINEs), long terminal repeat (LTR) retrotransposons, and DNA transposons[376]. These elements appear to be ancient with uncertain origins, though they have considerable homology with viruses[377]. The LTR retrotransposons resemble ancient retroviruses, and are commonly termed human endogenous retroviruses (HERV). Most HERV have multiple mutations and deletions which render them unable to replicate for general reviews see[212,378,379]. However, a few HERV copies appear to be complete, and sometimes produce viral proteins, though complete viral particles are only rarely produced[380]. Many women with breast cancer have increased expression of HERV in both their serum and cancers[381,382]. Melanoma biopsies have also been repeatedly reported to have high levels of expression of HERV[212,383].

LINEs are much more active than HERV. LINEs make copies of themselves which are inserted into random regions of the genome with deleterious effects[384]. Recent studies have reported a high frequency of novel insertions of LINEs in lung and colon cancer samples[385,386]. Transposable elements could cause cancer by mutagenesis and altering regulation of host genes[387,388].

LINEs and HERVs are silenced by epigenetic mechanisms, though they are frequently active during early development[389,390]. Do altered activation patterns of LINEs and HERVs during early development increase the risk of breast and other cancers?

NATURAL FACTORS AND NON-VIRAL INFECTIOUS AGENTS

When high rates of cancer were reported in a variety of occupations in the early 1900s, it seemed self-evident the cause of cancer was synthetic agents. Then research since the 1980s firmly established viruses as another important cause of cancer. In addition, there are a variety of other agents which cause cancer which the author has categorized as "natural factors and non-viral infectious agents".

Hormones

Interest in the notion that natural factors cause cancer is generally dated to have commenced in the 1930s. Estrone was identified in the urine of pregnant women in 1929[391], which was followed by an extensive research effort which resulted in the elucidation of the structures of cholesterol, bile acids, and the sex hormones[392]. In the early 1930s, Lacassagne administered weekly injections of estrone to three castrated male mice, and reported that every mouse injected with estrone developed mammary adenocarcinoma[162,163]. The experiment drew widespread interest among scientists, because estrone is a natural hormone which is produced endogenously in females. The experiment particularly was striking because male mice are not susceptible to spontaneous mammary tumors, and the cancers developed in each of the treated mice. The renowned Alexander Haddow considered Lacassagne's study to be the first report of a "natural" carcinogen[49]; though a variety of natural agents were recognized to cause cancer in animals by some well-studied cancer researchers when Lacassagne's publication appeared[393].

Bernardino Ramazzini was an Italian physician and professor who observed that each occupation was characterized by a distinct pattern of diseases. Ramazzini observed that nuns have high rates of breast cancer in 1713[9], but the reasons for this have been complex to decipher. Lane-Claypon[394] performed a case-control study which concluded that women who had multiple childbirths were less likely to develop breast cancer[395]. MacMahon *et al*[396] performed an international collaborative study and concluded that a younger age at the first childbirth was associated with a decreased risk. Breast feeding was also suspected of decreasing the risk, but it was difficult to definitively decipher. An international collaborative group recently estimated that the risk of breast cancer decreases by 4.3% for every 12 mo of breast feeding[397]. There is strong evidence that all of the above factors affect the risk of breast cancer, however the interaction of these factors has not been deciphered, and there is only a consensus for breast feeding reducing the risk[398].

Ultraviolet radiation

Paul Unna was a general physician who took a strong interest in skin diseases[399]. He built a clinic in order to diagnose and treat patients with skin diseases, and published a textbook of dermatology. In the publication he described the histological changes which accompanied the clinical conditions which were encountered by dermatologists. He described a "diffuse cyanotic redness" which occurred on the face and hands of

sailors after long term exposure to the "weather" which is implied to be due to ultraviolet light. He described both the clinical and histological changes as the lesions progressed to become cancerous. Following this, he compares the course of the sailor's skin disease with xeroderma pigmentosum in adults who had been exposed to the sun and developed skin cancer[400,401].

Parasites

Schistosoma haematobium (*S. haematobium*) is a parasitic fluke, which is endemic in Africa and Southwestern Asia. It is spread through human skin contact with infected water[402]. Ferguson reported a case series of *S. haematobium* and bladder cancer in Egypt in 1911[403]. He documented that 40% of males over 5 years of age were infected with the parasite, and described 40 patients with bladder cancer with ova of the fluke present mostly in the portal vein, bladder or tumor. *Opisthorchis viverrini* (*O. viverrini*) is another parasitic fluke, which is contracted by eating raw fish. The infection rates are endemic in Thailand and other east Asian countries. *O. viverrini* was suspected of being a carcinogen by Stewart[404] in 1931; however the first case series did not appear until the report by Bhamarapravati and Viranuvatti[405] in 1966, who reported that the infection was associated with the bile duct cancer known as cholangiocarcinoma in Thailand[406].

Dr. Burkitt began working in a teaching hospital in Kampala Uganda in the mid-1950s, when he encountered a high number of children with very rapidly growing lymphomas. The lymphomas were frequently characterized by massive malignant growths that occurred in the abdomen and jaw areas. With funds of only £ 25 ($75), he prepared leaflets and photographs, which were sent to health care workers across Africa. The leaflets described the clinical features of the lymphoma, in order to survey the geographical distribution of the cancer. This was followed up with a 10000 mile safari, for which he described his research resources as: A photograph album illustrating the tumor; a second hand Ford stationwagon; the companionship of Dr. Clifford Nelson, "a Canadian doctor"; and Dr. Ted Williams "a mission doctor with a life-time of African experience and an expert car mechanic"[338]. The lymphoma rates were found to be high in a belt stretching from 10° north to 10° south of the equator, and rare at altitudes of over 5000 feet above sea level. This corresponded to the distribution of the areas which were holoendemic for malaria which is attributed to cause the lymphoma[343].

Fungus

In 1960, turkey poults in England were dying at high rates of a mysterious acute hepatic necrosis, which resulted in dramatic economic loses. A Brazilian groundnut component of the diet was identified as the cause of the illnesses[407]. Lancaster *et al*[269] reported that rats were peculiarly resistant to the acute necrosis, but after extended feeding they developed liver tumors without cirrhosis. Adamson *et al*[270] reported that the toxin induced liver tumors in primates as well. The carcinogenic factor was identified as aflatoxin B1 (AFB_1), a toxin which is synthesized by the fungus *Aspergillus flavus*[408]. Following this, it was been difficult to ascertain whether aflatoxins are carcinogenic to humans. The rates of liver cancer are high in hot humid tropical regions of the world where aflatoxin contaminates stored foodstuffs. However, the prevalence of aflatoxin contamination is highly heterogeneous in areas of high contamination. It was necessary to determine the exposure of specific individuals, which was not performed in early studies. Furthermore, areas with high levels of aflatoxin contamination frequently have high rates of HBV infection, which also required consideration[409,410].

A study from Shanghai, China collected blood and urine samples from 18000 middle aged men and followed them prospectively for four years[411,412]. A nested case-control analysis of the cohort estimated a relative risk of 3.4 for men with urinary metabolites of aflatoxin only, a relative risk of 7.3 among men with seropositivity for HBsAg only, and a relative risk of 59 for men who had both urinary metabolites of aflatoxin and were seropositive for HBsAg. They concluded that aflatoxin is carcinogenic, and that there is a strong positive interaction between aflatoxin and HBV. Wu *et al*[413] have written a succinct review of the evidence that aflatoxin causes liver cancer.

Bacteria

Robin Warren was a pathologist at the Royal Perth Hospital who possessed an attention for detail. He was examining gastric biopsy specimens in 1979, when he thought he saw small spiral shaped bacteria growing on the surface[414,415]. Dogma stated that the stomach is sterile, so there was much opposition to the notion of viable bacteria growing in the stomach[414]. The editors at the Lancet wanted to publish the findings, but were delayed for months because no reviewer could be found who thought the work was credible[414,415]. The bacteria were reported to be present in 80% of patients with gastric ulcers and in 100% of patients with duodenal ulcers[416]. It was later found that *helicobacter pylori (H. pylori)* survives in the stomach by residing in the gastric mucosa which provides a partially effective physical barrier from the acidic pH and by synthesizing small amounts of ammonia which neutralizes hydrochloric acid[417,418]. *H. pylori* curiously have a trophy for healthy mucosal epithelium. Among patients with duodenal ulcers, the bacteria is interestingly present at considerably low frequencies in the ulcer crater and acutely inflamed edges, compared to the healthy gastric antrum where it is present at very high frequencies[419]. As gastric tissue progresses through atrophic gastritis to intestinal metaplasia and carcinoma, the bacterium is similarly detected at decreasing frequencies in metaplasias and carcinomas compared to the healthy gastric tissue[420-422].

Patients with gastric cancer have been reported to have a higher rate of seropositivity for *H. pylori* than normal persons. A meta-analysis of 19 studies (5

cohort and 14 case-control) reported a summary odds ratio of 1.92 (95%CI: 1.32-2.78) for the occurrence of gastric cancer among those who were seropositive for *H. pylori*[423]. The association was reported to be considerably stronger when the samples for serology were collected more than ten years before the diagnosis of cancer[424].

Wood dust, alcohol

Woodworkers in the furniture industry of the Oxford region of England were reported to have high rates of nasal adenocarcinoma[425]. The increased rates were observed mostly among persons exposed to hardwood rather than softwood dust[426]. Men who worked in trades which consumed high amounts of alcohol, such as brewers and inn keepers, were reported to have high rates of cancer of the oesophagus[427].

Food preparation technique

It was reported that the incidence of NPC was high in areas of Southern China, where the consumption of salted fish was high[428]. It was later found that the process of preparing salted fish in the high incidence areas is susceptible to bacterial contamination[429], and that nitrosamines are produced during the salting process[430,431]. Rats which were fed Cantonese style salted fish developed cancers of the nasal and paranasal regions[432,433]. Some studies have reported that the consumption of Cantonese-style salted fish during weaning is associated with a higher risk of development of NPC than consumption during adulthood[434].

Herb

A group of middle aged women were involved in a weight loss regimen in Belgium that involved consumption of a mixture of Chinese herbs which included the *Aristolochia* species. They developed high rates of renal fibrosis that frequently progressed to urothelial carcinomas of the renal pelvis and ureter after a very short latency[435]. In a series of ten patients who received renal transplants for nephropathy due to *Aristolochia* consumption, four were diagnosed with a multifocal high grade carcinoma *in situ* of the renal pelvis and ureter[435]. The exposure had lasted for an average of 20 mo, and the cancers were diagnosed after only 2-6 years after ceasing the regimen[435]. In another series, 17 of 39 patients with end stage nephropathy were diagnosed with upper urinary tract urothelial carcinomas[436]. Upper urinary tract urothelial carcinomas had been previously reported to occur at high rates in phenacetin analgesic abusers[86], and were characteristic of those living in the Balkans where *aristolochic* species contaminate wheat fields[437,438] and in Taiwan where consumption of *aristolochic* herbs is common[439,440]. Urothelial carcinomas of the upper urinary tract were rare in other regions of the world[441], so the imported herb was promptly identified as the cause.

WCRF/AICR analyses of diet, obesity, exercise, and supplements

The World Cancer Research Fund and the American Institute for Research on Cancer (WCRF/AICR) undertook comprehensive meta-analyses of the potential of foods, nutrition, and physical activity to prevent cancer (www.wcrf.org). Experimental work in the 1940s and 1950s demonstrated that caloric restriction dramatically reduced the incidence of most spontaneous and induced tumors of mice[442,443]. However it is difficult to accurately estimate caloric intake of humans on an individual level, and the epidemiologic studies yielded unclear results. Body weight is much more reliably measured, and is considered a reflection of total energy balance; the sum of energy intake and energy expenditure. Cancers of the oesophagus, kidney and endometrium have been consistently reported to be associated with overweight and obesity[444,445]. There is also convincing evidence that a sedentary lifestyle increases the risk of colon, and probably breast and endometrial cancers[446,447]. Consumption of red and processed meats has been estimated to cause around a 20% increase in risk of colorectal cancer with daily consumption of moderate amounts[448,449]. Increased adult height is also associated with an increased risk of colorectal cancer[450]. Consumption of a high fibre diet has been estimated to cause around a 10% decrease in risk of colorectal cancer[451].

There has been much interest in the potential of nutritional supplements to prevent cancer[452]. Epidemiological studies have shown that populations with higher serum levels of β carotene have reduced rates of lung cancer. However, investigators were confounded when a large randomized double-blind study reported that smokers who took β carotene supplements had higher rates of lung cancers compared to those who took a placebo[453]. Numerous studies confirmed that consumption of β carotene supplements increased the risk of lung cancer in smokers, though there is no evidence that β carotene supplements increase the rate among non-smokers[454].

The WCRF/AICR used the designation of "convincing" to indicate their highest level of certainty that an agent increases or decreases the risk of cancer[455]. The discussion above was limited to factors which received the designation of "convincing". Consensus concerning the effect of diet and nutritional supplement usage is an evolving area. As new studies are published which include additional patients and are better designed, the WCRF/AIRC expects their consensus to further evolve. They concluded that there was "convincing" evidence that diets high in fruits and vegetables decreased the rates of a variety of cancers, based predominantly on evidence of case-control studies in a 1997 publication. However a number of cohort studies have been published since 1997 which have weakened the evidence, such that the evidence is no longer considered to be "convincing", so it has been revised in subsequent publications[456,457]. There

is good evidence that consumption of dairy products reduces the risk of colorectal cancer, however there is also fairly consistent evidence that consumption of dairy products increases the risk of prostate cancer[458]. This is considered to be a complex problem, so dairy products have not been designated. Recent analysis has concluded that consumption of coffee reduces the risk of liver cancer among men, though the evidence is designated as "probable" rather than "convincing"[459].

Natural verses synthetic

It is evident that numerous natural agents cause cancer in humans. Many natural components of foods have been reported to be carcinogenic to rodents[460,461]. A variety of toxins are well recognized to be produced by plants[462-464]. Bruce Ames *et al*[462] surveyed the natural chemicals which have been tested to cause cancer in rodents, and concluded that about half of those tested induced tumors[465]. Concerning the synthetic chemicals which have been tested, about half of them similarly induced tumors[462,465] (Brambilla *et al*[466] independently concluded that around 50% of pharmaceuticals cause cancer). Ames *et al*[462] concluded that natural chemicals are just as carcinogenic as synthetic chemicals.

It is frequently not obvious which substances are synthetic. Soot, coal tar, asbestos, erionite, arsenic, uranium, radon, and radium could each be considered natural because they occur naturally, though usually at very low levels. The author posits that the level of exposure is more important than whether a substance is natural or synthetic. The dose-response relationship is strongly supported by studies with both experimental animals and humans[467-469]. According to the dose-response relationship, when the exposure levels are low, the rates of cancers are low, and when the exposures are high, the rates of cancer are high. Whether the dose-response curve is linear is controversial, and the shape of the curve may change at low dosages, but the principle of increasing rates of cancer for increasing levels of exposure is well established for most exposures[138].

Recognition of soot as a carcinogen only emerged when young boys were exposed to high levels with the advent of chimney sweeping as an occupation. Radium occurs naturally in the mineral pitchblende. Its carcinogenic activity only became obvious when it was refined from pitchblende, ingested by watch dial painters, and then induced osteosarcomas of the jaw bones among dial painters. It could be argued that occupational levels of exposure are unnatural, though it is relevant to mention that a great number of occupational exposures are not associated with cancer[470].

Emerging studies of possible new natural carcinogens

The variety of natural agents which have been documented to cause cancer is considerable. While viruses have received an intense amount of research for their possible roles in cancer, bacteria have received considerably less. The colon contains 10^{11}-10^{12} bacteria per gram of fecal material[471]. The recent development of high-throughput technologies is permitting detailed analysis of the microbial composition of the feces as well as the mucosa of the colon[472,473]. Interesting associations of certain bacterial species with colorectal cancers have been reported[474-476]. The microbiota of the colon can be modified with antibiotics, prebiotics and probiotics, so it will be interesting to observe how this research develops.

CONCLUSION

Some occupational carcinogens were readily identified, because they were akin to natural experiments, in which a few people in the community were exposed to uncommon substances with very rare types of cancer occurring in some occupations. Scrotal cancer was practically unheard of, except among chimney sweeps, and then later among men with occupational exposure to coal tar or shale oil[12]. Mesothelioma was encountered so seldom, that before the report of 33 cases among asbestos miners in South Africa, some pathologists had argued that tumors of the mesothelium did not occur[61,477,478]. It had been argued that tumors ascribed to mesothelial origin were likely misdiagnosed tumors of other cell types, which had metastasized to the mesothelium. Occupational exposures which caused very rare cancers were promptly recognized.

Other occupational exposures caused excessively high rates of common cancers. Around 50% of the early miners of Schneeberg and Joachimsthal were estimated to have developed lung cancer[479]. A small group of men who worked distilling β-naphthylamine in England were reported to have 90%-100% incidence of bladder cancer[480-482]. Barling reflected in 1926, that with the exception of Hall-Edwards, all of the other early radiologists whom he knew, had succumbed to skin cancers[483]. Occupational exposures which caused extremely high rates of common cancers were also readily recognized.

The notion that synthetic agents are the cause of cancer evolved from the early identification of occupational (or synthetic) agents. Infectious agents, as well as other naturally occurring agents, were usually more widespread geographically. The exposure to naturally occurring agents generally extended temporally for generations, so their effects were not readily evident.

Proof of most infectious causes of cancer required both technical advancements to identify the agents and large epidemiological studies to determine whether the agent is associated with increased rates of cancer. A few of the earliest infectious agents were identified with simple methods, but others could not be elucidated until more advanced methods were developed. The ova of *S. haematobium* and *O. viverrini* were identified with simple laboratory techniques and low powered light microscopes[403,484]. EBV was discovered using the EM[339], which only became consistently reliable in the

1950s[192]. HTLV and HIV were discovered using enzyme assays for reverse transcriptase, which were developed following the discovery that RNA viruses replicate using this enzyme[217,218]. HPV-16, HPV-18 and HCV were identified using recombinant DNA technology shortly after it became available in the mid-1970s. Modern techniques, such as next generation DNA sequencing introduced in 2005[174] have the potential to lead to the identification of additional infectious causes of cancer.

Each of the early theories concerning the cause of cancer which were described in the introduction, have been demonstrated to have some validity. Cancers are now considered to be caused by both irritation and infectious agents. Cohnheim's theory of embryonic rests also appears to have some validity, as there is evidence that tumor cells originate from stem cells[485]. The range of identified human carcinogens now includes occupational exposures, pharmaceuticals, X-rays, natural factors, non-viral infectious agents, and viruses.

Important contributions were sometimes made by persons who were not formally well qualified and occasionally by researchers not specifically searching for causes of cancer. Those who made important contributions were usually characterized by meticulous attention to detail and those who investigated viruses often persisted in their study in the presence of adversity.

It is interesting to reflect, that contrary to the dogma of the early 1900s, that infectious agents did not and could not cause cancer, it is now evident that they do. Discoveries of the roles of infectious agents as causes of cancer have contributed significantly to our ability to prevent cancer since 1980. Cervical cancer is the second most common cancer among women in the developing world[486]. Essentially all cervical cancers are now attributed to HPV, as cervical cancer seldom occurs without exposure to HPV[487]. Few chemical carcinogens share this distinction of being essential, for the development of specific cancer. Cervical cancer accounts for 5.2% of the world cancer burden cancer[488]. Liver cancer is the third most common cancer among men in the developing world[486]. Liver cancers caused by HBV and HCV are estimated to account for 4.9% of the total world burden of cancer[488]. *H. pylorus* has been estimated to cause around 2/3 of the cases of gastric cancer[486], and is estimated to cause 5.5% of the world cancer burden[488]. The global burden of cancers due to infectious agents has been estimated to be 17.8%[488].

Infectious agents were expected to be present in the diseased tissue according to Koch's postulates, but liver cancer caused by HBV is often devoid of the virus, except for the integrated virus which is truncated and unable to produce infectious particles. HPV follows a similar pattern in cervical cancers, while H pylora is usually completely devoid in gastric cancer tissues. Infectious agents did not cause cancer in a manner predicted by microbiologists of the early 1900s.

The IARC designated 109 agents as group 1 carcinogens in their update of November 7, 2012. Group 1 is defined as having "sufficient evidence of carcinogenicity in humans". About half of these agents have been discussed in this review. Selection of carcinogens for inclusion/exclusion was designed to include the widest possible variety of agents without producing an excessively long list. If a mixture was found to be carcinogenic and later a component of the mixture was also reported to be carcinogenic, then usually only the mixture or the pure agent was reviewed. If one route of exposure was determined to be carcinogenic (*e.g.*, dermal exposure to coal tar), then usually other routes of exposure were not discussed (*e.g.*, inhalation of fumes of coal tar). About half of the agents listed as group 1 carcinogens were excluded based on these principles. The complete list of the 109 group 1 carcinogens is available at the IRAC website (www.iarc.fr) or (http://monographs.iarc.fr/ENG/Classification/index.php last accessed January 27, 2013).

Additional information concerning the above discussed carcinogens can be found in the IARC monograph series. Cumulative indexes are provided at the end of recent volumes of the monographs, and also at their website (http://www.iarc.fr). The National Toxicology Program has independently compiled a similar list of carcinogens (http://ntp.niehs.nih.gov/ntp/roc/twelfth/roc12.pdf). Foods, supplements, physical activity, and obesity have been comprehensively reviewed in the publication titled Food, Nutrition, Physical Activity and Prevention of Cancer: A Global Perspective which is available at the World Cancer Research Fund website (http://www.wcrf.org).

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REVIEW

Overview, prevention and management of chemotherapy extravasation

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Abstract

Chemotherapy extravasation remains an accidental complication of chemotherapy administration and may result in serious damage to patients. We review in this article the clinical aspects of chemotherapy extravasation and latest advances in definitions, classification, prevention, management and guidelines. We review the grading of extravasation and tissue damage according to various chemotherapeutic drugs and present an update on treatment and new antidotes including dexrazoxane for anthracyclines extravasation. We highlight the importance of education and training of the oncology team for prevention and prompt pharmacological and non-pharmacological management and stress the availability of new antidotes like dexrazoxane wherever anthracyclines are being infused.

Key words: Chemotherapy; Extravasation; Vesicant; Tissue damage; Dimethyl sulfoxide; Dexrazoxane; Antidote; Hyaluronidase

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Core tip: Chemotherapy administration carries safety concerns, which include accidental extravasation, to patients. We review and update readers and health care providers on the risks of chemotherapy extravasation, prevention and management. We present the definitions, grading, classification and guidelines related to chemotherapeutic drugs and groups. We present an update on prevention and management and antidotes, particularly dexrazoxane for anthracyclines extravasation. We present summary statements of American Society of Clinical Oncology, European Society of medical Oncology, Oncology Nursing Society and European Oncology Nursing Society guidelines. We stress the importance of education and training of the entire oncology team members who share responsibility to ensure the safe

administration of chemotherapy and avoid extravasation.

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INTRODUCTION

Intravenous infusion is the principal modality of administration of anti-cancer drugs for most types of malignant disorders with numbers exceeding 1 million infusions each day worldwide[1]. Chemotherapy administration carries safety concerns to both patients and the medical team. These concerns include extravasation of chemotherapy, which is defined as the accidental infiltration of chemotherapy into the subcutaneous or sub-dermal tissue at the injection site[1-4], and can result in tissue necrosis[1,2,4,5]. The exact incidence of chemotherapy extravasation varies greatly due to the general lack of reporting and absence of centralized registry of chemotherapy extravasation events. While center-based guidelines and policies attempt to minimize its risk, chemotherapy extravasation still has a prevalence that can range from 0.1% to 6% when administered through a peripheral intravenous access[3] and from 0.26% to 4.7% when administered through a central venous access device (CVAD)[6-8]. Institution-based guidelines should be based on evidence, where available, but they are often vague and non-specific, if present[1,4].

In order to avoid additional chemotherapy adverse effects, every effort should be made to minimize the complications of chemotherapy administration. All the oncology team members share responsibility to ensure the safe administration of chemotherapy. In this article, we review the literature, provide clinical information on chemotherapy extravasation, and discuss guidelines and recommendations for its prevention and management. This article serves as a review of the clinical aspects of chemotherapy extravasation and latest advances in classification, prevention and management of chemotherapy extravasation.

This review includes a comprehensive literature search in the PubMed, Med-Line and Google Scholar databases was conducted for guidelines, case reports, clinical trials, retrospective studies and conferences on chemotherapy extravasation prevention and management. We used the following Medical Subject Headings terms: "Chemotherapy", "extravasation", "prevention", "management", "extravasation", and "guidelines" and combined them using boolean operators. Once we found a set of relative citations, we included citations using the "related articles" option as well. All references that we thought were relevant were printed and analyzed, and their main relevant ideas were paraphrased and noted. Literature review was focused on our research question: "What should a healthcare practitioner know about chemotherapy extravasation, its prevention, and its management based on the current literature?".

CLASSIFICATION

Classification of intravenously administered drugs

Intravenously administered drugs can be classified into five categories according to their damage potential: Vesicant, Exfoliants, Irritants, Inflammitants, and Neutrals. The drug damage from extravasation can range from skin erythema to soft tissue necrosis. We list below examples of intravenously administered drugs according to various categories and in decreasing order of damage potential[9-13].

Vesicants: Drugs that can result in tissue necrosis or formation of blisters when accidentally infused into tissue surrounding a vein[14]. They include Actinomycin D, Dactinomycin, Daunorubicin, Doxorubicin, Epirubicin, Idarubicin, Mitomycin C, Vinblastine, Vindesine, Vincristine, and Vinorelbine.

Exfoliants (may have low vesicant potential): Drugs that can cause inflammation and shedding (peeling off) of skin without causing underlying tissue death[15]. Drugs may cause superficial tissue injury, blisters and desquamation[12,13]. They include Aclacinomycin, Cisplatin, Docetaxel, Liposomal Doxorubicin, Mitoxantrone, Oxaliplatin, and Paclitaxel.

Irritants: Drugs that can cause inflammation, pain or irritation at the extravasation site[14], without any blister formation. Clinicians use the term irritant also to refer to drugs that can cause a burning sensation in the vein while being administered: Bendamustine, bleomycin, carboplatin, dexrasoxane, etoposide, teniposide, and topotecan.

Inflammitants: Drugs that cause mild to moderate inflammation, painless skin erythema and elevation (flare reaction) at the extravasation site[14]. They include bortezomib, 5-fluorouracil, methotrexate, and raltitrexed.

Neutrals: Drugs that neither cause inflammation nor damage upon extravasation[14]. Monoclonal antibodies (rituximab and trastuzumab) are also listed under this category: Asparaginase, bevacizumab, bleomycin, bortezomib, cetuximab, cyclophosphamide, cytarabine, eribulin, fludarabine, gemcitabine, ifosfamide, melphalan, rituximab, and trastuzumab.

Table 1 summarizes the different types of the above mentioned drugs according to their vesicant potential.

Vesicants may be sub-classified into DNA binding drugs and non-DNA binding drugs[16]. DNA binding drugs are capable of producing more severe tissue damage and mainly include anthracyclines and alkylating agents such as mechloretamine and bendamustine. Non-DNA

Table 1 The different types of the above mentioned drugs according to their vesicant potential

Neutrals	Inflammitants	Irritants	Exfoliants (may have low vesicant potential)	Vesicants
Asparaginase Bevacizumab bleomycin Bortezomib cetuximab, Cyclophosphamide	Bortezomib	Bendamustine	Aclacinomycin cisplatin Docetaxel liposomal Doxorubicin mitoxantrone Oxaliplatin paclitaxel	Actinomycin D
Cytarabine eribulin Fludarabine gemcitabine Ifosfamide	5-Fluorouracil methotrexate raltitrexed	Bleomycin		Dactinomycin daunorubicin Doxorubicin epirubicin Idarubicin mitomycin C Vinblastine vindesine
Melphalan rituximab Trastuzumab		Carboplatin dexrasoxane Etoposide Teniposide Topotecan		Vincristine vinorelbine

binding compounds are mainly vinca alkaloids and taxanes[13].

Drugs do not always fall under the strict definitions, and case reports of different extravasation potentials have been described. For example, taxanes have a poorly defined delineation between vesicants or irritants[13]. Docetaxel, though usually refered to as an irritant, has exfoliant and low vesicant properties described in 12 case reports[5,12,16-25]. While vinorelbinecan cause severe irritation inside the vein site of infusion, it is a moderate vesicant if extravasated into the surrounding tissue[17,18,25]. Alkylating agents like cyclophosphamide, ifosfamide and andmelphalan are considered neutrals[16]. Although etoposide and teniposideare usually classified as irritants, they may have low vesicant potential if a highly concentrated infused drug is extravasated[9].

Flare reaction, vessel irritation and venous shock, are other events that should be differentiated extravasation. Flare reaction is a not uncommon transient painless skin streaking erythema looking like urticaria with skin elevation that may occur with anthracycline administration. It is usually associated with itching, burning sensation and pain and that resolves within 1 to 2 h[26,27]. Vessel irritation causes pain, discomfort and tightness along the infused vessel with possible accompanied erythema and dark skin discoloration[27]. While both flare reaction and vessel irritation do not usually present with erythema, the extravasation is usually associated with swelling of the tissue surrounding the infused veinand predominantly manifests by erythema. The patient will complain of aches and burning sensation at the administration site. Unlike flare reaction and vessel irritation, extravasation is usually manifested with no or minimal blood return at the infusion site. Venous shock is due to the administration of very cold agents into the vein causing the loss of blood flow return due to venous muscle spasm, and it is managed by the application of warm compressors which can help to relax the vein[27].

RISK FACTORS

Risk factors of chemotherapy extravasation

Risk factors are related to the chemotherapeutic agent infused, patient factor, and iatrogenic causes. Factors related to chemotherapeutic agent itself and that increase the risk of chemotherapy extravasation include the vesicant properties of the drug, its concentration, volume and duration in which the infusion extravasated[28]. Factors related to patients and that increase the risk of chemotherapy extravasation include small and/or fragile veins, lymphedema, obesity, impaired level of consciousness, and having had previous multiple venipunctures. Iatrogenic causes include lack of training of nurses, poor cannula size selection, poor location selection and lack of time. Extravasation can occur upon accidental puncturing of the vein or upon movement of the cannula itself due to movement of the patient or insecure fixing. Prolonged peripheral line infusions of vesicants carry an increased risk of extravasation and vesicants should not be infused as prolonged unsupervised infusions *via* a peripheral vein[3].

CLINICAL MANIFESTATIONS

Tissue damage

Chemotherapy extravasation is manifested by a wide range of symptoms that can be mild and can present as an acute burning pain, swelling, at the infusion site. Symptoms vary according to the amount and concentration of extravasated drug. Pain and erythema, induration and skin discoloration progresses over few days and weeks, and may progress to blister formation. Blister formation or necrosis can lead to invasion and destruction of deeper structures[1,3-5]. Damage can reach tendons, nerves, and joints[19] depending on the location of the vein where extravasation occurs.

Grading of severity of extravasation

According to the latest Common Terminology Criteria for Adverse Events (CTCAE), published by the United States Department of Health and Human Services, National Institutes of Health, Natinal Cancer Institute, and widely used in Clinical Trials[20] (Version 4.0, May 2009), extravasation can be divided into four grades (Table 1) ranging from 2, which is manifested by erythema with associated edema, pain, induration, and phlebitis,

Table 2 Grades of Infusion site extravasation according to common terminology criteria for adverse events (V4.0, May 2009)

Adverse event	Grade				
	1	2	3	4	5
Infusion site extravasation	-	Erythema with associated symptoms (*e.g.*, edema, pain, induration, phlebitis)	Ulceration or necrosis; severe tissue damage; operative intervention indicated	Life-threatening consequences; urgent intervention indicated	Death

to grade 5, which refers to extravasation that leads to death. There is no grade 1. Table 2 shows the four grades of extravasation of chemotherapy (CTCAE V4).

Factors that determine the extent of tissue damage from chemotherapy extravasation

Factors that determine the extent of tissue damage from chemotherapy extravasation include its pH, osmolarity, vasoconstrictive potential, and duration for which it remains in tissue. Infusion solution whose pH is far from the physiologic pH (7.35-7.40) and/or osmolarity (281-282 mOsm/L) can irritate the venous endothelium and vessel wall and can damage the cell proteins and cause cell death[21]. Hypertonic solutions can further increase tissue injury and lead to necrosis. Vesicants with high vasoconstrictive potential can result in tissue necrosis by severe vasoconstriction of capillary smooth muscles and reducing blood flow. Vesicants that are retained in extravasation tissue area for a long duration lead to a vicious cycle of direct cell injury. Typical examples are anthracyclines which enter the cells and bind to DNA causing immediate and continuous tissue injury. On the other hand, vesicants that are easily metabolized and are not retained in tissue include vinca alkaloids and taxanes. Despite their ability to cause direct tissue damage, they cannot bind to DNA and are easily metabolized[21].

Manifestations of some commonly used chemotherapeutic drugs

Anthracyclines: Although all vesicants can cause tissue damage upon extravasation, anthracyclines, such as daunorubicin, doxorubicin, epirubicin, and idarubicin, have the greatest vesicant potential when compared to other chemotherapeutic agents. While all chemotherapeutic agents cause similar signs upon extravasation, anthracyclines are characterized by causing immediate pain and burning sensation, which can last up to hours and can be severe. Lesions form slowly over weeks and expand over periods of months due to tissue retention of the extravasant vesicant[9]. Weeks after the extravasation episode, surrounding tissue may become red, firm and tender. The resolution of redness depends on the size of the extravasation area. If the area is small in size, redness will gradually resolve over the following weeks. If extravasation is significant, the center of the redness area becomes necrotic and painful. The accidental leak of anthracyclines can cause severe tissue damage. By cellular uptake and remaining for an extended period of time in tissue, they cause a continuous vicious cycle of tissue damage[22-24,29]. In two prospective, open-label clinical trials[22,30], the patient with anthracycline extravasation who developed tissue necrosis had a large extravasation area of 253 cm^2. If the necrotic area is painful, surgical debridement may be required to remove any damaged and possibly infected necrotic tissue. In case no debridement is indicated, necrosis can progress to result in a thick, leathery eschar surrounded by a band of red painful skin, and can ulcerate to the underlying neurovascular tissue and tendons and cause pain. Ulceration is usually progressive and can result in persistent burning pain, nerve damage, and joint stiffness all of which may compromise the function of the involved organ or even cause its permanent disability[29]. Spontaneous healing rarely occurs after anthracyclines extravasation. In addition to surgical debridement, split-thickness skin graft is usually required when the necrosis extends deep into the tissue. In case the periosteum of underlying bone was involved, the skin graft cannot survive on cortical bone and the area of injury should be covered instead by a pedicle skin flap[23]. Dexrazoxane hydrochloride was FDA-approved for anthracyclines extravasation and has been reported to produce significant extravasation wound healing[4].

Liposomal encapsulation of doxorubicin reduces the toxicity of doxorubicin extravasation by decreasing its diffusion capacity and hence its toxicity to healthy tissue[31]. In phase Ⅱ and Ⅲ clinical trials assessing liposomal doxorubicin efficacy, two extravasations were reported and caused only inflammation with complete recovery and no tissue damage[31]. In a few case reports of liposomal doxorubicin extravasation, patients had reported pain, erythema, and edema but no necrosis or ulceration of extravasation area nor there were need to undergo surgical debridement[31].

Vinca alkaloids: Vinca alkaloids, which include vinblastine, vincristine, and vinorelbine, can cause direct cellular damage upon extravasation. Extravasation is known to cause a mostly painful ulceration, local paresthesia and slow healing[9,32]. It can cause significant irritation and usually presents with intense pain around intravenous line or port site, erythema and tenderness[32]. Erythema may be delayed by 1-2 h and even 3 d depending on the dosage of the vinca alkaloid administered[32]. This is followed by blister formation, swelling and induration and can be complicated by sloughing, ulceration and tissue necrosis. Vinorelbine, which is a moderate vesicant, also causes common irritation and burning sensations which are prevented by proper dilution, short infusion time and use of an adequately large vein[33].

Taxanes: Taxanes, including docetaxel and paclitaxel, are most often classified by literature as vesicants although there is no clear delineation. Most reactions following extravasation of taxanes consist of erythema, tenderness and swelling[12]. There are case reports of patients who had necrosis and skin exfoliation[34-38]. It is rare that taxane extravasation requires surgical debridement. In a paper that combined 35 case reports, only three patients developed ulceration two of whom required skin closure[11].

Oxaliplatin: Platinum compounds have been classified as irritants. Oxaliplatin has been recently reported to have vesicant properties[9]. Extravasation usually begins with a palpable swelling and discomfort upon palpation[9]. Lesion usually progresses to erythematous painful lesions and resemble erysipelas[9,10]. Long-term outcome is usually healing and necrosis and surgical debridement are rarely needed. The harm caused by oxaliplatin extravasation is not comparable to that of anthracyclines and vinca alkaloids[10].

PREVENTION

Medical team continuing education and training

Education and training are basic elements for licensing health care professionals and for good clinical practice. They are essential to improve management and patient outcome. Education and training among nurses and physicians remains the mainstay of safe chemotherapy administration and emphasizes the importance of being *preemptive* instead of reactive to extravasation[1,27]. In fact, the Joint Commission International emphasizes the standards of proper chemotherapy administration[39]. Knowledge of literature and international guidelines is essential. Local institution policies should be available and stress proper administration of Ⅳ chemotherapy and prevention of accidental extravasation[19,37]. Education of the medical team about extravasation prevention includes ensuring knowledge of risk factors, signs and symptoms, guidelines for prevention and management. Compliance to manufacturer's recommendations for each drug should be ensured byboth, nurses and physicians, as well as clinical pharmacists.

Appropriate vascular access

Consideration of the appropriate vascular access is crucial for the prevention of chemotherapy extravasation. Chemotherapy infusion can be either through a central venous access or through an adequate peripheral vein. Central venous access can be accomplished through a CVAD that is placed either as an implanted port or as a peripherally inserted central catheter[1]. CVADs are also known as Port-a-cath[40] or polysite[41] catheters. Veins that are small and/or fragile should be avoided as they might not withstand the required flow and rate of infusion and may have a lower threshold for extravasation. Locations that are also generally avoided include the dorsum of the hand, the antecubital fossa, and the radial and ulnar aspects of forearm[2,20]. Patients who do not have adequate peripheral venous access should have a central venous catheter placed[16].

Peripheral arm assessment consists of: (1) assessing location and fragility of the patient's veins that can be reflected by the inspection and palpation of the vein. Veins that have a small caliber and/or are superficial are generally considered fragileand should be avoided. In addition, assessment; also consists of (2) patient's age; (3) presence of diabetes; (4) steroid use; (5) history of previous venipunctures; (6) presence or absence of ecchymosis; (7) prior hospitalization or blood drawing history of axillary lymph nodes dissection; (8) lymphedema; (9)vascular accident in an extremity, which is the accidental puncturing of a vein. In parallel to peripheral arm assessment, the level of consciousness of the patient should be also assessed for the purpose of assuring immobility and compliance during catheter insertion[1,2,16].

Appropriate cannula and needle selection

Selection of the appropriate cannula type and size play an important role in chemotherapy extravasation prevention. The ideal cannula is one that can remain patent to allow blood flow and that does not dislodge from its place. The recommended choice is to use the smallest size of adequate and appropriate cannula in the largest vein available. Use of 1.2-1.5 cm long small bore plastic cannula and a clear dressing that shows any possible extravasation beneath it are recommended[42]. A butterfly needle should never be used for vesicant chemotherapy administration[16,43].

Patient education

Since patients are the first to feel any symptoms of possible extravasation and are relied upon to report them, their education is a crucial step in chemotherapy extravasation prevention. Risk of chemotherapy extravasation should be clearly explained to patients. Physician and nurses should emphasize to the patient the importance of providing accurate history regarding previous manipulation in extremities, cooperation with the person performing the venipuncture, and reporting any symptoms that may arise during the infusion[1,27]. Patients should be instructed to report any discomfort, pain, redness or swelling at infusion sites. Nurses and physicians should never underestimate the significance of any patient symptom and check the infusion site and venous patency immediately. Patients should also be aware of the class of drug and options of venous access and understand the higher risk of extravasation associated with it should be explained if they choose peripheral venous access over central[1].

Guidelines for chemotherapy administration and extravasation prevention

Although there are no prospective randomized clinical trials to establish treatment of chemotherapy extravasation, management of chemotherapy extravasation

Table 3 Overall summary of guidelines for prevention of chemotherapy extravasation

Continuous education of the medical team about all policies and protocols regarding chemotherapy administration
Classification of chemotherapeutic drugs: Knowledge of characteristics of the drug and compliance to the manufacturer's recommendations
Appropriate vascular access
In case a central vascular access is not possible, an adequate peripheral vein is used[16]
Veins that are small and/or fragile should be avoided[2,20]
It is not recommended to use veins located at the dorsum of the hand, the antecubital fossa, and the radial and ulnar aspects of forearm[2,20]
Appropriate peripheral arm assessment[1,2,16]
Palpation of the vein
History of previous venipunctures
Available extremities where veins can be punctured
Level of consciousness of the patient
Appropriate equipment selection[42,43]
Use of the smallest size of cannula in the largest available vein
Use of 1.2-1.5 cm long small bore plastic cannula
Use of a clear dressing
Avoiding the use of a butterfly needle
Educating the patient about all risks associated with chemotherapy administration
Devising and updating standards and policies regarding chemotherapy administration at each healthcare center
Documentation and reporting of any extravasation incident

have been learnt through case reports, animal models and international clinical studies. We present relevant important statements from North American and European Guidelines published by the European Oncology Nursing Society (EONS), Oncology Nursing Society (ONS), American Society of Clinical Oncology (ASCO) and European Society of medical Oncology (ESMO)[16,44]. In addition to International published guidelines, local institutions should have their own adapted guidelines and pathways for chemotherapy administration and also management of accidental extravasation.

ASCO and ONS: The ASCO and the ONS published safety standards for chemotherapy administration in outpatient[19,44] and inpatient settings[45,46]. These standards outlined the important steps in chemotherapy administration, including defining the "extravasation management procedures"[44] prior to administration. ONS published extravasation prevention and management guidelines in the book "Chemotherapy and Biotherapy Guidelines and Recommendations for Practice", Polovich *et al*[11] (2009). Examples of guidelines provided are close monitoring of the infusion site every 5 to 10 min and avoiding infusion of vesicants for more than 30 to 60 min[1]. In addition, ONS has an online course, ONS/ONCC Chemotherapy Biotherapy Certificate Course[47] that reinforces important information to safe administration of chemotherapy and provides links to online courses, such as "Access Device: The virtual clinic", which helps better train nurses and physicians[47]. The ASCO has a special emphasis on chemotherapy administration. It launched in 2010 the Quality Oncology Practice Initiative (QOPI) Certification Program (QCP). In the QCP report published in the Journal of Oncology Practice in March 2013, Gilmore *et al*[48] measured implementation of chemotherapy administration safety standards in the setting of outpatient cancer patients. Extravasation management procedures were met by 40.47% of practices. The report emphasized the importance of availability of up-to-date extravasation management standards at the sites[48].

ESMO and EONS: The EONS published in 2007 guidelines that can help nurses better understand extravasation[49,50]. It conducted its sixth Spring Convention in 2008 in Geneva, Switzerland, where it launched the new guidelines for chemotherapy extravasation prevention and management. Guidelines included nursese ducation, assessment of venous access, assessment of equipment used, and the importance of patient education[42,50]. This was followed by publishing guidelines developed jointly with the ESMO in 2012[16]. Details of guidelines published are included in the following section "Management".

Local institution guidelines: These should be encouraged and include definition and diagnosis of extravasation, risk factors, guidelines for prevention, and management[27]. For example, Cleveland Clinic has standards of chemotherapy administration clearly stated in its "Chemotherapy/Biotherapy Safe Handling Guidelines (Policy NPM-127), which was initially published in 1996 and revised in 2007[51]. Any local incidence of extravasation should be reported. While documentation may differ among institutions, certain items remain essential and should be documented for every incident. In addition to date and time and patient's name, name of the drug, characteristics of the solution infused, the Ⅳ access used, description of the extravasation area, signs and symptoms and management should always be documented[16].

Table 3 summarizes guidelines for chemotherapy extravasation prevention.

MANAGEMENT

Continuous monitoring at the beginning and during the infusion is essential every 5 to 10 min. Cancers centers should ensure the availability of "Extravasation

Kits" at the treatment units. These kits should contain disposable syringes and cannulas, cold-hot packs, gauze pads, adhesive plaster, gloves, and antidotes that can be used in cases of extravasation and that will be discussed below[3]. Management according to EONS and ONS, and few available clinical studies, are outlined below.

Initial non-pharmacologic management

In case of chemotherapy extravasation and as soon as the patient complains of pain or swelling, the first step should be immediate cessation of the infusion while keeping the cannula or port needle in place. This is followed by attempts at aspiration of the chemotherapeutic agent and removing the cannula or port needle[3]. Aspiration of the drug is usually done by a 10 mL syringe, percutaneous needle aspiration, liposuction, simple squeeze maneuver, or by surgical fenestration and irrigation[3,21]. Catheter can then be removed if there are no antidotes that need to be infused at the extravasated site. Elevation of the affected limb and thermal application by either cold or hot packs should follow[28]. Elevation of the limb helps in reabsorption of the extravasated agent by decreasing capillary hydrostatic pressure and It is recommended to during the first 24 to 48 h of the incident[21]. It is also recommended that thermal application is performed approximately four times daily for 20 min each for 1-2 d[42]. In addition, saline dispersion can help in diluting the vesicantby infiltrating normal saline *via* a large catheter[21]. Taking a photo of the extravasation area helps for follow up of progress or healing process. Cold compresses can be used to reduce pain and local inflammation by causing vasoconstriction and reducing drug further spread. Cold compresses should not be used in the cases of extravasation of vinca alkaloids because it may cause further tissue damage[14]; warm compresses and heat can be applied in incidents of vinca alkaloids extravasation as they may cause vasodilatation and absorption of extravasated drug from tissue sites.

Pharmacologic management

Dexrazoxane hydrochloride for anthracycline extravasation: Dexrazoxane is a member of the bisdioxopiperazine family and is an FDA-approved antidote for intravenous anthracycline extravasation[52]. The exact mechanism by which it reduces tissue damage resulting from chemotherapy extravasation is unknown. There is general belief that it works through two main mechanisms. Being an analog of the iron chelatorethylenediaminetetraacetic acid that can strongly bind Iron and displace it fromanthracycline, it is thought that dexrazoxane helps to reduce the oxidative stress caused by complexes of metal ions and anthracyclines[4,53]. Also, it can exerta catalytic inhibition of topoisomerase Ⅱ,the main target of anthracyclines[53]. Dexrazoxane has been initially used to reduce the incidence of cardiomyopathy associated with anthracyclnes and is approved in patients with breast cancer responding to doxorubicin and requiring continued therapy after they exceed 300 mg/m^2[52]. Dexrazoxane is administered as a 1-2 h intravenous infusion (Ⅳ) for 3 consecutive days through a large caliber vein in a limb other than the affected one[3,4,21] as follows: It is usually given at a dosage of 1000 mg/m^2 within 5 h of extravasation and then at a dosage of 1000 mg/m^2 on second day and 500 mg/m^2 on the third day following extravasation[3,43]. To date, in addition to several case reports[4,52], there are two large prospective multicenter clinical trialsabout the use of dexrazoxane in anthracyclines vesicant extravasation[21,22,30,52]. The overall efficacy of dexrazoxanewas 98%[53]. Langer *et al*[41] also reported prevention of complications of doxorubicin and epirubicin extravasation by dexrazoxane. In a case of port-a-cath chest wall massive extravasation, El-Saghir *et al*[4] reported the successful use of dexrazoxane, for immediate relief of pain and slowing down of necrosis, along with local infiltration of granulocyte-macrophage colony-stimulating factor at the borders of the ulceration site to promote the acceleration of wound healing and reduce the need for skin grafting[4]. The two prospective open-label single-arm studies in patients with anthracyclines extravasation were published in 2007 by Mouridsen *et al*[30]. Dexrazoxane was given within 6 h and repeated at 24 and 48 h. Efficacy was noted in 53 of 54 patients (98.2%) and only one patient required surgical debridement. Toxicity was manageable and includes transient elevation of liver enzymes and neutropenia that may be also due to chemotherapy itself. The use of dexrazoxane as an antidote to anthracyclines extravasation is now recommended by NCCN, EONS, ONS, and ASCO and has been formulated in a new preparation and has level Ⅲ-B evidence (Evidence Level Ⅲ: Evidence obtained from well-designed controlled trials without randomization; "B": Moderate strength of recommendation)[16,54]. Doxorubicin is one of the most widely used drugs and hence has the highest potential and risk for extravasation, and, therefore, dexrazoxane should be made available at all centers that administer anthracyclines chemotherapy.

Hyaluronidase: Hyaluronidase is an enzyme that degrades hyaluronic acid in tissues and promotes diffusion of the extravasated agent. The usual dose consists of multiple subcutaneous injections of hyaluronidase 150-100 IU given as five 0.2 mL injections[42,55]. When used for chemotherapy extravasation, it is recommended for vinca-alkaloids, etoposide[56] and taxanes extravasation mainly[16,23] and has level Ⅴ-C evidence (Evidence Level Ⅴ: Evidence from systematic reviews of descriptive and qualitative studies; "C": Poor strength of recommendation)[16,54]. It is injected locally subcutaneously into the extravasation area.

Dimethyl sulfoxide: Dimethyl sulfoxide (DMSO) is an organosulfar solvent that is topically applied to improve absorption of the extravasated solvent[21,49].

It also has free-radical scavenging properties[3]. Its efficacy was observedin few studies. In a prospective study by Cassagnol *et al*[3], patients withanthracycline extravasation, DMSO 99% was administered twice daily for a period of 14 d and no ulcers were described[3]. In another prospective study by Bertelli *et al*[56], out of a total of 122 assessable patients with extravasation of doxorubicin, epirubicin, mitomycin, mitoxantrone, cisplatin, carboplatin, ifosfamide or fluorouracil, only one patient suffered an ulceration. Treatment with DMSO was generally well tolerated with the only side effect being mild local burning and breath odor[57]. The use of topical DMSO (99%) as an antidote to anthracycline extravasation and to Mytomicin C has level Ⅳ-B evidence (Evidence Level Ⅳ: Evidence from well-designed case-control and cohort; "B": moderate strength of recommendation)[16,54]. DMSO is available as a solvent, and a dropper is usually used to instill drops over the affected skin. It is used asa topical application of DMSO 99% of four drops per 10 cm^2 to twice the size of the extravasation area[3,22]. In cases of anthracyclines extravasation, the combination of DMSO and cooling are most commonly described initial therapy for minor anthracyclines extravasation, especially when dexrazoxane is not available.

Sodium thiosulfate: It is an antidote generally recommended for mechlorethamine (nitrogen mustard) extravasation. A study conducted by Doellman *et al*[21] showed that the use of sodium thiosulfate was associated with significantly improved healing time in 63 patients who had a variety of chemotherapy induced extravasation injuries, including doxorubicin, epirubicin, vinblastine, and mitomycin C[21]. The use of sodium thiosulfate as an antidote to mechlorethamine extravasation has level Ⅴ-C evidence (Evidence level Ⅴ: Evidence from systematic reviews of descriptive and qualitative studies; "C": poor strength of recommendation)[16,54]. It is usually subcutaneously locally injected in a 2 mL solution at a concentration of 0.17 mol/L[16].

Acceleration of wound healing

Local injection of corticosteroids has been hypothesized to accelerate wound healing and prevent ulcer formation. While *in vitro* animal experimental studies showed no prevention of ulcer formation after corticosteroid injection, it was reported to have clinical benefit on ulcer prevention when used on humans[58-61]. Variable results have been reported regarding the success of wound healing after the use of local corticosteroids, which depends on the amount of inflammatory cells generated at the site of extravasation[62]. Local injection of granulocyte macrophage colony-stimulating factor, which is a glycoprotein growth factor, has been reported to be beneficial to wound healing in cases of doxorubicin extravasation[4,62]. The mechanism is believed to be through stimulation of cellular components such as fibroblasts and endothelial cells[62]. Also, local injection of normal saline has been also mentioned as beneficial in prevention of wound ulceration after extravasation[62].

Surgery and skin grafting

Indications for surgery in chemotherapy extravasation include full-thickness skin necrosis, chronic ulcer, and persistent pain. It is crucial that all necrotic tissue be removed until bleeding occurs and only healthy tissue left for wound coverage. To ensure complete excision, some surgeons use intraoperative fluorescent dye injection to detect the doxorubicin HCl in the tissue to ensure complete excision. After this, either immediate or delayed surgical reconstruction and skin grafting can be performed[63].

Extravasation in the presence of CVADs

Accidental cases of extravasation in the presence of CVADs is very rare and reported in 0.24% of cases[16]. Extravasation may occur in the subcutaneous tissue of the chest wall or neck, or in the mediastinum. Physicians and nurses should make sure that infusion needles are properly inserted in the port or chamber. In cases of extravasation in the subcutaneous tissue, infusion should be stopped immediately when patient complains of pain or swelling. Pharmacological management, including the use of dexrazoxane for anthracyclines extravasation should be instituted as reviewed in the above sections[16]. A recent report indicated benefit from immediate removal of the CVAD along with Subcutaneous Wash-Out Procedure if extravasation is detected early, to help minimize the exposure of tissue to extravasated agent and the risk of tissue necrosis[64]. In cases of mediatinal extravasation, ESMO guidelines include stopping the infusion, use of dexrazoxane for cases of anthracyclines, and possible surgical draining procedures for the remaining solution, antibiotics, steroids and analgesics to control symptoms from mediastinitis or pleuritic[64].

Experimental non-pharmacologic methods

Negative pressure wound healing: Also called vacuum-assisted closure (VAC) dressing, this method applies a negative pressure to the wound, aids in aspiration of extravasated vesicant, and improves its environment. There are only few reports in which negative pressure wound healing (NPWH) was used for vesicant extravasation. Lucchina *et al*[65] reported a case where surgical VAC dressing was used for vinorelbine extravasation, in addition to hyaluronidase and DMSO, resulted in complete healing of the wound. In an experimental animal study conducted by Evren *et al*[66] on rabbits with doxorubicin extravasation, there was smaller extravasation areas in rabbits subjected to NPWH, but no histological difference compared to control rabbits.

Hyperbaric oxygen therapy

Hyperbaric oxygen therapy (HBO) is defined by the Undersea and Hyperbaric Medical Society as a therapy consisting of intermittent breathing 100% oxygen in a

Table 4 Non-pharmacological management of chemotherapy extravasation

Institutions should always ensure availability of "extravasation kits" at floors in which chemotherapy can be given
Initial non-pharmacologic management
Continuous monitoring at the beginning and during the infusion is essential every 5 to 10 min
Aspiration of the vesicant by a 10 mL syringe, percutaneous needle aspiration, liposuction, simple squeeze maneuver, or by surgical fenestration and irrigation
Elevation of the affected limb and thermal application (cold or hot)

Table 5 Pharmacological management of chemotherapy extravasation

Dexrazoxane as an antidote to anthracyclines extravasation has level Ⅲ-B evidence[16]
Hyaluronidaseas an antidote to vinca-alkaloids and to taxanes extravasation has level Ⅴ-C evidence[16]
Topical DMSO (99%) as an antidote to anthracycline extravasation and to Mytomicin C has level Ⅳ-B evidence[16]
Sodium thiosulfate as an antidote to mechlorethamine extravasation has level Ⅴ-C evidence[16]

DMSO: Dimethyl sulfoxide.

chamber whose pressure is greater than atmospheric pressure[67]. Its role in chemotherapy extravasation is still unclear, but it is believed that HBO increases production of oxygen free radicals and thus can aid in extravasation wound healing. In an experimental animal study conducted by Aktas *et al*[67] on Wistar-Albino rats for adriamycin extravasation, there was complete wound healing for 16 animals out of the 36 animals in the HBO group but no complete wound healing in any of the control group[68].

Use of biologically synthesized nanoparticles

Recent advances in the development of chemotherapeutic agents that incorporates biologically synthesized nanoparticles have been associated with less toxicity to surrounding tissue[31]. Nanodrugs are based on the combination of chemotherapeutic molecules with nanoparticles carriers, which include liposome, polymer, and micelle[69]. Chemotherapeutic molecules which have been used so far for synthesis of nanoparticles include cisplatin, carboplatin, doxorubicin, 5-fluorouracil, paclitaxel, vinblastin and etoposide[69]. For example, the use of liposomal forms of chemotherapeutic agents such as doxorubicin was associated with decreased diffusion capacity of the drug and hence less toxicity to surrounding tissue[31,69].

Tables 4 and 5 summarize non-pharmacological and pharmacological management of chemotherapy extravasation, respectively.

CONCLUSION

Safe administration of chemotherapy and prevention of extravasation is a shared responsibility among medical team members. Education of patients about risks and manifestations are essential. Prevention of chemotherapy extravasation is an important quality indicator for certification of chemotherapy infusion centers (QOPI, ASCO). International guidelines have been published by ASCO and ONS in the United States and ESMO and EONS in Europe. While only some healthcare institutions devise their own policies and guidelines regarding extravasation prevention and management, there is a need to have local institution education, training and guidelines. All institutions that administer intravenous chemotherapy should have known antidotes available. In spite of all efforts to prevent, accidental extravasation still occurs and more research for antidote for many drugs is needed.

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MINIREVIEWS

Intensive care outcomes in adult hematopoietic stem cell transplantation patients

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Abstract

Although outcomes of intensive care for patients undergoing hematopoietic stem cell transplantation (HSCT) have improved in the last two decades, the short-term mortality still remains above 50% among allogeneic HSCT patients. Better selection of HSCT patients for intensive care, and consequently reduction of non-beneficial care, may reduce financial costs and alleviate patient suffering. We reviewed the studies on intensive care outcomes of patients undergoing HSCT published since 2000. The risk factors for intensive care unit (ICU) admission identified in this report were primarily patient and transplant related: HSCT type (autologous *vs* allogeneic), conditioning intensity, HLA mismatch, and graft-versus-host disease (GVHD). At the same time, most of the factors associated with ICU outcomes reported were related to the patients' functional status upon development of critical illness and interventions in ICU. Among the many possible interventions, the initiation of mechanical ventilation was the most consistently reported factor affecting ICU survival. As a consequence, our current ability to assess the benefit or futility of intensive care is limited. Until better ICU or hospital mortality prediction models are available, based on the available evidence, we recommend practitioners to base their ICU admission decisions on: Patient pre-transplant comorbidities, underlying disease status, GVHD diagnosis/grade, and patients' functional status at the time of critical illness.

Key words: Stem cell transplantation; Intensive care; Mechanical ventilation; Comorbidity; Outcome prediction

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Core tip: The outcome of hematopoietic stem cell transplantation (HSCT) patients admitted to intensive care remains poor but not "futile". While risk factors for intensive care unit (ICU) admission are mostly patient and transplant related, prognostic factors for HSCT patients admitted to ICU are primarily related to patients' functional status and interventions in ICU. Based on the available evidence, we recommend patient

selection for ICU to be based on patient pre-transplant comorbidities, underlying disease status, graft-versus-host disease diagnosis/grade, and patients' functional status at the time of critical illness.

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INTRODUCTION

The role of hematopoietic stem cell transplantation (HSCT) has been established in the treatment of various high-risk malignancies and non-malignant conditions. Due to the intense conditioning prior to HSCT and slow post-transplant immune recovery, patients undergoing HSCT are prone to develop infectious and other complications that may lead to death. In fact, transplant related mortality (TRM) is a significant cause of HSCT failure and TRM rates as high as 50% have been reported in high-risk transplants/patients[1]. The factors related to the patient (performance status, comorbidities) and the transplant (conditioning regimen intensity, donor and graft type) determine post-transplant immune recovery and organ damage extent, hence influence the risk and severity of the treatment complications[2-7]. While the pace of post-transplant immune recovery is variable; its pattern is more predictable and may be divided into 3 phases: Pre-engraftment, early post-engraftment, and late post-engraftment. The primary immune defects leading to infections are innate immunity in the pre-engraftment phase; cell-mediated immunity in the early post-engraftment phase; cell-mediated and humoral immunity in the late post-engraftment phase.

Post-transplant complications may be life-threatening and require intensive care due to respiratory failure, shock, organ failure, and others. Even though intensive care unit (ICU) outcomes have improved over the last few decades, ICU admission after allogeneic HSCT (AlloSCT) is still associated with poor prognosis. Consequently, the benefit of intensive care has been challenged in this patient population. In this manuscript, we review the outcomes of intensive care in adult patients undergoing HSCT with emphasis on the factors leading to intensive care and outcome prediction. As practices of intensive care and HSCT have evolved over years, we will focus on seventeen reports published since 2000 summarized in Table 1.

FACTORS ASSOCIATED WITH ICU ADMISSION RATES

In cohorts including only AlloSCT patients, ICU admission rates have been consistently reported to range between 9%-20%[8-12] with only two outliers[13,14]. Naeem *et al*[14] reported an ICU admission rate of 57% among patients who received umbilical cord blood grafts. However, the advent of double-cord blood transplants has improved the immune recovery since that study was published; the ICU admission rate after cord blood transplants is very likely lower now. The small variation in ICU admission rates among the rest of the studies may be explained by different patient selection criteria and varying patient/disease characteristics of the study cohorts.

The ICU admission rate reported in the only study including exclusively autologous HSCT (AutoSCT) patients was 3.3%[15]. Similarly, the reported ICU admissions rates in cohorts including both Allo and AutoSCT[12,16] are lower than those reported in exclusively AlloSCT cohorts[8,10,11,17,18]. The lower admission rates after AutoSCT are likely due to less frequent pulmonary post-transplant complications compared to AlloSCT[19].

The reported risk factors for ICU admission among AlloSCT patients are myeloablative conditioning, acute graft-versus-host disease (GVHD), and HLA mismatch between donor and recipient[9,13,14]; all of which are transplant-related and not surprisingly also increase TRM[6,20,21]. In the only recent study that methodologically assessed the ICU admission risk factors, Benz *et al*[9] did not find patient age, gender, disease type or stem cell source to affect ICU admission risk. While the association between patient pre-transplant comorbidities and ICU admission risk has never been evaluated, comorbidities have been shown to significantly increase patient's risk for critical illness as they influence both TRM[7] and ICU outcomes[8].

The most common reasons for ICU admission after HSCT are respiratory failure and septic shock; pulmonary infections can cause both simultaneously. Nevertheless, non-infectious pulmonary diseases, *i.e.*, diffuse alveolar hemorrhage and acute respiratory distress syndrome, may also lead to respiratory failure after HSCT. Other reported reasons for ICU admission include cardiac dysfunction, neurological disorders, and gastrointestinal bleeding. These may arise due to treatment itself, *i.e.,* busulfan induced seizures and alkylator induced congestive heart failure; development of GVHD; and patients' comorbidities.

PROGNOSTIC FACTORS AND ICU OUTCOMES

Despite the improvement in general ICU outcomes, prognosis for HSCT patients admitted to ICU is still poor with reported hospital mortality ranging from 46% to 84% in series published between 2000 and 2015 (Table 1). The wide range is likely due to inclusion of AutoSCT patients in some of the cohorts and different patient selection criteria between centers. In cohorts including only AlloSCT patients, hospital mortality and overall

Table 1 Summaries of the studies of adult hematopoietic stem cell transplantation patients admitted to intensive care unit published between 2000-2015

Ref. (study period)	No. of patients admitted to ICU [total N of HSCTs (%)], ICU admission risk factors	Reasons for ICU admission (%)	Interventions (%)	Outcomes	Factors evaluated for outcome prediction	Predictors of outcome on mutlivariate analysis	Notes
Boyaci *et al*[31] (2007-2010)	48 patients (7 Auto and 41 AlloSCT)	Respiratory failure 86%, sepsis/septic shock 75%, renal failure, liver failure, AMS	MV 75%	Mortality: 79% in hospital	Age, gender, underlying disease, remission status, HSCT type, HLA match, conditioning intensity, cause of ICU admission, GVHD, SOS, APACHE Ⅱ, GCS, SOFA, # of organ failures, various vitals and lab values, VA, MV	APACHE Ⅱ score and VA in ICU a/w higher mortality	
Bayraktar *et al*[8] (2001-2010)	389 AlloSCT patients a/to ICU within 100 d of HSCT [Of 3039 patients (13%)]	Respiratory failure 61%, septic shock 12%, AMS 9%, arrhythmia 5%, non-GI, non-CNS bleeding 4%	N/R	Mortality: 64% in hospital	Age > 55, underlying disease, year of HSCT was, HSCT period at ICU admission, graft source, HLA match status, donor relation, conditioning intensity, aGVHD at ICU admission, HCT-CI score	HCT-CI ≥ 2, ablative conditioning, aGVHD at ICU admission a/w higher mortality. ICU admission during conditioning regimen a/w lower mortality	HCT-CI score, a measure of pre-transplant comorbidities, can be calculated even prior to HSCT
van Vliet *et al*[17] (2004-2009)	49 AlloSCT [Of 319 (15%)]	Infectious complications 86%, respiratory failure 67% Ablative conditioning and unrelated donor grafting a/w increased risk for ICU admission	N/R	1-yr OS: 15% Mortality: 33% in ICU, 53% in hospital		NR	Univariate analyses demonstrated improved 100-d survival between 2004-2005 to 2008-2009
Agarwal *et al*[30] (1998-2008)	123 HSCT patients (73% AlloSCT)			Mortality: 41% in ICU, 62% in hospital. OS @ 1yr: 24%	Age, underlying disease, type of HSCT, GVHD, neutropenia, hospital admission-ICU interval, organ failures, sepsis type, APS, APACHE Ⅱ, MV	Fungal infection and number of organ failures a/w higher ICU mortality	Hard to explain why GVHD was a/w lower ICU mortality
Depuydt *et al*[33] (2000-2007)	44 AlloSCT	Bacterial infections 32%, non-bacterial infections 30%, non-infectious causes 39%. Overall, pulmonary related causes 39%	MV (73%), RRT (27%)	Mortality: 61% in ICU, 75% in hospital, 80% @ 6 m	Age, gender, bacterial infection, GVHD grade, HSCT-ICU interval, SOFA	Bacterial infection as the cause of ICU admission a/w lower hospital mortality	Improvement in SOFA score by 5th d of ICU was sig better in patients with bacterial infections
Benz *et al*[9] (1998-2007)	33 AlloSCT [Of 250 (13%)] ICU admission risk factors: aGVHD grade Ⅱ-Ⅳ and HLA mismatch	Pulmonary complications 33%, sepsis 24%, neurological disorders 18%, cardiovascular problems 6%,	MV 64%, VA 42%, RRT 27%	OS @ 1yr: 28%		NR	SAPS Ⅱ and SOFA scores did not reliably predict survival
Townsend *et al*[13] (1996-2007)	164 AlloSCT (majority TCD) [Of 552 (30%)]. ICU admission risk factors: Ablative conditioning	Sepsis 67%, respiratory failure 55%	MV 50%	Survival: 32% in ICU OS @ 1yr: 19% overall, 61% in patients who survived ICU	Donor type, conditioning intensity, reason for ICU admission, NIV, MV, VA, RRT, various labs, APACHE Ⅱ, duration of ICU stay, duration of MV	MV, raised BUN at admission and ablative conditioning a/w worse ICU survival	
Trinkaus *et al*[15] (2001-2006)	34 AutoSCT patients admitted within 100 d of SCT [Of 1013 (3.3%)]	Sepsis 32%, respiratory failure 29%, cardiovascular failure 26%		ICU mortality: 38%		NR	

Neumann *et al*[18] (1999-2006)	64 AlloSCT [Of 319 (20%)]	Pulmonary complications 53%, Sepsis 22%, renal failure 9%, bleeding 3%, status epilepticus 3%		ICU mortality: 66%	Age, gender, underlying disease, remission status, conditioning intensity, HLA match status, GVHD, ICU admission indication, HSCT-ICU interval, SOFA, various labs, SOS	SOFA ⩾ 12 and BUN > 60 a/w higher ICU mortality	
Gilli *et al*[10] (1995-2005)	91 AlloSCT (29% < 18 yrs old) [Of 661 (14%)]	Respiratory failure 41%, septic shock 31%, neurological events 12%	MV 48%, RRT 5%, VA 58%	Mortality: 58% in ICU, 70% @1m	Conditioning intensity, reason for ICU transfer APACHE II, SOFA, VA, RRT, IMV	SOFA score a/w 30 d mortality	APACHE Ⅱ underestimated mortality
Naeem *et al*[14] (1998-2003)	25 UCBT [Of 44 (57%)] ICU admission risk factors: Ablative conditioning	Pneumonia 52%, GI bleeding (12%), Sepsis 8%, renal failure 8%	MV 48%	ICU mortality: 72%		NR	
Pène *et al*[11] (1997-2003)	209 AlloSCT [Of 1025 patients (20%)]	Repiratory 67%, hemodynamic 23%, neurologic 18%, renal 17%, other 5%	MV (58%), RRT (28%), VA (47%)	Survival: 48% in ICU, 32% in hospital, 27% @ 6 m, 21% @ 1 yr MV patients: 18% in ICU, 16% in hospital	Age, gender, underlying disease, remission status, conditioning intensity, graft source, HSCT-ICU interval ⩽ 30 d, corticosteroid Rx, serum bilirubin level, MV, VA, RRT	Corticosteroid Rx, serum bilirubin level at ICU admission, MV	None of the 35 patients with admission LOD score > 10 survived the hospital stay
Scale *et al*[41] (1992-2002)	504 patients (264 AlloSCT) who were admitted to ICU following the BMT hospitalization [Of 2653 (19%)]		MV 51%, RRT 7%	1-yr mortality: 67%		NR	
Kim *et al*[42] (1999-2001)	18 AlloSCT [Of 210 (9%)]	Respiratory failure 50%, renal failure 39%, septic shock 11%		ICU mortality: 94%			
Soubani *et al*[16] (1998-2001)	85 HSCT patients (45 AlloSCT) [Of 745 (11%)]	Respiratory 48%, Sepsis 23%, cardiac 19%, neurologic 6%, bleeding 2%	MV in 60%	Mortality: 39% in ICU, 59% in hospital, 72% @ 6 m CU mortality 63% among patients with MV	Age, gender, smoking history, race, underlying disease, HSCT type, HLA match, HSCT-ICU interval, GVHD, various labs	High lactate level, MV, > 2 MOFs during ICU stay a/w higher ICU mortality	
Kew *et al*[12] (1992-2001)	37 HSCT patients (28 AlloSCT) [Of 440 (9%)]	Respiratory failure 65%, hemodynamic instability 57%,	MV in 68%	29 patients died within 1 yr	Pre-ICU patient characteristics, MV, VA	VA a/w shorter OS	
Afessa *et al*[32] (1996-2000)	111 patients (62 Auto, 50 AlloSCT)	Respiratory failure 40%, cardiac reasons 26%, sepsis 14%, CNS dysfunction 5%, GI bleeding 5%	MV 55%	Mortality: 33% in ICU, 46% in hospital 30-d mortality was 78% among AlloSCT patients	Type of HSCT, graft source, post-transplant days @ ICU admission, GVHD, APACHE III, APACHE II, ARDS, MOF, sepsis, MV, VA	Higher APACHE III score @ ICU admission, AlloSCT, MV, ARDS, MOF, sepsis, VA a/w higher 30-d mortality	AUC of receiver operating characteristic curve for APACHE III and hospital mortality was 0.704

A/to: Admitted to; a/w: Associated with; AlloSCT: Allogeneic HSCT; AutoSCT: Autologous HSCT; AMS: Altered mental status; APACHE: Acute Physiology and Chronic Health Evaluation; APS: Acute physiological score; ARDS: Adult Respiratory Distress Syndrome; BUN: Blood urea nitrogen; CNS: Central nervous system; GCS: Glasgow Coma Score; GI: Gastrointestinal; GVHD: Graft-versus-host disease; HCT-CI: Hematopoietic cell transplantation-specific comorbidity index; HSCT: Hematopoietic stem cell transplantation; LOD: Logistic Organ Dysfunction score; m: Months; MOF: Multiorgan failure; MV: Invasive mechanical ventilation; NIV: Non-invasive ventilation; NR: Not reported; OS: Overall survival; pts: Patients; RRT: Renal replacement therapy; Rx: Treatment; SAPS: Simplified Acute Physiology Score; SOFA: Sequential Organ Failure Assessment; SOS: Sinusoidal obstruction syndrome; UCBT: Umbilical cord blood transplantation; VA: Vasoactive drug treatment.

survival (OS) at one year range from 53% to 75% and from 15% to 28%, respectively.

The ICU outcomes in HSCT patients were even more dismal prior to 2000. In reports published prior to 2000,

Table 2 Possible reasons for improved outcomes in patients who are admitted to intensive care unit after hematopoietic stem cell transplantation

Improvements in HSCT
Reduced intensity conditioning
Better antimicrobial prophylaxis
Pre-emptive therapy of cytomegalovirus infections
Improved antifungal therapy
Improvements in intensive care
Early use of non-invasive ventilation
Early goal-directed therapy for septic shock
Better patient selection
Improved recognition of clinical deterioration and earlier ICU admission
Use of palliative care for patients with a slim chance of recovery

HSCT: Hematopoietic stem cell transplantation; ICU: Intensive care unit.

Table 3 Factors that were found to be associated with outcomes of intensive care among hematopoietic stem cell transplantation patients

Patient/disease related factors
Pre-transplant comorbidities
Transplant related factors
Type of HSCT (allogeneic *vs* autologous)
Conditioning regimen intensity
Graft-*vs*-host disease
Patient functional status at ICU admission
Serum bilirubin level
Serum lactate level
Blood urea nitrogen level
APACHE Ⅱ / Ⅲ scores
SOFA
Type of infection (bacterial *vs* fungal)
Post-ICU admission factors
Mechanical ventilation
Vasopressor support

APACHE: Acute Physiology and Chronic Health Evaluation; SOFA: Sequential Organ Failure Assessment; HSCT: Hematopoietic stem cell transplantation; ICU: Intensive care unit.

hospital mortality ranged from 77% to 98% and long-term survival ranged from 3% to 10%[22-29]. Similarly, Agarwal *et al*[30] reported higher ICU mortality rates between 1988-1998 compared to 1998-2008 in a single center. The improvement in ICU outcomes over the last few decades may have been due to improvements in intensive care, HSCT, and patient selection (Table 2).

Poor outcomes among HSCT patients admitted to ICU is not universal. Moreover, HSCT patients surviving ICU stay do not necessarily perform worse in the long-run compared to those who never require intensive care[13]. On the other hand, non-beneficial intensive care has costs to patients, families, and the healthcare system overall. Preventing non-beneficial intensive care may provide comfort and dignity for the patient, increase bed availability for those who would benefit from intensive care, and reduce the economic burden. To identify the factors affecting outcomes in HSCT patients admitted to ICU and guide patient selection for intensive care, various small retrospective cohort studies have been performed (Table 1). These factors may be divided into four categories (Table 3).

Patient/disease related factors

Patient age, gender, and underlying disease type were not found to be associated with ICU outcomes. The only patient related factor that has been shown to affect ICU outcomes is the presence of pre-transplant comorbidities. In one of the largest cohorts reported to date, we have shown that an Hematopoietic Cell Transplantation-Specific Comorbidity Index (HCT-CI) of more than or equal to 2 was significantly and independently associated with increased hospital mortality[8]. A higher HCT-CI score, an index of pre-transplant comorbidities, was also associated with shorter OS. Available at the time of HSCT planning, HCT-CI may help providers and patients make informed decisions regarding intensive care before the need arises.

Transplant related factors

Conditioning regimen intensity and the type of HSCT (autologous *vs* allogeneic) affects a patient's risk for critical illness[13]. Whether they affect the ICU outcomes is not as clear. Of the few studies that included both allogeneic and autologous HSCT[30-32], only one demonstrated significantly worse prognosis after AutoSCT compared to AlloSCT[32]. However, these included small cohorts, and in the only recent study including exclusively AutoSCT patients[15], ICU mortality was numerically lower than that in most AlloSCT patient cohorts. Similarly, while Townsend *et al*[13] and our group have observed that ablative conditioning is associated with higher hospital mortality, other researchers have not found such association[10,11,18,31]. Additionally, Pène *et al*[11] and we observed that patients who had active GVHD or were being treated with corticosteroids at the time of ICU admission had worse prognosis than those who did not. There likely is a genuine cause-effect relationship between GVHD and ICU mortality as GVHD causes tissue damage and its treatment suppresses immune system cultivating severe infections. Finally, none of the recent studies found an association between ICU outcomes and patient age, underlying hematological diagnosis, donor type, HLA match or stem cell source.

Patient functional status at the time of ICU admission

The patient's severity of illness at the time of ICU admission is known to be predictive of general ICU survival, hence various predictive scoring systems, such as Acute Physiology and Chronic Health Evaluation (APACHE) and Sequential Organ Failure Assessment (SOFA) score, based on organ function are commonly used in clinical practice. It is evident that organ function at the time of ICU admission is also prognostic for HSCT patients. However, as results have been discrepant, there is no agreement on which measures are optimal. Elevated serum lactate[16], bilirubin[11], and blood urea nitrogen[13,18] have been inconsistently

found to be associated with worse prognosis. As for the organ function indexes, APACHE Ⅱ was not found to be predictive of ICU outcomes in HSCT population in various studies[10,13,30] except one[31]. In a cohort of 112 patients, Afessa *et al*[32] did not find APACHE Ⅱ to have prognostic value while they found APACHE Ⅲ to have a moderate discrimination and good calibration in predicting hospital mortality. Gilli *et al*[10] observed that APACHE Ⅱ underestimated mortality while higher SOFA scores were associated with higher hospital mortality. In their cohort, none of the 20 patients with a SOFA score > 11 survived. On univariate analyses, Trinkaus *et al*[15] also found SOFA to be predictive of mortality. Similarly, Neumann *et al*[18] found a SOFA score > 12 to be significantly and independently associated with higher mortality; of 45 patients with SOFA >11, only 4 survived. On the other hand, Boyaci *et al*[31] and Benz *et al*[9] did not find SOFA to be predictive of mortality in multivariate analyses. Finally, Depuydt *et al*[33] reported that patients who were admitted to ICU with bacterial infection had a better prognosis than others, likely due to the more rapid improvement of bacterial infections with antibiotic treatment. In contrast, Agarwal *et al*[30] found fungal infections to be associated with higher mortality.

Post-ICU admission factors

Several authors demonstrated that short-term outcomes are worse in patients who required endotracheal intubation[11,13,16,32]. In fact, mechanical ventilation (MV) is the most consistently shown prognostic factor in HSCT patients admitted to ICU. Unfortunately, 48%-78% of HSCT patients require MV during their ICU stay (Table 1). Similar to MV, vasopressor support has also been found to be associated with worse short-term prognosis in a few studies[12,31]. Although significantly associated with outcomes, events that happen in ICU cannot be used for outcome prediction before a patient's ICU admission.

Overall, it can be deduced that: (1) patient/disease related factors do not play a major role in determining ICU outcomes with the exception of patient comorbidities; (2) while transplant related factors affect ICU admission risk, they do not necessarily influence ICU outcomes with the exception of transplant type and GVHD diagnosis at the time of ICU admission; (3) the major determinant of ICU outcome seems to be the patients' functional status at the time of ICU admission; (4) the value of traditional prognostic indexes has not been validated in HSCT patients but may be useful in identification of patients with a very slim chance of survival.

FUTURE OBJECTIVES AND RECOMMENDATIONS

As physicians and researchers continue improving the HSCT process and outcomes, optimization of the delivery of a comprehensive intensive care plan should become an important component of the overall patient management. This goal, requires establishment of adequate communication channels among patients, relatives, transplant physicians and intensivists. In addition, we believe that there is a need for further development of clinical algorithms to assess benefits and risks of intensive care, alternative palliative care, and appropriateness of life support and resuscitation at multiple points in time: (1) prior to HSCT; (2) when early warning signs of critical illness appear; (3) upon development of critical illness; (4) every third to fifth day of intensive care; and (5) prior to initiation of life supportive measures such as endotracheal intubation and MV.

Prior to HSCT

Transplanters should assess patients' comorbidities, calculate their HCT-CI scores, and talk to the patient about the possibility and prognosis of intensive care/intubation beforehand.

Early warning signs

Retrospective studies demonstrated HSCT patients admitted to ICU demonstrated early warning signs that could be detected by early warning score systems (EWSS) consisting of nursing observations[34] and suggested improvements in ICU outcomes among HSCT patients after such systems and early outreach teams were implemented[35]. Accordingly, we recommend implementation of EWSS and outreach teams in transplant centers.

Upon development of critical illness

In the literature, there is a lack of agreement on which factors should be used to predict ICU outcomes of HSCT patients. We believe patient pre-transplant comorbidities, underlying disease status, GVHD diagnosis/grade, and patient's functional status at the time of critical illness should be taken into account while deciding on benefits of intensive care. Although none of the previous studies showed the underlying disease or its remission status to affect short-term ICU outcomes -similar to the studies done in cancer patients[36,37]; the remission status significantly affects the long-term survival of HSCT patients[20,21], and likely would affect the long-term outcome after ICU admission. To establish a clinical algorithm for patient selection, transplanters and intensivists need a prognostic index specific for critical HSCT patients. Hence, more studies on large HSCT cohorts with multi-center validation are needed. We believe hospital mortality should be the primary outcome assessed in such future studies as long-term outcomes of HSCT patients surviving ICU is similar to those of patients who never required intensive care[13] and, in our experience, the number of patients who died on their second ICU admission but during the same hospitalization is not insignificant.

Every third to fifth day of intensive care

Although none of the recent studies showed the length of ICU stay to affect short-term ICU outcomes, in our experience the longer the patient stays in ICU, the less likely he/she is to survive. Therefore, we believe the intensivist and the transplant physician should review the benefits of intensive care every three to five days in ICU.

Prior to endotracheal intubation

Initiation of MV is a turning point in the intensive care of HSCT patients. MV is associated with shorter survival and also suffering of patient and the family[11,16,32]. The combination of hepatic and renal failure in mechanically ventilated patients is almost universally fatal[38-40]. Therefore, for HSCT patients with renal and/or hepatic failure requiring MV, a frank discussion should be made with the patients' family prior to intubation and initiation of ventilation.

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MINIREVIEWS

Role of copper transporters in platinum resistance

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Abstract

Platinum (Pt)-based antitumor agents are effective in the treatment of many solid malignancies. However, their efficacy is limited by toxicity and drug resistance. Reduced intracellular Pt accumulation has been consistently shown to correlate with resistance in tumors. Proteins involved in copper homeostasis have been identified as Pt transporters. In particular, copper transporter receptor 1 (CTR1), the major copper influx transporter, has been shown to play a significant role in Pt resistance. Clinical studies demonstrated that expression of CTR1 correlated with intratumoral Pt concentration and outcomes following Pt-based therapy. Other CTRs such as CTR2, ATP7A and ATP7B, may also play a role in Pt resistance. Recent clinical studies attempting to modulate CTR1 to overcome Pt resistance may provide novel strategies. This review discusses the role of CTR1 as a potential predictive biomarker of Pt sensitivity and a therapeutic target for overcoming Pt resistance.

Key words: Resistance; Cisplatin; Copper transporter receptor 1; Copper transporter; Copper transporter receptor 2; ATP7A; ATP7B

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Core tip: Platinum (Pt)-based chemotherapy is the backbone of treatment for various solid malignancies in both curative and palliative settings. However, the efficacy of Pt is limited by toxicity and inevitable resistance. Hence, it is important to understand the mechanisms of Pt resistance to not only identify treatment non-responders, but more importantly to help develop strategies to overcome resistance and improve efficacy. We herein discuss our current understanding of the mechanisms of Pt resistance, with a particular emphasis on the role of copper transporter receptor 1 in Pt resistance.

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INTRODUCTION

Role of platinum chemotherapeutics in cancer

Cisplatin, also called the "penicillin of cancer", has

remained the mainstay of treatment for a variety of solid tumors over the last four decades and is an essential component of both curative-intent and palliative chemotherapy regimens[1,2]. First described in 1845 as Peyrone's salt and subsequently noted to inhibit binary fission in *Escherichia coli* bacteria, cisplatin is platinum (Pt)-based alkylating agent that binds to DNA and causes intra/inter strand crosslinking which interferes with cell division and causes apoptosis. Carboplatin and oxaliplatin are newer members of the Pt family of compounds with similar mechanisms of action as cisplatin but with different toxicity profiles.

Pt agents have a number of toxicities that limit their clinical use. The most common adverse effects from cisplatin include nephrotoxicity, ototoxicity, neurotoxicity and myelosuppression. Cisplatin is also highly emetogenic. Carboplatin is less emetogenic and has a lower risk of nephrotoxicity and ototoxicity; however, it is more myelosuppressive than other Pt compounds. Oxaliplatin which is significantly neurotoxic has the lowest risk of nephrotoxicity and ototoxicity amongst Pt compounds.

Despite the same class, each Pt drug has a unique role in the management of individual cancers, and in most circumstances these agents are not interchangeable. Cisplatin is the most active Pt agent against testicular, lung, ovarian and bladder cancers, and is the only Pt drug recommended in curative-intent treatment for these malignancies. In contrast, carboplatin may be substituted for cisplatin in the palliative setting for advanced solid tumors where cisplatin may not be tolerated due to adverse effects. In general, oxaliplatin is the Pt of choice for colon cancer.

Pt resistance is an inevitable occurrence with rare exception. Aside from germ cell tumors, metastatic solid tumors are generally thought to be incurable with cytotoxic chemotherapy due to the development of resistance and subsequent disease progression. While advances in molecular biology and genomic (personalized) medicine have driven an exponential increase in therapeutic options and improved outcome in various malignancies, Pt-based chemotherapy remains the backbone of treatment for a variety of solid tumors. Therefore, it is crucial to understand mechanisms of Pt resistance in order to develop strategies to overcome this nearly universal phenomenon.

Mechanisms of Pt resistance

The clinical utility of Pt agents is limited by both intrinsic and acquired resistance. For example, cisplatin-based treatment is associated with up to 80% response rates in patients with limited stage small cell lung cancer; however, the median overall survival is less than a year due to lack of durable response[3]. Understanding the mechanisms of Pt resistance may improve clinical outcomes. Pt resistance is complex and is regulated by a cascade of events that interfere with any of the multiple steps involved in its cytotoxic actions, from initial drug entry into the cell to the final stages of apoptosis. While not fully understood, identified mechanisms of resistance include: Increased glutathione and metallothionein, which inactivates the reactive forms of Pt[4-6], activation of nucleotide excision repair pathway and other pathways associated with DNA repair[7,8], and dysregulation of the tumor suppressor *p53* gene that is required for apoptosis[9-12]. Dysregulation of the Ras and MAPK pathway[13,14] and the heat-shock proteins[15] have also been implicated in Pt resistance.

Despite the multifactorial nature of Pt resistance, reduced intracellular drug accumulation is one of the most consistently identified features of cisplatin-resistant cell lines[4,16]. Reduced influx or increased efflux of the drug is associated with decreased intracellular accumulation. Pt drug influx has been attributed to both non-saturable as well as energy-dependent active transport processes[17,18]. Currently identified Pt influx transporters include copper transporter receptor 1 (CTR1) and organic cation transporters belonging to the soluble carrier (SLC) SLC22A2 family. On the contrary increased levels of the multidrug resistance associated transporter protein MRP2 (cMOAT), adenosine triphosphate (ATP) binding cassette (ABC) multidrug transporters, CTR2 and copper-transporting P-type adenosine triphosphates (ATPase's) have been observed to confer resistance[19,20]. In this review we will focus on the importance of intratumoral Pt levels in promoting chemosensitivity and the role of CTRs, specifically CTR1, in contributing to Pt resistance.

INTRACELLULAR PT AND TUMOR PT-SENSITIVITY

It has been hypothesized that reduced intracellular Pt concentration may confer resistance to Pt-based chemotherapy. Both *in vitro* and *in vivo* studies provide data to support this hypothesis.

In vitro studies

Lanzi *et al*[21], demonstrated that a reduction of drug accumulation in cisplatin-resistant (A431/Pt) human cervix squamous cell carcinoma compared to Pt-sensitive squamous cancer cells directly correlated with the extent of cisplatin-induced DNA damage. Mann *et al*[22], noted that, in human ovarian cancer cell lines, decreased Pt drug accumulation is associated with resistance. Several other investigators observed similar positive correlations between accumulation of Pt and cytotoxicity in cancer cell lines derived from ovarian, leukemia and lung cancer tissues[23-26]. All these studies support drug accumulation as a contributing factor to Pt resistance. However, cell line studies represent only a single phenotype and do not take into account complex tumor- host interaction that may allow for other mechanisms of chemoresistance.

In vivo studies

It has been demonstrated that the elimination of Pt compounds is triphasic in nature, with a terminal

plasma half-life of 5.4 d for cisplatin. In contrast, Pt has a long half-life in human tissue that is yet to be quantified[27]. Pt and DNA adducts were detectable in autopsy tumor samples from patients who had received Pt up to 15 mo ante mortem[28,29]. In a prospective study of two groups of advanced non-small cell lung cancer (NSCLC) patients receiving cisplatin at two different doses, plasma Pt concentration correlated with the dose of cisplatin administered, however tissue Pt concentration did not. In this study there was a weak correlation between simultaneous plasma and tumor tissue concentration[30]. In 44 patients with NSCLC who had received neoadjuvant Pt-based therapy and subsequently underwent surgical resection, tissue Pt concentrations in resected tumor specimens significantly correlated with percent reduction in tumor ($P < 0.001$). The same correlations were seen irrespective of the Pt drug utilized, number of cycles and histologic subtype. Patients with higher intratumoral Pt concentrations also had longer time to recurrence ($P = 0.034$), progression-free survival ($P = 0.018$), and overall survival ($P = 0.005$). This was the first clinical study to establish a relationship between tissue Pt concentration and tumor response, and supports Pt accumulation as an important mechanism of resistance even in the clinical setting[31]. In another study of 19 patients with muscle invasive bladder cancer who had received Pt-based neoadjuvant therapy, total Pt concentration in normal adjacent bladder tissue significantly differed by tumor pathologic response ($P = 0.011$). Specimens with pathologic complete responses had the highest Pt concentrations compared to those with a down-staging to non-muscle invasive disease ($P = 0.0095$) or no response/progression ($P = 0.0196$)[32]. These findings suggest that intratumoral Pt accumulation may be an important determinant of Pt sensitivity and tumor responses across tumor types.

CTRS

Pt chemotherapeutics cross the cell membranes by passive diffusion and transporters. Various ion pumps and transporters have been implicated in the transport of Pt agents, some of which are well-characterized[33]. More recently, transporters involved in copper homeostasis have been identified as important in Pt influx and efflux. Copper is an essential micronutrient and a cofactor for many enzymes. However, its intracellular form is highly toxic, and hence, a complex network of proteins have evolved to chaperone copper to the copper-dependent proteins. Chaperone proteins include CTR1, CTR2, antioxidant protein (ATOX 1), ATP7A and ATP7B. All of the above discussed transporters possess a metal binding sequence that binds both copper and Pt[34].

CTR1 is the most extensively studied Pt influx transporter and will be described in detail in the next section. CTR2, another copper uptake protein, has a substantial structural homology to CTR1but functions as a Pt efflux transporter. Higher CTR2 levels correlated with Pt resistance in ovarian cancer cell lines[35]. It was also noted that in a human 2008 epithelial cancer cell model, higher expression of CTR2 was noted to be associated with increased intracellular copper and Pt resistance[36]. Further studies are needed to better understand the role of CTR2 in cisplatin resistance in human cancers.

ATP7A and ATP7B are two copper transporting P-type ATPase that also maintain copper homeostasis and have been implicated in Pt efflux[37,38]. ATP7A is thought to regulate Pt accumulation, primarily by sequestering Pt intracellularly, whereas ATP7B located in the Trans Golgi network mediates Pt drug efflux *via* a process that involves its transport into vesicles involved in the secretory pathway[39]. In human epidermoid carcinoma KB-3-1 cell(a derivative of HELA–cervical cancer line), transfection with ATP7B conferred cisplatin resistance[40]. Similarly in prostate cell lines, overexpression of ATP7B correlated with Pt resistance[40]. The observation that human tumor cells transfected with ATP7B acquire resistance to cisplatin lends credence to the hypothesis that drug efflux plays a role in resistance[41]. Several cell line studies, including one of fibroblasts derived from a patient with Menkes disease, which is characterized by copper deficiency, confirmed the role of efflux proteins in enhancing Pt resistance[42,43]. ATP7B silencing resulted in enhanced cisplatin sensitivity and increased DNA adducts formation in cisplatin-resistant cells; however this was not observed with ATP7A silencing[44]. In both NSCLC xenografts exposed to cisplatin and colorectal cancer patients treated with oxaliplatin, increased levels of ATP7B were associated with Pt resistance[45,46]. In the only study to simultaneously assess influx and efflux transporters, expression of CRT1, ATP7A and ATP7B were measured in three pairs of parent cell lines and cisplatin-resistant cell lines derived from various types of invasive oral squamous cell carcinoma. ATP7B expression correlated with the acquisition of cisplatin resistance more closely than either CTR1 or ATPP7A[39].

PROFILE OF CTR1

Structure and localization

CTR1 is a 190 amino acid (aa) protein with three trans-membrane domains, a approximately 67 aa extracellular N-terminal (ecto) domain, and a approximately 15 aa C-terminal cytosolic tail[47,48]. Crystallographic analysis of human CTR1 noted that the permeation conduit formed by the association of three CTR1 molecules involves a series of rings of methionines capable of chelating copper in a trimeric configuration[48,49]. Two rings each containing three methionines are stacked on top of each other in the narrowest part of the pore, and a ring of three cysteines is located at the bottom of the pore. The aperture has a truncated cone shape measuring approximately equal 8 Å at the external entrance and approximately equal 22 Å at the intracellular end[50]. The expression of CTR1 is ubiquitous and localizes to the plasma membrane in some cell lines and perinuclear vesicles in others[51].

Role in copper transport

CTR1 is the primary influx transporter of copper in human cells. Transport of copper by CTR1 is energy-independent[52] and results in conformational changes in CTR1[53]. Knockout of both CTR1 alleles results in an embryonic lethal phenotype thought to be secondary to deficiency of copper[54]. Organ-specific knockout of CTR1 in the intestine and liver confirms the role of CTR1 as an important copper transporter[55,56]. The exact mechanism of copper transport across CTR1 is not yet completely understood and further studies are warranted.

ROLE OF CRT1 AS A PT TRANSPORTER

Despite the "narrowest opening" of trimetric CTR1 being smaller than the molecular size of cisplatin, studies suggest that prior to entering a cell, cisplatin is activated by interacting with the extracellular methionine clusters of CTR1, which results in the formation of an intermediate that is smaller than the radius of the narrowest opening CTR1[57,58].

In vitro studies

Ishida *et al*[59], described CTR1 as a significant uptake transporter of cisplatin. They used a mutagenized wild type yeast cell library to select for mutants that grew in the presence of toxic doses of cisplatin. Cells with a CTR1 mutation that decreased CTR1 cell expression were noted to have profound Pt resistance compared to other mutants. In order to determine the mechanistic role of CTR1 in cisplatin resistance, cisplatin - DNA adducts were measured. They observed that decreased Pt uptake is responsible for lower Pt adduct levels and resistance. They also demonstrated that cisplatin, similar to copper, down-regulated CTR1 expression in yeast cell lines.

CTR1 knockout in intestinal epithelial mouse cell lines also led to a decrease in intracellular Pt levels and resistance[60]. Similarly, overexpression of CTR1 was associated with cisplatin sensitivity in ovarian and colorectal cancer cell lines[61,62]. In cisplatin-resistant small cell lung cancer cells, sensitivity was restored when CTR1 was introduced into these cells[63]. Ivy *et al*[64] also noted that higher intracellular Pt correlated with higher CTR1 levels in human embryonic kidney cells and mouse embryonic fibroblasts. However contrary to other studies, the investigators noted that in ovarian tumor cells uptake of Pt was linear and non-saturable, suggesting that there could be other mechanisms besides CTR1 involved in Pt transport, including proteins involved in copper homeostasis[64].

In vivo studies

In rat dorsal root ganglion, CTR1 expression by immunohistochemistry (IHC) and RT-PCR correlated with Pt uptake and treatment-induced cell body atrophy[65]. Similarly, in a murine model utilizing mouse embryo fibroblasts, CTR1 knockout completely eliminated responsiveness of cells to Pt agents[66]. In a mouse model of human cervical cancer (HPV16/E), the levels of cisplatin-induced DNA adducts correlated with CTR1 mRNA in most organs tested, including skin, lung, liver, pancreas, and uterus[67].

CTR1 EXPRESSION AND CLINICAL OUTCOME

To date, several human studies have investigated the role of CTR1 in Pt sensitivity. In 15 patients with stage Ⅲ/Ⅳ serous epithelial ovarian tumors who underwent optimal cytoreductive surgery (residual masses 1 cm or less) and subsequent Pt-based therapy, tumor CTR1 mRNA correlated with Pt sensitivity. Patients with no evidence of disease progression within 6 mo had higher CTR1 mRNA levels than in patients with refractory or resistant disease. Using clinical and array based expression data from the cancer genome atlas; the same investigators were able to independently validate the correlation of CTR1 m RNA levels with clinical outcomes in patients with advanced ovarian tumors who also underwent Pt-based therapy[67]. Higher CTR1 expression by IHC in patients with stage Ⅲ endometrial cancer who had received carboplatin also correlated with longer disease free and overall survival (P = 0.009)[68].

In a study of 30 patients with NSCLC who had received neoadjuvant Pt-based chemotherapy, patients with undetectable CTR1 expression in their tumors had reduced intratumoral Pt concentrations and tumor response[69]. In another study of 54 patients with stage Ⅲ non-small lung cancer who received Pt-based combination chemotherapy, higher CTR1 expression correlated with longer progression free survival and overall survival (P = 0.01 and 0.047, respectively)[70]. More recently, we demonstrated that tumor CTR1 expression in cystectomy specimens of patients with muscle invasive bladder cancer correlated significantly with pathologic downstaging after Pt- based neoadjuvant chemotherapy[71].

REGULATION OF CTR1

CTR1 expression has been shown to be regulated at both transcriptional and post-translational levels by various factors including transcription factor specificity protein 1 (Sp1) as well as copper and other heavy metals such as Cd, Zn and Ag[72,73]. Sp1 is a zinc finger transcription factor that binds to GC-rich motifs in promoters and is involved in many cellular processes including cell differentiation, cell growth, apoptosis, immune responses and response to DNA damage. Song *et al*[74] demonstrated that three binding sites in the CTR1 promoter of Sp1 are involved in the basal and copper concentration-dependent regulation of CTR1 expression. The zinc-finger domain of Sp1 serves as a sensor of copper that regulates CTR1 expression in response to fluctuations in copper concentration[74,75]. In addition,

modulation of Sp1 levels also affected the expression of CTR1. Cisplatin competes with copper for CTR1-mediated transport and trigger the rapid degradation of CTR1. It has been postulated that this mechanism serves to limit the toxic accumulation of the metal that it transports[76,77]. The down-regulation of CTR1 expression after Pt exposure has been confirmed in various cell lines and is considered functionally significant as subsequent copper uptake, despite Pt absence, is noted to be decreased[49,77].

In a study of 282 Chinese patients with NSCLC who received Pt-based therapy, genetic polymorphisms of CTR1 at reference single nucleotide polymorphism (rs) rs7851395 and rs12686377 were associated with Pt resistance and poor clinical outcomes. Patients with a GT haplotype had increased susceptibility to Pt resistance, whereas AG haplotype conferred longer overall survival[78]. In a second study of 204 Chinese patients, CTR1 polymorphism (rs10981694 A > C) correlated with Pt toxicity in patients with advanced stage NSCLC and could be potentially used for pretreatment evaluation of toxicity. However, the survival times of patients with different rs10981694 genetic polymorphisms were not significantly different[72]. Functional implications of these polymorphisms are not clear.

CTR1 AS A THERAPEUTIC TARGET

Cisplatin-induced degradation of CTR1 was noted to be reversible with the proteasome inhibitor bortezomib in both mouse fibroblast and human ovarian carcinoma cell lines. This in turn correlated with increased cellular uptake and the cytotoxicity of cisplatin in a synergistic manner[73]. Cells lacking CTR1 had no change in cisplatin uptake with bortezomib suggesting that bortezomib may act primarily through blocking CTR1 degradation. NCT01074411 is an ongoing phase 1 trial of intraperitoneal bortezomib and carboplatin that tests this hypothesis in patients with recurrent ovarian cancer.

Ag and zinc have been noted to induce CTR1 expression. In CTR1-transfected or nontransfected HEK293 cells Ag, Zn inhibited CTR1-mediated copper uptake[52]. Also in IGROV1 and SKOV-3 cells treated with different concentrations of Zn, and Ag, there was a concentration-dependent increase in expression of CTR1 and Sp1[79]. In a double-blind, placebo-controlled study of 34 patients with stage Ⅲ/Ⅳ nasopharyngeal carcinoma receiving cisplatin-based chemotherapy, zinc supplement (75 mg/d) was associated with longer overall survival, local-free survival and disease-free survival compared with placebo (P = 0.044, P = 0.007, and P = 0.033, respectively)[77]. More recently in ovarian cancer cells and xenograft mice, (-)-epigallocatechin-3-gallate (EGCG), a major polyphenol from green tea was noted to increase CTR1 m RNA and protein expression. These findings translated into EGCG enhancing the sensitivity of ovarian cancer SKOV3 and OVCAR3 cells to Pt through increased Pt accumulation and DNA-Pt adducts[80]. Copper chelators are a class of compounds that preferentially bind either cuprous or cupric forms of copper and potentially modulate copper redox-activity without removing copper from the system. They are characterized as either membrane-permeable or - impermeable and serve as an organ-selective copper delivery or deprivation system to manipulate the biological function of copper. Tetrathiomolybdate (TTM), a specific and effective copper chelator was initially developed as a therapeutic agent to treat Wilson's disease, which is characterized by excessive copper accumulation in liver and brain[81]. TTM demonstrates antiangiogenic, antifibrogenic, and anti-inflammatory actions in preclinical studies. While TTM has a good safety index, most of its toxicity in animals is due to copper deficiency that is easily reversible with acute copper supplementation[82]. Daily treatment with TTM has been shown to safely reduce bioavailable copper in 2-4 wk in humans and mice, likely through formation of a high-affinity tripartite complex with copper and proteins[83]. Liang *et al*[76] demonstrated that in cisplatin-resistant ovarian cancer cell lines derived from patients, resistance associated with reduced expression of the CTR1 could be overcome by copper-lowering agents (TTM, D-penicillamine and trientine) which enhanced CTR1 expression. In a murine model of human cervical cancer, combined therapy with TTM and cisplatin enhanced therapeutic efficacy by increasing tumor-specific uptake of Pt[67]. Similarly, in oxaliplatin-resistant cell lines derived from human cervical carcinoma, D-penicillamine in combination with cisplatin and oxaliplatin overcomes resistance through increased CTR1 expression by up regulation of Sp1[84]. These studies provided the mechanistic rationale for using copper chelation to overcome Pt resistance in cancer patients. Trientine was combined with carboplatin in ovarian cancer patients[85]. NCT01837329 is an ongoing phase 1 study combining TTM with Pt-doublet in advanced NSCLC patients. Further studies are needed to validate these findings in order to use the above agents as adjuncts to conventional Pt based therapy and improve outcomes.

CONCLUSION

Pt-based chemotherapy is the backbone of both curative and palliative treatment for numerous malignancies. Copper transporters, in particular CTR1, play a significant role in intracellular Pt accumulation and have the potential to be used as predictive biomarkers of Pt sensitivity. In addition, modulation of copper transporter expression may be a novel therapeutic strategy to enhance the efficacy of Pt chemotherapy by increasing intratumoral Pt concentration. Specifically, copper chelators and agents that prevent degradation of CTR1, such as bortezomib, are currently being studied in combination with Pt in a variety of solid tumors known to develop Pt resistance.

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MINIREVIEWS

Recent advances in microvascular autologous breast reconstruction after ablative tumor surgery

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Abstract

Breast cancer is a ubiquitous disease and one of the leading causes of death in women in western societies. With overall increasing survival rates, the number of patients who need post-mastectomy reconstruction is on the rise. Especially since its psychological benefits have been broadly recognized, breast reconstruction has become a key component of breast cancer treatment. Evolving from the early beginnings of breast reconstruction with synthetic implants in the 1960s, microsurgical tissue transfer is on the way to become the gold standard for post oncology restoration of the breast. Particularly since the advent of perforator based free flap surgery, free tissue transfer has become as safe option for breast reconstruction with low morbidity. The lower abdominal skin and subcutaneous fat tissue typically offer enough volume to create an aesthetically satisfying breast mound. Nowadays, the most commonly used flap from this donor site is the deep inferior epigastric artery perforator flap. If the lower abdomen is not available as a donor site, the gluteal area and thigh provide a number of flaps suitable for breast reconstruction. If the required breast volume is small, and there is enough tissue available on the upper medial thigh, then a transverse upper gracilis flap may be a practicable method to reconstruct the breast. In case of a higher amount of required volume, a gluteal artery perforator flap is the best choice. However, what is crucial in addition to selecting the best flap option for the individual patient is the timing of the operation. In patients with confirmed post-mastectomy radiation therapy, it is advisable to perform microvascular breast reconstruction only in a delayed fashion.

Key words: Breast cancer; Microsurgery; Autologous tissue transfer; Breast reconstruction; Flap; Transverse musculocutaneous gracilis; Fasciocutaneous infragluteal; Deep inferior epigastric perforator

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Core tip: Mastectomy is a frequent sequela of the treatment and prophylaxis of breast malignancies. Autologous microvascular breast reconstruction is becoming the gold standard in correcting these disfiguring interventions. The lower abdomen, as well as the gluteal and thigh area, is the source of multiple flaps usable for breast reconstruction. With the advent of perforator flap surgery, today's reconstructive surgeons are able to minimize donor site morbidity whilst maximizing patient outcomes. If timed and performed correctly, microsurgical breast reconstruction is a safe procedure with low donor site morbidities and excellent aesthetic results.

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INTRODUCTION

Breast cancer is a ubiquitous disease and one of the leading causes of death in women in western societies. While breast conservation techniques together with postoperative radiation often are successful treatment strategies for local control, mastectomy often remains unavoidable in breast cancer therapy[1-3]. Reconstructive options are divided into implant based reconstruction and reconstruction using autologous tissue or a combination of both. Spanning from the early beginnings of breast reconstruction with implants in the 1960s to the autologous microvascular breast restoration of today, plastic surgery continues to offer a great variety of options for women having suffered from mastectomy. Today, with many different methods available, it is important to find a procedure that fits the specific needs of every individual patient[4-6].

HISTORICAL PERSPECTIVES

The history of breast reconstruction dates back to Vincenz Czerny, who was the first ever to successfully accomplish breast augmentation in 1895, by using a removed lumbar lipoma[7]. One year later, the latissimus dorsi myocutaneous flap was the first described autologous muscle flap for post-mastectomy breast reconstruction[8]. However, for the following decades autologous breast reconstruction was not the gold standard. Surgeons used paraffin injections and polyvinylic alcohol sponge implantation for breast augmentation, with terrible outcomes for the patients[9]. Silicone implants were first introduced in 1961 by Cronin *et al*[10] for aesthetic breast augmentation. Henceforth silicone implants became the leading option in breast reconstruction. Despite their considerable risk for capsular fibrosis, alloplastic implants are still used in the majority of breast reconstruction procedures worldwide at this day[12].

The rediscovery of the latissimus dorsi flap in the late seventies led to a renaissance of autologous breast reconstruction[13]. The rather limited amount of soft tissue volume that is available by this technique, however, often is not enough to replace the excised breast tissue sufficiently. Therefore, reconstruction of the breast mound with the latissimus dorsi musculocutaneous flap usually demands the addition of a silicone implant.

Striving to overcome the volume deficit of the latissimus dorsi flap and to be able to solely rely on autologous tissue, Scheflan *et al*[14] pioneered a pedicled abdominal flap with a transverse skin island in 1982. Not only its robust vascularization and the large arc of rotation, but also the concomitant contouring benefits of an abdominoplasty, made this technique popular among surgeons and patients alike[11]. On the contrary, herniation of the abdominal wall, bulk in unwanted regions due to its muscular pedicle as well as venous congestion are well known disadvantages of the pedicled transverse rectus abdominis muscle (TRAM) flap[15]. With the advent of perforator flaps, Koshima *et al*[16] revolutionized breast reconstruction by completely sparing the rectus muscle in 1989. This new technique, the deep inferior epigastric perforator (DIEP) flap, allowed the raising of large flaps without weakening of the abdominal wall. Consisting exclusively of fat tissue and skin, this autologous flap resembles the real breast tissue more closely and produces a more natural look of the reconstructed breast mound. However, experience has shown that technical challenges, variable anatomy and certain patient characteristics are limiting its applicability. Therefore, alternative flap donor sites also had to be taken into consideration.

Gluteally-based flaps such as the superior gluteal artery perforator (SGAP), the inferior gluteal artery perforator (IGAP) and the fasciocutaneous infragluteal (FCI) flap, as well as medial thigh based-flaps such as the transverse musculocutaneous gracilis (TMG) flap or the profunda artery perforator (PAP) flap have emerged as valuable alternatives if the use of abdominal flaps is precluded. Each of these flaps has particular advantages and disadvantages and indications must be assessed carefully. However, microvascular transplanted flaps should no longer be considered as a last resort, but rather begin to represent the first choice for post mastectomy breast reconstruction. Their ability to resemble a very natural breast mound has made autologous flaps an invaluable addition to the armamentarium of the reconstructive plastic surgeon.

FLAPS FORM THE LOWER ABDOMEN

Of all donor sites available, the lower abdomen has evolved as the gold standard in microsurgical breast reconstruction. The lower abdominal skin and subcutaneous fat tissue typically offer enough volume to create

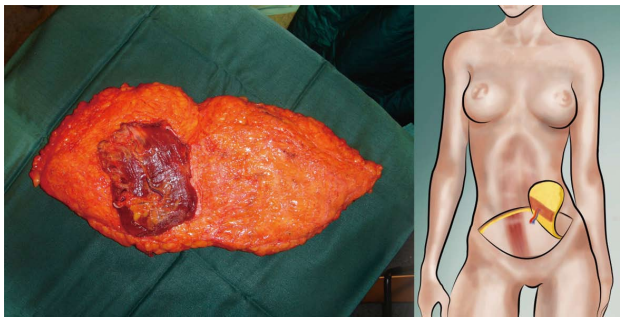


Figure 1 Typical example of a free transverse rectus abdominis muscle-flap showing the undersurface with its vascular pedicle and the completely excised rectus abdominis muscle.

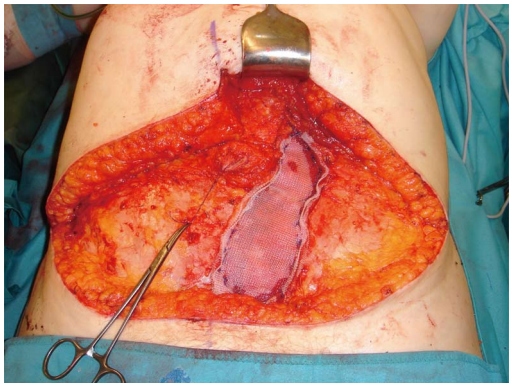


Figure 2 After harvest of a full transverse rectus abdominis muscle-flap, the fascial defect of the rectus fascia usually has to be closed with a synthetic mesh.

an aesthetically satisfying breast mound. Nowadays, the most commonly used flap from this donor site is the deep inferior epigastric artery perforator (DIEAP) flap[17]. Prerequisites for the usage of lower abdominal flaps are enough surplus tissue in this area to match the volume of the remaining breast and the acceptance of a relatively long scar.

TRAM flap

Both, the pedicled TRAM-flap by Scheflan *et al*[14] and the free TRAM flap by Holmström[18] have been widely used for autologous breast reconstruction in the past decades. The free TRAM flap is composed of a lower abdominal skin island overlying usually one rectus abdominis muscle (Figure 1). The overlying skin island and its subcutaneous fat tissue is supplied by the perforating vessels from the deep inferior epigastric artery (DIEA) travelling through the muscle. It covers approximately the area typically excised in an aesthetic abdominoplasty procedure. The DIEA is dissected together with a 5-6 cm strip of rectus muscle and anastomosed microscopically to the recipient vessel, which is typically the internal mammary artery (IMA) or, less frequently, the thoracodorsal artery. The fascial defect in the lower abdominal wall is typically repaired with an alloplastic mesh (Figure 2) and the wound is closed similarly to an abdominoplasty incision. Significant donor-site morbidity

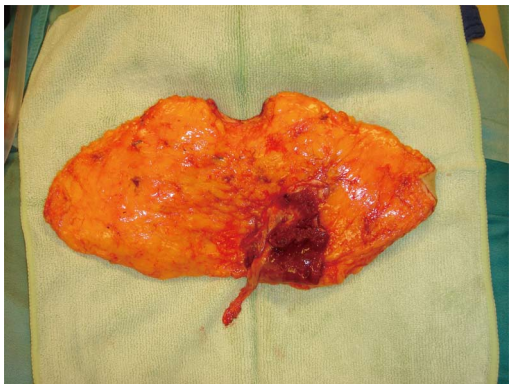


Figure 3 In a muscle-sparing free transverse rectus abdominis muscle, only a portion of the rectus muscle is included in the flap.

remained a major concern with this technique and resulted in an effort towards conserving as much muscle as possible[19]. Over time, the free TRAM flap sacrificing the whole muscle evolved into the muscle-sparing (ms-) TRAM flap and in further consequence into the DIEP flap with the main goal to minimize donor-site morbidity by harvesting less muscle and fascia. The ms-TRAM flap, in which only a small cuff of muscle around the vascular pedicle is harvested (Figure 3), is still a widely used method in breast reconstruction as it is technically less challenging than the DIEP flap with a comparable donor site morbidity[20].

DIEAP flap

The popularity of the DIEP flap has increased widely over the last decade. Currently it is the gold standard in autologous breast reconstruction not only because of its low donor site morbidity but also because of the satisfying aesthetic outcomes it can achieve. The DIEP flap utilizes the same skin island as the TRAM flap without harvesting the abdominal rectus muscle and anterior rectus sheath. Harvesting DIEP flaps requires considerable expertise in perforator pedicle dissection. Especially during the intramuscular part of the pre-paration one must dissect carefully to avoid damaging of the perforator vessels, which would subsequently lead to compromised flap circulation[21]. The DIEP flap is generally based on 1-3 perforating vessels and their connection to the DIEA (Figure 4). Unlike the TRAM flap dissection, these perforating vessels are carefully traced through the abdominal rectus muscle to their connection with the DIEA. Prior to surgery, the major perforating vessels can be identified *via* computed tomographic (CT)-angiography (Figure 5), Doppler ultrasonography or, if available, magnetic resonance imaging-angiography. Preoperative CT-angiography has been shown to reduce operative time, and can help to avoid injuries of the vulnerable pedicle[20]. DIEP flaps are typically outlined similar to aesthetic abdominoplasty procedures and surgical marking takes place in a standing position.

Simultaneous raising of the flap and preparation

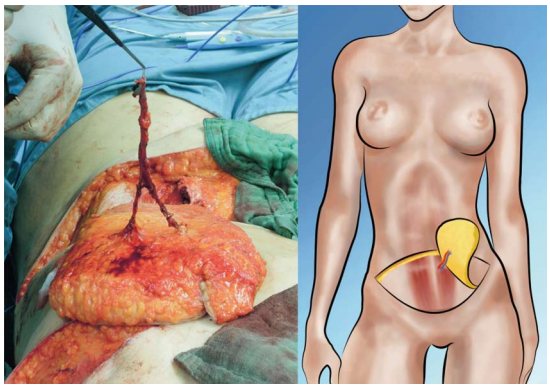


Figure 4 Example of a deep inferior epigastric perforator-flap with 2 perforators merging into the deep inferior epigastric vessels.



Figure 5 Computed tomographic-angiography to better elucidate the exact anatomy of perforators of the deep inferior epigastric perforator-vessels. The red arrow points out the piercing of the perforator through the rectus abdominis muscle on the sagittal (left) and transverse (right) view.

of the recipient vessels typically requires a two team approach. The IMA and vein are also here the preferred recipient vessels, while the thoracodorsal artery and vein remaining second choice. The IMA is typically approached in the second or third intercostal space. In case of a tight rib interspace, a small part of the rib cartilage can be removed for a better insetting of the pedicle.

After the skin incisions are made the superficial inferior epigastric artery (SIEA) and vein are first approached. In case of sufficient size and quality, they can be dissected down to their origin from the common femoral artery and a SIEA flap can be performed (see below). In case of an unsuitable SIEA, the abdominal skin island is raised carefully from lateral to medial until the lateral row of perforators is reached. Vessel diameter is the key factor in selecting a perforator. Once a perforator has been chosen, the anterior rectus fascia is incised and the perforator is dissected through the muscle until sufficient caliber of the pedicle is reached. Additional perforators in the same row may also be dissected out and included for further blood supply. However, intercostal motor nerves supplying the rectus muscle have been shown to enter the muscle just medial to the lateral row perforators and therefore harvesting of the lateral row perforators may decrease abdominal wall stability.



Figure 6 Pre- and post-operative pictures of a patient who underwent delayed reconstruction on her right side with a hemi-deep inferior epigastric perforator-flap and immediate reconstruction of her left side with the second hemi-deep inferior epigastric perforator-flap. She already had reconstruction of the nipple-areola-complex with tattooing and a local skin flap.

After completion of dissection, the flap is divided from its pedicle and passed to the chest for anastomosis and inset. The recipient vein and the vein of the flap are typically connected under magnification using an anastomotic coupling device. The arterial anastomosis is generally performed with a 9.0 suture. Upon completion of the anastomosis, the flap is checked for capillary refill. Finally, the incision in the anterior rectus fascia is closed directly without the need of a mesh.

DIEP flaps can be used in uni- and bilateral post-mastectomy breast reconstructions (Figure 6) and may also be used for reconstruction of congenital thoracic defects. There are only a few contraindications for the use of a DIEP flap. In the past, absolute contraindications included history of previous abdominoplasty, liposuction, or active smoking. Large transverse or oblique abdominal incisions were regarded as relative contraindications. Today, as a result of technical refinements, the only real absolute contraindications to DIEP flap reconstructions are non-compliant patients and/or a poor general condition. Obesity itself is not a contraindication, although it may lead to less aesthetically pleasing outcomes[22-24].

Complications occur infrequently. Partial flap loss has been reported as low as only 2.5%, total flap loss with less than 1%[25]. Problems regarding the venous anastomosis appear much more likely than problems with the arterial anastomosis. The most common complications include necrosis of the fat tissue of the flaps and seroma formation. The risk of bulging in DIEP flaps is reported as two thirds lower in comparison to TRAM flap reconstructions. Abdominal herniation occurred in fewer than 1% of all DIEP flaps compared with 3.9% in TRAM flaps[20,24,25]. However, current literature fails to prove a significant difference in the risk of developing abdominal bulge/hernia between ms-TRAM flap and DIEP flap based reconstructions[26]. Additionally, there is no significant difference in the risk of donor site morbidity between bilateral and unilateral DIEP flap breast reconstruction[26].

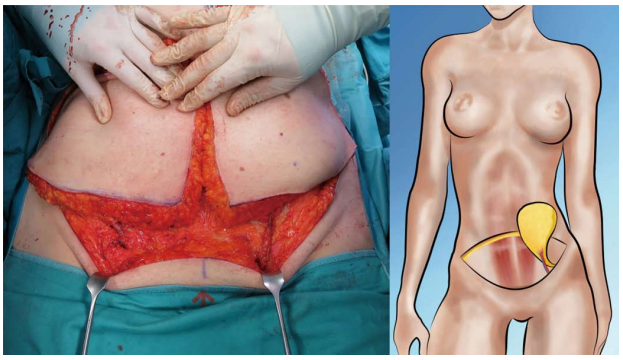


Figure 7 Example of a split superficial inferior epigastric artery-flap with both pedicles clearly visible. Since there is no injury to the rectus fascia, this the most desirable choice of flap from the lower abdomen.

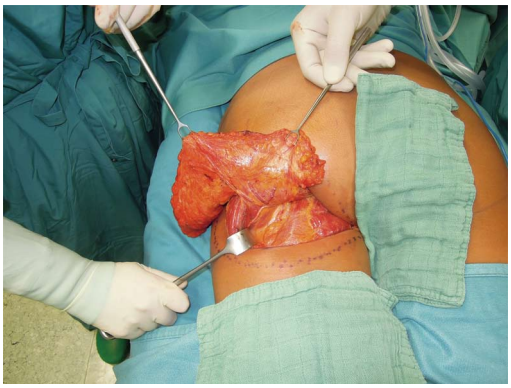


Figure 8 Example of a fasciocutaneous infragluteal-flap almost completely raised. At the undersurface of the flap, the flap's main vessels, *i.e.*, the descending branch of the inferior gluteal vessels, can be seen clearly. Additionally, a branch of the posterior femoral cutaneous nerve is visible and spared.

SIEA flap

The SIEA flap is the least invasive option of free abdominal tissue transfer for breast reconstruction. It provides the same tissue as the DIEP flap, without injuring the anterior rectus sheath and no vessel dissection needs to be performed through the muscle (Figure 7). Consequently, the SIEA flap causes the least donor site morbidity of all abdominal flaps, eliminating the risk of developing unwanted abdominal bulk or herniation[27]. However, the dissection of the vascular pedicle requires a high level of expertise, mainly because of its variability in existence, location, and caliber. The superficial inferior epigastric vessels have noted to be absent in 13%-42% of dissections. Furthermore, vessels are often not in appropriate caliber to sufficiently support the perfusion of the tissue volume needed for breast reconstruction. As a consequence, the amount of skin and fat tissue, which may be transferred utilizing a SIEA flap, is limited[22,24,28].

GLUTEAL AND THIGH FLAPS

If the lower abdomen is not available as a donor site, the gluteal area and thigh provide a number of flaps suitable for breast reconstruction. If the required breast volume is small, and there is enough tissue available on the upper medial thigh, a transverse upper gracilis flap may be a practicable method to reconstruct the breast. In case of a higher amount of required volume, a gluteal artery perforator flap is the best choice[22,29].

Other more exotic examples of free flaps in use for breast reconstruction, which will not be reviewed here in detail, include the anterolateral thigh flap[30,31] and the recently described PAP flap[32].

Gluteal artery perforator flaps and the FCI flap

The first free gluteal myocutaneous flap for breast reconstruction was performed by Fujino *et al*[33] in 1975. As the development of the perforator flap technique revolutionized the applicability of soft tissue transfer, gluteal flaps became popular in the 1990s[11]. Gluteal perforator flaps offer a great alternative for breast reconstruction in women who have more tissue available in the buttock area than in the lower abdomen. Similar to the DIEP and SIEA flaps, no sacrifice of muscle is required using this technique.

The superior and inferior GAP (S/I-GAP) flaps have been specified and modified by Allen *et al*[34-36]. The surgical procedure is similar to the DIEP flap. The gluteal vessel pedicle is dissected throughout the gluteus maximus muscle to their origin in the internal iliac artery. The pedicle is generally about 5-8 cm long, which provides sufficient length for a comfortable anastomosis and inset at the chest wall. A clear disadvantage of this method is in the necessary rearrangement of the patient from abdominal to supine position during surgery. GAP flaps are generally a safe reconstructive option. Vascular complications are reported in approximately 6%, total lap loss in 2% of all cases. However, approximately 4% of all patients require revisions because of donor site problems, such as contour deformity after SGAP, or scar issues after IGAP flaps[12,22,24].

Another, in our opinion more valuable flap of the gluteal region is the FCI flap. The FCI flap is based on the descending branch of the inferior gluteal artery with a very reliable location, good caliber and a pedicle length ranging up to 18 cm (Figure 8). Unlike other gluteal flaps, the vessels curl around the lower border of the gluteus maximus muscle. Therefore, no perforator dissection through the muscle is necessary. Studies demonstrated that even thin patients offer enough tissue to allow breast reconstruction with adequate volume[37], making the FCI flap a convincing alternative to standard free tissue breast reconstruction in certain cases. The tissue from this region is more compact and not as soft compared to the lower abdomen making it more suitable for reconstruction of firm breasts in younger patients (Figure 9).

Gracilis flaps

Since its first description for free tissue transfer in 1976[38] the gracilis flap has become a workhorse in autologous breast reconstruction. The TMG flap is an alternative method for patients who do not have adequate abdominal tissue, or seek to avoid abdominal



Figure 9 A 49-year-old patient after bilateral nipple-sparing prophylactic mastectomy and immediate breast reconstruction with bilateral fasciocutaneous infragluteal-flap.

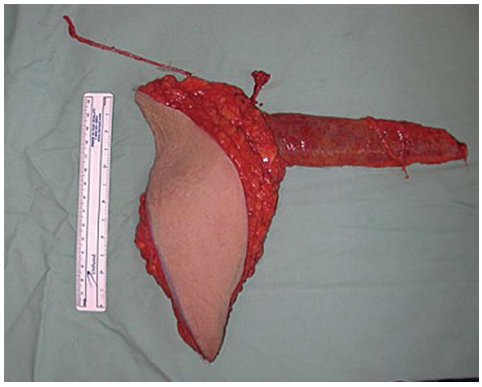


Figure 10 Intraoperative image of a completely harvested transverse myocutaneous gracilis flap with flap pedicle (short) and the relatively long saphenous vein as a venous supercharge.



Figure 11 Pre- and post-operative pictures after immediate bilateral skin-reducing breast reconstruction with a transverse musculocutaneous gracilis-flap and reconstruction of the nipple-areola-complex.

scarring. The TMG flap is ideally suited for reconstruction of small to moderate sized breasts, providing excellent contour and projection[39]. The donor site is well hidden and harvesting the flap has no functional consequences[40].

The flap is designed with a semilunar skin island in the inner thigh with a mean volume of around 300 cc. The raising of the skin island is started close to the groin and harvested with the underlying fascia. The average pedicle length is reported as 6-8 cm, with an arterial diameter of 1.2 mm and a venous diameter of 2.8 mm. The artery is usually slightly smaller than the IMA, which usually does not preclude a safe anastomosis[41] (Figure 10).

Performing a unilateral TMG flap may cause asymmetry. The use in bilateral reconstruction is reported as one of the strongest advantages of the flap and even recommended as first choice, except for patients that would clearly benefit from an abdominoplasty (Figure 11). As with all flaps including a muscular component, the TMG flap is prone to volume loss resulting from neurogenic atrophy, but a true impact on cosmetic appearance has yet to be demonstrated[22,42]. A true disadvantage of the gracilis flap is the small size of the skin island. A single TMG flap may provide not enough tissue for sufficient reconstruction of a large breast. In this case, two TMG flaps or the usage of a combined infragluteal-transverse myocutaneus gracilis flap can be a viable option[43].

TIMING OF RECONSTRUCTION

Breast reconstruction can be divided into 3 approaches: Immediate, delayed-immediate and delayed[22]. The advantage of immediate breast reconstruction is the preservation of the breast skin and inframammary fold, which results in optimized aesthetic outcomes and minimized psychological burden of the patient by eliminating a period without a breast. The disadvantage is the uncertainty regarding radiotherapy and chemotherapy and their effects on autologous breast reconstruction. An increased incidence of fat necrosis and a higher rate of surgical revisions are reported in patients with free tissue transfer and further anti cancer therapy[44].

Therefore, a delayed-immediate reconstruction may be a good alternative. Expander implants are inserted as a temporary placeholder to maintain the breast skin envelope. Breast reconstruction is performed once radiotherapy is complete. In contrast, a fully delayed reconstruction offers the advantage of a typically very motivated patient with completed oncological treatment. The obvious disadvantage is the reduction of skin tissue

and the presence of significant scarring[45].

The optimal timing of breast reconstruction in relation to radiation therapy is controversially discussed. Therefore, it is advisable to perform autologous microvascular breast reconstruction in patients, who are confirmed for post-mastectomy radiation therapy, only in a delayed fashion. If post-mastectomy radiation therapy is likely but not certain, delayed-immediate breast reconstruction may be the better choice. Nonetheless, because of the mostly satisfying aesthetic outcome and the contradictory literature regarding this topic, immediate autologous breast reconstruction should at least be considered and discussed with the patient, even when post-mastectomy radiotherapy is anticipated.

CONCLUSION

Autologous microvascular breast reconstruction is becoming more and more accepted as a key component of breast cancer treatment after mastectomy. The abdominal wall or, alternatively, the gluteal and thigh areas provide numerous flaps suitable for breast reconstruction with appealing results. Recent advances in surgical technique enable today's reconstructive surgeons to minimize donor site morbidity whilst maximizing patient outcomes. If timed and performed correctly, microsurgical breast reconstruction is able to provide the best aesthetic outcomes with low complication rates and donor site morbidity.

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MINIREVIEWS

Targeting metabolism in breast cancer: How far we can go?

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Abstract

Adjuvant therapies for breast cancer have achieved great success in recent years and early breast cancer is now a curable or chronic disease. Targeted therapies, including endocrine therapy and human epidermal growth factor receptor-2 targeted therapy, marked a new era of breast cancer treatment. However, except for chemotherapy, an efficient drug treatment to improve the overall survival of breast cancer patients is still lacking for triple negative breast cancer. Furthermore, a certain proportion of breast cancer patients present with resistance to drug therapy, making it much more difficult to control the deterioration of the disease. Recently, altered energy metabolism has become one of the hallmarks of cancer, including breast cancer, and it may be linked to drug resistance. Targeting cellular metabolism is becoming a promising strategy to overcome drug resistance in cancer therapy. This review discusses metabolic reprogramming in breast cancer and the possible complex mechanism of modulation. We also summarize the recent advances in metabolic therapy targeted glycolysis, glutaminolysis and fatty acids synthesis in breast cancer.

Key words: Breast cancer; Targeted therapy; Metabolism; Drug resistance; Chemotherapy

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Core tip: Breast cancer cells display distinct metabolic characteristics according to different molecular phenotypes. There may be crosstalk with the estrogen receptor and human epidermal growth factor receptor-2 signal pathways in the metabolic regulation in breast cancer cells that make it more complex to evaluate the efficiency of an anti-metabolic drug. On the other hand, the research on target metabolism in breast cancer will also largely help us to understand the complicated mechanism by which an anti-metabolic drug improves the efficacy of cancer therapy or overcomes drug resistance.

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INTRODUCTION

Breast cancer now has the highest incidence of cancer in women. This is attributed to the molecular classification of breast cancer based on the hormonal receptor and human epidermal growth factor receptor-2 (HER-2), targeted therapy and other adjuvant therapies that prolong the overall survival and greatly decrease the mortality of this disease. However, mortality remains high for locally advanced and metastatic cancer. We still lack effective methods for treatment when drug resistance occurs and recurrence and metastasis develop, especially in triple negative breast cancer (TNBC).

Females have a specific energy metabolic pattern compared to males[1]. Estrogens, progesterone-to-estrogen ratio and androgen levels affect the energy material transporter and metabolic enzyme expressions in cells[2]. Estrogens may increase the expression of peroxisome proliferator activated receptor, Akt and activate AMP-activated protein kinase (AMPK), which consequently influence the metabolic process, including glucose utility, lipid uptake, storage, lipogenesis and lipid oxidation[3,4]. Endocrine therapy plays a pivotal role in estrogen receptor (ER) positive breast cancer treatment. Rapamycin, which inhibits the mammalian target of rapamycin (mTOR), is a downstream target of Akt and enhances the susceptibility of breast cancer cells to endocrine therapy[5]. However, there is still a certain proportion of breast cancer patients that present with primary resistance to endocrine therapy and some patients could develop secondary resistance which makes it much more difficult to control the disease progress[6]. A similar condition occurs in chemotherapy and HER-2 targeted therapy in breast cancer. Therefore, researchers are looking for new strategies or compounds to reduce drug resistance and enhance the efficacy of therapy.

Metabolic reprogramming is the primary and basic factor during cell transformation[7,8]. Foreign stress forces tumor cells to accommodate new circumstances through metabolic reprogramming caused by epigenetic change and gene mutation. Altered energy metabolism has become one of the hallmarks of cancer[7]. Mounting evidence also attributes the drug resistance to dysregulated cellular metabolism[9,10]. Recently, much more interest has focused on targeting metabolic enzymes for cancer therapy or reversing drug resistance[11-13]. Cancer cells have distinct metabolic properties, including enhanced aerobic glycolysis, fatty acid synthesis and glutaminolysis, to sustain immortal proliferation[7,14]. This review will discuss the metabolic reprogramming and advances in metabolic targeted therapy in breast cancer.

METABOLIC REPROGRAMMING IN BREAST CANCER

To meet the abundant requirement of energy and materials for proliferation, most malignant cells present with increased aerobic glycolysis, fatty acid synthesis and glutaminolysis, which are distinctive from normal cells[15] (Figure 1). In 1956, Warburg[16] first postulated that cancer cells had a significantly higher rate of glycolysis than normal cells to produce ATP for proliferation. He also hypothesized that due to the defective function of mitochondria (this was proved wrong afterwards), pyruvate produced from glycolysis was converted to lactate more than acetyl CoA through the tricarboxylic acid (TCA) cycle. This phenomenon is now called the Warburg effect and it exists regardless of oxygen availability. For the adaption of the Warburg effect, cancer cells exhibit altered expression of different glucose transporters and glycolysis enzymes. Glucose crosses the plasma membrane *via* glucose transporter proteins (GLUTs) and fourteen types have been identified. Although little is known about the role of glucose transporters in cancer biology, GLUT1, GLUT2, GLUT3, GLUT4, GLUT5 and GLUT12 have been detected in breast cancer cells[17-20]. Different expression patterns of GLUT isoforms in breast cancer may have an association with pathological grade, cancer cell differentiation and prognosis. According to the molecular subtype of invasive breast cancer, HER-2 positive and TNBC mostly exhibit higher levels of glycolysis which need higher levels of expression of GLUT[21]. As the most invasive type in breast cancer, TNBC had the highest expression of GLUT-1 when compared to other types[21]. Increased activity of enzymes involved in glycolysis, like hexokinase (HK) and lactate dehydrogenase-A (LDHA), have also been studied and their expression may affect cancer cell growth[22,23].

Increased glutamine metabolism is another alternative energy origin for cancer cells, including breast cancer, and is thought to be a central metabolic pathway cooperating with glycolysis[24,25]. Most cancer cells cannot proliferate without a glutamine supply and glutamine addiction provides intermediates for amino acid and lipid synthesis[26]. Under hypoxic conditions, proliferating cells, including breast cancer cells, mostly employ reductive metabolism of glutamine-derived alpha-ketoglutarate to synthesize acetyl CoA for lipid synthesis that normally enters into the canonical TCA cycle. That pathway is isocitrate dehydrogenase 1 dependent[27,28]. Intermediate metabolites derived from glutamine metabolism, such as antioxidants NADH, glutathione and ammonia, could change the reduction-oxidation status in cancer cells, promote stromal cell autophagy and increase tumor growth and drug resistance[25,29]. Cell studies showed that a high glutamine supply protected MCF7 cells from tamoxifen-induced apoptosis[30]. Amino acid transporter-2, glutaminase 1 (GLS) and glutamate dehydrogenase are three key enzymes involved in glutamine metabolism[31]. Immunohistochemical staining of breast cancer tissues indicated that HER-2 positive and TNBC exhibited the most frequent expression of glutamine metabolism related proteins than other types[32]. Glutamine produces glutamate under the catalytic effect of glutaminase, thus the ratio of glutamate

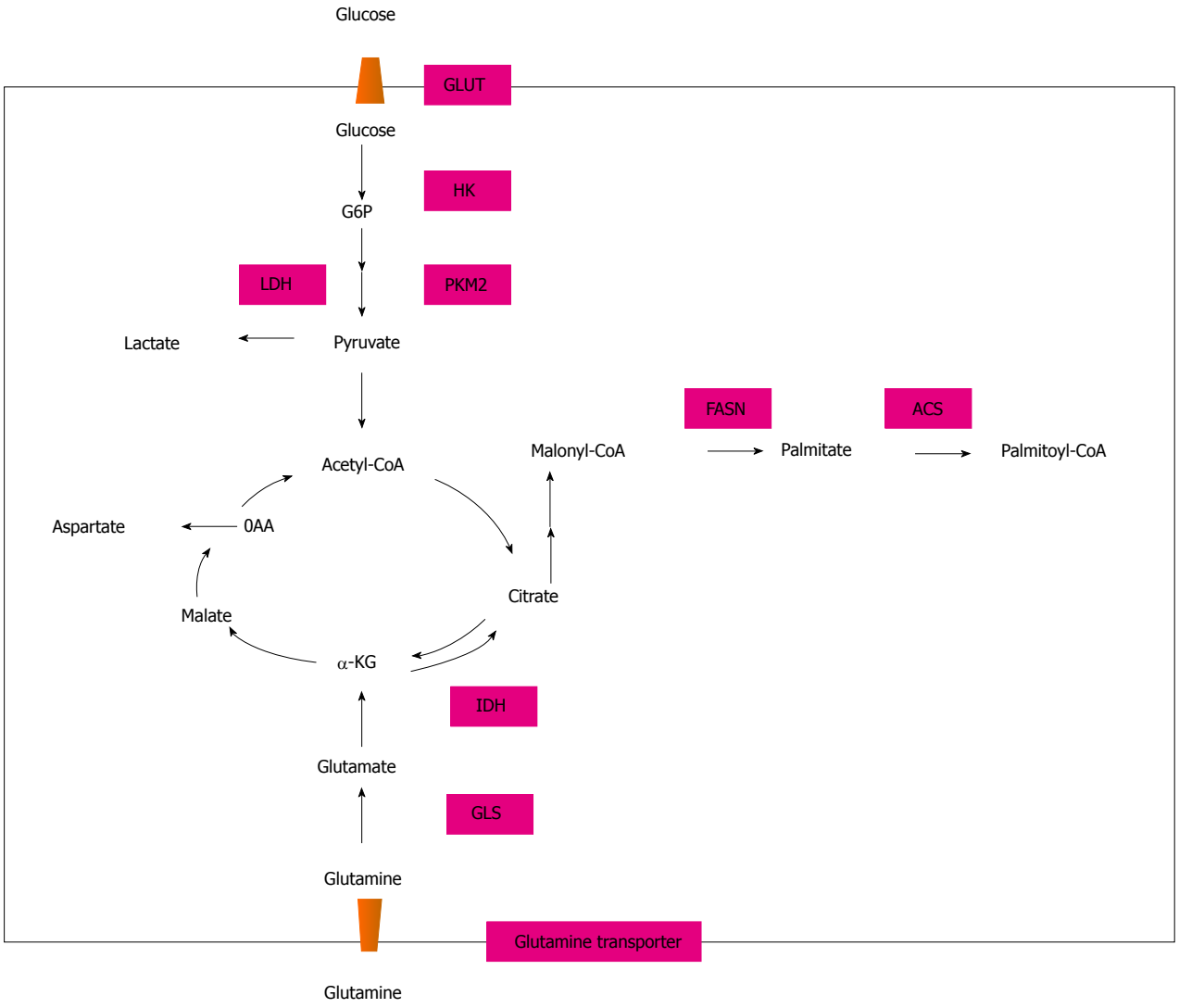


Figure 1 Metabolic reprogramming in malignant cells. Most malignant cells present with increased aerobic glycolysis, fatty acid synthesis and glutaminolysis. The pink circles in the figure show the possible metabolic targets of enzymes or receptors. GLUT: Glucose transporter proteins; HK: Hexokinase; PKM: Pyruvate kinase M; LDH: Lactate dehydrogenase; FASN: Fatty acid synthase; ACS: Acetyl-CoA synthase; IDH: Isocitrate dehydrogenase; GLS: Glutaminase.

to glutamine may indicate the glutamine metabolic activity[33]. Asiago *et al*[34] reported that an elevated level of glutamate was associated with disease outcome in breast cancer patients. Metabolomic analysis of 270 clinical breast cancer samples and 97 normal breast samples showed that breast cancer cells had a higher glutamate-to-glutamine ratio than normal cells, particularly ER- tumor cells[35]. A cell study showed that highly invasive and drug-resistant breast cancer cells were characterized by increased glutamine metabolism with an increased glutamate-to-glutamine ratio and greater expression of glutaminase compared with noninvasive breast cancer cells[36].

Under normal conditions, breast cells utilize circulating lipids for the synthesis of new structural lipids, while breast cancer cells mostly synthesize fatty acids by themselves. The biosynthetic enzyme fatty acid synthase (FASN) is the key enzyme required for the synthesis. FASN expression in breast cancer was first explored during the 1980s when its expression was increased after progestin treatment[37]. Recently, FASN expression has been recognized as an oncogene for its role in carcinogenesis. Upregulation of FASN has been reported in many different tumors, including breast cancer, and it may be associated with tumor development, recurrence and prognosis[38]. Immunohistochemistry staining revealed the highest FASN expression in HER-2 breast tumors and lowest in TNBC tumors, with the studies in breast cancer cells obtaining the same results[39,40]. Vazquez-Martin *et al*[41] postulated a "HER2-FASN axis" that indicated the bidirectional regulation mechanism between FASN and HER2 which could enhance cancer cell proliferation, survival, chemoresistance and metastasis in breast carcinomas.

MODULATION OF METABOLIC REPROGRAMMING IN BREAST CANCER

Breast cancer is classified into four molecular subtypes: Luminal A, luminal B, HER-2 overexpression and basal

types, with type luminal A accounting for about 70%[42]. The estrogen and HER-2 signal pathways play critical roles in breast cancer carcinogenesis, progression and prognosis. They may interact with each other as well as other signal pathways. Since most cancer cells have a high nutrition intake requirement to accommodate cell proliferation and altered metabolism may be a hallmark of cancer development, different molecular subtypes of breast cancer should exhibit distinct metabolic phenotypes. However, to date, we still know much less about the modulation mechanism of tumor-specific metabolic changes, especially in breast cancer[43]. We also know less about how these changes may change molecular phenotypes of breast cancer and affect response to drug treatment.

Although scientists are trying hard to find how signal pathways control the energy metabolism of cancer cells, little is known about the complex network. Hypoxia-inducible factors (HIF) and the proto-oncogene c-Myc are two major regulators in energy metabolism, including glucose, protein and fatty acid metabolisms[44]. Other genes, including *Akt*, *Ras*, *Raf*, *Src* and *EGFR,* may also be involved in glycolysis and activating these genes could cause increased glucose uptake. mTOR inhibitor rapamycin may inhibit cancer cell glucose metabolism by downregulating pyruvate kinase M2 and restoring the susceptibility of breast cancer cells to tamoxifen treatment effectively may be one mechanism of rapamycin[45]. On the other hand, estrogen-induced HIF-1 accumulation in breast cancer cells stimulates glucose uptake *via* the PI3K/Akt signaling pathway[19,46] which also leads to increased mTOR phosphorylation[47]. Another clinical study found that HIF-1 had the highest expression in HER-2 positive breast cancer[21]. It indicated that HIF-1 has crosstalk with the ER and HER-2 signal pathways.

The *c-MYC* gene controls cancer cell glutaminolysis through several targeted genes. MYC is overexpressed in 30%-50% of high-grade breast tumors[48,49]. Increased MYC expression often indicates increased dependency on glutamine and glucose for survival, may have a correlation with drug resistance in breast cancer cells and inhibition of MYC could reverse the drug resistance[50-52]. In antiestrogen resistant breast cancer cells, MYC could activate an unfolded protein response through glucose-regulated protein-78 (GRP78/HSP5A/BiP) and inositol-requiring enzyme-1α (IRE1α/ERN1) and increase c-Jun N-terminal kinase activation and spliced X-box protein-1 to support cell survival[45]. The inhibition of MYC was shown to decrease glutaminase activity, although there were different results in drug resistant breast cancer cells and other cells[50,53,54]. Inhibition of glutaminase reversely could decrease MYC expression[51]. Activation of the Akt/mTOR signal pathway also stimulates uptake of glutamine through increased glutaminase activity[55] and the underlying mechanism may be through eIF4B dependent control of c-Myc translation[56]. In both ER and HER-2 positive breast cancer cells, upregulation of HER-2 is one possible mechanism for endocrine treatment resistance. The crosstalk between ER and HER2 may regulate MYC-mediated glutamine metabolism[52]. ER downregulator fulvestrant may decrease glutamine consumption through inhibition of MYC and glutaminase and consistent expression of MYC may abrogate the effect of rapamycin on glutaminase[52,56], although the highest glutamine metabolic activity was seen in HER2-type breast cancer, which meant a possible correlation between glutamine activity and the HER-2 signal pathway[32].

Although the mechanism of overexpression of FASN in breast cancer cells is still uncertain, it has been proved that the potent lipogenic transcription factor sterol regulatory element-binding protein 1 (SREBP-1), can regulate FASN expression through binding with the site of the FASN promoter with co-activating transcription factors such as NF-γ Sp1 and Spot14[57,58]. Dietary polyunsaturated fatty acids suppress FASN expression through the modulation of NF-γ binding to the FASN promoter by SREBP-1c[59]. PI3k-Akt and the MAPK signal transduction pathway are also thought to be involved in FASN modulation[60,61]. Under hypoxic conditions, *FASN* gene is upregulated *via* the activation of Akt followed by the induction of the *SREBP-1* gene[62]. Inhibition of MAP kinase also decreases transcription from the FASN promoter and reduces FASN expression in MCF7 cells[63]. The mTOR inhibitor rapamycin may also inhibit FASN in breast cancer cells[64]. Recently, a "HER2-FASN axis" is thought to exist which indicates the bidirectional regulation mechanism between FASN and HER2. The highest level of FASN expression in the HER-2 positive breast cancer type also confirms this hypothesis. FASN could also be regulated by estrogen in ER-positive breast cancer cells. Estrogen stimulates FASN expression and inhibiting FASN augments E2-stimulated transcriptional activity and enhances the E2-mediated ER expression synergistically[65].

TARGETING GLYCOLYTIC ENZYMES

As a basic energy resource for cancer cells, many enzymes are involved in glucose metabolism. The efficiency of target metabolism therapy has been proved in enhancing anticancer treatments or overcoming drug resistance in breast cancer cells, including chemotherapy resistance, endocrine therapy resistance and HER-2 targeted therapy resistance. Besides searching for a new agent to block glucose metabolism or induce a switch from glycolysis to mitochondrial respiration, researchers are also making much effort to find the underlying effect of existing agents on metabolic changes. Sorafenib is a multikinase inhibitor and may downregulate GLUT-1 expression in breast cancer cells through AMPK-dependent inhibition of the mTORC1 pathway, inhibit cell proliferation and induce apoptosis[66].

The glucose transporter family consists of 14 sodium-independent facilitative glucose transporters (SLC2A1-14 or GLUT1-14). GLUT1 appears to be the predominant glucose transporter in many types of cancer cells, inclu-

ding breast cancer[67]. A small compound, WZB117, has shown its inhibitory activity on GLUT1 in MCF-7 breast cancer cells[68]. Synergistic anticancer effects of combined WZB117 with other anticancer drugs, cisplatin or paclitaxel, were also observed. Added to the mitochondrial inhibitor, WZB117 was more efficient in inhibiting cell proliferation, which indicated WZB117 may be more effective in aggressive cancer cells that invariably had mitochondrial dysfunction[68].

HK-2, the first regulatory enzyme in glycolysis, has an important role in glycolysis. 2-DG, a glucose analog, binds with HK competitively and inhibits glycolysis. Although as a single agent the antitumor effect was not significant, a study showed that 2-DG combined with trastuzumab inhibited trastuzumab-sensitive and resistant breast cancers in *in vitro* and *in vivo* models of HER-2 positive breast cancers with more efficient inhibition of glycolysis *via* downregulation of heat shock factor 1 and LDHA[69].

LDHA is the enzyme that catalyzes the conversion of pyruvate to lactate. LDHA knockdown stimulates the switch of HER-2-initiated breast cancer cells to mitochondrial oxidative phosphorylation, decreases cell proliferation to hypoxic conditions and interferes with tumorigenicity[70]. Dichloroacetate (DCA), an inhibitor of pyruvate dehydrogenase kinase (PDK), may activate pyruvate dehydrogenase, which is governed by PDK, and facilitate the conversion of pyruvate to acetyl Co-A, which demonstrates the antiproliferative properties in highly metastatic diseases of DCA[71]. The inhibitor of LDH-A selectively inhibits the growth of HER-2-overexpressing cells and enhances the sensitivity of trastuzumab-resistant breast cancers to trastuzumab treatment[69,23]. Furthermore, downregulation of LDH-1 by oxamate shows a synergistical inhibitory effect on taxol-resistant breast cancer cells by promoting apoptosis when combined with taxol[9].

TARGETING GLUTAMINE METABOLISM

In many cancer cells, glutamine is used to replenish the TCA cycle and oxidative phosphorylation instead of glucose to produce enough ATP to support cell proliferation[72]. Glutamine addiction is a common strategy for some cancer cells like breast cancer cells to escape drug treatment. Glutamine transporters or glutaminolysis are becoming a potential pharmacological target to revert resistant cancer cells to respond to the initial therapy. An amino acid transporter SLC6A14, also known as $ATB^{0,+}$, is upregulated specifically in ER-positive breast cancer. Blockade of SLC6A14 in ER-positive breast cancer cells could inhibit mTOR activity, cause cell apoptosis and activate autophagy[73].

Glutaminase, the enzyme that catalyzes glutamine to glutamate has attracted much interest for targeted cancer therapy recently. Two novel glutaminase inhibitors have been discovered: CB-839[74] and 968[51]. CB-839 showed the most potent antiproliferative activity in a TNBC cell line, while no antiproliferative activity was observed in an ER–positive cell line. In xenograft models, CB-839 displayed significant antitumor activity, both as a single agent and in combination with paclitaxel. Compound 968 showed the greatest cytotoxic effect in MDA-MB-231 breast cancer cells. Genome analysis proved that compound 968 could induce changes in many anti-apoptotic and/or promote metastasis-related gene expression and histone modifications as well, which subsequently activate apoptosis and decrease the invasiveness of MDA-MB-231 cells. It also enhanced chemotherapy sensitivity of breast cancer cells when combined with the chemotherapeutic drug doxorubicin.

TARGETING FATTY ACID METABOLISM

FASN is the key biosynthetic enzyme in the fatty acid synthesis pathway that synthesizes long-chain fatty acids palmitate from malonyl-CoA. Acetyl-CoA carboxylase (ACC) carboxylates acetyl-CoA to malonyl-CoA. Upregulation of FASN has been reported both in premalignant lesions and most human cancers. In normal cells, fats are absorbed freely and FASN is downregulated, except in the lactating breast and cycling endometrium. The unique distribution of FASN in different tissues makes FASN an attractive target for cancer therapy. The inhibition of FASN causes depletion of the end product long chain fatty acids and the accumulation of the substrate malonyl-CoA. Evidence showed that inhibition of ACC did not induce cancer cell apoptosis, which meant the accumulation of malonyl-CoA may be the reason for the antitumor effect of FASN inhibition[75,76].

A bidirectional regulation mechanism between FASN and HER2 was illustrated[41,77]. FASN blockade suppresses HER2 overexpression at the transcriptional level with the upregulation of the expression of PEA3, a transcriptional repressor of HER-2. HER-2 overexpression stimulates FASN expression and fatty synthesis and this HER-2 mediated induction can be inhibited by trastuzumab. The combination of FASN inhibitor and trastuzumab stimulates MDA-MB-231/HER-2 cell apoptosis and re-sensitizes trastuzumab-resistant breast cancer through the downregulation of HER-2 expression[78,79]. Menendez *et al*[77] hypothesized that FASN inhibition would result in major changes in the synthesis of phospholipids, which should increase the degradation of HER-2 and enhance the action of the anti-HER-2 antibody trastuzumab.

Furthermore, FASN inhibitor cerulenin demonstrated a strong synergism with docetaxel in HER-2 overexpressing and docetaxel-resistant SK-Br3 cells, which indicated the role of FASN in HER-2-induced breast cancer chemotherapy resistance[80]. FASN blockade also could induce a synergistic chemosensitization of breast cancer cells to other chemotherapy agents, such as paclitaxel, adriamycin, 5-FU and vinorelbine[81-84].

CONCLUSION

Breast cancer is a heterogeneous group of neoplasms

originating from epithelial cells that can be divided into various molecular phenotypes. Targeted therapy, such as endocrine therapy and HER-2 targeted therapy, has achieved great success in breast cancer treatment. However, like chemotherapy resistance, resistance to endocrine therapy and HER-2 targeted therapy can produce discouraging results. Recently, cancer research has focused on dysregulated metabolism in cancer cells and metabolic reprogramming is now considered a hallmark of cancer. More and more evidence supports the idea that dysregulated cellular metabolism may be associated with drug resistance in cancer therapy. In breast cancer, many agents that target specific enzymes in the metabolic pathways, including glycolysis, glutaminolysis and fatty acid synthesis, have been developed or proposed. Some of them have shown the ability to enhance the efficacy of current therapies and resensitize resistant cancer cells and have now been progressed to clinical trials. However, to date, none have been put into routine clinical practice for a couple of reasons. The main reason may be the extremely complex modulation of metabolism and their crosstalk with other signal pathways. Hence, there are three key problems that need to be elucidated: (1) energy pathways may be employed by cancer cells as well as normal cells. The influence or toxicity of metabolic drugs on normal cells should be evaluated carefully besides its antitumor effect. This question is prominent when combining metabolic drugs targeting different pathways to avoid insufficient effects or drug resistance; (2) for breast cancer, different molecular types may possess a specific metabolic phenotype. Even a "good" molecular type of breast cancer, like luminal A, may have recurrent metastasis caused by drug resistance in a relatively short period and so it is critical to find which specific enzymes for specific molecular phenotypes could be promising targets. This understanding will help us better distinguish which altered metabolic phenotypes may have a poorer prognosis and higher invasiveness than other types; (3) it has been postulated that metabolic regulation may have crosstalk with ER and HER-2 signal pathways. The genetic regulators such as c-myc, PI3k/Akt /mTOR and MAPK regulate metabolism as well as ER and HER-2 signal pathways. They form a complex framework, like the "FAS-HER-2 axis" and "c-myc-mTOR axis", which determines the growth, apoptosis and drug resistance of cancer cells. Completely understanding the framework for breast cancer is still a challenge for developing a successful metabolic therapy. Nevertheless, much effort and progress has been made in this field and we hope that, in the near future, targeting tumor metabolic pathways may become an important component of the comprehensive treatment of breast cancer.

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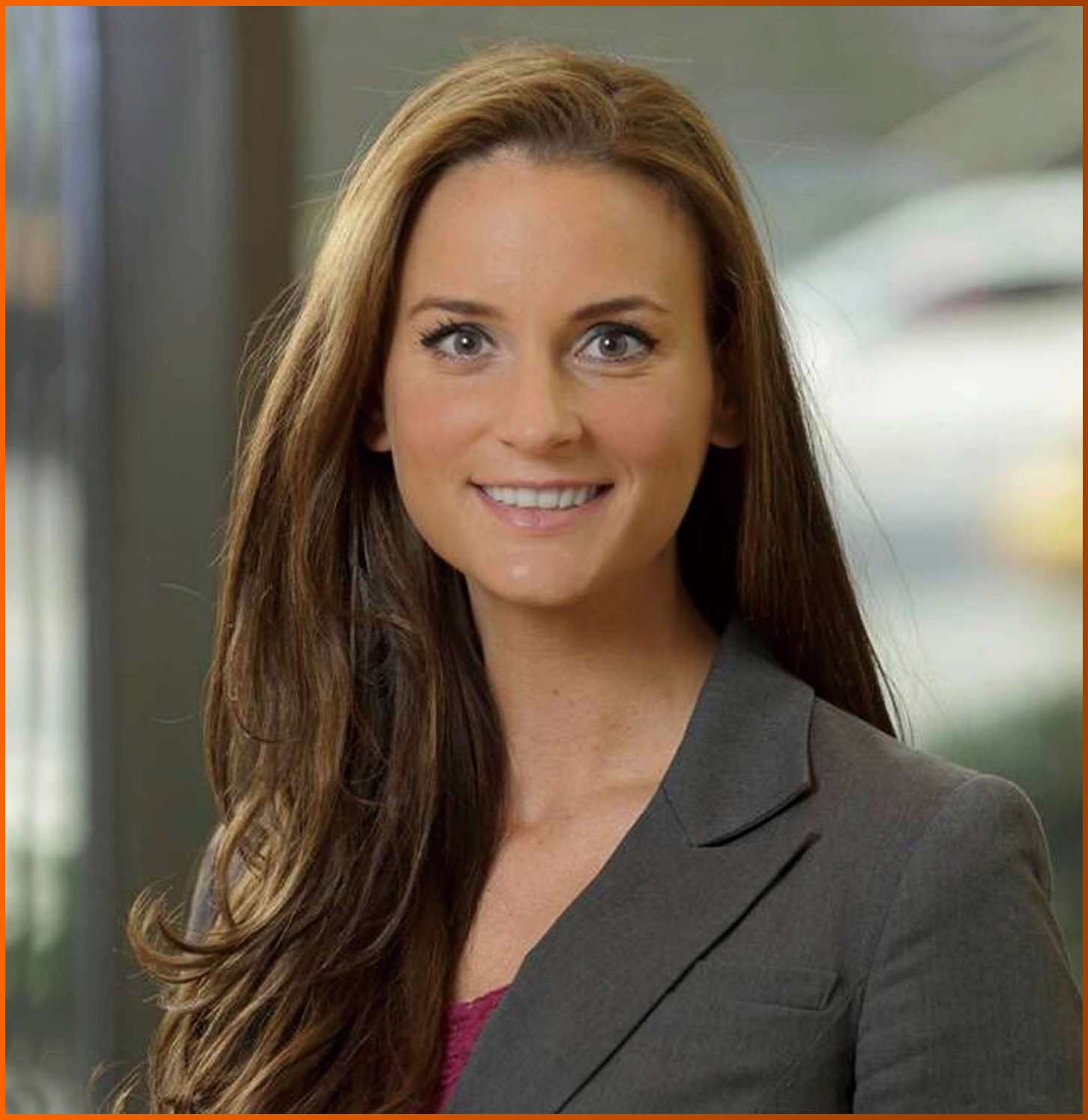


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EDITORIAL

Gastro-entero-pancreatic neuroendocrine tumors: Is now time for a new approach?

Rossana Berardi, Mariangela Torniai, Agnese Savini, Silvia Rinaldi, Stefano Cascinu

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Abstract

Gastro-entero-pancreatic tumors (GEP-NETs) are rare neoplasms often characterized by an overexpression of somatostatin receptors. Thus, radiolabeled somatostatin analogues have showed an increasing relevance both in diagnosis and treatment, especially in low- and intermediate-differentiated GEP-NETs. These evidences have led to a growing development of new functional imaging techniques as 68Ga-DOTATATE positron emission tomography/computed tomography (PET/CT) proved useful in the management of these neoplasms. However these tumors have a heterogeneous behavior also modifying their aggressiveness through time. Therefore sometimes 18F-fluorodeoxyglucose PET/CT appears to be more appropriate to obtain a better assessment of the disease. According to these considerations, the combination of different functional imaging techniques should be considered in the management of GEP-NETs patients allowing clinicians to choose the tailored therapeutic approach among available options.

Key words: 18F-fluorodeoxyglucose positron emission tomography/computed tomography; Gastro-entero-pancreatic neuroendocrine tumor; 68Ga-DOTATATE positron emission tomography/computed tomography; Diagnosis; Imaging

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Core tip: Our paper stressed the importance of combined 68Ga-DOTATATE and 18F-fluorodeoxyglucose positron emission tomography/computed tomography in the management of gastro-entero-pancreatic tumors (GEP-NETs). In fact we underlined that the association of these functional imaging techniques showed an important role in redefining the disease after progression, especially in intermediate-grade GEP-NETs, allowing clinicians to choose the tailored therapeutic approach among available options.

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INTRODUCTION

Gastro-entero-pancreatic neuroendocrine tumors (GEP-NETs) are uncommon neoplasms including a wide range of anatomical, clinical, histological and molecular malignant entities.

Diagnostic approach in this setting is crucial in order to manage the therapeutic strategy.

Development of novel positron emission tomography (PET) tracers (68Ga-DOTApeptides), specifically binding to somatostatin receptors overexpressed on the surface of NET cells, determined the individualization of this kind of neoplasm on 68Ga-DOTA-peptide PET/computed tomography (PET/CT) scans.

Functional imaging of GEP-NETs still plays an important role in the diagnosis and management of this condition, largely due to its ability to provide information for therapy planning.

THE ROLE OF FUNCTIONAL IMAGING

68Ga-DOTATATE PET/CT showed high accuracy in G1-G2 GEP-NETs, especially in bone metastasis and in occult primary tumor detection[1] than other imaging procedures (particularly CT, performed usually as first-line investigation, and other functional assessment [somatostatin receptor scintigraphy (SRS)].

Gabriel *et al*[2] confirmed the diagnostic role of 68Ga-DOTATOC PET, in comparison with SRS, CT, in the detection of unknown primary tumor in the presence of clinical or biochemical suspicion of neuroendocrine malignancy initial tumor staging, and follow-up after therapy for NETs patients, reporting a sensitivity of 97% and specificity of 92%.

Ambrosini *et al*[1] retrospectively studied the sensitivity, specificity and accuracy of 68Ga-DOTA-NOC PET/CT in comparison with CT alone for the evaluation of bone metastasis in patients with neuroendocrine tumor. PET was performed for staging, unknown primary tumor detection, reevaluation of disease, post-therapy assessment and follow-up. In particular PET seems to detect more bone lesions than CT, showing a higher sensitivity (100% *vs* 80%), specificity (100% *vs* 98%), positive predictive value (100% *vs* 92%), and negative predictive value (100% *vs* 95%)[1].

Furthermore, 68Ga PET/CT allows molecular imaging of NETs with very high diagnostic sensitivity and specificity especially to identify earlier occult metastases not manifest with other procedures. It contributes to accuracy of data by facilitating: The selection of patients for a somatostatin analogues therapy with curative/palliative/"neoadjuvant" intent; to assist in finding appropriate surgical option and for evaluation of treatment response [especially to peptide receptor radionuclide therapy (PRRT)] through clinical symptoms improvement or worsening, and prediction of time to progression.

In fact, the greater sensitivity of 68Ga-DOTATOC could provide new clinical information resulting in altered surgical plans in some patients (occasionally preventing unnecessary surgery)[3].

SUV of 68Ga-DOTA-NOC had, also, a prognostic role in patients with NET. A SUVmax ≥ 19.3 was found to be a significant predictor of survival[4], and a SUVmax > 4 seemed to be significantly associated with progression free survival on multivariate analysis[5].

Then the change in tumor-to-spleen SUV ratio appeared an independent predictor of progression free survival after PRRT)[3].

The use of 18F-fluorodeoxyglucose (18F-FDG) PET/CT in NETs, instead, is emerging to detect tumors with an increased propensity for invasion and metastasis and with overall poorer prognosis, usually less differentiated neuroendocrine tumors with high Ki67 index (neuroendocrine carcinomas) or G1-G2 NETs developed a de-differentiation[6].

In particular Binderup *et al*[7] found that 18F-FDG-PET positivity was stronger than currently used Ki67 index, representing a different sensitivity depending on grading and proliferation rate: 41% for NETs with Ki67 < 2% and 92% when Ki67 at or above 15%.

Garin *et al*[8] conducted a prospective study of patients with metastatic gastrointestinal and thoracic well-differentiated neuroendocrine tumor to compare principally SRS to 18F-FDG PET imaging. 18F-FDG PET gave excellent negative and positive predictive values for early tumor progression of 91% and 93%, respectively. 18F-FDG PET and SRS resulted associated with progression-free survival (both $P = 0.001$) and overall survival ($P = 0.001$ and $P = 0.03$, respectively). At multivariate analysis, only 18F-FDG PET was predictive for progression-free survival[8].

Then, the use of 18F-FDG PET/CT seems to be auspicious to define prognosis of disease, by directing to a potential more aggressive therapeutic approach.

A routinely use of combined 68Ga and 18FDG PET/CT in patients with GEP-NET still represents matter of research. Many studies are investigating whether those new imaging modalities, alone or in combination, are able to provide more precise information about diagnosis, disease extension, restaging, selection of therapy, patients' response to treatment and disease course, taking into account the heterogeneity of NETs.

Naswa *et al*[9] compared these two molecular imaging technique in a recent retrospective analysis and confirmed that Ga-DOTA-NOC PET-CT is superior to 18FDG PET/CT in the detection of lymph node, liver and skeletal metastases - known to be associated with negative prognostic implication on clinical outcomes, moreover underling their potential complementary role to segregate patients into proper therapeutic groups[9].

A similar study by Has Simsek *et al*[10] compared 68Ga-DOTATATE and 18F-FDG PET/CT in 27 GEP-NET

Table 1 Indirect comparison between studies investigating in the role of dual positron emission tomography/computed tomography

Ref.	Type of study	Patients enrolled	Sensitivity in detection of liver metastasis	Sensitivity in detection of lympho-node metastasis	Sensitivity in detection of bone metastasis	Relationship between grading and PET/CT positivity	Impact of dual PET/CT on therapeutic decision
Naswa *et al*[9]	Retrospective study	51 GEP-NETs	No statistical differences between 68Ga and 18F-FDG	68Ga was superior to 18F-FDG ($P < 0.003$)	No statistical differences between 68Ga and 18F-FDG	No data	Dual PET/CT helped in selecting therapies
Has Simsek *et al*[10]	Prospective study	27 GEP-NETs: 10 G1 17 G2	68Ga: 95% 18F-FDG: 40%	68Ga: 95% 18F-FDG: 28%	68Ga: 90% 18F-FDG: 28%	In 74% of patients, 68Ga predominated in patients with lower Ki-67 index, while 18F-FDG in higer ki-67 index GEP-NETs	Dual PET/CT influenced treatment decision in 59% of cases
Partelli *et al*[11]	Retrospective, bi-institutional study	49 P-NETs	18F-FDG: Described 1 false negative	No data	18F-FDG: described 1 false negative	Median Ki-67 for 68Ga positive tumors: 7%. Median Ki-67 for both 68Ga and 18F- FDG positive tumors: 10% ($P = 0.130$)	No significant differences

GEP-NETs: Gastroenteropancreatic neuroendocrine tumors; P-NETs: Pancreatic neuroendocrine tumors; 18F-FDG: 18F-fluorodeoxyglucose; PET/CT: Positron emission tomography/computed tomography.

Table 2 Comparison between 68Ga-DOTATATE and 18F-fluorodeoxyglucose positron emission tomography/computed tomography sensitivity in neuroendocrine neoplasms

Ref.	68Ga-DOTATATE PET/CT	18F-FDG PET/CT
Kayani *et al*[13]	Sensitivity 82%	Sensitivity 66%
Kayani *et al*[14]	Sensitivity 100%	Sensitivity 54%
Conry *et al*[15]	Sensitivity 72%	Sensitivity 78%

18F-FDG PET/CT: 18F-fluorodeoxyglucose positron emission tomography/computed tomography.

patients, investigating the relation between the complementary PET/CT results and histopathological grading; the impact of the combined PET/CT on the therapeutic decision was globally of 59%.

According to these studies, the use of these molecular techniques in combination within clinical practice presents an increasing role in GEP-NETs diagnosis.

Also Partelli *et al*[11] showed that the positivity of both 68Ga PET/CT and 18FDG PET/CT was higher in tumors with median Ki67 index equal to 10% in pancreatic NETs. However this result did not reach statistical significance and the Authors concluded that combined dual tracer PET/CT does not influence the choice of treatment strategy (Table 1).

On the basis of the findings described above, is now time for a new diagnostic approach including the dual tracer PET/CT in GEP-NETs ?

In our opinion, the higher contribution of the combined PET/CT scan can be obtained in the management of intermediate-grade tumors, due to their heterogeneity in response to treatment and prognosis. In particular, in G2 tumor with high Ki-67, near to the 20% cut-off value, the 18F-FDG PET/CT could help to identify patients with a more aggressive disease, who should benefit of conventional chemotherapy.

Furthermore, GEP-NETs' histological grading can change through time. Ki-67 index assessment might show discrepancies between primary tumor and metastatic sites even in more than 35%[12].

There were 3 other studies examining the 68Ga-DOTATATE PET/CT in comparison with 18F-FDG PET/CT in neuroendocrine neoplasms (Table 2). The sensitivity of the 68Ga-DOTATATE PET/CT and 18F-FDG PET/CT described to be 72.2%-100% and 66%-77.8%, respectively[13-15].

In these cases, the real value of combined 68Ga and 18FDG PET CT mainly consists in the possibility to demonstrate areas of different tumor grading or developed de-differentiation and to evaluate post-treatment response assessment, conditioning therapeutic decisions.

CONCLUSION

In conclusion, we believe that combined 68Ga-DOTATATE and 18F-FDG PET/CT at the diagnosis are helpful in the tailored therapeutic approach of GEP-NETs and can overcome the shortcomings of histopathological grading, especially in intermediate-grade GEP-NETs, selecting candidates who would undergo the appropriate mode of treatment, whether SSA analogues, targeted therapies or cytotoxic agents.

In addition, combined dual tracer PET/CT imaging could have an interesting role to redefine the disease after progression, allowing clinicians to choose the most appropriate management after the first line of therapy. Further research is necessary to confirm and validate our hypothesis.

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EDITORIAL

Targeting Enhancer of Zeste Homolog 2 as a promising strategy for cancer treatment

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Abstract

Polycomb group proteins represent a global silencing system involved in development regulation. In specific, they regulate the transition from proliferation to differentiation, contributing to stem-cell maintenance and inhibiting an inappropriate activation of differentiation programs. Enhancer of Zeste Homolog 2 (EZH2) is the catalytic subunit of Polycomb repressive complex 2, which induces transcriptional inhibition through the tri-methylation of histone H3, an epigenetic change associated with gene silencing. EZH2 expression is high in precursor cells while its level decreases in differentiated cells. EZH2 is upregulated in various cancers with high levels associated with metastatic cancer and poor prognosis. Indeed, aberrant expression of EZH2 causes the inhibition of several tumor suppressors and differentiation genes, resulting in an uncontrolled proliferation and tumor formation. This editorial explores the role of Polycomb repressive complex 2 in cancer, focusing in particular on EZH2. The canonical function of EZH2 in gene silencing, the non-canonical activities as the methylation of other proteins and the role in gene transcriptional activation, were summarized. Moreover, mutations of EZH2, responsible for an increased methyltransferase activity in cancer, were recapitulated. Finally, various drugs able to inhibit EZH2 with different mechanism were described, specifically underscoring the effects in several cancers, in order to clarify the role of EZH2 and understand if EZH2 blockade could be a new strategy for developing specific therapies or a way to increase sensitivity of cancer cells to standard therapies.

Key words: Enhancer of Zeste Homolog 2; Polycomb group proteins; Histone methyltransferase; Enhancer of Zeste Homolog 2 inhibitors; Anticancer drugs; Cancer therapy; Epigenetics

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Core tip: Epigenetics modifications are key players in differentiation programs and are frequently altered in cancer. Since chromatin changes can be reversed with specific drugs, in the last years several studies explored

the possibility to target epigenetics alteration as a new strategy for cancer treatment. This editorial focuses on Enhancer of Zeste Homolog 2 (EZH2), the catalytic subunit of Polycomb repressive complex 2 in cancer, analyzing different roles of this protein in various cancers. Several different classes of EZH2 inhibitors are also highlighted, giving distinct thoughtfulness to small molecules that are now under consideration as potential candidates for cancer treatment alone or in combination with other drugs.

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INTRODUCTION

The chromatin structure in eukaryotes depends on covalent modifications that distinguish transcriptionally active and silent regions of the genome and affects the genetic material functionality. Histone post-translational modifications play a key role in the regulation of chromatin structure and gene expression. For instance, acetylation of histone H3 tail, on several lysines, is associated with gene expression, whereas the trimethylation of histone H3 on Lys27 (H3K27me3) or trimethylation of histone H3 on Lys9 is a mark of silent chromatin[1,2].

Several enzymes dynamically deposit or remove specific marks on the chromatin regulating gene expression and drive numerous biological processes. For instance, the differentiation of distinctive cell types in an organism is rigorously related to the establishment, while the maintenance of the correct epigenetic status and alteration of chromatin structure is frequently associated with different disease disorders, including cancer[1-3].

Numerous cancer types are associated with specific patterns of histones H3 and H4 modification and several epigenetic patterns enable to distinguish disease subtypes[4,5].

Epigenetic changes are reversible and specific drugs are capable to recover the correct chromatin status of a normal cell and, in turn, promote differentiation, cellular senescence or apoptosis. Therefore, the inhibition of epigenetic enzyme could be a good strategy for cancer treatment[6].

This editorial focuses on the role of the Enhancer of Zeste Homolog 2 (EZH2), the catalytic subunit of Polycomb repressive complex 2, which catalyzes the addition of methyl groups to lysine 27 of the N-tail of histone H3. This enzyme is responsible for the silencing of various genes involved in several processes as cell cycle progression, apoptosis and differentiation and it is frequently deregulated in cancer[7].

POLYCOMB GROUP PROTEINS

Polycomb group proteins (PcG) are a family of proteins highly conserved among eukaryotes, involved in development, stem cell biology and cancer[8-13]. They are regulators of the epigenetic gene silencing required in many processes, like mammalian X-chromosome inactivation and imprinting[14,15]. Moreover, the PcG-dependent epigenetic silencing controls the timely expression of genes involved in stem cell fate and lineage commitment[9-11,16], ensuring the establishment and maintenance of the correct transcriptome during development[17].

In mammals, PcG proteins form two main complexes: Polycomb-repressive complex 1 (PRC1) and 2 (PRC2)[18-22]. PRC1 is formed by BMI1, RING1A/B, CBX, and PHC subunits[23]. As of now, the mechanism of PRC1 dependent gene silencing is not completely clear. RING1A/B is an ubiquitin E3 ligases which catalyze the monoubiquitylation of histone H2A at lysine 119 (H2AK119ub1), an histone post translational modification associated with gene silencing[13,24]. Nevertheless, transcriptional silencing has been detected also in absence of ubiquitylation[18]. Other studies *in vitro* demonstrated that PRC1 prevents the SWI/SNF-dependent chromatin remodeling, competing for the binding with target nucleosomes. Indeed PRC1 complex is able to bind three nucleosomes, resulting in the chromatin compaction[25]. PRC1 affects also the transcription while inhibiting a correct assembly of RNA Polymerase Ⅱ preinitiation complex[26].

PRC2 is composed by EZH2 or EZH1, EED, SUZ12 and RbAp46. EZH2 is the most studied catalytic subunit of PRC2 and contains the SET domain responsible for the histone methyltransferase activity on lysine-27 of histone H3[19-22]. SUZ12 and EED stimulates H3K27 histone methyltransferas increasing more than 1000 fold the catalytic activity of EZH2 alone whereas RbAp46 is responsible for the histone binding[13]. EZH1 is a homologue of EZH2, which originates an alternative PRC2 complex, however, data about this protein are sometimes contrasting[27]. It has been demonstrated that EZH1, in embryonic stem (ES) cells, is able to trimethylates H3K27, contributing to the silencing of a subset of developmental genes. Its activity partially complements EZH2 role in the maintenance of the ES cells pluripotency[28]. On the other hand, other findings showed that EZH1 and EZH2 are recruited at the same set of target genes but EZH1 is ubiquitously expressed, whereas EZH2 expression is associated with proliferating cells[29]. There is also evidence that EZH1, compared to EZH2, exhibits a weaker histone H3 methyltransferase activity, while its depletion does not affect the global H3K27me2/3 levels; despite this, it is able to efficiently compact the chromatin through a mechanism independent of the presence of the methyltransferase cofactor S-Adenosyl methionine (SAM)[29].

Remarkably, during muscle differentiation several evidences showed a role of EZH1 in transcriptional activation. Indeed, EZH1 occupies transcriptionally active

genes marked with H3K4me3 and interacts with RNA Pol Ⅱ, promoting transcriptional elongation[30]. It has also been shown that the interchange between PRC2-EZH2 and PRC2-EZH1 complexes controls the correct timing of transcriptional activation of muscle specific genes such as myogenin[31]. A similar mechanism has been discovered at the promoter of *PSD-95* gene during the development of hippocampal neurons[32].

The most common PcG-dependent gene silencing mechanism is the cooperation between PRC1 and PRC2; in fact, the establishment of H3K27me3 by PRC2 complex induces the recruitment of PRC1 by binding the chromodomain of the PHC subunits[21,33]. Once recruited, PRC1 brings transcriptional repression of target genes through the mechanisms described above.

PRC2 is also able to cooperate with other epigenetic silencing enzymes, for instance, it acts upstream of DNA methyltransferases (DNMTs) in order to induce a more stable transcriptional silencing characterized by the methylation of di-nucleotides CG. Although the mechanism is not completely clarified, essential proofs supported that DNMTs recruitment depends on the presence of the active form of EZH2, suggesting a context-dependent crosstalk between EZH2 and DNMTs. However, much remains unknown about this interaction, particularly, it is not clear if DNMTs bind directly EZH2 or H3K27me3 or if other factors are involved[34-37].

PRC2 is also able to associate with histone deacetylases, reinforcing transcriptional repression of target genes[6,19-22,38-40].

Other than transcriptional repression, EZH2 has a role in the promotion of gene activation[41-43], this mechanism has been discovered in breast and prostate cancer and it is described below.

EZH2 AND CANCER

Epigenetic modifications have a key role in the normal mammalian development and are required in all somatic cells. In ES cells and in precursor, PRC2 contributes to silence the principal genes involved in the differentiation promotion, preventing the premature activation of the differentiation processes and maintaining their pluripotency[9-11]. In addition, the main targets of EZH2 are genes involved in cell cycle regulation, as for instance Ink4b/Arf/Ink4a*locus*; its inhibition impedes cell cycle arrest and contributes to preserve the proliferative potential[9,44-50].

Because of its importance in various aspects of cellular development and tissue differentiation, EZH2 expression is strictly regulated. For instance high levels are detected in stem cells and undifferentiated cell progenitors, while its expression decreases during the differentiation process[6,7,51]. EZH2 activity could be regulated, other than transcriptionally, also by different mechanisms as several miRNA and post translational modifications (reviewed in[7]). Furthermore, the recruitment of PRC2 complex at target promoters covers a very important role: PRC2 binds DNA with low affinity and recruiting factors are supposed to be necessary to drive the complex to target genes[52]. This hypothesis could also explain why EZH2 is recruited, in different tissue, at different set of genes.

Epigenetic abnormalities result in an inappropriate gene expression that drives to an altered cellular physiology in several diseases. The first evidences of the involvement of EZH2 in cancer were found in breast and prostate[39,53] but a number of human tumors are nowadays associated with EZH2 alteration[7]. Frequently, EZH2 expression is correlated with metastatic cancer cells and poor prognosis[6,7,51].

The role of EZH2 in cancer could be linked to its activity in self-renewal promotion and in the maintenance of undifferentiated state of cells.

EZH2 target genes are generally crucial regulators of the balance between cellular differentiation and cell cycle progression, and their deregulation is able to promote cancer progression[6]. For instance, EZH2-dependent silencing of Ink4b/Arf/Ink4a*locus* leads to the downregulation of p16, p15 and p14, resulting in uncontrolled proliferation and inhibition of apoptosis[54,55]. Furthermore, EZH2 inhibits other tumor suppressor genes such as p21, PTEN, DAB2IP, and Bim[56-60].

PRC2 complex inhibits also several miRNA involved in cell cycle regulation, for instance mir-31 in melanoma[61], miR-139-5p, miR-125b, miR-101, let-7c, and miR-200b in metastatic liver cancers, promoting cell motility and metastasis[62].

The other class of EZH2 target genes is composed by differentiation-related factors. Genome wide assays showed that factors as Gata, Sox, Fox, Pou, Pax, components of Wnt, TGF-β, Notch, FGF and retinoic acid pathways are silenced by EZH2. The activity of EZH2 inhibits differentiation and promotes carcinogenesis[8-12]. In embryonal rhabdomyosarcoma, for example, high levels of EZH2 inhibit the activation of muscle specific genes and its depletion promotes muscle specific genes transcription and a partial recovery of the muscle differentiation program[63].

Activity of EZH2 independent of H3K27me3

EZH2 activity is not restricted to H3K27 trimethylation, in fact several studies reported that it is also able to methylate other proteins[64-68].

EZH2 and other PRC2 subunits have been found in the cytoplasm, where they control actin polymerization and cell proliferation of T-lymphocytes and fibroblasts[64]. Aberrant EZH2 overexpression has been detected in both nuclei and cytoplasm of human prostate cancer cells. The cytoplasmic fraction, responsible for the reduction of the pool of insoluble F-actin, influences cell adhesion and migration, therefore contributes to invasiveness and metastatic ability of tumor cells[65].

Previous studies showed that EZH2 is able also to methylate other histones as the histone H1 at lysine 26 when associated with a different isoform of EED[66]. Recently it has been discovered that EZH2 is also able to methylate GATA4, inhibiting its transcriptional

activity in heart[67]. This is the first evidence that PRC2 influences the function of transcription factors involved in the developmental processes not only modulating their expression levels but also regulating their post-translational modifications.

This evidence is also supported by another study showing that in breast cancer, EZH2, in association with other PRC2 components, plays an essential role in the regulation of p38 pathway. p38 mitogen-activated protein kinase signaling pathway is involved in the promotion of epithelial-to-mesenchymal transition, cell invasion and motility. EZH2 is able to bind the phosphorylated and activated p38 counterpart, increasing its downstream signaling. This study highlighted a novel fundamental role of EZH2 in breast cancer. EZH2 overexpression enhances the levels of phospho-p38 while EZH2 knockdown induces a mesenchymal-to-epithelial transition and decreases cell motility. Clinical breast cancer specimens reveal that EZH2 is overexpressed, and co-expressed with phospho-p38 in about two-third of cases, while EZH2 inhibition results in a reduction of spontaneous breast cancer metastasis *in vivo*[68].

Finally, EZH2 is also able to promote transcriptional activation interacting with different transcription factors[41,42,69]. In breast cancer, for instance, EZH2 interacts with ERα, Wnt signaling components TCF, and β-catenin at the promoter of target genes, enhancing transcriptional activation of c-Myc and cyclin D1 genes; this mechanism is independent of the methyltransferase activity[41]. Still in breast cancer, EZH2, independently from other PRC2 subunits, is also able to activate NF-κB signaling, interacting with its components Rel A and Rel B, and inducing the activation of genes implicated in oncogenesis such as IL6 and TNF[42]. Similarly, in castration-resistant prostate cancer the oncogenic functions of EZH2 are not dependent on its transcriptional silencing activity but on the transcriptional activation of a subset of genes. EZH2 does not bind these genes by recruiting other PRC2 components, but rather through the association with the androgen receptor (AR), which in turn, after EZH2 dependent methylation, leads to increase the transcriptional activation of these genes. It has been proposed that the methylation of AR is dependent on AKT that phosphorylates EZH2 at serine 21, promoting the binding with AR[43,70]. Interestingly, it has been shown that AKT-dependent phosphorylation decreases the affinity of EZH2 with histone H3, resulting in a reduction of the H3K27 methylation[71]; this event can promote the binding of EZH2 with AR and the role of the methyltrasferase as transcriptional activator.

Finally, EZH2 is also able to promote cyclin A transcription[72]. Cyclin A gene transcription is inhibited by pRb2/p130, a member of Rb family with an onco-suppressor role[73]. pRb2/p130 is able to recruits HDAC1 at cyclin A gene inducing gene silencing and G1 arrest[74]. EZH2 competes with HDAC1 for its binding with pRb2/p130, disrupting the occupancy of both proteins on cyclin A promoter and inducing gene activation and cell cycle progression[72,75].

Mutations

The activity of EZH2 in cancer is also influenced by mutations. In diffuse large B-cell lymphoma, an heterozygous mutation of EZH2 at Tyrosine 641, (Y641), which affects its catalytic domain, was initially associated with a loss of functions, but other studies showed that this mutation results in a limited capacity to carry out H3K27 monomethylation but augmented ability for di- and tri-methylation. In these tumors, wild type Tyrosine can be substituted with different amino acids (Phenylalanine Y641F, Histidine Y641H, Asparagine Y641N and Serine Y641S) and mutants cooperate with wild type protein to increase EZH2 activity[76-78]. Another mutation, called A677G, has been discovered in lymphoma cell lines and primary tumors. This mutation, that replaces Alanine with Glycine, as the mutation in Y641, increases the trymethylation of H3K27 but, on the contrary, displays similar affinity for all three substrates: Unmethylated, mono-edy-methylated H3K27[79]. A687V is another gain-of-function (GOF) mutation discovered in lymphoma, it substitutes Alanine 687 with Valine, and it is similar to other mutations since enhances EZH2 ability to perform dimethylations, whereas the ability of catalyzing trimethylations remains the same[80]. Parallel mutations have been discovered also in melanoma, where they contribute to the promotion of tumor growth[81,82].

EZH2 INHIBITORS

The peculiar role of PRC2 in the promotion of tumor growth, deregulation of apoptosis, and alteration of proper proliferation and differentiation programs, suggests that EZH2 can be a good target for therapy in cancer. Several inhibitors of EZH2 have been designed and they can be classified based on the different mechanism of inhibition (Figure 1 and Table 1).

Regulators of EZH2 levels

3-Deazaneplanocin A (DZNep) is a S-adenosyl-l-homocysteine (AdoHcy) hydrolase inhibitor able to deplete EZH2 and to reduce H3K27me3 at PRC2 target genes. The mechanism is not completely understood but it seems that an absent or reduced activity of AdoHcy hydrolase and the successive AdoHcy accumulation causes an inhibition of SAM-dependent methyltransferases. DZNep treatment reduces levels of EZH2 and other PRC2 components thought a proteosome-mediated degradation, while RNA transcription does not change[83]. DZNep treatment induces apoptosis in breast, colorectal, prostate cancer and hepatocellular carcinoma whereas apparently, it is able to discriminate between cancerous and non-cancerous cell lines; consequently, it does not induce cell death in breast and lung epithelial cells, primary lung fibroblast, and human skin fibroblast cells[83]. Other studies, focusing on several non-small cell lung cancer (NSCLC) cell lines, showed that DZNep induces p27 accumulation, cell cycle arrest and apoptosis, while immortalized bronchial epithelial and fibroblast cell lines are less sensitive to apoptosis[84]. Interestingly, studies

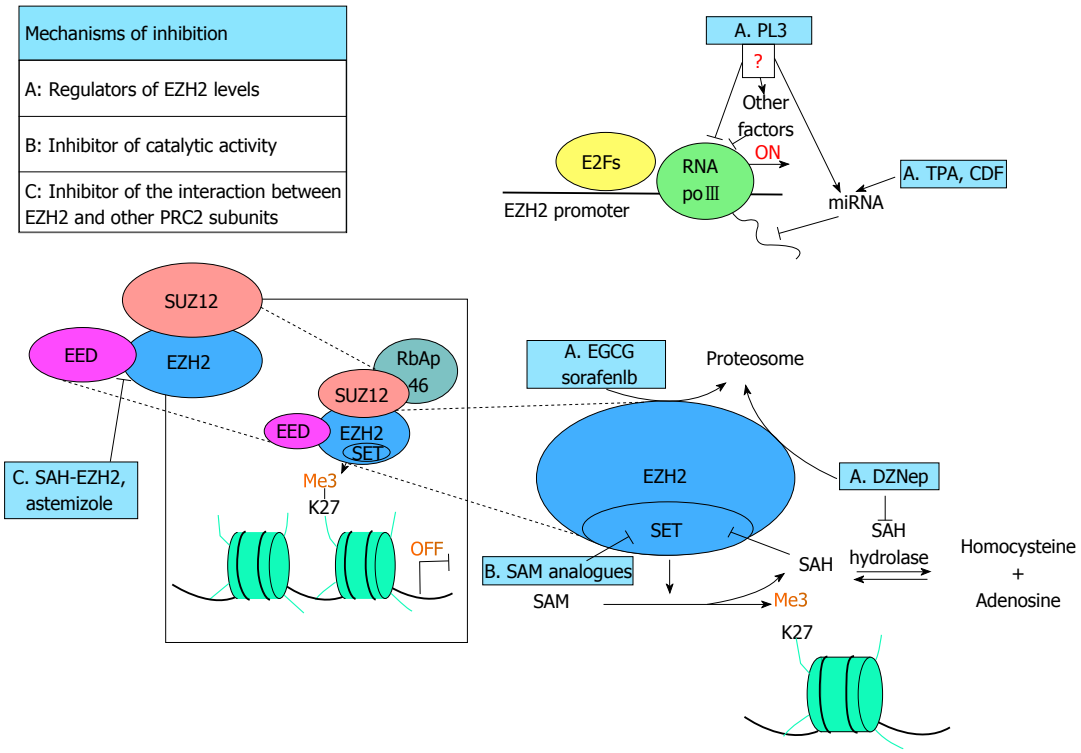


Figure 1 Schematic representation of polycomb-repressive complex 2 inhibition mechanisms. The image enclosed in the square represent the PRC2 active complex, able to methylate the lysine 27 of the histone H3 (H3K27me3), suppressing the transcription of target genes. Dashed lines pointed a part of the complex or the methylation pathway. The main PRC2 inhibitors (blue square) are included in the figure and labeled with the letters A, B or C depending on the mechanism of action (drugs marked with A are responsible for EZH2 depletion, with B are inhibitors of the catalytic activity of EZH2 and with C are able to destroy PRC2 complex. In group A PL3 is able to repress EZH2 expression but is not clear if the mechanism is direct or involves other factors or miRNAs. TPA and CDF reduce levels of EZH2 inducing the expression of some miRNA. EGCG, Sorafenib and DZNep promote proteasome-dependent EZH2 degradation. On the other hand DZNep, inhibiting SAH-Hydrolase, leads to an accumulation of SAH that, in turn, hampers try-methylation of H3K27me3 inhibiting EZH2 SET domain. Group B, SAM analogues compete with SAM for the binding with the substrate pocket blocking SET domain activity. Finally in the group C both the peptide SAH-EZH2 and Astemizole destroy the binding between EZH2 and EED event required for the activity of PRC2 complex. SAM: S-adenosylmethionine; SAH: S-adenosylhomocysteine; PRC2: Polycomb-repressive complex 2; EZH2: Enhancer of Zeste Homolog 2; EGCG: (-)-epigallocatechin-3-gallate; DZNep: 3-Deazaneplanocin A; SAH-EZH2: Stabilized alpha-helix of EZH2.

performed in several gastric cancer cell lines and in primary human gastric cancer cells showed that the effects of DZNep are related to p53 status, and cells with p53 wild type are more sensible to the treatment. p53 genomic status could be a potential predictive marker of DZNep response in this specific cell type[85].

Effects on the EZH2 protein levels have been detected also during the treatment with (-)-epigallocatechin-3-gallate (EGCG), a green tea-derived bioactive polyphenol. In skin cancer cells, treatment with EGCG induces a global reduction of H3K27me3 and reduces the levels of two PcG proteins: Bmi-1 and EZH2. Reduced levels of these PcG proteins are associated with decreased expression of several cyclin dependent kinases (CDKs) and cyclins (CDK1, CDK2, CDK4, cyclin D1, cyclin E, cyclin A and cyclin B1) and increased levels of p21 and p27 that, in turn, induce cell cycle arrest. Apoptosis is also stimulated by the treatment, indeed levels of caspase 3, 8 and 9 and PARP cleaved and Bax are higher compared with not treated cells whereas level of Bcl-xL expression decreased. Bmi-1 over expression reverses these EGCG-dependent changes[86] indicating that effects are dependent on PcG proteins. Similarly to DZNep, EGCG reduces the levels of EZH2 and Bmi-1 through a mechanism proteasome-dependent but, in skin cancer cells, the combination of EGCG with DZNep is more effective than each single agent[87]; indicating that the two molecules can also cause responses in different pathways.

The functions of PRC2 are tissue-specific and its expression is strictly regulated during normal development. The main regulators of PRC2 expression are proteins of pRb/E2F pathway; it has been demonstrated that E2F factors are required for the expression of EZH2 and EED in mouse embryonic fibroblasts and ectopic expression of pRb and p16, that are involved in E2F target gene repression, induces transcriptional repression of PRC2 subunits; silencing of pRb, on the contrary, increases their transcription[88-90]. Also c-Myc binds EZH2 promoter and induces transcription through the acetylation of histones H3 and H4[91]. Furthermore, several miRNA are also involved in EZH2 regulation[92].

It has been discovered that a natural compound isolated from the bark of Polyalthia longifolia, 16-Hydroxy-cleroda-3,13-dien-15,16-olide (PL3), induces apoptosis in leukemia cells[93]. The effects of this compound, already known for its anti-inflammatory activity and for its potential cytotoxicity in breast cancer cells and

Table 1 Enhancer of Zeste Homolog 2 inhibitors classified for Enhancer of Zeste Homolog 2 inhibition mechanism

Mechanism of inhibition	Drug	Level of development	Ref.
Regulators of EZH2 levels	DZNep		[83-85,87]
	EGCG		[86,87]
	PL3		[93]
	CDF		[94]
	TPA		[98]
	Sorafenib		[99]
SAM analogues	EPZ005687		[100]
	EI1		[101]
	GSK126		[102,109]
	GSK343		[103]
	GSK926		[103]
	EPZ-6438	Phase 2 (ClinicalTrials.gov Identifier: NCT01897571)	[104,110]
	UNC199		[105,109]
	Tanshindiols		[106]
	5-Methoxyquinoline Derivatives		[107]
	Tetramethylpiperidinyl Benzamides		[108]
Inhibition of the interaction between EZH2	SAH-EZH2		[111]
with other PRC2 subunits	Astemizole		[112]
Unknown	CPI-1205	Phase1 (ClinicalTrials.gov Identifier: NCT02395601)	
	GSK2816126	Phase 1 (ClinicalTrials.gov Identifier: NCT02082977	

PRC2: Polycomb-repressive complex 2; EZH2: Enhancer of Zeste Homolog 2; PL3: 16-Hydroxycleroda-3,13-dien-15,16-olide; CDF: Diflourinated-curcumin; SAM: S-Adenosyl methionine; DZNep: 3-Deazaneplanocin A; EGCG: (-)-epigallocatechin-3-gallate; TPA: 12-O-tetradecanoylphorbol-13-acetate; SAH-EZH2: Stabillized alpha-helix of EZH2.

hepatocellular carcinoma cells, seem dependent on the repression of EZH2 and Suz12 expression. PL3 treatment reactivates the tumor suppressor targeted by PRC2 while inducing apoptosis[93]. The concentration required for pro-apoptotic effects in cancer cells is high, therefore additional studies focused in designing novel PL3-derived compound are required to improve the pharmacological properties and decrease the side effects of the molecules. Alternatively, the compound can be used in combination with other drugs to increase the effects at lower concentration[93].

Another natural compound called diflourinated-curcumin (CDF), a synthetic derivative of curcumin characterized by natural antitumor activity, is able to decrease EZH2 expression in pancreatic cancer cells. This molecule exhibits a particular mechanism of action, which involves the upregulation of a set of microRNAs (miRNA) such as let-7a,b,c,d, miR-26a, miR-101, miR-146a, and miR-200b,c, typically downregulated in pancreatic cancer[94]. Some of these miRNA are, at the same time, target and regulator of EZH2[7]. Although it has been confirmed that the re-expression of miR-101 is able to significantly decrease the expression of EZH2[95,96], however the peculiar ability of CDF to inhibit pancreatic tumor growth remains controversial[94]. Numerous evidences underscored that miR-101 plays a key role in the regulation of EZH2 in a wide panel of cancer and that miR-101 is linked to EZH2 through a negative mutual feedback loop. Indeed re-expression of miR-101 downregulates EZH2 expression, while the inactivation of EZH2 leads to the upregulation of miR-101[94]. In hepatocellular carcinoma, EZH2 is a direct target of miR-101, the expression level of this latter is negatively correlated with the protein level of EZH2; in fact, miR-101 is frequently underexpressed. MiR-101 overexpression downregulates EZH2, repressing proliferation, invasion, colony formation and cell cycle progression *in vitro,* while suppresses tumorigenicity *in vivo*[97]. miR-101 is also able to increase sensitivity to doxorubicin or fluorouracil, improving the activity of chemotherapeutic drugs in liver cancer cells[97]. Remarkably, in hepatocellular carcinoma, treatment with 12-O-tetradecanoylphorbol-13-acetate (TPA) promotes miR-101 expression reducing levels of EZH2, EED and H3K27me3. In liver cancer cells, TPA induces G0/G1 cell cycle arrest with a mechanism mediated by PKCα and ERK pathways[98].

Finally, Sorafenib, a multikinase inhibitor used for the treatment of advanced-stage hepatocellular carcinoma, reduces level of EZH2, accelerating the proteasome-mediated EZH2 degradation. Sorafenib induces cell growth arrest and apoptosis and its effects can be reversed by the overexpression of EZH2. The combination of Sorafenib with DZNep has synergic effects in cell growth arrest and apoptosis and these properties could be evaluated in the future as a new combination therapy for the treatment of advanced hepatocellular carcinoma[99].

SAM analogues

The development of indirect methods inhibiting EZH2 protein levels could be recognized as a promising strategy for therapy, although it is crucial to consider the potential involvement of other pathways that may lead to a decrease of the specificity and an increase of the side effects.

The specific targeting of EZH2 catalytic domain could prove to be a powerful tool in gene based therapy, which could solve the problems related to the indirect

mechanisms. Specific molecules, competitors of S-adenosyl-methionine (SAM), have been developed and are able to inhibit methyltransferase activity of EZH2 competing for the binding with the active site of EZH2, without affecting its expression levels.

One of these compounds called EPZ005687 has been designed in 2012 and it specifically inhibits H3K27me3 in lymphoma cells. Specificity for EZH2 is greater than 500-fold compared to a panel of 15 other methyltransferases analyzed, and 50-fold compared to EZH1. Particularly, this inhibitor is able to induce specifically G1 arrest and apoptosis in lymphoma cell lines, carrying point mutations of Y641 and A677 residues within the catalytic domain of EZH2, but it establishes minimal effects on the cell lines containing wild-type EZH2[100].

In 2012, Novartis developed an EZH2 inhibitor, EI1, which showed a strong selectivity for EZH2 (more than 10000-fold compared to other methyltransferases and about 90-fold compared to EZH1). This inhibitor decreases the global level of H3K27me3 in both EZH2 wild type and mutant lymphoma cell lines; however, it triggers cell cycle arrest and apoptosis specifically in mutant cells[101].

GSK126, another EZH2 inhibitor discovered in 2012, is more than 1000-fold selective for EZH2 compared to 20 other methyltransferases and more than 150-fold compared to EZH1. This inhibitor has a strong effect in EZH2 mutated diffuse large B-cell lymphoma cell lines and also inhibits the growth of EZH2 mutant large B-cell lymphoma in xenografts models[102].

Other SAM-competitive inhibitors have been proposed in the last years and their activity has been tested in several tumors, for instance GSK343 and GSK926[103], EPZ-6438[104], UNC199[105], Tanshindiols[106], 5-Methoxyquinoline Derivatives[107], Tetramethylpiperidinyl Benzamides[108]. UNC199 is the first orally bio-available inhibitor characterized by having a strong activity *in vitro*. Although it is an analogue of GSK126, it is less selective for EZH1[105,109]. Remarkably, EPZ-6438 is recently going through clinical testing for Non-Hodgkin Lymphomas patients[110].

Inhibition of the interaction between EZH2 with other PRC2 subunits

Interactions with other components as EED or Suz12 are necessary for the canonical activity of EZH2[6]. Another strategy for EZH2 inhibition is to block the interactions between the methyltransferase and other PRC2 subunits.

A peptide called SAH-EZH2 has been designed starting from the alpha-helical domain of EZH2 (aa 40-68), a 27-mer-peptide domain responsible for the binding between EZH2 and EED. This peptide, short enough to cross the cellular membranes, is able to disrupt the EED/EZH2 complex and to inhibit H3K27me3 in a dose-dependent manner; moreover, it decreases levels of EZH2 possibly impairing its protein stability. MLL-AF9 leukemia cells treated with SAH-EZH2 underwent cell cycle arrest and monocyte/macrophage differentiation, while were not driven to apoptosis[111].

Peptides or peptidomimetic inhibitors are generally considered metabolically unstable and have poor bioavailability to be suitable as therapeutic drugs. Recently, it has been identified that Astemizole, a drug previously used in the treatment of seasonal allergic rhinitis, is an inhibitor of the EED/EZH2 protein-protein interaction. Astemizole competes with EZH2 for the binding with EED, destabilizing PRC2 complex and inducing cell cycle arrest in leukemia cells[112]. Interestingly, the combination treatment of SAH-EZH2 or astemizole and various SAM analogues produced a significant synergistic effect on lymphoma cells[111,112], indicating that the suppression of catalytic activity and the disruption of PRC2 complex could influence different pathways and a combinatorial drug therapy can be a more effective therapeutic strategy.

Combination of EZH2 inhibitor with other drugs

Chromatin alterations are considered excellent candidates to explain how different factors may increase the risk of cancer, for that reason they represent an important aspect of tumor biology and may constitute good targets for future epigenetic-based therapies. For instance, several histone deacetylase and DNA methyltransferase inhibitors are already under evaluation as potential anticancer drugs, and several clinical trials are underway[113,114]. Various studies are exploring the possibility to combine the effects of EZH2 inhibitors with other epigenetic drugs in order to develop new strategies for cancer therapy. The combination of 5-AZA-2'-deoxycytidine (5-AZA-CdR), a DNA methyltranserase inhibitor with DZNep to treat human and murine leukemia cells, revealed similar synergic effects accompanied by a meaningful reduction in clonogenicity. Additionally, microarray analysis showed that the combination therapy increases the expression of more than 150 genes, including *CDKN1A* and *FBXO32*[115]. Histone deacetylases are also frequently altered in cancer and can contribute to tumor progression. A synergic interaction was observed with a three-drug combination of trichostatin-A (TSA: A histone deacetylase inhibitor) plus DZNep and 5-AZA-CdR in acute myeloid leukemia, showing a significant activation of several tumor suppressor genes and inhibition of cell growth and cell survival[116].

EZH2 inhibitors can be also used in combination with classical chemotherapeutic drugs to solve problems related to side effects (combined treatment consent to reduce concentration of drugs) or resistance of cancer cells often associated to the treatments with the only chemotherapeutic agents. The discovery of heterozygous EZH2 GOF mutants, identified in non-Hodgkin Lymphomas, which proved to be more sensitive to EZH2 inhibitors, leaded to the hypothesis that combination of classical treatment with PRC2 inhibitors could improve the efficacy of the cancer therapy. Standard Non-Hodgkin Lymphoma therapy is a combination of several drugs, called CHOP (Cyclophosphamide, Hydroxyldaunorubicin, Oncovin, and Prednisone). Studies *in vitro* and *in vivo* demonstrated that the combination of EPZ-6438 with

CHOP increases anti-proliferative benefits compared to treatment with the EZH2 inhibitor alone, and this effect is thought to be mediated by glucocorticoid receptor agonists such as Prednisolone, the active metabolite of Prednisone[110].

Moreover, other studies investigated the inhibition of EZH2 in combination with conventional therapies in prostate cancer. Etoposide, an inhibitor of topoisomerase Ⅱα (Top2a), is used in combination with other drugs in the standard therapy for castrate resistant prostate cancer. Levels of Top2a are higher in aggressive cancer and in these patients EZH2 expression correlates positively with Top2a expression. It has been demonstrated that the combined inhibition of EZH2 and Top2a, increases anti-tumor response both *in vitro* and *in vivo* models of prostate cancer, indicating that combination therapy can be a new strategy for aggressive prostate cancer[117].

Finally, various studies explored the interplay between Myc and EZH2, both involved in cancer. In Myc-driven prostate cancer, EZH2 directly suppresses interferon-γ receptor 1 (IFNGR1) in a Myc-dependent manner. EZH2 depletion restores the expression of IFNGR1, increasing the sensitivity of these cells to interferon-γ with consequent activation of IFN-JAK-STAT1 tumor-suppressor signaling that leads to apoptosis. Activity of EZH2 in Myc-driven tumors is strictly correlated to Myc amplification. Myc knockdown reduces levels of EZH2 and H3K27me3 at IFNGR1 promoter only in Myc-amplified prostate cell lines; while in Myc-independent tumor growth, IFNGR1 inhibition seems to be related on DNA hypermethylation[118]. This evidence suggested that combination of EZH2 inhibition with interferon-γ could be a specific strategy for Myc-driven prostate tumors and can help to improve the outcome of patients.

Numerous evidences confirm that EZH2 activity is strongly correlated with Myc. Definitely, Myc induce the expression of EZH2, downregulating miR-26a/b[119]; inhibits AKT-dependent phosphorylation of EZH2 at Serine 21 increasing its H3K27me3 activity[120]. Remarkably, Myc recruits EZH2 to miR-26a promoter and cooperatively suppresses miR-26a expression with consequent EZH2 upregulation. EZH2 in turn, inhibits miR-494, a repressor of Myc. Consequently, Myc and EZH2 generate a positive feedback loop to assure persistent high protein levels of both proteins[121]. In several hematological malignancies, Myc expression is inhibited by a BET bromodomain inhibitor, JQ1[122,123]. In B-cell Lymphoma cells, the pharmacological inhibition of EZH2 with DZNep in combination with JQ1 has a synergic effect in the suppression of cell growth and clonogenicity with a mechanism mediated by miR26a re-expression. The combined inhibition of Myc and EZH2 expression levels could result in an effective therapeutic strategy to successively suppress tumor growth in aggressive B-cell Lymphoma[124].

Synthetic lethality

Synthetic lethality occurs when two mutations result in cell death when acting in combination, but when acting separately they do not have any effect on cell viability[125]. Based on the distinctive genomic features of each tumor, current cancer research focus on finding targets that are able to kill exclusively cancer cells. Synthetic lethality screenings such as Synthetic genetic array, synthetic lethality by microarray, and genetic interaction mapping, are high-throughput methods to identify tumor mutations, or altered pathways that can lead to the synthetic lethality. Consequently, the presence of one of these mutations in cancer cells, but not in normal tissues, can make them ready to be selectively killed by mimicking the effect of the second alteration with targeted therapy[126]. Inhibition of EZH2 is under evaluation as a strategy to induce synthetic lethality. Recent findings have underlined that inactivating mutations in the gene encoding the AT-rich interacting domain containing protein 1A (ARID1A), a SWI/SNF complex subunit, are frequently detected in a large selection of cancers[127,128]. The first evidence, capable of proving a synthetic lethality between EZH2 inhibition and ARID1A mutations, has been reported in ARID1A-deficient ovarian clear cell carcinomas (OCCCs), an aggressive human cancer that commonly develops resistance to treatments[129,130]. ARID1A mutated OCCCs treated with EZH2 inhibitor GSK126 exhibited significantly cell growth arrest and apoptosis, however ARID1A wild type cells are not sensitive to the treatment, even if the reduction of H3K27me3 is comparable[131]. The same data set demonstrated that gene PIK3IP1, an inhibitor of PI3K–AKT signaling, is a direct target of both ARID1A and EZH2. These results suggested a specific implication of the PI3K/AKT signaling in ARID1A-mutated cells. ARID1A-deficient tumors appeared to be addicted to EZH2 activity, and the pharmacological inhibition of EZH2 promoted the upregulation of PIK3IP1 and contributed to the synthetic lethality through the inhibition of the PI3K–AKT pathway. Remarkably, the EZH2 inhibitor GSK126 induced regression of ARID1A-mutated ovarian tumors also *in vivo*[131]. Similarly, in rhabdoid tumors pharmacological inhibition of EZH2 induced apoptosis in SMARD1 (another subunits of SWI/SNF) mutated cells[104].

CONCLUSION

In the last years many researchers focused their studies on the design of new EZH2 inhibitors and in trying to clarify if EZH2 modulation can be a specific therapeutic strategy for cancer treatment, alone or in combination with other standard drugs or other epigenetics inhibitors. Three EZH2 inhibitors are currently under observation in clinical trials: CPI-1205 in B-Cell Lymphomas (ClinicalTrials.gov Identifier: NCT02395601) E7438 (previously called EPZ-6438) in Advanced Solid Tumors or B Cell Lymphomas (ClinicalTrials.gov Identifier: NCT01897571) and GSK2816126 in Relapsed/Refractory Diffuse Large B Cell and Transformed Follicular Lymphomas (ClinicalTrials.gov Identifier: NCT02082977). These studies will help to clarify if EZH2 inhibition is

a good strategy for cancer treatment. Indeed the importance of polycomb protein during development and differentiation processes can lead to side effect. Data obtained *in vitro* in breast cancer showed that EZH2 specifically induces cell death in cancer cells but not in normal cells[83]. Moreover, *in vivo* studies showed that, treatments with some EZH2 inhibitors alone or in combination with other chemotherapeutic agents are well tolerated by mice resulting in minimal toxicity or modest weight loss of 10%[105,117,118].

Nevertheless, the studies described above demonstrated that EZH2 activity can behave differently in distinguishing neoplasms, and a singular inhibitor can be effective in certain kind of cancer but not in others. For instance, it is well known that Top2a inhibitor Etoposide is actually effective in a minority of patients with non-small-cell lung cancer. Recent findings validated that in this particular type of cancer, EZH2 inhibition drives differential effects in response to the Top2a inhibitors *in vitro* and *in vivo*. BRG1 (LOF) and EGFR (GOF) mutant cancer cells in response to EZH2 inhibition increased Top2a inhibitor sensitivity, thus shrinking and fragmenting into apoptotic bodies. On the contrary, EGFR and BRG1 wild-type NSCLC cells responded to EZH2 inhibition with upregulation of BRG1 and ultimately become more resistant to the Top2a inhibitor[132]. Furthermore, in rhabdomyosarcomas, treatment with EZH2 inhibitors has different effects in embryonic or alveolar subtypes where induces respectively differentiation or apoptosis[133,134].

Another interesting example concerning the selective inhibition of EZH2 was displayed in prostate cancer. It has been shown that in Myc-driven prostate cancer, EZH2 exhibited a significantly Myc-dependent downregulation of IFNGR1. The pharmacological depletion of EZH2 by the inhibitor DZNep restored the expression of IFNGR1 and, when treated in combination of interferon-γ, as discussed previously, provided a remarkable synergic antitumor effect with interferon-γ. Conversely, EZH2 catalytic inhibitors, although they efficiently reduced H3K27me3, failed to mimic EZH2 depletion. Therefore, patients with advanced prostate cancer driven by Myc could have benefits from a therapeutic depletion of EZH2, indicating that the ability of EZH2 to increase the sensitivity of cancer cells to interferon-γ is independent by its catalytic activity[118].

An important point for consideration, in order to understand if a drug is suitable for therapy, concerns taking into account its penetration into the organs. A panel of five EZH2 inhibitors was recently tested to evaluate the affinity with P-glycoprotein (P-gp/ABCB1) and breast cancer resistance protein (BCRP/ABCG2) two members of ABC transporter superfamily, both present in blood-brain barrier. All of them were able to bind the transporters, resulting in a limited brain penetration[135].

Another key point to be contemplated concerns the real possibility that cancer cells could develop new mutations, making themselves resistant to EZH2 inhibitors as it was highlighted recently for SAM analogues[136].

Moreover, the role of EZH1 in tumorigenesis is not completely understood and because functions change in different tissues, it will be necessary to evaluate, depending form the tumor, inhibitors that target both EZH1 and EZH2, or drugs that specifically target EZH2 maintaining EZH1 activity.

For all these reasons, further studies are needed to better clarify the different roles of EZH2 in cancer, in addition noteworthy efforts are required in developing new generation of EZH2 inhibitors in order to design specific therapeutic strategies that alone or in combination with classical therapy could provide novel potential clinical approaches aimed at eradicating a wide variety of human cancers.

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EDITORIAL

Sequential treatment in disseminated well- and intermediate-differentiated pancreatic neuroendocrine tumors: Common sense or low rationale?

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Abstract

Fortunately, the landscape of the systemic treatment for grade 1 and 2 pancreatic neuroendocrine tumors has changed in the last decade with at least four different alternatives approved in the field. Chemotherapy, somatostatin analogues, sunitinib and everolimus remind valid options according to the most referenced international guidelines. However, and although this is something done in the routine practice, there is a lack of evidence for the use of any of these strategies after failure to the others. Moreover, further sequential alternatives in third or fourth line have never been tested prospectively. The need for a better understanding of the rationale to sequence different systemic options is even greater in non-pancreatic neuroendocrine tumors since available therapies are scarce. Sequential strategies in other solid tumors have led to a clear improvement in overall survival. This is also believed to occur in neuroendocrine tumors but no clear data on it has been delivered yet. We postulate that the different mode of action of the systemic options available for the treatment of neuroendocrine tumors may avoid the complete resistance of one option after the other and that sequential use of these agents will be translated into a longer overall survival of patients. Prospective and randomized trials that seek for the activity of drugs after failure to another systemic alternatives are highly needed in this field of neuroendocrine tumors.

Key words: Carcinoids; Everolimus; Neuroendocrine tumors; Pancreas; Overcoming resistance; Sequential-treatment; Sunitinib

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Core tip: There is a need to improve the rationale we use when approaching to a sequential systemic treatment strategy in disseminated neuroendocrine tumors. Up to now, we do not have level 1 evidence to use any systemic alternative after failure to a prior one. Widely heterogeneous populations have been recruited in larger phase Ⅲ pivotal trials in neuroendocrine tumors. Therefore, it is difficult to find final conclusions from the registration trials. In this article we aim to summarize the

available evidence behind the use of different alternatives after failure to standard somatostatin analogs.

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INTRODUCTION

The sequencing of different systemic options is something that is routinely done in metastatic solid tumors where other alternatives are available. Moreover, the use of successive lines of treatment have led to an improvement in the overall survival of all major tumors like breast cancer[1], colorectal cancer[2], non-small cell lung cancer[3] or renal cell carcinoma[4] among others. In addition, the enlargement of survival is accompanied by the maintenance of patient's quality of life[5-8]. Both goals, survival and quality of life, are the two main objectives that we are looking for in our patients with disseminated solid tumors.

The systemic management of pancreatic neuroendocrine tumors (pNETs) has dramatically changed in the last few years. Everolimus and sunitinib are novel targeted agents that have been approved recently based on a significant improvement in progression free survival against placebo[9,10]. Caplin and cols. Recently showed that somatostatin analogues, particularly lanreotide, may have also a role in tumor growth control and not only as a symptom-relievers in pNETs[11]. Streptozocin-based schemes of chemotherapy have been widely used worldwide for the last three decades to treat metastatic pNETs on the basis of high tumor and symptom responses found in prospective and retrospective studies[12-14]. Temozolomide is an alkylating agent that has shown impressive response rates according to objectives RECIST criteria but in non-randomized trials and retrospective series in pNETs[15,16]. Based on the induced-rate of tumor shrinkage, temozolomide is also considered an option for the systemic treatment of pNETs in the most referenced international guidelines[17-19] (Figure 1).

OVERCOMING RESISTANCES IN PNETS: THE BASIC SCIENCE PERSPECTIVE

The deep exomic sequences analysis of tumor samples from pNETs patients showed that there are at least two genetic key drivers that are leading the development of these tumors[20]. The phosphatidylinositol 3-kinase/AKT/mammalian target of rapamycin (mTOR) pathway is directly or indirectly affected by mutations in patients with pNETs[20]. Indirect mutations related with the mTOR pathway are related with the biallelic inactivation of the *MEN1* tumor supressor gene. The activity of the protein encoded by *MEN1* gene, the menin, is involved in the regulation of AKT access to the cell membrane. Therefore, a loss of function of the menin activity translates into a highest availability of AKT in tumor cell membrane that activates the mTOR signalling pathway[21].

The second most frequently mutated genes in patients with pNETs are affecting to domain-associated protein gene (*DAXX*) and ATR-X gene (*ATRX*). A total of 43% of the patients are harbouring mutations in either *DAXX* or *ATRX* genes. Both genes are encoding proteins involved in chromatin remodelling process and are also related to the activation of alternative lengthening of telomeres[22].

From the histology analysis and phenotype features perspective, these tumors are also characterized by high presence of newly tumor-formed blood vessels and somatostatin receptors on the surface of tumor cells. Overexpression of vascular endothelial growth factor receptors not only in the endothelial cells or in the stroma but also in the surface of the neuroendocrine cells has been extensively observed[23,24]. The presence of somatostatin receptors over the surface of pNETs tumor cells is over 90%[25,26]. Somatostatin receptors are playing a crucial role not only in the production and release of active peptides by the tumor cells but also in the growth and tumor cell survival process.

The complexity of the underlying biology behind the development of neuroendocrine tumors makes that it is difficult to believe that by targeting one of these involved mechanisms we can control the growing of the tumor for a prolonged period of time in the majority of the patients. At the same time, this complexity means that it is also very likely that one agent targeting one of these pathways could easily revert the resistance to the action of another agent against other mechanism. Overlapping resistance is not frequent among different approaches in the mode of action.

OVERCOMING RESISTANCES IN PNETS: THE CURRENT CLINICAL PERSPECTIVE

A wide range of agents with distinct mode of actions is used in the systemic treatment of pNETs. In renal cell carcinomas (RCC) it is established that mTOR inhibitors can overcome the resistance to tyrosine kinase inhibitors[27]. Moreover, there is a clear strategy for sequencing treatments in RCC; tyrosine kinase inhibitors should go first and after progression mTOR inhibitors are leading to a higher overall survival[28]. This clear superiority of sunitinib *vs* everolimus has not been established in neuroendocrine tumors so far.

In the RADIANT-3 trial, everolimus significantly prolonged median progression-free survival compared with placebo in 410 patients with pNETs (11.0 mo *vs* 4.6 mo; HR = 0.35; 95%CI: 0.27-0.45; $P < 0.001$)[9]. In an

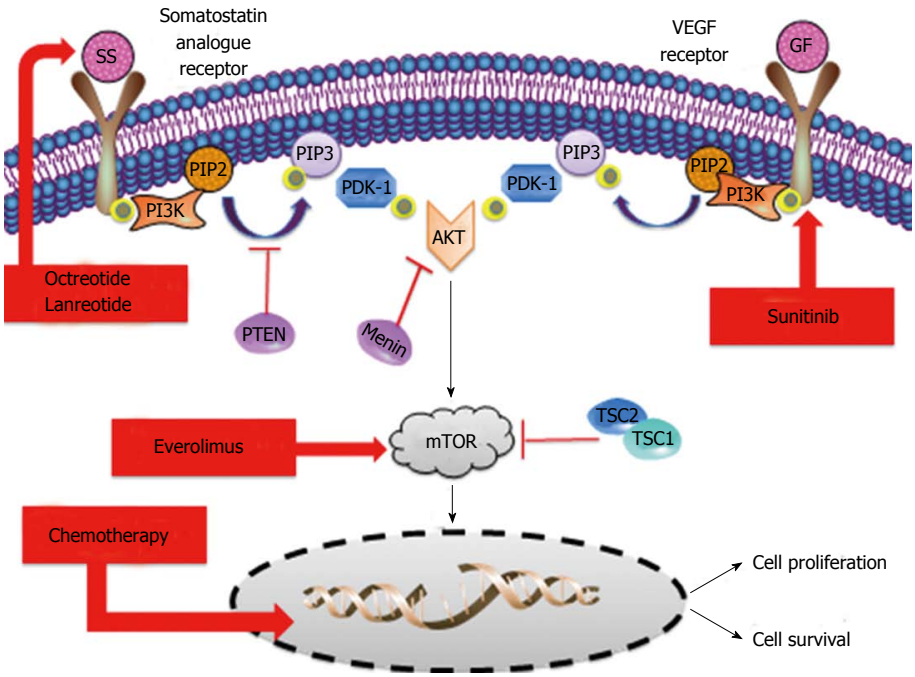


Figure 1 Summary of the mode of actions of the different available alternatives for the systemic management of pancreatic neuroendocrine tumor patients. PNET: Pancreatic neuroendocrine tumor; VEGF: Vascular endothelial growth factor; PI3K: Phosphatidylinositol 3-kinase; mTOR: Mammalian target of rapamycin.

ulterior retrospective analysis of the RADIANT-3 trial, it was shown that everolimus improved median progression free survival by 7.8 mo in patients who previously received chemotherapy (11.0 mo *vs* 3.2 mo; HR = 0.34; 95%CI: 0.25-0.48; $P < 0.0001$) and by 6.0 mo (11.4 mo *vs* 5.4 mo; HR = 0.42; 95%CI: 0.29-0.60; $P < 0.0001$) in patients who were chemonaive. It is important also to highlight that patients recruited for the RADIANT-3 trial were stratified according to if they received or not prior treatment with chemotherapy. Therefore, no significant differences are found in baseline characteristics of the two subgroups of patients[29]. Same findings were found in the retrospective subgroup analysis of those patients who were recruited for the RADIANT-3 trial and received prior treatment with somatostatin analogues (0.40: 95%CI: 0.28-0.57) *vs* those who did not receive prior treatment with either lanreotide or octreotide (0.36: 95%CI: 0.25-0.51)[9].

Similar findings were observed at the SUN-1111, the pivotal trial for sunitinib in advanced pNETs patients. When we take a look in deep to the forest plot analysis of the clinical outcome of patients treated at the SUN-1111 trial we observe that those patients who were treated before with somatostatin analogues had very similar HR for progression free survival (0.43: 95%CI: 0.21-0.89) than those patients recruited who were naïve for somatostatin analogues treatment (0.41: 95%CI: 0.22-0.75). In addition, there was also a highly significant benefit in terms of progression free survival in favour to those patients who did not receive or only received one systemic prior treatment and were randomized to sunitinib *vs* placebo (0.33: 95%CI: 0.19-0.59). Although not statistically significant there was observed also a trend to a higher benefit for sunitinib *vs* placebo in those patients who received more than two prior lines of systemic treatments (0.61: 95%CI: 0.27-1.37)[10].

To our knowledge, there are not prospective data to support the clinical activity of sunitinib followed by everolimus or the other sequence around in advanced pNETs patients. In an evidence-based sense, there is no available evidence to support the use of somatostatin analogues or chemotherapy after failure to novel targeted agents. The fact that novel targeted agents have shown activity after failure to somatostatin analogues and/or chemotherapy does not necessarily means that novel drugs should be used later in our sequential strategy of management of these patients. Actually, everolimus and sunitinib could be sequenced before the use of other systemic alternatives.

ROLE OF OTHER TARGETED AGENTS IN THE RESISTANCE OF SUNITINIB AND EVEROLIMUS

The Spanish Task Force Group for Neuroendocrine tumors (GETNE) has recently published the results of the PAZONET trial. We recruited 44 patients with advanced NETs that were treated with the multitargeted tyrosine kinase inhibitor pazopanib as a single agent (n = 14) or in combination with somatostatin analogues (n = 30) according to the investigators decision. Eleven (25%) patients had previously received everolimus, 16 (36.4%) previously received a tyrosine kinase inhibitor, and 8 patients previously had failed to both mTOR and antiangiogenic systemic treatments. The

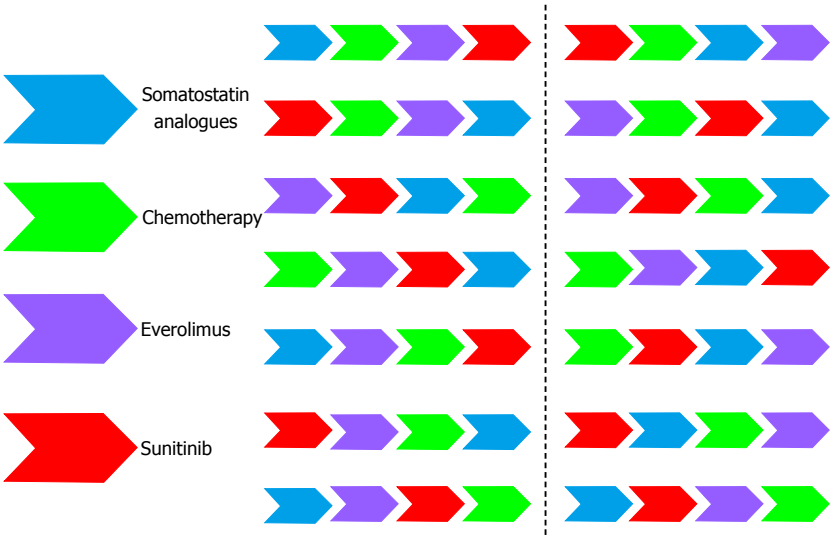


Figure 2 Different sequential alternatives to be used in advanced pancreatic neuroendocrine tumor patients based on approved agents. The term "chemotherapy" may include streptozocin ± 5-fluorouracil or doxorubicin, or temozolomide ± capecitabine.

median progression free survival that we achieved in the intention to treat population was 9.5 mo (95%CI: 4.8-14.1), however the median progression free survival in the subgroup of patients with one prior multitargeted inhibitor o a prior mTOR treatment were 12.4 mo (95%CI: 11.3-13.5) and 6.8 mo (95%CI: 0.0-15.3) respectively. The time to treatment failure was much lower in those patients who received both different alternatives before since median progression free survival was only 4.0 mo (95%CI: 1.3-6.8). Interestingly, those patients who received pazopanib plus somatostatin analogues did it better in terms of progression free survival that those who received pazopanib alone (11.7 mo, 95%CI: 9.7-13.7 *vs* 4.2 mo, 95%CI: 3.3-5.1; $P = 0.043$)[30].

Bevacizumab has been widely studied either alone[31] or in combination with octreotide[32,33], temsirolimus[34], everolimus[35,36], chemotherapy[37-39] or sorafenib[40] in patients with disseminated NETs. Although there were some of these patients previously treated with chemotherapy or other targeted agents, no data regarding clinical outcome for these subgroup populations has been released yet.

THE CRYSTAL BALL: WHAT WOULD BE THE FUTURE OF SEQUENTIAL TREATMENT IN DISSEMINATED PNETS?

There are four different strategies currently approved or widely accepted for the treatment of advanced pNETs: Somatostatin analogues (lanreotide and octreotide), chemotherapy schemes (streptozocin- and temozolamide-based), everolimus and sunitinib. In addition, there are colleagues that are using interferon as well for pNETs patients and in those sites with availabi–lity the use of peptide receptor radionuclide therapy is also common. Therefore, we can count on at least six different systemic approaches for the routine management of disseminated pNETs in addition of local techniques. How we can best order these alternatives is crucial to achieve the longest survival with the higher preservation of patient's quality of life.

There is a wide range of different sequential alternatives that can be followed in our patients with advanced pNETs (Figure 2). It is not reliable to conduct one trial in each of these situations. To our knowledge, the only randomized sequential trial in pNETs that is now undergoing is the European Neuroendocrine Tumors Society/Spanish Task Force Group for Neuroendocrine Tumors (GETNE) trial called SEQTOR (NCT02246127). The SEQTOR trial seeks to compare the efficacy and safety of everolimus followed by chemotherapy with streptozocin-fluorouracil (STZ-5FU) upon progression or the reverse sequence in advanced progressive pNETs.

The GETNE group is also leading the PALBONET trial that consists on a phase Ⅱ trial in which the cyclin-dependent kinase-4 (CDK4)/CDK6 inhibitor palbociclib is assessed as single agents in disseminated pNETs patients after progression to at least one prior novel targeted agent.

Nowadays, and in order to be pragmatic, we should consider classical clinical parameters like if the tumor is functioning or not, presence of symptoms because of tumor bulk, rapidness of tumor growth, Ki67 proliferation index, histology grade of differentiation, comorbidities, *etc*., to customize our treatment. After progression to our first approach we need to consider once again all these parameters and customize the treatment one more time in a common sense based more than in evidence based sense. There is no single sequential strategy that has proved to be superior to others as of yet. Moreover, there is no single treatment that has shown to be superior to the others. This remains the challenge of neuroendocrine tumors in which "art" is still the standard of care line after line of treatment. There is an urgent need for increasing our systemic armamentarium against these tumors and

for ordering our sequential strategies. More evidence is needed across different lines of treatment for pNETs. In addition, the concomitant use of the available options could be synergistic but we do not know if the total time to tumor growth is superior to the administration of the different strategies alone one after the other. Probably, the greatest advances and efforts that are being performed in the field of molecular biology of these tumors may translate sooner than later into a better management of our patients in the clinic.

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EDITORIAL

Present laparoscopic surgery for colorectal cancer in Japan

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Abstract

In many clinical studies, laparoscopic surgery (LS) for colon cancer has been shown to be less invasive than open surgery (OS) while maintaining similar safety. Furthermore, there are no significant differences between LS and OS in long-term outcomes. Thus, LS has been accepted as one of the standard treatments for colon cancer. In the treatments of rectal cancer as well, LS has achieved favorable outcomes, with many reports showing long-term outcomes comparable to those of OS. Furthermore, the magnification in laparoscopy improves visualization in the pelvic cavity and facilitates precise manipulation, as well as providing excellent educational effects. For these reasons, rectal cancer has seemed to be well indicated for LS, as has been colon cancer. The indication for LS in the treatment of locally advanced rectal cancer, which is relatively unresectable (*e.g.*, cancer invading other organs), remains an open issue. In recent years, new techniques such as single-port and robotic surgery have begun to be introduced for LS. Presently, various clinical studies in our country as well as in most Western countries have demonstrated that LS, with these new techniques, are gradually showing long-term outcomes.

Key words: Laparoscopic surgery; Colorectal cancer; Colectomy; Total mesorectal excision; Randomized controlled trial; Robotic surgery; Single-port surgery

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Core tip: Our findings describe the merits of laparoscopic surgery (LS) over open surgery. We present some new LS techniques. We conclude with an explanation of the safety and curability of LS for colorectal cancer.

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INTRODUCTION

Laparoscopic surgery (LS) for bowel disease was first reported in 1991 in the United States[1]. In Japan, the

first such surgery was performed in 1992 for a patient with cecal cancer[2]. Subsequently, the indications for LS were gradually expanded to include colorectal cancer and inflammatory bowel diseases such as appendicitis and diverticulitis[3]. Around 1994, however, frequent port site recurrences (PSR) after LS for colon cancer were reported, resulting in LS temporarily being deemed to be contraindicated[4]. On the other hand, few reports on PSR were reported in Japan at that time. The reason LS had a very low incidence of PSR was that the indication of LS was limited to early stage cancer in Japan. PSR was, at that time, reported as arising from the spreading of cancer cells during LS due to inappropriate manipulations of the tumor. After this realization, the principles of surgical oncology were strictly followed resulting in decreased port site recurrence; and presently, there have been no such cases reported. As the use of LS spread, clinical studies began to be carried out comparing its short- and long-term outcomes with those of open surgery (OS)[5]. On the basis of these results, LS spread rapidly in Japan becoming another standard therapy for bowel diseases, in addition to the conventional OS. Herein, we outline the current status of LS for colorectal cancer in Japan and its perspectives for the future.

COLON CANCER

Regarding colon cancer, randomized controlled trials (RCTs) comparing LS with OS have been carried out, and numerous meta-analyses of data from such trials have been reported. These reports demonstrated the superiority of LS over OS in terms of short-term outcomes and the non-inferiority of LS to OS in terms of long-term outcomes. As LS has increasingly become a standard procedure, the difference in operative time *vs* OS has gradually been reduced. In Japan, a randomized controlled trial to confirm the non-inferiority of LS to OS in terms of overall survival was conducted. And the primary endpoint of 5-year overall survival was demonstrated in ASCO-GI 2014[6]. Eligibility criteria included: Colon cancer; tumor located in the cecum, ascending, sigmoid, or recto-sigmoid colon; T3 or T4 without involvement of other organs; N0-2; and M0. Patients were randomized preoperatively and underwent tumor resection with D3 dissection. A total of 1057 patients were randomized (OP 528, LAP 529) from October 2004 through March 2009. Conversion to OS was only needed for 29 patients (5.4%) in the LS arm. The low conversion rate indicated a high quality of surgeons in this study group. JCOG0404 and results of other large clinical trials are shown in Table 1. The 5-year OS was 90.4% (95%CI: 87.5%-92.6%) in the OS arm, and 91.8% (95%CI: 89.1%-93.8%) in the LS arm. The non-inferiority of laparoscopic complete mesocolic excision in overall survival was not demonstrated[4]. Additionally, patients assigned to LS had less blood loss ($P < 0.001$), although LS lasted 52 min longer ($P < 0.001$). The short-term results in this trial are shown in Table 2. LS was associated with a shorter time to the first flatus, decreased use of analgesics after 5 postoperative days, and a shorter hospital stay. Morbidity [14.3% (76/533) *vs* 22.3% (117/524), $P < 0.001$] was lower in the LS arm[7]. Unfortunately, the non-inferiority of laparoscopic complete mesocolic excision in overall survival was not demonstrated for stage Ⅱ, Ⅲ colorectal cancer, however, because the overall survival of both arms was relatively identical and better than expected. Furthermore, the safety of LS in elderly patients and those with Stage Ⅳ disease, for whom less invasive surgery is desirable, has been demonstrated retrospectively, and another RCT is now underway[8,9]. Therefore, during the two decades since its initial introduction, data unique to Japan, serving as evidence for the validity of LS as a standard therapy for colon cancer, have steadily been accumulated.

RECTAL CANCER

Standard treatment procedures for advanced rectal cancer have yet to be established in most Western countries and Japan. Control of local recurrence, a characteristic of advanced rectal cancer, is an important treatment goal, along with the improvement of overall survival. Total mesorectal excision (TME) has been accepted as a standard procedure for the reduction of local recurrence throughout the world. As for the clinical significance of prophylactic lateral lymph node dissection, which is aggressively performed in Japan, patient enrollment in an RCT comparing this procedure with TME has been completed. The results of this trial are awaited.

Whether or not LS is an appropriate procedure for rectal cancer remains unclear. In many RCTs conducted in Western countries, LS is not indicated for the treatment of rectal cancer. The Medical Research Council CLASIC trial, an RCT of patients with colorectal cancer, reported a higher rate of tumor-positive circumferential resection margins after LS, despite no significant differences in the local recurrence rate or overall survival rate compared to LS. Oncologic safety was therefore not demonstrated.

Numerous clinical research investigations including RCTs comparing LS and OS in patients with rectal cancer, and meta-analyses have been conducted in recent years. COLOR Ⅱ (2004-2010) designed in the Netherlands and the COREAN trial (2006-2009) in South Korea exemplify RCTs focusing on advanced rectal cancer (cT3, T4)[10-13]. Both of these trials showed more significance of LS between groups in short-term outcome, and no significant differences were found in the complication rate. The long-term outcome of the CREAN trial reported no statistical differences in 3-year event-free survival rate at the primary end point, local recurrence rate at the secondary endpoint, overall survival rate, and quality of life. The same applies to the COLOR Ⅱ trial targeting 1044 cases of rectal cancer, where no statistical significances were shown between the two groups in 3-year local recurrence rate at the primary end point, and overall survival rate and event-free survival rate at the secondary end point.

In Japan, phase Ⅱ studies are being performed to

Table 1 Trial JCOG0404 and other large clinical trials

Trials	Cases Open:Laparoscopy	Conversion rate (%)	Overall survival (%) Open:Laparoscopy
COST	428:435	21	85:86
Braga	201:190	4	83:84
CLASIC C	268:526	16	68:67
COLOR	621:627	19	84.2:81.8
JCOG 0404	533:524	29 cases 5.40%	> 90

evaluate the safety and effectiveness of LS for clinical Stage 0/Ⅰ lower rectal cancer. As the first step, studies were designed to assess the technical safety of LS. The primary endpoint was the incidence of adverse events. If the safety is confirmed, the second step will focus on oncologic outcomes, with overall survival as the primary endpoint. Secondary endpoints in both the first and second steps included recurrence-free survival, operative mortality, the rate of histologically curative surgery, and the rate of conversion to OS. The results of these clinical studies are very important for determining the future indications of LS for rectal cancer.

CERTIFICATION SYSTEM FOR SURGEONS

In 2005, the Japan Society for Endoscopic Surgery began a certification system for the fields of gastrointestinal and general surgery. This certification system initially focused on surgical technique. Accreditation in the fields of gastrointestinal and general surgery required experience as the head surgeon or an instructive assistant in at least 20 advanced operations, as well as the ability to independently perform advanced endoscopic surgery in a specialized field, and to provide "procedural guidance" on technique. Along with having the technical skills of a head surgeon, certification required that the candidate could act as a coordinator of LS. Certification is also based on a detailed review of unedited videotapes of the candidates' LSs. About 20%-40% of the applicants who apply receive accreditation. Review criteria are made public, and all reviews are conducted on an impartial basis.

Minimal invasiveness of laparoscopic colectomy

Compared with OS, LS offers many benefits, such as a small surgical wound, good esthetic results, less pain, decreased use of analgesics, early recovery of intestinal peristalsis, and a shorter hospital stay[14-23]. In terms of inflammatory cytokine levels, however, the minimal invasiveness of LS remains controversial. Some studies have reported significantly lower inflammatory cytokine levels after LS[15,16], whereas others have found no significant difference in such levels between LS and OS[17,24,25]. Further studies are needed to objectively evaluate the minimal invasiveness of LS for colorectal cancer.

Table 2 Short-term results in the JCOG0404 trial

Variables	Laparoscopic surgery	Open surgery	*P* value
Bleeding (mL)	50	85	< 0.001
Operation time (min)	211	159	< 0.001
Lymph node dissections	=		Not significant
First postoperative flatus	<		
Postoperative hospital stay	<		
Surgical site infection (superficial layer)	<		
Complications (anastomosis leakage/ileus)	=		

NEW OPERATIVE TECHNIQUES

New laparoscopic procedures such as natural orifice specimen extraction (NOSE), single-port surgery, and robotic surgery have begun to be attempted for colorectal cancer as well as other diseases covered by endoscopic surgery[26-28]. Regarding NOSE for colorectal disease, a procedure involving removal of the resected bowel *via* the vagina or anus has frequently been reported. The procedure performed *via* the vagina is applicable to all bowel resection techniques including right hemicolectomy, but the procedure *via* the anus is applicable only to resection of rectal cancers located at low levels. NOSE requires resection and anastomosis within the peritoneal cavity and is therefore more difficult and time-consuming than LS. In terms of short-term outcomes (*e.g.*, safety), it has been reported that NOSE is not inferior to LS. However, despite the complex manipulations required, the only significant advantage of NOSE is the esthetic outcome, according to the data collected to date.

In Japan, robotic surgery for colorectal cancer is not covered by the national health insurance, so patients receiving this surgery must pay all the related hospital expenses themselves. During robotic surgery, the surgeon remotely controls the robot three-dimensionally from a console, with the use of a binocular magnifier. Physiological tremor of the surgeon is erased electronically through the automation of the robot. The three-dimensional visual field and the manipulation of the forceps with a high degree of freedom can evidently shorten the learning curve for surgeons. However, a large-scale system is needed, preoperative manipulations are complex, and the devices and materials are expensive. Robotic surgery has been reported to be excellent as a means of preserving nerves during pelvic surgery and improving the precision of total mesenteric resection. On the other hand, smoothly dealing with accidental events during surgery (*e.g.*, bleeding) is anticipated to be difficult because this surgery requires such a large-scale system. Therefore, it would be desirable to clarify the features if in which robotic surgery is superior to LS.

CONCLUSION

The colon and rectum are rich in elasticity, and their

resection and anastomosis are possible, leaving only a small surgical wound and enabling segments to easily be exposed for surgery. The visual field magnification of a laparoscopy allows high precision surgery in the narrow pelvic cavity. The colon and rectum can, therefore, be regarded as organs suitable for LS. If further efforts are made to achieve standardization of LS procedures and improvement of the LS educational system, LS will undoubtedly become a standard therapy for many bowel diseases. Furthermore, it is anticipated that new techniques such as reduced port surgery and robotic surgery will be proven safe in the near future. In any event, it is desirable to develop and advance operative procedures favorable from the viewpoints of low invasiveness, high safety, radical treatment capability, and cost effectiveness.

In Japan, the safety and curability of surgery for colorectal cancer are much better than in most Western countries, facilitating the rapid expansion of LS. LS is expected to gain further acceptance and progress even farther. However, daily efforts of colorectal surgeons to improve their surgical skills and to continuously collect and analyze data are considered most important.

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REVIEW

Estrogen receptor alpha gene amplification in breast cancer: 25 years of debate

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Abstract

Twenty-five years ago, Nembrot and colleagues reported amplification of the estrogen receptor alpha gene (*ESR1*) in breast cancer, initiating a broad and still ongoing scientific debate on the prevalence and clinical significance of this genetic aberration, which affects one of the most important genes in breast cancer. Since then, a multitude of studies on this topic has been published, covering a wide range of divergent results and arguments. The reported prevalence of this alteration in breast cancer ranges from 0% to 75%, suggesting that *ESR1* copy number analysis is hampered by technical and interpreter issues. To date, two major issues related to *ESR1* amplification remain to be conclusively addressed: (1) The extent to which abundant amounts of messenger RNA can mimic amplification in standard fluorescence *in situ* hybridization assays in the analysis of strongly expressed genes like *ESR1*, and (2) the clinical relevance of *ESR1* amplification: Such relevance is strongly disputed, with data showing predictive value for response as well as for resistance of the cancer to anti-estrogen therapies, or for subsequent development of cancers in the case of precursor lesions that display amplification of *ESR1*. This review provides a comprehensive summary of the various views on *ESR1* amplification, and highlights explanations for the contradictions and conflicting data that could inform future *ESR1* research.

Key words: Estrogen receptor alpha gene; Breast cancer; Tamoxifen; Gene amplification; Methodology

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Core tip: The estrogen receptor alpha gene gene (*ESR1*) is one of the most important genes in breast cancer, but the prevalence of *ESR1* amplification is matter of ongoing debate. A number of studies suggest that technical issues and lack of standards contribute to the discrepant findings. Future studies should focus on the potential clinical relevance of this phenomenon.

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INTRODUCTION

In 1990, when Nembrot *et al*[1] reported on amplification of the estrogen receptor alpha encoding gene *ESR1* in breast cancer, it was not possible to foresee that, two and a half decades later, conflicting data on the prevalence and possible clinical significance of this alteration would lead to an ongoing debate[2,3]. The relevance of the controversy results from the importance of the gene for the treatment of breast cancer. *ESR1* encodes the estrogen receptor alpha (ERα), which is a cellular receptor for the steroid hormone estrogen, a key molecule that regulates the growth and differentiation of the mammary gland[4-8]. ERα is activated by estrogen and drives cell proliferation in breast cancer[9-11]. About two thirds of breast cancers express ERα at the time of diagnosis, making the ERα-protein the most frequently applied clinical biomarker and molecular therapy target for this tumor type[9,12-15].

Gene amplification is a critical mechanism for oncogenic activation of a gene[16,17], and is believed to be a marker for oncogene addiction[18,19]. The success of Herceptin® in treating breast and gastric cancers in which the *ERBB2* gene [that encodes the human epidermal growth factor 2 (HER2)] is amplified has impressively demonstrated the clinical value of gene amplification[20,21]. The report of frequent *ESR1* amplification as a candidate marker for optimal response of proliferating breast disease to anti-estrogenic Tamoxifen monotherapy, thus attracted considerable attention in the scientific community[22-29].

However, the accounts of *ESR1* amplification were challenged from the outset. Watts *et al*[30] used the same method as Nembrot *et al*[1], but reported an unexpected lower incidence of copy number increase in 1991. This already suggested that differences in laboratory protocols, interpretation of results, and tissue sampling may represent major challenges in the analysis of *ESR1* amplification. To date, articles that present a wide range of diverging data and arguments[22,31], continue to be published, and debate or address the topic of *ESR1* amplification. An intense dialog flared up following the report that frequent *ESR1* amplification was detected in a large series of breast cancers and that regarding clinical data suggested a particular benefit of Tamoxifen treatment for these patients[24-26,28,29,32-38]. This controversy was especially evident in response to the suggestion that pre-mRNA artifacts could explain the conflicting results reported in a 2012 fluorescence in situ hybridization (FISH) study on *ESR1* amplification[3,22,23,39,40], which appeared to be a self-fulfilling prophecy concerning mRNA artifacts that were discussed as far back as 2008[36]. Conclusions ranged from no *ESR1* amplification in breast cancer[38] and reports of *ESR1* amplification being "fictional"[39], to reports of frequent prevalence, with predictive significance for response or resistance to anti-estrogen therapy[24-26,40,41]. In addition, whether amplification of *ESR1* is an early or late event, and whether it can be implemented as a potential marker for prophylactic anti-estrogen treatment[3,42], is also unresolved.

PREVALENCE OF *ESR1* AMPLIFICATION

Definitions and references

Various studies have published *ESR1* amplification frequencies that range from 0% to 75% (Figure 1, Appendices A-D), depending on methods used, sample cohorts, and threshold definitions[31]. The amplification is typically described as occurring in a mosaic pattern, indicating heterogeneous and low level increases in copy number. Nuclei often show only few additional gene copies in tight clusters of the homogeneously staining region (HSR) type[40] (Figure 2).

This pattern of amplification is of particular relevance for understanding the scientific debate on *ESR1* amplification in breast cancer. To gain clarity on the prevalence of gene amplification requires first that the term be defined. In general, "gene amplification" is defined as an increase in the gene copy number in a cell, independent of the ratio of gene copy number to centromere copy number[43,44]. However, as testing for human epidermal growth factor receptor 2 (HER2, *ERBB2*) became more frequent, the term "gene amplification" was reserved for amplifications with an average gene to centromere ratio of $\geqslant$ 2.0 or $\geqslant$ 2.2 (or > 6 copies per nucleus), simply because the threshold for predicting the response to therapy was determined at this level[45-47]. As a consequence, low level gene amplification - with a ratio less than 2.0 but greater than 1.0 - was neglected. However, for studies in which low copy number increases associated with gene amplification are investigated, the exclusion of amplifications with these low ratios[48] can have major consequences with respect to the prevalence of the genetic alteration, and can decrease the frequencies determined to considerably lower numbers in study cohorts, as shown in Figure 1 and Appendices A-D.

Low-level gene copy number alterations such as *ESR1* amplifications often present as a continuum of one-to-several additional *ESR1* copies, and minor changes of the threshold cut off value can have a major impact on study outcome. For example, using a cut off of > 2.0 instead of $\geqslant$ 2.0 for amplification calling, or a ratio 2.2 instead of 1.8, can change the amplification frequency by almost 50%[22,49,50]. In a recent study done with use of next-generation sequencing (NGS), the threshold of $\geqslant$ 6 average copies [as recommended for *ERBB2* (HER2) testing[46]] in tissue samples with tumor purity of > 20% resulted in only 0.8% *ESR1* amplification across samples[51].

The low level and heterogeneous character of *ESR1* amplification suggests that "classical *ERBB2* (HER2)" thresholds may not be optimal for *ESR1* analysis[26,40]: This is even more true when non-morphologic methods are applied for analyzing isolated DNA, wherein the choice of normalization references has a critical impact on analysis outcome. Indeed, several investigators employing quantitative polymerase chain reaction (qPCR) have demonstrated that the prevalence of *ESR1* amplification depends massively on the choice of the reference genes or sequences[32,34,40], and have suggested that variable

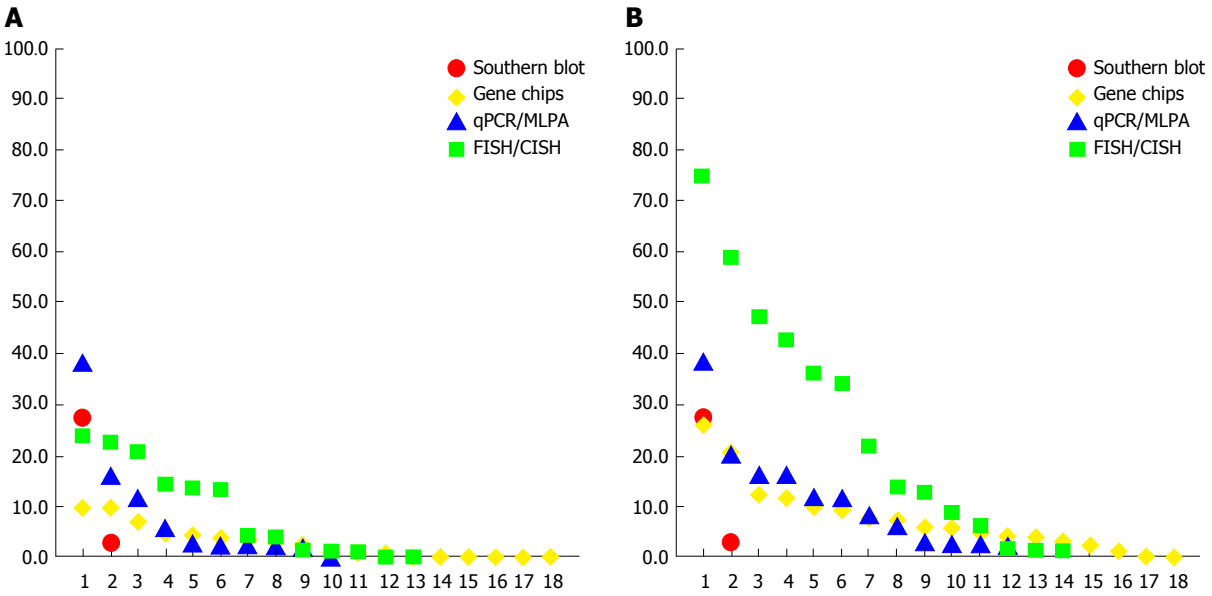


Figure 1 Prevalence of estrogen receptor alpha gene amplification in the literature. The fraction of altered cases (y-axis) is indicated across published studies (x-axis) and is shown separately for different detection methods. The studies are sorted in descending order of *ESR1* amplification frequency, as detected. A: Prevalence of *ESR1* amplification defined according to the diagnostic criteria for *ERBB2* (HER2) amplification; B: Prevalence of *ESR1* copy number increase including amplification and gain. For study citations see Appendices A-D. *ESR1*: Estrogen receptor alpha gene; MLPA: Multiplex ligation-dependent probe amplification; FISH: Fluorescence in situ hybridization; CISH: Chromogenic *in situ* hybridization.

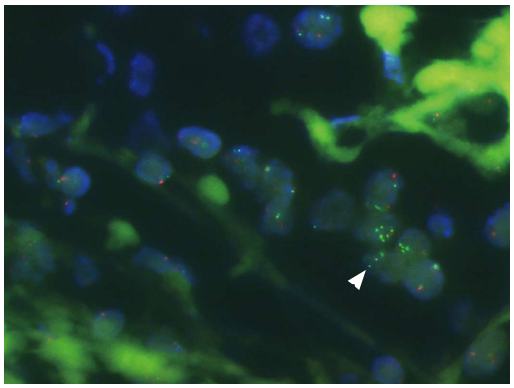


Figure 2 Heterogeneous estrogen receptor alpha gene amplification detected by fluorescence *in situ* hybridization analysis. Green and red spots represent estrogen receptor alpha gene (*ESR1*) gene probe and centromere 6 probe, respectively. White arrowhead points to tumor cell nuclei (blue) with increased numbers of *ESR1* fluorescence *in situ* hybridization signals next to tumor cell nuclei without increased numbers of signals. From Moelans *et al*[40].

deletion frequencies of reference genes are responsible for this phenomenon[32,34].

In fact, use of some assays with reference genes that have a lower frequency of deletion in breast cancer (approximately 18% *vs* approximately 30%) - according to The Cancer Genome Atlas (TCGA)[52] - also led to the detection of lower frequencies of *ESR1* amplification (*ASXL2*, *EIF5B* and *PVR vs ESR2*). Note, however, that when reference genes such as *PIEZO2* (*FAM38B*) are used, which have higher deletion frequencies (approximately 30%), the frequency of *ESR1* amplification remains low[34], suggesting that factors other than reference gene alterations may also contribute to the outcome of qPCR studies. This is also exemplified in cases when two qPCR assays lead to different *ESR1* amplification results, even though the different reference genes (*ESR2* and *SOD2*) used had similar deletion frequencies (approximately 30%) according to TCGA.

These assays highlight a huge difference (approximately half a dimension) in the dynamic range for the same samples, pointing to the impact of technical issues, in addition to the status of the reference gene, on study outcome[53] (Supplementary Tables S1-S3 and Supplementary Graphs S1-S4).

Heterogeneity and counting

Tumor heterogeneity and plasticity have been increasingly recognized over the last few years as common properties of cancer[54-64] that make molecular diagnosis difficult[22,29,40,57,58,64-66]. Heterogeneity is an obvious issue in the analyses of isolated DNA, where mixtures of cells with normal copy numbers and low level amplification may easily result in an "average" copy number that ranges below the definition threshold for the amplification status or even within the background noise of measurement[40]. In the case of heterogeneous cancers analyzed with use of *in situ* methods (such as FISH), the choice of the tumor area(s) for analysis is of the utmost importance[40]. Accordingly, approaches that classify the tumor gene status based on a minimum of successful hybridized tumor cells[67] might fail to detect aberrations that are only present only in a minor portion of the cancer bulk.

Signals and copies

Besides the purity and analyzability of tumor cells, the interpretation of FISH signals also contributes to analysis outcome. For example, automated FISH scoring

systems are limited in their ability to count individual signals in tight clusters, and instead measure the ratio of fluorescence intensities[68]. But because fluorescence intensities and signal numbers can vary due to various technical issues that are independent of copy number, automated analysis may be considerably less sensitive than the human eye. This may be particularly true in the case of the low-level clusters that are typical of *ESR1*[31,32], because identification of individual gene copies is highly dependent on the interpretation of signal patterns. A major challenge in determining low level gene copy numbers that are also heterogeneously distributed is the identification of FISH signals that represent a single gene copy. For example, distinguishing dense clusters of multiple gene copies (which often appear as one large signal) from true single gene copies is difficult; and additionally it is virtually impossible to detect tandem gene duplications (which occur commonly in breast cancer genomes[69]) with use of established FISH scoring criteria that recommend that "doublets" (*i.e.,* two tightly adjacent signals) be interpreted as a single gene copy.

Such a FISH signal-counting recommendation is often applied, based on the interpretation guidelines for *ERBB2* (HER2) FISH testing[70]. However, the recommendation to count two adjacent signals as one is based on studies that determine numerical chromosome aberrations, not focal changes in gene copy number[71,72]. This approach is warranted for chromosome enumeration that uses probes that do not hybridize within the centromere region: Following the s-phase of the cell cycle, chromosomes consist of two chromatids, each of which contains one gene copy. Accordingly, FISH analysis displays two signals nearby for these two gene copies[73] (Figure 3), even though chromosome number is not pathologically increased in these cases and such signals should be counted as one.

In contrast to chromosome enumeration, the increased number of gene copies on one chromatid or chromosome is relevant for determining gene copy number: In this case, signal doublets should be considered to represent two gene copies. Studies that combine FISH and gene chip or Southern blot technologies show that FISH signal doublets represent two gene copies on one chromosome, and as such constitute gene amplification[74-77] (Figure 3 and Supplementary Figures S1-S7).

Gene chip challenges

Gene chip technology and NGS are powerful techniques for detecting genomic alterations with high accuracy and objectivity, compared to morphologic methods that evaluate results based on the interpretation of individual observers. Nevertheless, use of these methods is associated with serious pitfalls. In 2008, a published summary of gene chip analyses listed a huge range of amplification frequencies reported in different studies and different tumor populations, ranging from 7% to 35%, even for *ERBB2* (HER2)[33]. Over the years, various limitations have led to a reconsideration of these methods. Most importantly, it has to be taken into account that the isolated DNA was analyzed as an average of many different cells. But also methodological limitations due to technology-related background of measurement and the quality of the gene chip hybridization (call rates) have to be considered[22,29,36,40,78]. The large amount of data involved in these studies also makes these methods strongly dependent on the specific computational approaches and algorithms that are used[78-80].

The general impact of the normalization reference and the computational method used to analyze raw data played a key role in the rediscovery of *ESR1* amplification, based on the analysis of 22 breast cancers with use of Affymetrix 10K SNP gene chips[24,81]. This is illustrated in Supplementary Figures S1-S7 and the Supplementary Optical Dataset S1, as well as in a video documentation (Supplementary Video Clips S1 + S2).

TCGA provides the largest and most advanced gene chip copy number database for isolated DNA from tumor samples[82,83]. A meta-analysis of TCGA dataset offers new insights into *ESR1* copy number alterations beyond the published FISH studies. For example, two tumor entities with the most frequently reported *ESR1* amplification (by FISH analysis), *i.e.,* breast and endometrial cancers[24,84], are top ranked also by Genomic Identification of Significant Targets in Cancer (GISTIC) analysis (2016-06-01 stddata_2015-04-02 regular peel off)[52]. For GISTIC, TCGA defines gene amplification as a linear copy number increase that exceeds the genome-wide median (adjusted to diploid) by more than 0.1 copies[83,85]. Overall, focal (smaller than half a chromosome arm) and high level (increase of more than one copy) *ESR1* amplifications are determined by GISTIC in 15.6%, 5.9% and 1.9% of breast cancers (n = 1080), respectively. Furthermore GISTIC analysis demonstrates that *ESR1* undergoes focal amplifications significantly (threshold q = 0.25) above the genome-wide average rate in breast cancer (q = 0.096)[52,86,87]. TCGA also confirms the existence of very small amplifications, with *ESR1* and *CCDC170* being the only genes in the GISTIC peak[52] (Figure 4): Although some peaks are due to structural alterations that involve only part of the *ESR1* gene, others are limited specifically to entire *ESR1* gene and its flanking regions (Figure 4)[52,88], providing additional strong support for a clonal selection process that targets the *ESR1* locus[86]. Amplifications that were limited to *ESR1* - but included parts of *CCDC170* - were also found in another SNP gene chip study[81] (supporting Figures 1 and 5) and by NGS, in a breast tumor that was sensitive to estradiol treatment[89] (Figure 5). Obviously, novel and publicly available databases such as TCGA collection[52] were not available at the time when the debate on *ESR1* amplification started, but the latest upgrade is still not considered in all publications to date[90].

Fact or phantom

FISH allows the analysis of gene copy number variations at a single cell level, and its morphological localization.

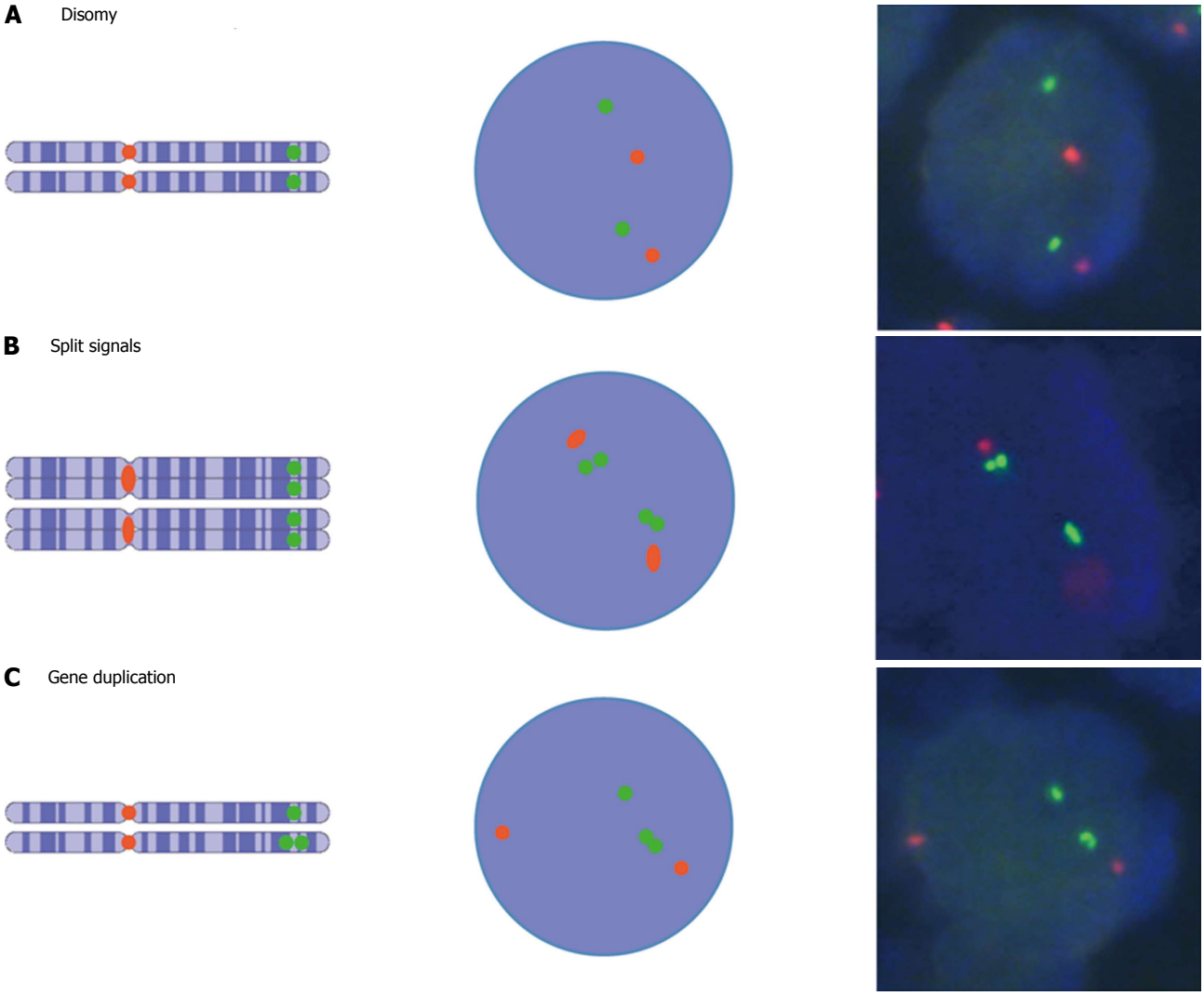


Figure 3 Estrogen receptor alpha gene single and split signal patterns and their supposed appearance as detected with fluorescence *in situ* hybridization assays. Green and red spots represent estrogen receptor alpha gene gene probe and centromere 6 probe, respectively. A: Normal disomy with two chromosomes and two gene copies; B: Normal disomy after s-phase with four chromatids and four gene copies; C: Disomy harboring mono allelic gene duplication with two chromosomes and three gene copies[38]. Photos from Moelans *et al*[40].

This makes FISH especially well-suited for taking tissue heterogeneity into account. But signal interpretation with use of FISH is dependent on observer subjectivity and is prone to influence by signal artifacts; FISH probes may also detect pre-mRNA transcripts of *ESR1*, and such hybridization could mimic DNA hybridization signals[39,40]. Such mRNA artifacts may lead to *ESR1* amplification calling by FISH that is false positive in cases with amplification signals that are exclusively limited to the nucleotide sequence of *ESR1*[24]. These artifacts may also explain the lower rates of amplification found by other methods[39]. The significance of this phenomenon has, however, not yet been fully evaluated, and some reports indicate that it does not have a major impact: These include cases with *ESR1* amplifications whose extensions have been mapped (by FISH) to the gene locus only[24]. For example, FISH amplification signals are detectable even after RNase treatment of the tissue section prior to the FISH analysis, indicating that DNA is targeted[40] (Figure 6).

Also, in at least 50% of tested cancers, *ESR1* amplification identified by FISH can be confirmed by multiplex ligation-dependent probe amplification PCR[40] (Figure 6), and additionally, a qPCR could show that tumors with *ESR1* amplification (determined by FISH) average significantly higher *ESR1* copy numbers than do tumors without *ESR1* amplification (also detected by FISH) (Supplementary Tables S1-S3, Supplementary Graphs S1-S4)[53].

It is important to understand that failed validation of FISH-determined *ESR1* amplifications by qPCR or gene chip analysis may be due to tumor purity and heterogeneity. Quantitative analysis of DNA isolated from tissue samples is always prone to underestimation of gene copy numbers - especially when tumor samples are not microdissected. This is because, due to the presence of non-cancerous cells present in a tumor sample, cancer cell purity typically ranges between 20%-80% in breast cancer, and is often overestimated in histological analyses[91]. The "contaminating" non-

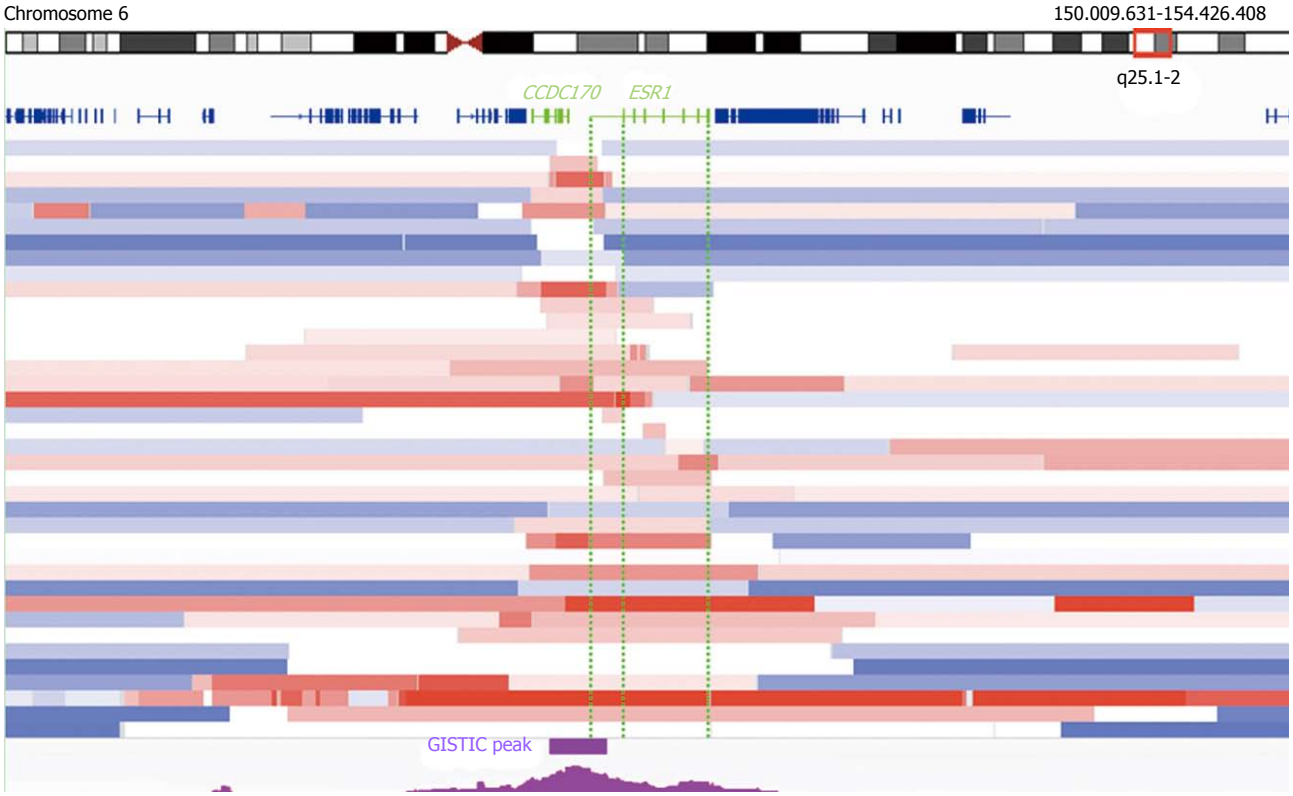


Figure 4 Architecture of the most focal estrogen receptor alpha gene amplifications in the cancer genome atlas. Segmented log2 copy number ratios in 38 TCGA breast cancers represented as horizontal bars (red: Increased, white: Neutral/normal, blue: Decreased/deleted). Focal amplifications that are smaller than the region that is 2 Mb up and downstream of estrogen receptor alpha gene (*ESR1*) (150.009.631-154.426.408 bp) and that harbor any amplified *ESR1* sequences in relation to their flanking regions are shown. The 15 cases at the top of the figure show amplifications that overlap any CCDC170 sequences and only parts of *ESR1*. In the lower 22 cases, the amplicon peak includes either the full *ESR1* sequence, or parts of *ESR1* without overlapping CCDC170. Positions of genes are indicated in dark blue; *ESR1* as well as CCDC170 are highlighted in green. The position of the GISTIC-log10 q-value peak is indicated in magenta. The position of the significant GISTIC *q*-value (< 0.25) region is indicated as a separate magenta colored bar (95%CI)[52,83,86]. TCGA: The Cancer Genome Atlas; GISTIC: Genomic Identification of Significant Targets in Cancer.

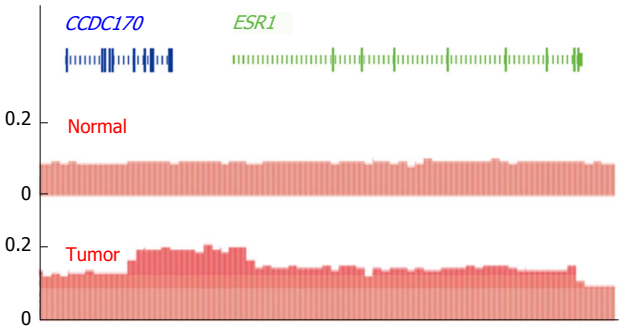


Figure 5 Estrogen receptor alpha gene amplification in a breast cancer tumor responding to estradiol treatment. DNA copy number profiles (red) in relation to the genomic position of *CCDC170* (blue) and estrogen receptor alpha gene (*ESR1*) (green) in normal and a breast cancer tumor tissue that harbors amplification of the *ESR1* gene. The amplified DNA sequence extends from *CCDC170* throughout the promoter region and the coding sequence of *ESR1*. The mapping of amplification was performed using read counts obtained during whole genome sequencing. Read counts above normal and max reads including *ESR1* are indicated in increased darker red shading. Data and graphic illustration according to Li *et al*[89].

cancerous tissue inevitably "dilutes" the tumor DNA, and leads to underestimation of changes in gene copy number[22,29,40,52,91]. Clearly this dilution effect is even greater when low-level and heterogeneous alterations (*e.g.*, the *ESR1* amplification) are analyzed[52,62,88].

CLINICAL SIGNIFICANCE OF *ESR1* AMPLIFICATION

Gains for expression

A causal relationship between *ESR1* copy numbers and increased expression of ERα protein, which drives cell proliferation[9-11], provides the molecular underpinning of the potential clinical relevance of *ESR1* amplification. Expression of the ERα-protein itself has long been used for decades as a biomarker for initiating anti-estrogen treatment in breast cancer[9]. However, regardless of the prevalence of gene amplification across a tumor type, the increase in copy number for a given gene is a well-known mechanism for increasing its expression[16,17,44,92,93]; accordingly, gene amplification is assumed to be a marker for a tumor's addiction to the expression of the amplified gene[18,19].

Several reports, including those that use DNA-specific methods for *ESR1* copy number determination, have documented a significant correlation between *ESR1* gene amplification and ERα protein expression[24,25,28,50,67,83-97] (Table 1).

Nevertheless, it is important to keep in mind that other

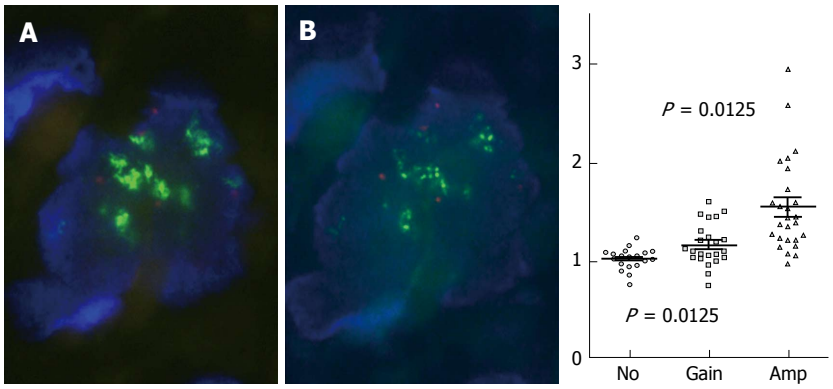


Figure 6 Effect of RNase treatment on fluorescence *in situ* hybridization signal patterns. RNase pretreatment resulted in a higher fraction of tumor cells that showed point-shaped fluorescence in situ hybridization (FISH) signals, when fuzzy clouds of estrogen receptor alpha gene (*ESR1*) signals detected by standard FISH were eliminated (A). *ESR1* copy number ratios determined by multiplex ligation-dependent probe amplification (MLPA) (y-axis) of "not increased" *ESR1* copy number (no), *ESR1* copy number gain (gain) and *ESR1* amplification (amp) determined by FISH (x-axis) according to ERBB2 (HER2) testing criteria (B). Results suggest an association of increased DNA copies of *ESR1* determined by MLPA with increased *ESR1* signals detected by FISH (B). MLPA ratios of groups "no" and "amp" as well as of "no" and "gain" are significantly different (see dot plot). Modified from Moelans *et al*[40].

mechanisms in addition to amplification also regulate a gene's transcription, and its translation into protein. In fact, some studies[1,34,39,98,99] challenge the correlation between *ESR1* copy number and expression levels (Table 1). Nonetheless, it is clear that here, too, the majority of discrepant findings are likely due to technical and methodological reasons. These could include a low number of cases analyzed, false negative ERα expression test results, or lack of statistical power due to low frequencies of tumors with *ESR1* copy number alterations detected. However, *ESR1* amplification has been described in ERα-protein negative breast cancers with poor survival[99] and it is conceivable that general genetic instability drives ERα-independent 6q amplification in these tumors. Accordingly, such cases are unlikely to be associated with ERα-protein expression and could contribute to the findings that challenge the correlation between *ESR1* copy number and ERα expression levels.

Response or resistance

The controversy about the relevance of *ESR1* amplification in breast cancer derives from claims that this genetic aberration is a potential predictive marker for optimal response to endocrine therapy. Three studies used FISH for gene copy number determination, and reported that breast cancer patients that who were treated with Tamoxifen, and who showed *ESR1* amplification in their tumors, had a better disease-specific survival than did patients without this alteration. These studies also included a retrospective analysis of the Tamoxifen-only arm of the prospective randomized ABCSG-06 trial (Figure 7)[24,25,41]. Additionally, a qPCR study found a worst outcome for patients whose tumors were ERα-negative and had *ESR1* amplification, while there was no association between to survival in patients with ERα-positive cancers that received Tamoxifen treatment[99]. In contrast, another study suggested that *ESR1* amplification predicted resistance to Tamoxifen therapy[26], although the results were not reproduced in a follow-up study of the randomized Danish cohort of the BIG-98 trial[27].

These discrepant results can be explained by differences in the kinds and mechanisms of amplification. For example, gene amplification driven by general genetic instability may be a marker for aggressive tumors[100] with unfavorable prognosis, that are less likely to respond to any therapy. Such amplifications, with lack of ERα protein expression[99], would dominate the results of survival analysis in aggressive tumor subsets[26]. Other tumors might amplify a gene specifically driven by the tumors' addiction to the respective pathway[19]. Indeed, this mechanism has been suggested in two independent studies that observed focal *ESR1* amplifications of low-level copy number change in long-term estrogen-deprived (LTED) MCF7 breast cancer cell lines, with use of DNA-specific gene chips and qPCR for *ESR1* copy number determination. And yet another experimental study showed that breast-cancer-derived xenografts respond to estrogen treatment of tumor cells that harbor *ESR1* amplification, as determined by NGS[89,101].

Furthermore, in one clinical phase Ⅱ study for evaluating anti-estrogen treatment, a focal *ESR1* amplification appeared after therapy in one out of 49 tumors analyzed by NGS[102]. These functional studies provide strong evidence for the potential clinical relevance of *ESR1* amplification as a mechanism of ERα pathway regulation. One additional study used LTED MCF7 cells to show a change of *ESR1* gene status detectable by FISH; however, the FISH signals were RNase-sensitive and no *ESR1* copy number increase was detectable by *ESR1* qPCR, suggesting that the FISH results may have been due to probe hybridization to abundant RNA[103].

Gene amplifications in human cancers are markers of the tumor's dependence on the encoded protein, and point to a potential target of therapy[18,20,21,45-47,104,105]. However, the effects of therapy depend on effective target neutralization, and indicate that target levels must be relevant for effective inactivation by antagonistic drugs.

Table 1 Published studies testing interrelations of estrogen receptor alpha gene (*ESR1*) amplification with estrogen receptor alpha protein expression over cases, studies are separated for test results by correlation or association and no correlation or association found

	Ref.	Patients (*n*)	*ESR1* CNI (%)	ERα-negative CNI (%)	Method for CNI detection
Correlation or association found					
1	Nembrot *et al*[1]	22	27.3	0	Western blot
2	Holst *et al*[24]	1652	36.1	1.3	FISH
3	Tomita *et al*[25]	133	33.8	0	FISH
4	Moelans *et al*[28]	135	8.1	27.3	MLPA
5	Tsiambas *et al*[50]	60	21.6	-	FISH
6	Dunbier *et al*[95]	44	20.5	0	Gene chip
7	Laenkholm *et al*[96]	220	42.4	8.8	FISH
8	Singer *et al*[41]	394	47.5	1	FISH
9	Lin *et al*[67]	150	12.7	5.9	FISH
10	Pentheroudakis *et al*[97]	1010	58.8	12.5	FISH
11	Li *et al*[93]	219	-	-	Gene chip
12	Soysal *et al*[3]	58	15.5	0	FISH
No correlation or association not found					
1	Watts *et al*[30]	37	2.7	0	Western blot
2	Reis-Filho *et al*[34]	70	11.4	25	Gene chip
3	Vincent-Salomon *et al*[35]	341	0.9	66.70%	Gene chip
4	Moelans *et al*[125]	39	Approximately 20	-	MLPA
5	Ooi *et al*[39]	51	5.9	0	FISH/MLPA
6	Markiewicz *et al*[99]	281	11.7	66.7	qPCR
7	Chen *et al*[2]	301	8.6	46.2	FISH

Studies are separated with regard to correlation or association and no correlation or association. Frequency of *ESR1* copy number increase (CNI), size (*n*) of study cohort, and proportions of ERα negative (ERα-) tumors in cases with *ESR1* CNI, as available. MLPA: Multiplex ligation-dependent probe amplification; FISH: Fluorescence in situ hybridization; qPCR: Quantitative polymerase chain reaction.

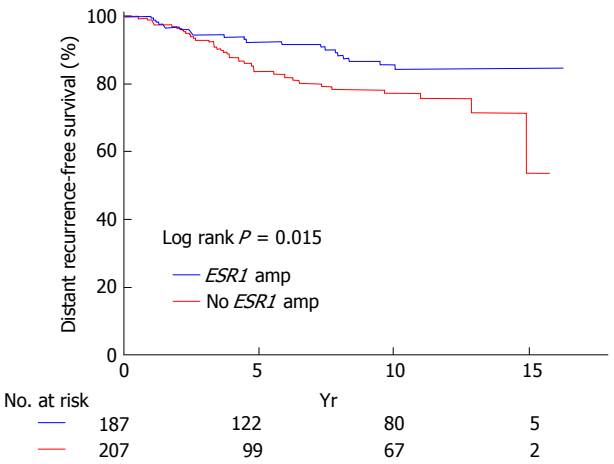


Figure 7 Kaplan-Meier plot for distant recurrence-free survival of 394 breast cancer patients treated with Tamoxifen. Patients with (blue) and without (red) estrogen receptor alpha gene amplification in primary tumor. From Singer *et al*[41].

In other words, the success of therapy might depend on the fold change in the amplified gene's copy number.

Accordingly, gene amplification is a well-known mechanism that underlies drug resistance[106-113]. Even in the case of HER2 (*ERBB2*), the mechanism of "receptor overcrowding" (and thus the level of receptor gene expression) was believed to be responsible for turning a marker for response into one for resistance depending on the level of gene expression[113].

And while the threshold for therapy response was determined at a doubled gene dose in the case of *ERBB2* (HER2), amplifications of other genes [*e.g.*, *EGFR*, *ERBB3* (HER3), and *PIK3CA* in lung cancer] might be relevant at lower levels[40,73,114-123]. This, as well as a tumor's heterogeneity regarding the amplification status of a gene, should be taken into account when considering gene amplification as a maker for therapeutic response or resistance.

Early or late

There is growing evidence that low-level gene copy number amplifications represent an adaptation by tissues to selective pressures[89,101], even in normal (non-transformed) cells[62]. It is obvious that such alterations in growth-regulating pathways can increase the risk for cancerous outgrowth[10,93]. The appearance of *ESR1* amplification in precancerous lesions, and their increased frequency during neoplastic transformation, suggest that such amplification is an early event that is potentially cancer-initiating and that drives cell proliferation[3,24,42,124,125]; what is not as clear is whether *ESR1* amplification alone is sufficient to transform cells. Nevertheless, detection of *ESR1* amplification in breast cancer precursor lesions might help to identify patients at high cancer risk, and it has thus been suggested that such patients could benefit from prophylactic anti-estrogen treatment[3].

FUTURE DIRECTIONS AND CHALLENGES

Rat runs and maps of malignancy

The debate on *ESR1* amplification in breast cancer is mainly based on methodological issues, including technical limitations, quality of application, and interpretation of results using the standard methods that are available today. The controversy about the frequency of

low level *ESR1* amplifications in particular, highlights the need for methodically advanced and sensitive approaches that will allow consistent findings.

The power of high throughput screening methods for a bird's eye perspective of the schematic landscapes has enabled us to draw integrated maps of malignancy. But cancer is not yet vanquished, and zooming in to details of these landscapes could open new dimensions of insights into hidden and undiscovered molecular pathways of malignancy (rat runs) that might be missed from a bird's eye viewpoint.

A future perspective could comprise a combination of the existing cancer landscapes and detailed information derived from sensitive targeted approaches that will enable us to develop eagles eyes. Use of established morphological imaging methods such as FISH, as well as newly developed NGS-based approaches, could combine the objectivity of computational analysis algorithms with the resolution of single-cell analyses. These methods could integrate spatial and morphological, objective and high resolution measurement within tissues[126], and are in the process of being developed. Initial results have been published with use of single-nucleus sequencing[56,61,127], but challenges of using NGS data processing and whole genome amplification still remain to be tackled[79,80,128].

Gnosis and medicine's 5 sigma

The reproducibility of research on potential drug targets is low - successful only in about a fifth of studies published[129-131]. While the established FISH method seems suggested to be a valuable approach for studying the clinical relevance of *ESR1* amplification or gene status in breast cancer[3,24-26,40,41,50,96] (Figure 7), there is no established consensus on how the interpretation of signal patterns or of gene status classification thresholds and definitions. As such, the nature of the *ESR1* gene status on the level of nucleic acids (DNA or RNA) might appear to be of secondary importance when considering a reproducible phenomenon that has an established standard diagnostic method and that is potentially applicable as a clinical marker[3]. In contrast, studies on the potential clinical significance and status definitions of detectable phenomena seem to be rather reasonable. In this context, the robustness and predictive power of a clinically applicable marker may be more important than its molecular properties.

Richard Horton recently commented that "much of the scientific literature, perhaps half, may simply be untrue", pointing to the recent *P*-values of "5 sigma" set in particle physics. And the idea, that, regarding scientific publications, "something has gone fundamentally wrong with one of our greatest human creations", highlights the need for open debates and paper critiques in science[132]. However, scientific debates will be rewarded when, besides the *P*-values and technical methodology, we do not lose sight of the goals of medical research[133].

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REVIEW

Clinical utilities and biological characteristics of melanoma sentinel lymph nodes

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Abstract

An estimated 73870 people will be diagnosed with melanoma in the United States in 2015, resulting in 9940 deaths. The majority of patients with cutaneous melanomas are cured with wide local excision. However, current evidence supports the use of sentinel lymph node biopsy (SLNB) given the 15%-20% of patients who harbor regional node metastasis. More importantly, the presence or absence of nodal micrometastases has been found to be the most important prognostic factor in early-stage melanoma, particularly in intermediate thickness melanoma. This review examines the development of SLNB for melanoma as a means to determine a patient's nodal status, the efficacy of SLNB in patients with melanoma, and the biology of melanoma metastatic to sentinel lymph nodes. Prospective randomized trials have guided the development of practice guidelines for use of SLNB for melanoma and have shown the prognostic value of SLNB. Given the rapidly advancing molecular and surgical technologies, the technical aspects of diagnosis, identification, and management of regional lymph nodes in melanoma continues to evolve and to improve. Additionally, there is ongoing research examining both the role of SLNB for specific clinical scenarios and the ways to identify patients who may benefit from completion lymphadenectomy for a positive SLN. Until further data provides sufficient evidence to alter national consensus-based guidelines, SLNB with completion lymphadenectomy remains the standard of care for clinically node-negative patients found to have a positive SLN.

Key words: Melanoma; Metastasis; Review; Biologic characteristics; Sentinel lymph node; Sentinel lymph node biopsy

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Core tip: This review examines the development of sentinel lymph node biopsy for melanoma as the quintessential technique for determining a patient's nodal status, the efficacy of sentinel lymph node biopsy in patients with melanoma, and the biology of melanoma metastatic to sentinel lymph nodes.

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INTRODUCTION

An estimated 73870 people will be diagnosed with melanoma in the United States in 2015, resulting in 9940 deaths from the disease[1]. The treatment for localized primary cutaneous melanoma is wide local excision (WLE) with dedicated 1-2 cm margins depending on the depth of the melanoma. WLE is curative in the majority of patients when melanoma is found and treated at an early stage. However, 15%-20% of melanoma patients develop nodal metastasis, which portends a significantly worse prognosis[2,3]. Lymph node basins are the most common site of melanoma metastasis and is often the first site involved with metastatic disease[4,5]. The presence of nodal disease, even at the microscopic level, predicts worse melanoma-specific survival (MSS), and nodal status comprises an important component of the 7th edition American Joint Committee on Cancer staging system for melanoma[2].

In particular, the presence or absence of nodal micrometastases is the most important prognostic factor in early-stage melanoma, particularly in intermediate thickness melanoma, and as such, evaluation of regional lymph nodes and detection of nodal metastasis provides powerful staging data[3,6]. Furthermore, clinically undetectable melanoma micrometastasis in the lymph nodes, which if left untreated, may develop into macrometastases which ultimately and theoretically may promote the development of metastatic distant disease[7,8]. Therefore, early detection of melanoma metastasis to the lymph nodes allows for early control of regional disease. Because of the prognostic value of nodal status in patients with melanoma, there has been extensive work over the past several decades into the development of surgical methods to ascertain nodal status in clinically node-negative patients with melanoma. This review specifically examines the development of sentinel lymph node biopsy (SLNB) for melanoma as the quintessential technique for determining a patient's nodal status, the efficacy of SLNB in patients with melanoma, and the biology of melanoma metastatic to sentinel nodes.

SENTINEL LYMPH NODE BIOPSY

Development and validation

Prior to SLNB, the management of clinically negative nodes in patients with melanoma was accomplished by means of either nodal observation alone or elective lymph node dissection (ELND) of the draining nodal basin. Although nodal observation after wide excision avoided unnecessary ELND at the index operation, patients who recurred with macroscopic nodal disease would require a delayed therapeutic lymph node dissection when these nodes became clinically evident, sometimes as long as 8-10 years later[8,9]. Prior to the introduction of SLNB, ELND at the time of WLE was the only available method to identify nodal micrometastases. The practice of ELND was challenged by prospective randomized trials that demonstrated no survival benefit over nodal observation. In addition, ELND exposed patients to increased morbidity in a group where up to 80% or more of patients were found to have no lymph node metastases after ELND[10-12]. Because staging of lymph nodes plays an important role in overall melanoma prognosis, SLNB was developed as a technique to determine the biology of the lymph node basin with significantly lower morbidity. Subsequently, ultrasound evaluation of the lymph node basin has been studied for determination of nodal metastases that can be confirmed by fine needle aspiration[13]. Although ultrasound is shown to be useful when compared with physical examination in the diagnosis of regional melanoma recurrences, the evidence for preoperative use of ultrasound in lieu of SLNB is lacking and is limited by the fact that relatively large metastatic deposits in the lymph node need to be present for visualization by ultrasound[14,15].

Morton *et al*[16] first developed the technique of intraoperative identification of the sentinel lymph nodes by taking advantage of the orderly draining pattern of lymph nodes surrounding a cutaneous melanoma. They postulated that migrating tumor cells from a primary lesion would first metastasize to a single node, or a select few nodes, before continuing on to the rest of the nodal basin and beyond. Thus, by examining the SLN for metastatic disease, one could infer that if the sentinel node is negative for malignant cells, the remaining nodes of the basin are as well. Since the first description in 1992, several studies have confirmed this theory and demonstrated that a negative SLN predicts the negative status of the remaining nodes in at least 96% of cases[17-20]. Conversely, when a SLN contains micrometastases, approximately 20% of positive SLN cases will have additional nodes beyond the sentinel node which also contain metastatic melanoma[21,22]. It should be emphasized that SLNB is not a simple biopsy procedure and it requires coordination between Nuclear Medicine

physicians to perform preoperative lymphoscintigraphy to localize the sentinel lymph nodes, surgeons to identify and resect the sentinel lymph nodes and pathologists to identify micrometastasis in the sentinel lymph nodes. Therefore, it is more appropriate to use the terminology of selective sentinel lymph node dissection. However, SLNB has been widely used in the literature, therefore, we use SLNB throughout this review.

Morton *et al*[16] first published on SLNB in 1992 and subsequently validated the role of this technique in the treatment of melanoma patients through the groundbreaking Multicenter Selective Lymphadenectomy Trial-Ⅰ (MSLT-Ⅰ). MSLT-Ⅰ was a prospective, randomized trial designed to determine whether SLNB performed in newly diagnosed, clinically lymph node negative melanoma patients conferred a survival advantage compared with observation alone of the draining nodal basins. The trial commenced in 1994 with a primary study population of patients with intermediate-thickness melanomas (the trial was subsequently expanded to include patients with thick melanomas and thin melanomas). The final analysis examined 2001 randomized patients (1347 intermediate thickness, 340 thin, and 314 thick melanomas) and was reported in 2014.

The final report of MSLT-Ⅰ demonstrated in the intermediate thickness group that the positive SLN rate was 16%. Furthermore, 10-year disease-free survival (DFS) rates were significantly higher in the SLNB arm when compared with the nodal observation arm (71.3% ± 1.8% *vs* 64.7% ± 2.3%, respectively; HR = 0.76; 95%CI: 0.62-0.94; *P* = 0.01), but no significant differences in 10-year MSS or overall survival (OS) were found between the SLNB arm and the nodal observation arm. In the thick melanoma group, the positive SLN rate was 32.9%, and a significant difference in 10-year DFS between the SLNB arm and the nodal observation arm was also seen (50.7% ± 4.0% *vs* 40.5% ± 4.7%, respectively; HR = 0.70; 95%CI: 0.50-0.96; *P* = 0.03), although again there were no significant differences in MSS and OS between the SLNB and nodal observation arms[3,20]. Due to a low number of patients, survival analyses could not be performed in patients with thin melanoma. Although a significant difference in DFS was found in MSLT-Ⅰ, it must be remembered that the primary endpoint of MSLT-Ⅰ was to determine if there was a difference in MSS between the two treatment arms (WLE with nodal observation *vs* WLE with SLNB). Unfortunately, due to the low event rate, the study proved to be underpowered to determine whether SLNB had an impact on OS or MSS.

The most critical analysis that came out of MSLT-Ⅰ was determining the prognostic value of SLN status. The study confirmed the value of SLN status as the most important prognostic factor for survival in patients with localized melanoma. Patients with a positive SLN had a significantly lower 10-year MSS (62.1% ± 4.8%) when compared with negative SLN patients (85.1% ± 1.5%; HR = 3.09; 95%CI: 2.12-4.49; *P* < 0.001). Furthermore, multivariate analysis found that SLN status was the most important prognostic predictor for melanoma recurrence and melanoma-specific death in the intermediate thickness group. The prognostic value of SLN status and the significant difference in 10-year MSS based on SLN status was also seen in patients with thick melanomas (48.0% ± 7.0% for positive SLN *vs* 64.6% ± 4.9% for negative SLN; HR = 1.75; 95%CI: 1.07-2.87; *P* = 0.03). Therefore, the status of a patient's SLN provides powerful prognostic information in patients with intermediate thickness and thick melanomas, although it is unknown if removal of microscopic disease confined to lymph nodes through SLNB has a therapeutic effect on survival.

In addition to demonstrating the prognostic significance of SLN status, MSLT-Ⅰ also showed that SLNB correctly identified patients who would later develop macroscopic nodal disease. This was shown by comparing the total rate of nodal disease that developed in both the SLNB arm and the nodal observation arm. In the intermediate thickness group, the total rate of nodal disease in the SLNB arm (positive SLN and nodal recurrence in negative SLNB patients) was 21.9% which was similar to the nodal recurrence rate of 19.5% in the nodal observation arm. Similarly, in the thick melanoma group, the total rate of nodal disease was 42% in the SLNB arm which again was similar to the nodal recurrence rate of 41.4% in the nodal observation arm. These results demonstrate that SLNB identifies the vast majority of patients with nodal micrometastases who would ultimately later develop macroscopic nodal recurrences. This allows for surgical intervention at a point when there is a lower burden of disease and when surgery may technically be less challenging.

Another observation that came from MSLT-Ⅰ is the suggestion that treatment of nodal disease at the microscopic level may impart a survival advantage compared with treatment of nodal disease at the macroscopic, clinically palpable level. This was based on the finding that 10-year MSS was significantly higher at 62.1% ± 4.8% in the SLNB group, in which a completion lymph node dissection (CLND) was performed immediately for microscopic nodal disease, *vs* 41.5% ± 5.6% in the nodal observation group, where a nodal dissection was performed for a macroscopic and clinically evident or palpable nodal recurrence (HR = 0.56; 95%CI: 0.37-0.84; *P* = 0.006). This difference remained significant even after accounting for false-negative results in the SLNB arm. However, this same significant difference was not seen in the thick melanoma group. These data potentially support findings of prior studies demonstrating an improvement in survival for immediate lymphadenectomy when compared with delayed lymphadenectomy. The potential survival advantage of early CLND after a positive SLNB is still controversial and may be due to lead time bias, but it is undeniably a prognostic marker and leads to improved local control[10,23].

These findings have been confirmed by a large, retrospective examination of 5840 patients from the Melanoma Institute Australia database[24]. In that large

study, DFS and regional recurrence-free survival were significantly improved in patients who had WLE with SLNB compared with patients who only had WLE, although MSS was not significantly different between these same two groups. However, for patients with melanomas >1-4 mm in thickness, MSS was significantly improved on univariate analysis for patients who had a SLNB, and distant metastasis-free survival was also significantly improved in patients who had a WLE with SLNB compared with patients who only had WLE.

There have also been studies that have investigated prognostic factors that predict the presence of SLN micrometastases in patients with melanomas 1.0 mm thickness. These factors help to guide selection of patients with melanoma who are most likely to benefit from SLNB, although current guidelines generally recommend SLNB for patients with intermediate thickness and thick melanomas who are clinically node-negative and medically fit for the procedure. The Sunbelt Melanoma Trial was a prospective, randomized trial designed to evaluate the role of high-dose interferon therapy in patients found to be SLN positive. In addition, it also provided a nonrandomized evaluation of SLNB[25]. A positive SLN was seen in 19.8% of patients, and in their population of 961 patients with melanomas of 1.0 mm thickness who underwent SLNB, multivariate analysis identified decreasing age, increasing Breslow thickness, Clark level, and ulceration as being significantly associated with a positive SLN.

Multiple prior studies also identified increasing Breslow thickness and decreasing age, as well as a high mitotic rate and lymphovascular invasion as predictors of a positive SLN in patients undergoing SLNB for melanoma[26-29]. The presence of ulceration is found to independently predict SLN status in many studies and, in fact, is included in current staging system because of its association with survival[19,26,29-32]. Other clinicopathologic factors have been evaluated, but the results of studies analyzing regression and tumor infiltrating lymphocytes as prognostic markers for SLN status have been mixed and inconsistent[33-37]. In 2011, a large study by the Sentinel Lymph Node Working Group, analyzing 3463 patients, confirmed many of these independent predictive factors for a positive SLN, including Breslow thickness, age, and lymphovascular invasion[37].

Technical aspects of sentinel node biopsy

The initial technique reported for SLNB made use of vital blue dye alone infiltrated into the skin at the site of the primary lesion. The blue colored draining lymphatic channel was traced to the blue sentinel node in the associated nodal basin. In the initial report, Morton *et al*[16] reported that the SLN was successfully identified in 82% of cases. Subsequently, radiolabeled colloid, which was also infiltrated at the primary lesion site in the same manner, was then utilized to aid in the detection of sentinel nodes. Furthermore, use of radiotracer introduced the use of lymphoscintigraphy, which allowed for preoperative identification of draining sentinel nodes, in addition to allowing for intraoperative identification of sentinel nodes using a handheld gamma probe. Use of both vital blue dye and radiotracer increased the identification of sentinel nodes to 97% to 99%[17,38,39]. Importantly, several studies also highlighted the importance of harvesting not only the SLN with the highest radiotracer count, but also nodes with lower counts, so as to not miss associated sentinel nodes that may be positive for melanoma micrometastases. The current literature supports the "10% rule", which entails removing all sentinel nodes with ≥ 10% radioactivity of the highest count node in order to optimize the detection of nodal metastases[25,38,40].

Morton's original description of SLNB utilized an isosulfan blue dye (lymphazurin), which was found to have adequate lymphatic uptake with a particle size large enough to become trapped in the SLN without readily traveling beyond[41,42]. Lymphazurin was found to be safe in the MLST-I trial, being associated with a very low complication rate, however subsequent studies cited an increased risk of anaphylactic reaction. Specifically, the use of lymphazurin was associated with a 1% risk for an anaphylactic reaction while other milder adverse allergic reactions included pruritis and localized swelling[43]. Methylene blue dye was used as a substitute when lymphazurin was in short supply nationally, and was found to be equally as effective in SLN identification and less expensive. However, controversy still exists over the use of lymphazurin compared with methylene blue, with studies conflicting in their efficacy and safety[44-46].

Today, the majority of sentinel nodes are identified using intraoperative radiotracer detection with or without the use of vital blue dyes, with a reported proportion of successfully mapped sentinel nodes ranging from 87% to 100%[38,47]. New radiotracers are being studied that have specific radioactive properties and that bind to specific receptors within lymph nodes. These newer radiotracers are being developed for use in both lymphoscintigraphy and intraoperative SLN detection[48,49]. Specifically, Tilmanocept was developed as a radiotracer which binds tightly not only to technetium, but more importantly, to mannose receptors *via* its attached mannose molecules. Mannose receptors are expressed in reticuloendothelial cells that are present in lymph nodes, and Tilmanocept is readily picked up and retained in these draining lymph nodes. A phase Ⅲ study demonstrated the efficacy of Tilmanocept during SLNB for melanoma patients and showed that Tilmanocept identified more sentinel nodes in more patients and also identified more sentinel nodes with melanoma when compared with vital blue dye[48]. Several additional technologies are in development, including indocyanine green (ICG) conjugated with human serum albumin, ICG labeled with Technetium-99m, and superparamagnetic iron oxide tracer[50-52].

Given the often complicated lymphatic drainage patterns, particularly of the head and neck, single photon emission computed tomography with integrated computed tomography (SPECT/CT) has been investigated as an additional modality to identify sentinel nodes.

Radiotracer is injected up to 24 h preoperatively and the sentinel nodes are then identified using SPECT. The integration of CT allows for identification of the anatomic location of the sentinel nodes. Several studies demonstrate a potential benefit of using SPECT/CT for preoperative planning and intraoperative decision making[53-56].

False negative rate of sentinel node biopsy

SLNB is a valuable staging and prognostic tool in patients with melanoma, however the efficacy of any test is in part dependent on the number of positive cases missed by a test or the false-negative rate (FNR) of that test. True positives are patients with metastatic disease in a SLN that has been verified on pathology. False-negatives are the number of patients with a negative SLNB who later develop a nodal recurrence in the dissected nodal basin due to missed microscopic nodal disease. The FNR is calculated as the number of false-negatives divided by the total number of true positives plus false negatives multiplied by 100 [false negatives/(true positives + false negatives) × 100]. There is some variation in the reported FNR across studies published since the introduction of SLNB in the early 1990's. However, the largest meta-analysis on SLNB to date by Valsecchi *et al*[47] reports data from 71 studies and includes over 25000 patients. These data demonstrated a FNR ranging from 0% to 34%, with a weighted summary estimate of 12.5%. The FNR was inversely associated with patients who underwent successful SLN mapping using lymphoscintigraphy with or without the use of dyes. This meta-analysis also demonstrated an additional association of FNR with an increasing length of follow-up and studies found to be of higher quality.

Complications from sentinel node biopsy

SLNB is a less morbid procedure when compared with CLND, which is the standard of care for patients with melanoma nodal metastasis. Prior studies describe the morbidity associated with CLND, including wound separation, cellulitis, hematoma/seroma, lymphedema, and nerve injury, with some studies citing a complication rate as high as 65%[57-59]. The Sunbelt melanoma trial reported on the complications seen in patients undergoing SLNB alone *vs* patients who underwent a SLNB followed by CLND for a positive SLN[60]. The overall complication rate in the SLNB alone group was approximately 5% and the most frequent complications were hematoma/seroma and wound infection. The most frequent complications in the group that had SLNB followed by CLND included lymphedema, wound infection, hematoma/seroma formation, and sensory nerve injury, with a total complication rate of 23.2%. The difference in complications was particularly pronounced for patients undergoing inguinal CLND (31.5%) compared with patients undergoing axillary CLND (4.6%). MSLT-Ⅰ also reported on the complication rate seen in patients having SLNB. When all of the various complications are totaled for patients who had SLNB alone, the complication rate was approximately 12% to 13% compared with the nearly 40% rate of complications seen in patients who also had CLND for a positive SLN.

SPECIFIC CLINICAL SCENARIOS

Thin melanoma

In the United States, the majority (> 70%) of patients who present with melanoma are diagnosed with thin melanomas (up to 1 mm in Breslow thickness)[61]. Patients with thin melanomas have a low risk of harboring a lymph node metastasis due to their early diagnosis[29]. The role of SLNB in thin melanoma patients is not clearly defined and current guidelines do not recommend its routine use, but rather recommend discussion at the individual patient level[6,62]. The risk of SLN metastases in thin melanoma patients is reported to range from as low as 1% to up to 18%, however the majority of these studies report a positive SLN in approximately 5% to 10% of thin melanoma cases[29,37,63-66]. Despite many studies describing independent predictive markers for a positive SLN in thin melanoma patients, there has been no consensus reached over which factors to utilize, and the factors found to be significant vary from study to study[29,65-69]. Additionally, there are inconsistent results when reporting the significance of SLN status in thin melanoma patients[29,64]. The most frequently associated risk factors for SLN metastases in patients with thin melanoma are Breslow thickness, Clark level, ulceration, mitotic rate, and younger age[29,63-66,69,70].

The decision to offer nodal staging depends in part on the risk threshold utilized. For SLNB, many surgeons utilize a 5% risk threshold for nodal metastasis as a criteria for potentially offering nodal staging. This is based in part on the low complication rate (approximately 5%-10%) and the low FNR (approximately 10%-15%) for SLNB. A 5% risk for a positive SLN is generally seen in melanomas with a Breslow thickness $\geqslant$ 0.75 mm and this criteria is frequently used as a threshold for offering SLNB in patients with thin melanoma. Conversely, the rate of SLN metastases in melanomas < 0.75 mm falls below 5% and the prognostic information gained from nodal staging becomes limited in these cases[29,63,64,71,72]. Clark level has also been shown to be prognostic for SLN metastasis, however it is unknown if this is a truly independent predictive marker in the face of Breslow thickness. Ulceration appears promising as a predictive marker for a positive SLN in thin melanoma patients, but ulceration is rarely seen in melanomas < 0.75 mm. More importantly, if a thickness $\geqslant$ 0.75 mm is utilized as the primary criterion for offering nodal staging, ulceration status becomes less crucial as a marker to predict SLN disease since the vast majority of ulcerated thin melanoma cases already are in melanomas $\geqslant$ 0.75 mm. Mitotic rate has also been evaluated as a potential predictive factor and has recently been incorporated into the AJCC staging system as prognostic for MSS in thin melanoma patients. However the predictive value of mitotic rate for SLN metastases is inconsistent in studies done on thin melanoma patients, possibly due to the diffe-

rences in evaluating and classifying mitotic rate across studies[29,32,63,72-77]. Based on current evidence, Breslow thickness ⩾ 0.75 mm appears to be the most consistent factor that independently predicts a > 5% risk for SLN micrometastases.

Thick melanoma

Thick melanomas (greater than 4 mm in Breslow thickness) represent approximately 5% of melanoma cases, but carries an approximately 50% survival rate in patients with thick melanoma, compared with over 90% for patients with thin melanoma[2,61]. Given that distant disease develops in a relatively high percentage of patients with thick melanoma (approximately 30% to 40% will develop distant metastasis), use of SLNB in this population is debated[78]. Indeed, many retrospective studies demonstrate that patients with thick melanoma have a dramatically increased risk of occult metastases. These studies also report variable results on the clinicopathologic factors that impact SLN positivity and on the prognostic significance of SLN status for patients with thick melanoma[20,78-85].

Approximately 25% to 40% of patients with thick melanoma will have nodal disease, and it is this population of thick melanoma patients who may potentially benefit from nodal staging. The majority of studies that have looked at SLNB in thick melanoma patients, particularly more recent studies, appear to show that SLN status is still prognostic in patients with thick melanoma. Data from the Sunbelt Melanoma Trial and the study by Gajdos *et al*[78] demonstrate that there is a significant difference in OS based on SLN status. A recent study by Yamamoto *et al*[86], the largest to date to examine SLNB in thick melanoma patients, demonstrated an overall and disease-specific advantage for patients found to be SLN negative, suggesting that SLNB offers valuable prognostic information for patients with thick melanoma. Based in part on the results of these studies, the published guidelines state that SLNB may be recommended for patients with thick melanoma to allow for accurate staging of disease[62,87].

Desmoplastic melanoma

Desmoplastic melanoma (DM) represents less than 4% of all cutaneous melanomas and is more frequently seen in older patients. It is found most commonly on the head and neck and is often a thicker tumor at presentation when compared with non-DM[88-90]. DM is divided into two histologic subtypes based on the extent of desmoplasia. Based on the Memorial Sloan Kettering Cancer Center classification system, pure DM consists of a spindle cell melanoma with ≥ 90% desmoplasia while a mixed DM has desmoplasia involving < 90% but > 10% of the spindle cell melanoma. DM is often described as being locally aggressive and having a high potential for local recurrence.

The role of SLNB in the management of DM is debated. Older studies on DM report nodal metastasis rates of approximately 30% to 40%, however contemporary single institution series demonstrate nodal metastasis rates of 9% to 18%. Furthermore, studies cite lower rates of nodal metastases for DM compared with conventional melanoma of equivalent thickness[90-95]. The positive SLN rate reported in the literature for DM ranges relatively widely from 0% to 18%[92-101]. If one excludes the small studies that report a zero rate of a positive SLN and also exclude smaller studies with less than 50 patients, the positive SLN rate for DM then ranges from 6% to 14%[92-94,96,98,101]. A large SEER database study on DM demonstrated a positive SLN rate of 2.8%[99].

In addition, clinicopathologic predictors of SLN disease in DM have also been studied. Several studies demonstrate a significantly higher SLN metastasis rate in patients with mixed DM[90,92,93,96-99,101]. The positive SLN rates in these studies for patients with mixed DM ranges from 14% to 25% while the positive SLN rate for patients with pure DM is lower at 2% to 9%. Gene expression profiling demonstrates that DM is molecularly distinct from non-DM and most closely mimics sarcomas molecularly which would explain the reason for the low incidence of lymph node metastases among patients with pure DM[102]. Again, if a 5% risk threshold for nodal disease is used as a criteria for offering nodal staging, SLNB should in general be offered to patients with mixed DM. However, controversy exists as to whether SLNB should be offered to patients with pure DM, particularly since some studies demonstrate that the SLN metastasis rate falls below 5%.

Acral lentiginous melanoma

Acral lentiginous melanoma (ALM) is the least common of the four major histologic subtypes of cutaneous melanoma, representing approximately 2% to 10% of all cases. ALM accounts for a markedly increased proportion of melanoma cases in darker-skinned populations[103,104]. ALM is shown to exhibit more aggressive features and is associated with poorer survival when compared with non-ALM[105]. The role of SLNB in ALM is unclear, however, the three largest studies all demonstrate a survival advantage in patients with a negative SLNB at the time of resection, suggesting that SLNB plays an important prognostic role in the management of patients with ALM[105-107]. Clinicopathologic factors predictive of SLN disease in ALM have not been well studied, and no study to date has elucidated independent predictive factors.

Head and neck melanoma

Head and neck melanomas (HNM) are shown to be more aggressive and carry an increased mortality when compared with melanomas in other anatomic locations. The role of SLNB and the clinicopathologic factors that predict a positive SLN in head and neck cases are not well defined in the literature. The difficulty of SLNB in HNM is partially attributable to the inconsistent lymphatic drainage patterns of the head and neck, as well as technical and anatomic considerations. For these reasons, lymphoscintigraphy and now SPECT/CT is frequently used preoperatively for HNM[108,109]. These difficulties also likely contribute to the variation in the FNR reported for

SLNB performed for HNM[110-112].

Recent publications by Parrett *et al*[111] and Fadaki *et al*[112], which represent two of the larger studies of SLNB in HNM, confirm the predictive value of younger age, ulceration, and Breslow thickness for SLN metastasis in HNM, which is consistent with results found for melanomas at other sites. Additional findings in these two studies are lower rates of SLN metastases for HNM but worse DFS and OS, findings that are also confirmed in prior studies[25,113]. Interestingly, the lower positive SLN rate seen in some studies does not portend improved survival, as would be expected given that SLN status is shown to be the most significant prognostic indicator in melanomas of other sites[111,112]. Although the mechanism of this paradoxical finding remains unclear, the use of SLNB for HNM is still recommended. In addition, the lentigo maligna melanoma subtype frequently occurs on the sun-exposed head and neck areas of older patients and has been shown to carry an improved prognosis. However, despite the improved prognosis, lentigo maligna melanoma is treated similarly to all other melanomas of the head and neck with regard to nodal staging and SLNB is also indicated for this subtype of melanoma[114].

Truncal melanoma

Melanoma of the trunk is the most common site for melanoma in men[115,116]. While risk factors for the development of truncal melanomas have been evaluated, few studies exist assessing the value of SLNB specifically for truncal melanomas. The MSLT-Ⅰ study demonstrated that truncal melanomas predicted a worse prognosis when compared with extremity melanomas in patients who underwent SLNB (HR for death from melanoma: 1.91; 95%CI: 1.26-2.88; P = 0.002)[20]. The use of SLNB in truncal melanoma can present a particular challenge, as the draining lymph node basin and sentinel nodes may be present in more than 1 nodal basin and drainage may occur in more than one direction: Cranial or caudal, and may cross the midline, emphasizing the vital role of lymphoscintigraphy in performing SLNB in this location[117,118]. Despite these issues, guidelines recommend use of SLNB for truncal melanomas.

Extremity melanoma

The lymphatic drainage of the upper and lower extremities is typically described as more predictable, although some have described more variable drainage patterns. For instance, drainage in the upper extremities can be to the epitrochlear nodes while drainage in the lower extremities can be to the popliteal nodes. Previous studies show conflicting and inconsistent results regarding the significance of the location of a primary melanoma on SLN status and survival. Some of the larger studies to compare the prognostic value of SLNB in extremity melanoma as compared with other sites include MLST-I, the Sunbelt Melanoma trial, and the large single institution study by Fadaki *et al*[112] MSLT-Ⅰ and the Sunbelt Melanoma trial both demonstrated that location of a melanoma on the extremity was prognostic for recurrence, while Fadaki *et al*[112] showed improved MSS and OS for patients with extremity melanoma when compared with patients with truncal and head and neck melanomas[20,25,112].

IS COMPLETION LYMPHADENECTOMY INDICATED FOR ALL POSITIVE SENTINEL NODE PATIENTS?

CLND is currently recommended for all patients with a positive SLN. Nodal recurrence after a positive SLNB ranges from approximately 4% to 5% in MSLT-Ⅰ and Sunbelt Melanoma trials to 15% as reported by Wong *et al*[119]. The goal of CLND is to improve regional disease control as well as to improve survival. However, no direct evidence demonstrates that CLND definitively imparts a survival benefit, although MSLT-Ⅰ does suggest improved survival for patients who underwent CLND after a positive SLNB when compared with patients who underwent a delayed nodal dissection for a macroscopic nodal recurrence (62% *vs* 41% MSS, HR = 0.56, 95%CI: 0.37-0.84; P = 0.006)[20]. This survival benefit in MSLT-Ⅰ was seen only when all node-positive patients were compared as opposed to analyzing the entire study population. In comparing the initial treatment arms (WLE with SLNB followed by CLND for a positive SLN *vs* WLE with nodal observation followed by nodal dissection for a nodal recurrence), there was a significant difference in DFS favoring the arm treated with SLNB followed by CLND, however there was no significant difference in MSS or OS. Therefore, no definite conclusions can be made based on the results of MSLT-Ⅰ as to whether performing CLND provides a survival benefit. Because the survival benefit of CLND is controversial, the routine use of CLND has been challenged[120,121].

The primary reason CLND is recommended for a positive SLN is for regional disease control. Approximately 15% to 20% of patients with a positive SLN are found to have additional disease in the CLND specimen. Furthermore, results from MSLT-Ⅰ also suggest that treatment of disease at the microscopic level through CLND for a positive SLN has less morbidity, specifically less lymphedema, than a nodal dissection performed for a macroscopic nodal recurrence[80]. Possible reasons for this may be that more nodal tissue may be involved with tumor and more tissue may need to be dissected for macroscopic disease, thereby increasing the amount of lymphatics that are disrupted. However, some argue that 80% to 85% of positive SLN patients are needlessly exposed to the morbidity of CLND since no additional nodal disease is found in these cases[119,122]. There have been numerous studies evaluating clinicopathologic factors that may predict additional nodal disease after CLND[123-131]. The ability to predict which positive SLN patients are at higher risk for additional nodal disease would allow one to offer CLND to patients who may benefit the most from this procedure. However, no factors are consistently reported, and predictive markers are extensively debated.

Several classification systems to predict non-SLN disease are reported in the literature, although none are universally accepted[125,126,128-131]. The most promising factor to predict which patients may not need a CLND is a subcapsular nodal deposit in the SLN measuring < 0.1 mm in maximal diameter. This factor predicts for an approximately 5% chance for additional nodal disease, however other studies have not shown the same results[123,127,131]. Currently, there is no consensus and no reliable way to predict which patients with a positive SLN will have additional nodal disease. As a result, a blanket recommendation for CLND for all positive SLN patients is seen in current guidelines.

The MSLT-Ⅱ trial is an ongoing randomized trial, which aims to clarify the role of CLND in patients with a positive SLN. Accrual has just completed and MSLT-Ⅱ will analyze prospective survival data between patients with tumor-positive sentinel nodes who undergo CLND with those who are randomized to nodal observation. Based on the current controversy surrounding CLND, the results of MSLT-Ⅱ are eagerly awaited although it will be several years before any results are reported. Additionally, the European Organization for Research and Treatment of Cancer - Minimal Sentinel Node Tumor Burden study (EORTC - MINITUB) is also underway to evaluate survival in patients with minimal tumor burden who undergo nodal observation compared with those who undergo CLND. There has already been a similar trial known as the German Dermatologic Oncology Group (DeCOG)-SLT trial. The results of the DeCOG-SLT trial were recently presented as an abstract at the American Society of Clinical Oncology Annual Meeting in 2015. This was a phase Ⅲ study which randomized positive SLN patients to either CLND or nodal observation. The trial showed that CLND after a positive SLNB did not prolong survival in positive SLN patients when compared with nodal observation alone[132]. In addition, there were no differences in 3 and 5 year recurrence-free survival, distant metastasis-free survival (DMFS) and MSS after a median follow-up of 35 mo. However, it is important to note that the authors did not present data in terms of regional disease control. Although the authors suggest these data provide sufficient evidence to end the practice of CLND for all positive SLN patients, further data must be presented to fully interpret the findings particularly in terms of regional disease control. The clinical and pathologic characteristics of the study population must also be considered, taking into account the location of the primary melanomas, heterogeneity of micrometastases, and melanoma thickness. While it is likely that certain subgroups of patients may not benefit from CLND, caution is warranted against abandoning the standard of care until the prospective data of the MSLT-Ⅱ and DeCOG-SLT trials are fully available and critically reviewed.

BIOLOGY OF SENTINEL NODES

Morton *et al*[133] described an "incubator" model in which melanoma metastasizes in an orderly process first to the SLN, which serves as the "gateway", followed by spread to other non-sentinel nodes in the draining nodal basin. However, in approximately 15%-20% of cases, melanoma will spread to distant sites without the development of disease in the draining nodal basin[134]. This phenomenon gives credence to the "marker" model in which metastatic disease in the SLN serves as a marker of disease that has already spread microscopically to distant sites[133]. The "incubator" model of SLN disease theoretically suggests that surgical treatment of nodal metastasis can stop further spread of disease while the "marker" model suggests that systemic therapy is required to treat distant disease. It is debated as to which model accurately represents the biology of SLN metastasis, however it is likely that both models are correct and that SLN disease correlates to each model in specific subsets of patients.

As the mechanism of metastatic melanoma spread is most commonly through lymphatic flow, understanding the implications of these factors in the primary tumor and associated lymph node environment is key[135-138]. Studies evaluating patients with melanoma and breast cancer, as well as studies in animal models, demonstrate the contribution of the molecular, cellular, and anatomic aspects of tumor cells towards the development of nodal micrometastases. Several studies highlight the ability of identifying growth factor overexpression and increased tumor vascularity to predict which patients are more likely to have a positive SLN[139-143]. Increased angiogenesis and lymphangiogenesis occur both at the site of primary malignancy and in the lymph node basin *via* the release of growth factors, most notably, VEGF[140,142,144,145]. Not only does increasing the number of lymphatic vessels increase the likelihood of tumor cell delivery to lymph nodes, but recent evidence also suggests these growth factors promote tumor cell recruitment in lymph nodes and modify the local immune environment to aid in cancer cell survival[146,147]. Additionally, increased tumor cell flow towards lymph nodes has been demonstrated to be augmented by increased intratumoral interstitial fluid pressure causing widened inter-endothelial openings, thus allowing easier entry into lymphatics[136,148].

Tumor infiltrating cells (TILs) are thought to be a host response to tumor cells and may play a role in controlling tumor growth in the lymph node basin[149]. Cells include tumor-specific cytotoxic T-cells and antigen-presenting dendritic cells. One mechanism of decreased TILs within the associated lymph nodes is a reduction in the number of antigen-presenting cells and activated T-cells, and an increase in the number of suppressor T-cells caused by immune suppressing cytokines originating from the primary tumor cells[150-152]. *Via* the mechanisms of increased lymph flow, these immune suppressing cytokines create a susceptible local environment in the nodal basin by inhibiting the tumor-specific cytotoxic T-cells described above. The immune suppressed environment is ideal for the growth of metastatic tumor cells[138,151,153]. The presence of increased TILs and, conversely, the

paucity of TILs in the primary melanoma have been demonstrated to be independently predictive of SLN status and also independently associated with survival in melanoma patients[34,154-156].

BIOMARKERS OF MELANOMA METASTASIS IN THE SENTINEL NODE

Advancement in the molecular understanding of melanoma and its gene expression profile has identified a variety of genetic, epigenetic, and protein biomarkers that show great promise as both predictive and prognostic markers of disease[21,157]. For example, expression levels of the NCOA3, SPP1, and RGS proteins each serve as independent predictors of SLN metastasis and DFS[158-160]. When combined as a multimarker index, the marker overexpression index is the most significant independent predictor of SLN metastases and DFS in two cohorts of melanoma patients. Such molecular markers show great promise in identifying patients who are high-risk for SLN metastases and would potentially benefit from SLNB[21,161].

Molecular evaluation of sentinel nodes at the time of SLNB also serves as a predictive indicator of disease outcomes. The use of quantitative real-time reverse transcriptase-polymerase chain reaction (qRT-PCR) assay of sentinel nodes at the time of operation can identify clinically relevant metastases that are missed by traditional histopathology[162,163]. Known as molecular upstaging, this method utilizes RNA biomarkers, and was evaluated in 214 melanoma patients with 10 years of follow-up data. Patients with qRT-PCR positive sentinel nodes had significantly worse OS and DFS compared with histopathologically negative sentinel nodes, demonstrating its potential value in detecting metastases in the sentinel nodes of patients with melanoma[163,164].

A 28-gene signature platform was developed that stratifies patient with localized cutaneous melanoma into low and high risk groups for the development of metastatic disease[165]. This study was subsequently expanded to evaluate this gene signature platform in 217 patients who had a SLNB[166]. Both the gene signature platform and SLNB were evaluated in terms of ability to predict DFS, DMFS and OS. Both the gene signature platform and SLNB were significant predictors of DFS and DMFS on multivariate analysis, while only the gene signature platform was a significant predictor of OS on multivariate analysis. Furthermore, utilizing both the SLNB results and the gene signature platform appeared to improve risk stratification. However, these results should be viewed carefully and have only been shown in a relatively limited number of patients. Further study is needed to validate the results of these studies.

Recently, the Cancer Genome Atlas Network published a framework for genomic classification of cutaneous melanoma. Four subtypes were identified based on the most significantly mutated genes in 333 melanomas: Mutant BRAF, mutant RAS, mutant NF1, and triple-wild-type. Although there was no survival association with the genomic classification, improved survival was found with samples enriched for immune gene expression associated with lymphocyte infiltration. These data support the correlation of tumor infiltration by lymphocytes and survival in melanoma patients described previously[167].

Microphthalmia transcription factor (MITF), a transcription factor involved in melanocyte differentiation and homeostasis, has been previously found to play an important role in controlling carcinogenic transformation[168,169]. Recently, Naffouje *et al*[170] demonstrated that MITF immunostaining in the primary tumor is associated with SLN status, suggesting its potential as a predictor of occult lymph node metastases. In addition, increased MITF expression was a significant prognosticator of DFS and OS in this study of 94 melanoma patients.

SUMMARY

The majority of patients with cutaneous melanomas are cured with WLE, however current evidence supports the use of SLNB given that 15% to 20% of patients will develop regional node metastasis. The results of prospective, randomized trials have clearly demonstrated the prognostic value of SLNB and have guided practice away from more invasive nodal staging techniques. Use of SLNB for melanoma is now standard of care, and given the rapid advancement in molecular and surgical technologies, the technical aspects of diagnosis, identification, and management of regional lymph nodes in melanoma will continue to evolve and to improve, particularly in identifying patients who should and should not be offered SLNB in specific clinical situations. Additionally, with ongoing high-quality trials examining the role of SLNB in melanoma, patients may be identified who may specifically benefit from CLND or who may undergo nodal observation for a positive SLN. Until further data provide sufficient evidence to alter consensus-based practice guidelines, SLNB with CLND remains the standard of care for clinically node-negative melanoma patients. Future histologic and molecular studies of the primary melanoma microenvironment and SLN micrometastasis may yield new insight into the molecular mechanisms that promote spread of melanoma cells to sentinel lymph nodes and beyond.

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REVIEW

Review of anticancer mechanisms of isoquercitin

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Abstract

This review was based on a literature search of PubMed and Scielo databases using the keywords "quercetin, rutin, isoquercitrin, isoquercitin (IQ), quercetin-3-glucoside, bioavailability, flavonols and favonoids, and cancer" and combinations of all the words. We collected relevant scientific publications from 1990 to 2015 about the absorption, bioavailability, chemoprevention activity, and treatment effects as well as the underlying anticancer mechanisms of isoquercitin. Flavonoids are a group of polyphenolic compounds widely distributed throughout the plant kingdom. The subclass of flavonols receives special attention owing to their health benefits. The main components of this class are quercetin, rutin, and IQ, which is a flavonoid and although mostly found as a glycoside, is an aglycone (lacks a glycoside side chain). This compound presents similar therapeutic profiles to quercetin but with superior bioavailability, resulting in increased efficacy compared to the aglycone form. IQ has therapeutic applications owing to its wide range of pharmacological effects including antioxidant, antiproliferative, anti-inflammatory, anti-hypertensive, and anti-diabetic. The protective effects of IQ in cancer may be due to actions on lipid peroxidation. In addition, the antitumor effect of IQ and its underlying mechanism are related to interactions with Wnt signaling pathway, mixed-lineage protein kinase 3, mitogen-activated protein kinase, apoptotic pathways, as well proinflammatory protein signaling. This review contributed to clarifying the mechanisms of absorption, metabolism, and actions of IQ and isoquercitrin in cancer.

Key words: Drug screening assays; Antitumor; Cancer; Flavonoids; Flavonols; Antioxidants; Neoplasms

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Core tip: Flavonoids have gained a great deal of attention over the years, and their health benefits are

the focus of the new research studies. This review contributed to clarifying the mechanisms of absorption and metabolism of isoquercitin, as well as emphasizing its chemopreventive and therapeutics effects in cancer. Overall, we presented a hypothesis of the underlying mechanisms of this biocompound in cancer.

Orfali GC, Duarte AC, Bonadio V, Martinez NP, de Araújo MEMB, Priviero FBM, Carvalho PO, Priolli DG. Review of anticancer mechanisms of isoquercitin. *World J Clin Oncol* 2016; 7(2): 189-199 Available from: URL: http://www.wjgnet.com/2218-4333/full/v7/i2/189.htm DOI: http://dx.doi.org/10.5306/wjco.v7.i2.189

INTRODUCTION

Flavonoids, which are widely distributed throughout the plant kingdom, are commonly found in fruits and vegetables. In addition, they are an accessible alternative preventive therapy or supplement for the treatment of numerous diseases. The importance of flavonoids is even more evident in developing countries where the technological challenges often hinder access to modern medicines and limit their therapeutic effectiveness[1].

Flavonoids are a group of polyphenolic compounds and as a part of the human diet, they have very low toxicity in addition to various beneficial health effects[2]. Among the numerous groups of flavonoids, the flavonols have recently been the focus of special attention for their potential actions on different organic reactions and biological pathways. Structurally, flavonols have one hydroxyl (OH) group on the third carbon (C3) and one carbonyl group (C=O) on the fourth carbon (C4) of the C ring (Figure 1)[2,3].

The best-known flavonols are (1) quercetin, an aglycone molecule widely found in nature; (2) rutin, a hydrophilic molecule; and (3) isoquercitin (IQ), a naturally occurring glycoside of quercetin also known as hirsutrin, isoquercetrin, quercetin-3-glucoside (Q3G), quercetin-3-O-β-D-glucoside (Figure 2). The molecular formula and molar mass of IQ are C21H20O12 and 464.38 g/mol, respectively. IQ is also sometimes called isoquercetin, which is a nearly identical quercetin-3-monoglucoside. Although they are technically different because isoquercetin has a pyranose ring whereas IQ has a furanose ring, functionally, the two molecules are indistinguishable. Published literature often considers them to be the same compound and uses the names interchangeably, as is the case in this present review. In addition, quercetin and IQ differ in their structure, bioavailability, absorption, and biological actions[2,4].

The absorption of flavonoids in the small intestine is limited owing to their molecular weight and hydrophilicity of their glycosides. Naturally occurring quercetin compounds are mainly glycosides such as IQ and are commonly found in plants and the human diet. Glycosides are not easily absorbed in the digestive tract, and most of their absorption occurs after transformation to the aglycone form[2].

IQ can be obtained by enzymatic hydrolysis of rutin with hesper kinase (produced by *Penicillium* sp), which has α-l-ramnosidase activity when applied at 58 ℃ for 30 min, and removes the rhamnose radical of the rutin molecule (Figure 2). Enzymatically modified IQ is the product of this procedure and consists of a mixture that includes Q3G, Q4G, rutin, and other small metabolites[5-7].

Recent *in vitro* and *in vivo* studies with flavonols, particularly IQ, have demonstrated their potential activities including antioxidants, anti-inflammatory, anti-allergic, and antiproliferative among other effects[2,7]. This review contributed to clarifying the absorption, bioavailability, and chemopreventive effects of IQ as well as its use in the treatment of cancer. In addition, we present a hypothesis of the underlying anticancer mechanism of this biocompound.

RESEARCH

This study was based on literature published between the years 1990 and 2015, found in online databases such as Scielo and Pubmed. We used search terms including "quercetin, rutin, isoquercitrin, isoquercitin, Q3G, bioavailability, flavonols, flavonoids, and cancer" as well as a combination of all the terms.

ABSORPTION OF IQ

Several studies have attempted to elucidate the pathway of IQ intestinal absorption. Thus far, the most widely accepted hypothesis involves lactase phlorizin hydrolase (LPH) as the major step[8,9,10], and sodium-dependent glucose transporter 1 (SGLT1) as a second step. LPH is an extracellular enzyme localized on the outer surface of the small intestinal brush-border membrane (BBM)[10,11]. When IQ is ingested, it is first hydrolyzed in the small intestine by the lactase domain of LPH, releasing the quercetin aglycone[12,13], which then passively diffuses to the enterocity throughout the BBM (apical surface). The deglycosylation of IQ leads to a higher concentration of the aglycone at the apical enterocyte membrane, thereby increasing the rate of absorption[9]. A small amount of IQ is transported by the SGLT1[8] present in the BBM of the small intestine, thereby transporting the intact glycoside into the cell[14]. In enterocytes, cytosolic β-glycosidase hydrolyzes the intact IQ, which is then transported *via* the SGLT1 route, into the quercetin aglycone. Quercetin and other flavonoids are substrates for uridine diphosphate-glucuronosyltransferases (UDP-GT) in the human intestine[14]. UDP-GT glucuronidates the quercetin aglycone into quercetin glucuronides (conjugated quercetin metabolites) and then it finally reaches the bloodstream[4,9] (Figure 3).

Intact quercetin glucoside is not detected in the plasma and portal blood[10] even shortly after consumption because the quercetin glucuronides are the main

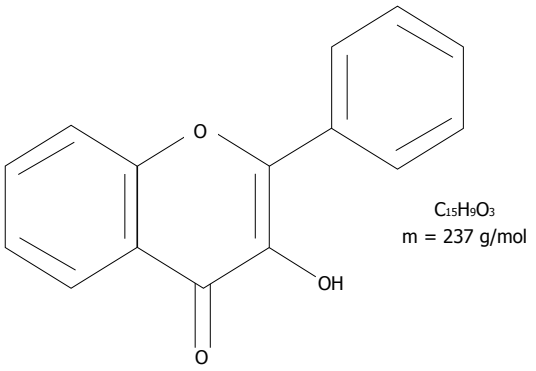


Figure 1 Basic structure of Flavonols. Flavonols have one hydroxyl (OH) group on the third carbon (C3) and one carbonyl group (C=O) on the fourth carbon (C4) of the C ring [2].

metabolites[14]. Lactase supplementation increases the hydrolysis and thereby the bioavailability of IQ and hence, may be a useful strategy to improve the metabolism and absorption of IQ[9]. The strategy might be especially useful in lactose-intolerant individuals with a diminished capacity to achieve this hydrolytic action[9]. A fat-enriched diet enhances quercetin-3-O-glucoside bioavailability, which might be owing to improved solubility and enhanced absorption of the lipophilic quercetin aglycone *via* lipid micelles, as well as delayed elimination of quercetin from the plasma owing to a prolonged enterohepatic circulation[12]. It is important to note that following the enzymatic hydrolysis by hesperidinase, rutin is mainly bioconverted into Q3G[4,7,8,10-12], thereby increasing the absorption[15] and bioavailability[16].

BIOAVAILABILITY OF IQ

Bioavailability is a measure of the extent to which a therapeutically active drug reaches the systemic circulation and is available at the site of action. Some studies have attempted to elucidate the bioavailability of IQ (Table 1); however, we were, unfortunately, unable to access these studies while other aspects were not found in the literature such as drug specificity (the availability at different sites of action or excretion data). Nevertheless, peak plasma time (first and maximum) was found in some studies, which are described in Table 1.

ANTIOXIDANT AND CHEMOPREVENTIVE EFFECTS OF IQ IN CANCER

The antioxidant effect is the best-described property of phenolic compounds[1]. Antioxidant effect is based on the ability of certain molecules to retard or inhibit oxidative damage, which is responsible for cell dysfunction as well as the onset of various health problems such as cardiovascular diseases, neurodegenerative diseases, autoimmune disorders, diabetes, and cancer. It is well established that one of the mechanisms for cancer development is oxidative stress and therefore, the chemopreventive effects of IQ could be attributed to its antioxidant and oxidative damage preventive activities.

The role of antioxidants is to block oxidative reactions induced by highly reactive oxidant molecules that damage other molecules by capturing electrons and modifying the chemical structure. These damaging molecules are the so-called free radicals or reactive oxygen species (ROS). The antioxidant properties of substances such as vitamins, minerals, enzymes, plant pigments, and other natural compounds protect the membrane and other components of the cellular structure.

IQ exerts antioxidative effects by inhibiting lipid peroxidation *via* interference with enzyme activity (xanthine oxidase), chelation of redox-active metals, increased absorption of vitamin C, and direct scavenging of ROS such as singlet oxygen, hydroxyl, peroxyl, peroxynitrite, and superoxide radicals[1,2,7,26,27].

In vitro studies comparing the antioxidant effect of flavonols (glycosides and aglycones) confirmed that their protective efficacy against lipid peroxidation reaction were mediated by free radical-scavenging activities[3,7]. However, it has been reported that these substances may also be cytotoxic and damage the cell membrane (mainly erythrocytes) in the presence of ROS, and thereby become extremely harmful to living organisms themselves[28]. Boligon *et al*[29] disagreed with this study and, therefore, analyzed the protective effects of flavonoids against chromosome damage induced by hydrogen peroxide (H_2O_2) n human lymphocytes. They concluded that flavonoids decreased the chromosomal damage by reducing the oxidative stress. Furthermore, Razavi *et al*[30] showed that Q3G has cytotoxic and antibacterial effects that are lower than its antioxidant potential, which justifies its application as a pharmacological agent to protect against ROS responsible for exacerbating tissue damage.

In vitro studies comparing the antioxidative effects of quercetin, IQ and other quercetin glycosides in liposomal phospholipid suspensions also demonstrated that the quercetin aglycone is a more efficient antioxidant than its glycoside[7,31,32]. This result should not reduce the credibility of IQ as an antioxidant compound since quercetin (aglycone) is not the dominant form in nature and has an inferior bioavailability to Q3G[2,33].

In 2004, Murota *et al*[4] measured the antioxidant activity of IQ, Q3G, and Q4G against ion-induced lipid peroxidation of rat intestinal mucosa. Q4G showed higher inhibition of lipid peroxidation than the other compounds did, although Q3G possessed a greater ion chelation capacity.

The protective effects of IQ against lipid peroxidation have been demonstrated several times. This action is mediated by numerous mechanisms including the following: Protecting the cell membrane by inhibiting LDH efflux[34]; maintaining erythrocyte deformability, which plays an essential role in circulatory system by preventing vascular diseases[35]; accumulating in the arterial wall and preventing hyperlipidemia[36]; inhibiting fatty acid formation resulting from lipid peroxidation[37]; protecting neural cells against oxidative stress induced by H_2O_2 by

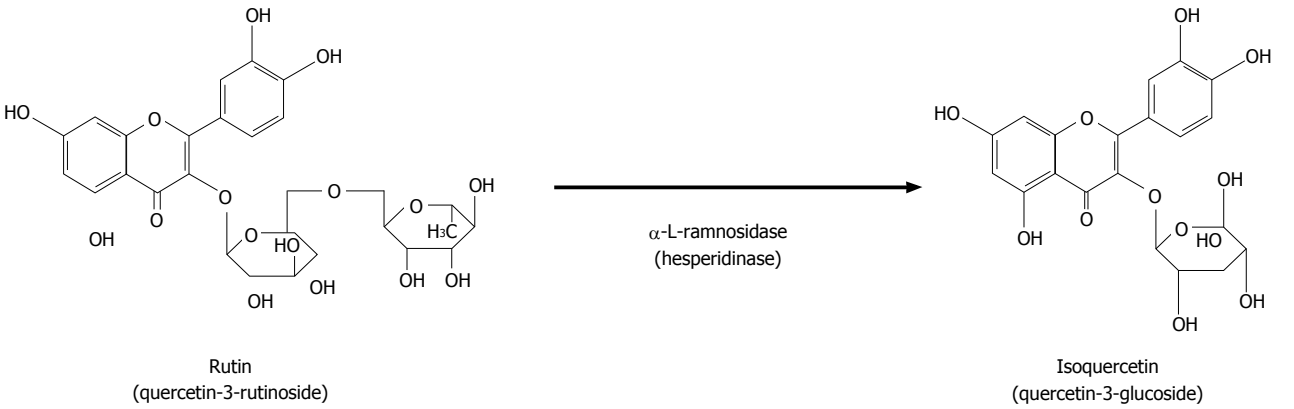


Figure 2 Enzymatic hydrolysis of rutin and formation of isoquercitin. The rutin (quercitin-3-rytinoside) is a hydrophilic molecule and isoquercitin (quercitin-3-glucoside), a naturally occurring glycoside of quercetin also known as isoquercetrin. Isoquercetrin can be obtained by enzymatic hydrolysis of rutin with hesperedinase, which has α-l-ramnosidase activity and removes the rhamnose radical of the rutin molecule.

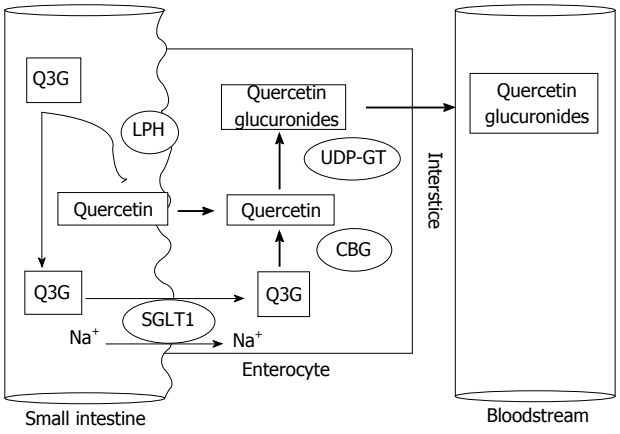


Figure 3 Accepted hypothesis pathway of isoquercitin intestinal absorption. The most widely accepted hypothesis of isoquercitin intestinal absorption involves lactase phlorizin hydrolase (LPH) as the major step, and sodium-dependent glucose transporter 1 (SGLT1) as a second step. LPH is an extracellular enzyme localized on the outer surface of the small intestinal brush-border membrane. When quercetin-3-glucoside (Q3G) is ingested, it is first hydrolyzed in the small intestine by the lactase domain of LPH, releasing the quercetin aglycone, which then passively diffuses to the enterocity throughout the apical surface. The deglycosylation of Q3G leads to a higher concentration of the aglycone at the apical enterocyte membrane, thereby increasing the rate of absorption. A small amount of Q3G is transported by the SGLT1 present in the apical surface of the small intestine, thereby transporting the intact glycoside into the cell. In enterocytes, cytosolic β-glycosidase (CBG) hydrolyzes the intact Q3G, which is then transported *via* the SGLT1 route, into the quercetin aglycone. UDP-GT glucuronidates the quercetin aglycone into quercetin glucuronides (conjugated quercetin metabolites and then it finally reaches the bloodstream. UDP-GT: Uridine diphosphate-glucuronosyltransferases.

decreasing lipid peroxidation and increasing cellular cholesterol concentrations[38]; reducing glutathione depletion and decreasing catalase and glutathione peroxidase 1 activities[39]; ROS and nitrite-scavenging activities[40]; and by regulating the oxidative complex NADPH and NO production[41], lipid-lowering activities in hepatocytes[42], induction of apolipoprotein A-I gene expression[43], and free radical scavenging[44-64].

IQ AND CANCER TREATMENT

An alternative strategy for reducing the risk of cancer is a dietary modification. Although phytochemicals naturally occur as complex mixtures, little information is available regarding possible additive, synergistic, or antagonistic interactions among these compounds. The cytotoxicity of quercetin, hyperoside (quercetin 3-O-galactoside), IQ (quercetin 3-O-glucoside), quercitrin (quercetin 3-O-rhamnoside), and spiraeoside (quercetin 4'-O-glucoside) were compared *in vitro* using cancer cell viability assays. The results indicate that IQ is a promising drug candidate for cancer therapy because its glycosylation conferred more advantageous pharmacological changes than quercetin had[65].

Amado *et al*[66] described the action of flavonoids on the Wnt signaling pathway, which plays an important role in the control of cellular differentiation, proliferation, and death as well as organogenesis and homeostasis in adults. The cancers most commonly associated with this pathway are colorectal[67], melanoma, hepatocellular carcinoma, gastric carcinoma, glioblastoma, leukemia, and breast cancers[66]. The main component of this pathway is β-catenin protein, which mainly functions to induce nuclear transcription. In the absence of Wnt ligands, β-catenin is phosphorylated by various enzyme complexes, and is able to bond with the ubiquitin-proteasome complex, which degrades it. However, in the presence of Wnt ligands, a cascade of reactions stabilizes cytoplasmic β-catenin, which can then translocate to the nucleus and mediate the stimulation of transcription factors. Numerous have studies demonstrate that inappropriate regulation and activation of this pathway is related to various diseases including cancer. The antiproliferative activity of flavonoids is related to their ability to modulate the activity of Wnt signaling at different levels. Thus, each flavonoid acts at different levels of the pathway making its actions specific against tumor cell lines. IQ for example, acts directly by inhibiting the nuclear translocation of β-catenin protein and exhibits significant anti-proliferative effects on glioblastoma cells[67].

DNA topoisomerases are important targets for cancer chemotherapy. Extracts of *Helichrysum pamphylicum* considerably inhibited the mammalian type I DNA topoi-

Table 1 Studies performed to elucidate bioavailability of isoquercitin and time to attain peak plasma time

Ref.	Animal	Ingestion form	Compound	Peak plasma time (min)		Half-life of excretion (min)
				First	Maximum	
Olthof *et al*[15], 2000	Human	Pure compound	Isoquercitin	-	37 ± 12	1110 ± 48
Chang *et al*[17], 2005	Rat	Pure compound	Isoquercitin	-	10	-
Lesser *et al*[18], 2006	Pig	Standard diet	Quercitin (standard)	± 30	95	-
			Quercitin (high fat content)		65	
Lan *et al*[19], 2007	Rat	Pure compound	Quercitin	-	114-294	-
Krogholm *et al*[20], 2010	Human	"Juice mix"	Quercitin	-	216 ± 96	-
Berger *et al*[21], 2012	Cow	Standard diet	Quercitin	-	30.0 ± 0.0	-
Gohlke *et al*[22], 2013	Cow	Standard diet	Quercitin	60-90	About 115	-
Cermak *et al*[23], 2003	Pig	Standard diet (supplemented)	Isoquercitin (standard)	< 60	210	-
			Isoquercitin (high fat content)	> 90	30	
			Quercitin	-	120	-
Lesser *et al*[12], 2004	Pig	Diet (different % of fat)	Isoquercitin (3%)	30	70 ± 7.9	-
			-32%		45.0 ± 7.9	
			Quercitin (3%)	-	102.9 ± 8.0	-
			-32%		51.4 ± 8.0	
Wiczkowski *et al*[24], 2008	Human	Shallot flesh	Isoquercitin	15	13980 ± 30	-
		Dry shallot skin	Quercitin	15	16680 ± 9	-
Reinboth *et al*[16], 2010	Dog	Standard diet (supplemented)	Isoquercitin	48	246	-
			Quercitin	72	234	-
Lee *et al*[25], 2012	Human	Mixture of apple peel and onion powder enriched applesauce	Isoquercitin	-	144 ± 90	-
			+			
			Quercitin	-	144 ± 90	-

somerase activity in a dose-dependent manner and this effect might be correlated with the antioxidant capacity of the plant[68]. There are few studies on the carcinogenicity induced by IQ. Studies in F344/DuCrj rats to evaluate the potential carcinogenicity of enzymatically modified IQ showed no apparent effects on the development of kidney neoplasms, hyperplasias, chronic nephropathy, or mammary fibroadenomas[69].

The first report we found in PubMed on IQ and cancer was published in the year 2000. It was about the protective effects of flavonoid glycosides (quercitrin-3-glucoside and isrhamnetin-4-glicoside) against an anion meal. The study showed that increases in flavonoid plasma levels were associated with high resistance of lymphocyte DNA to strand breakage[70]. After this paper, some other *in vivo* and *in vitro* reports were published with conflicting results. The antiproliferative activities of quercetin derivatives and rutin were compared using different cancer cell lines and the results demonstrated that IQ had more potent antiproliferative effects than the other flavonoids did, especially in colon, breast, and hepatocellular cancers[71]. Similar studies demonstrated that hydrolyzed rutin, which is obtained following the enzymatic hydrolysis of rutin by an hesperidinase and mainly composed of IQ (70%), exerted markedly potent antiproliferative effect *in vitro* compared to the effects of quercetin and rutin in various cancer cell lines including ovarian adenocarcinoma (OVCAR-3), breast adenocarcinoma (MCF-7), and glioma (U-251). It is suggested that the most potent antiproliferative effect induced by IQ mixtures might be related to its specific glucose transport carrier SGLT-1[7].

There are some studies about effect of flavonoids on specific cancer, see as following content.

Fibrosarcoma cancer

The inhibitory effects of *Salicornia herbacea* on matrix metalloproteinase-9 and 2 were evaluated in a human fibrosarcoma cell line (HT1080). The study suggested that the flavonoid glycosides in this plant have potential value as natural chemopreventive agents[72].

Prostatic cancer

Polyphenols purified from the Brazilian aroeira plant (*Schinus terebinthifolius*, Raddi), which contain IQ, induced apoptotic and autophagic cell death of an androgen-insensitive DU145 human prostatic carcinoma cell line[73].

Pancreatic cancer

Recently in 2015, *in vivo* and *in vitro* studies investigated the effect of IQ on the progression of pancreatic cancer. *In vitro*, IQ inhibited proliferation, promoted apoptosis, and induced cell cycle arrest in the G1 phase in pancreatic cancer cells. IQ activated caspase-3, 8, and 9 and reduced the mitochondrial membrane potential. In addition, IQ inhibited the expression level of the δ opioid receptor; however, it had no effect on the κ and μ opioid receptors. Furthermore, IQ inhibited the phosphorylation of extracellular signal-regulated kinase (ERK) and promoted the phosphorylation of c-Jun N-terminal kinase (JuNK). *In vivo*, IQ inhibited xenograft growth in nude mice[74].

Breast cancer

In 2005, Matysik *et al*[75] showed that *Calendulae officinalis* flos (Asteraceae), which contains IQ, had two biological effects in human skin fibroblast and human breast cancer cells (T47D). Extracts at concentrations

above 25 μg/mL, stimulated cell proliferation and cellular metabolism by increasing mitochondrial activity. However, concentrations higher than 75 μg/mL were cytotoxic[75]. Yang *et al*[76] assessed the synergistic effects of Q3G and apple extract in breast cancer cells. They proved that this combination significantly increased the antiproliferative activity against the growth of MCF-7 cells *in vitro*, compared to the effects of each compound alone. The antiproliferative activity of the apple extracts and Q3G combination was assessed by measuring the inhibition of MCF-7 human breast cancer cell proliferation, and the results demonstrated a synergistic effect against cell proliferation[76].

Cerebral cancer

Q3G pretreatment elevated both the expression and activation of sterol regulatory element-binding protein-2 (SREBP-2) and protected SH-SY5Y cells against H_2O_2-induced oxidative stress in neuroblastoma/SH-SY5Y cells. This data suggested a novel mechanism involving SREBP-2-mediated sterol synthesis that decreases lipid peroxidation by maintaining membrane integrity in the presence of oxidative stress[39].

IQ isolated from the aerial parts of *Hyptis fasciculata* was evaluated based on its ability to interfere with glioblastoma cell growth and the results suggested that beta-catenin-mediated signaling may be involved in the antiproliferative activity of IQ[77].

Lymphoproliferative cancer

Abnormal Savda Munziq is an herbal preparation used in Traditional Uighur Medicine for the treatment and prevention of diabetes, cardiovascular diseases, chronic asthma, and cancer. IQ and other flavonoids were identified as constituents of the preparation. The compound demonstrated antiproliferative activity in leukemia HL-60 cells[78].

Flavonoids (quercetin, quercitrin, IQ, and rutin) isolated from the leaves of *Scutia buxifolia* were evaluated against chromosomal damage induced by H_2O_2 in human lymphocytes and the result demonstrated that quercetin, IQ, and rutin recovered the mitotic index and chromosomal instability more than quercitrin did after treatment with H_2O_2[30].

Studies intended to evaluate the plant-associated quercetin glycosides and human phase Ⅱ quercetin metabolites, actually found by analyzing human biological fluids that following the consumption of quercetin containing foods, these compounds can interact with the estrogen receptors (ER). It showed that IQ displays ERα- and ERβ-dependent estrogenic activity, and the results correlated with the protective activity of diets rich in quercetin glycosides[78].

Colon cancer

In 2002, Salucci *et al*[79] suggested that the antioxidant activity of flavonoids was not involved in their inhibition of the growth of colon adenocarcinoma cells (Caco2). They concluded that IQ is a promising antioxidant agent but that it did not inhibit Caco2 cellular growth[79].

In 2005, Kern *et al*[80] reported a perspective on the antiproliferative effect and demonstrated the potent EGFR-inhibitory properties of polyphenol-rich apple juice extract in adenocarcinoma colon *in vitro* model (HT29)[80].

Another study suggested that the beneficial effects of red wine on human health were attributable to the flavonoid content, which channeled the body metabolism towards O-methylation to yield compounds with potential protective effects against cancer[81].

In vivo assays in *Xenopus* embryos, a functional model of canonical Wnt signaling studies, and *in vitro* models were used to demonstrate the inhibitory effect of IQ on Wnt/β-catenin. The flavonoid was shown to act downstream of the translocation of β-catenin to the nuclei. IQ affects *Xenopus* axis establishment, reverses the double axes and LiCl hyperdorsalization phenotype, and reduces Xnr3 expression. IQ showed antitumoral effects on colon cancer cells (SW480, DLD-1, and HCT116) whereas it exerted no significant effect on non-tumor colon cells (IEC-18), suggesting a specific effect on tumor cells *in vitro*[82].

Liver cancer

In 2008, a study in F344 rats demonstrated the chemopreventive potential of food additives containing IQ against liver carcinogenesis[83]. Similarly, *H. fasciculata* and IQ, which was identified in this species, showed activity as 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical scavengers, suggesting their antioxidant potential in human cancer[84].

Flavonoids are also foreign compounds (xenobiotics), which can potentially modulate the activity of cytochrome P450s (CYPs), the xenobiotic-metabolizing enzymes involved in the activation and detoxification of food and environmental carcinogens. In 2009, a study investigated the effects of model glycosylated and deglycosylated flavonoids IQ and rutin, respectively, on CYPs in the rat liver. The study demonstrated their differential effects on CYP expression, suggesting a possible increase in the risk of cancer development in humans and the danger associated with the consumption of these compounds in large quantities[85].

To clarify whether enzymatically modified IQ or melatonin supplementation reduces the oxidative stress-mediated hepatocellular tumor-promoting effect of oxfendazole, a benzimidazole, anthelmintic study was conducted in male rats. They were administered a single intraperitoneal injection of N-diethylnitrosamine and fed a diet containing oxfendazole with or without EMIQ or MLT in drinking water. It was concluded that co-administration of EMIQ or MLT suppressed the hepatocellular tumor-promoting activity of oxfendazole by decreasing ROS production *via* activation of CYPs[5].

In the same year, another study suggested that co-administration of enzymatically modified IQ suppresses the hepatocellular tumor-promoting activity of the synthetic flavonoid derivative β-naphthoflavone (BNF) in rats by the anti-inflammatory effects of enzymatically

modified IQ. In addition, the combination was shown to restore the cellular redox balance altered by BNF. The study compared the antiproliferative effects of EMIQ against the pro-oxidative and pro-tumor potential of the synthetic flavonoid derivative (BNF) in rat liver tissues. BNF is responsible for the maintenance of oxidative stress *via* production of ROS and activation of CYPs through the aryl hydrocarbon receptor. The results showed that the oxidative activity of BNF contributed to the formation of liver pre-neoplastic lesions while EMIQ demonstrated a protective effect by reducing the by reducing glutathione S-transferase P (GST-P)-positive cellular foci and cyclooxygenase (COX)-2 production. In addition, it suppressed encoding genes for proteins such as glutathione S-transferase mu 1 (GSTM1, drugs metabolizing enzyme), serpine1, COX-2, and nuclear factor kappa-light-chain-enhancer of activated B cells (Nf-κB), which are enzymes involved in inflammatory processes[86].

BNF is a strong inducer of CYP1A enzymes and exerts liver tumor-promoting activity through the enhancement of oxidative stress responses in rats. In 2011, a study investigated the role of the tissue environment surrounding hepatocellular preneoplastic lesions in the early tumor promotion stage induced by BNF in rats, using enzymatically modified IQ as an antioxidative chemopreventive agent. The results showed that most of the changes induced by BNF had disappeared after or were suppressed by IQ treatment, as well as the inhibition of tumor promotion. IQ also suppressed the transcription of tumor necrosis factor (TNF) induced by BNF. This observation suggested that BNF-induced oxidative stress causes single liver cell toxicity. This action further facilitated subsequent concomitant apoptosis and regeneration involving inflammatory responses including TNFα-signaling, which contributed to tumor promotion. In rat tissue, EMIQ reduces proinflammatory proteins (TNF-α) expression and cell cycle regulation molecules (Cdc20 and Cdkn2b)[87].

Enzymatically modified IQ suppressed the liver tumor-promoting activity of phenobarbital by inhibiting nuclear translocation of constitutive active/androstane receptor (CAR), and not by suppressing oxidative stress in rats. Morita *et al*[88] investigated the action of EMIQ in an experimental model of liver tumor induced by phenobarbital, a sedative and antiepileptic drug. The phenobarbital increased oxidative stress with the formation of ROS and activation of CAR. The group treated with EMIQ showed a significant decrease in oxidative stress markers in addition to inhibition of MAPK and CAR pathways, confirming the antioxidant and antiproliferative actions of this molecule[88,89].

A study investigated the effects of enzymatically modified IQ on preneoplastic liver cell lesions induced by thioacetamide promotion in a hepatocarcinogenesis rat model. The results suggested that thioacetamide (TAA)-induced tumor promotion involves activation of hepatic macrophages that produce proinflammatory factors. IQ may suppress the TAA-induced tumor-promoting activity by an anti-inflammatory mechanism mediated by suppressing the activation of these macrophages[90,91]. In addition, it may suppress tumor-promoting activity differentially between the inside and outside of GST-P(+) foci[90,91].

TAA induces oxidative stress and hepatocarcinogenicity in rats and downregulates p16 (Ink4a), which is associated with intraexonic hypermethylation in hepatocellular proliferative lesions. A study investigated the contribution of cell cycle aberrations associated with early hepatocarcinogenic processes induced by TAA using antioxidants, enzymatically modified IQ, and α-lipoic acid in a rat hepatocarcinogenesis model. The results suggested that downregulation of p16 (Ink4a) may allow the selective proliferation of preneoplastic cells promoted by TAA. However, antioxidants did not counteract this gene control. Moreover, effective suppression of TAA-induced cellular population changes within preneoplastic lesions by antioxidants, may reflect the facilitation of cell cycling and accumulation of DNA damage[91]. This damage activates cell cycle checkpoints, leading to G2 and M phase arrest at the early stages of the hepatocarcinogenesis promoted by TAA[91].

In 2014, Huang *et al*[92] investigated the impact of IQ from *Bidens bipinnata L.* extract on the progression of liver cancer *in vitro* and *in vivo*. IQ was shown to strongly inhibit proliferation, promote apoptosis, and induce G1 phase cell cycle arrest in human liver cancer cells. Additionally, IQ activated caspase-3, -8 and -9 while it inhibited expression levels of ERK and p38MAPK protein phosphorylation, promoted the phosphorylation of JuNK, and reduced the expression level of protein kinase C (PKC) in human liver cancer cells. Furthermore, *in vivo* experiments showed that IQs also significantly inhibited the growth of transplanted tumors in nude mice. The authors concluded that the molecular mechanism of IQ might be closely associated with the MAPK and PKC signaling pathways[92].

A study investigated the protective effect of bilberry extracts and enzymatically modified IQ on the hepatocarcinogenic process involving oxidative stress responses. The study used a two-stage hepatocarcinogenesis model in N-diethylnitrosamine-induced and piperonyl butoxide (PBO)-induced rats. They examined the modifying effect of co-administration of bilberry extracts and IQ on the liver tissue environment. Specifically, they determined their effects on oxidative stress responses, cell proliferation, and apoptosis. In addition, they also examined phosphatase and tensin homolog (PTEN)/Akt and transforming growth factor (TGF)-β/Smad signaling and evaluated the effects of all these phenomenon on the induction mechanism of preneoplastic lesions during early stages of hepatocellular tumor promotion. Their results suggested that PBO-induced tumor promotion potentiates PTEN/Akt and disrupts TGF-β/Smad signaling pathways, and is not related to oxidative stress responses. However, this promotion was suppressed by co-treatment with bilberry extracts and IQ, which

suppressed the proliferation activity of preneoplastic liver cells[93].

ANTITUMOR MECHANISMS OF IQ

The antitumor effect of IQ and its underlying mechanism of action remain unclear. However, based on literature reports it is possible to speculate on some likely mechanisms and create a plausible hypothesis. MAPK is a protein kinase family involved in various cell processes, and the most notable act attributed to these kinases involves cell proliferation and apoptosis signaling. Currently, three sub-families of MAPK have been discovered including ERK, JuNK/MAPK, and p38 MAPK and they each have their specific cellular targets and action sites. The activation of cell adhesion recognition sites is extremely important in the molecular adhesion of both extracellular membranes and nuclear structures. Cell adhesion recognition is mostly associated with catenin-cadherin complexes and play an important role in adhesion and pathway signaling. Numerous extracellular stimuli activate MAPK pathways, generating a chain of reactions leading to intracellular remodeling and biochemical cascades. These pathways have been associated with several diseases including cancer[88,89,92].

Mutations in the *MLK3* gene were first described in gastric and colorectal cancer[94] and the oncogenic effect was related to invasive induction of tumor cells. The study evaluated signaling pathways underlying the effect of *MLK3* gene. It described new Wnt pathway regulation mechanisms, which may be responsible for the induction of colorectal cancer invasion related to MLK3 mutations. A reasonable hypothesis for the IQ antitumor mechanism would be the involvement of signaling pathways mediating its modulation of Wnt signaling[74], which would reaffirm data presented by other studies[67,77]. The Wnt pathway in turn, is related to the p53 pathway (*via* apoptosis), and published literature has demonstrated its mediation of the apoptosis-inducing action of IQ[73]. The induction of apoptosis may be related to caspases, inhibition of ERK phosphorylation, and promotion of JuNK phosphorylation[74]. Another possibility with the apoptotic pathway would be an association with the GST-P(+) foci. IQ facilitates the apoptosis of preneoplastic cells by upregulating DR5. Outside the GST-P(+) foci, IQ suppresses apoptosis of non-transformed liver cells[90].

In addition, other possible signaling interactions of IQ could involve changes in the reduction of proinflammatory proteins such as TNF-α and other cytokines such as TGF-β[87]. It is evident that numerous mechanisms are possible mediators of the actions of IQ. Therefore, further studies are crucial to clarify the specific mechanism underlying the anticancer effects of IQ, which currently remain unclear.

CONCLUSION

Flavonoids have gained a great deal of attention over the years, and their health benefits are the focus of the new research studies. Flavonoids, which are widely distributed throughout the plant kingdom, commonly occur in fruits and vegetables. Therefore, these compounds are an accessible alternative to conventional therapies in the prevention or supplementation of treatments for numerous diseases. Flavonoids are particularly important in developing countries where the technological challenges often hinder access to modern medicines, limiting their therapeutic effectiveness[1,2,27].

This review contributed to clarifying the mechanisms of absorption and metabolism of IQ, as well as highlighting the possible mechanisms of its various physiological effect. In addition, we corroborated its wide therapeutic applicability and stressed the importance of further studies to elucidate its specific anticancer mechanisms.

Based on our findings, the flavonoid IQ possesses adequate bioavailability and inhibits the pathogenesis of various diseases and, therefore, warrants further study to enhance the understanding of its beneficial actions. In addition, further studies would facilitate the discovery of new therapeutic applications and highlight the essential contribution of IQ to ensuring a more balanced diet, which would contribute to a healthier lifestyle. Further studies on the metabolism of flavonoids, their actions, and effects of their metabolites in various tissues are necessary to facilitate their development and better application in clinical management.

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REVIEW

Role of the microbiome in non-gastrointestinal cancers

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Abstract

"The forgotten organ", the human microbiome, comprises a community of microorganisms that colonizes various sites of the human body. Through coevolution of bacteria, archaea and fungi with the human host over thousands of years, a complex host-microbiome relationship emerged in which many functions, including metabolism and immune responses, became codependent. This coupling becomes evident when disruption in the microbiome composition, termed dysbiosis, is mirrored by the development of pathologies in the host. Among the most serious consequences of dysbiosis, is the development of cancer. As many as 20% of total cancers worldwide are caused by a microbial agent. To date, a vast majority of microbiome-cancer studies focus solely on the microbiome of the large intestine and the development of gastrointestinal cancers. Here, we will review the available evidence implicating microbiome involvement in the development and progression of non-gastrointestinal cancers, while distinguishing between viral and bacterial drivers of cancer, as well as "local" and "systemic", "cancer-stimulating" and "cancer-suppressing" effects of the microbiome. Developing a system-wide approach to cancer-microbiome studies will be crucial in understanding how microbiome influences carcinogenesis, and may enable to employ microbiome-targeting approaches as part of cancer treatment.

Key words: Microbiome; Non-gastrointestinal cancers; Carcinogenesis; Dysbiosis; Microbial agent

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Core tip: "The forgotten organ", the human microbiome, comprises a community of microorganisms that colonizes various sites of the human body. A complex host-microbiome relationship has emerged in which many functions became codependent. This coupling becomes evident when disruption in the microbiome composition, termed dysbiosis, is mirrored by the development of pathologies in the host. Among the most serious consequences of dysbiosis, is the development of cancer. As many as 20% of total cancers worldwide are caused by a

microbial agent. Here, we will review the available evidences implicating microbiome involvement in the development and progression of non-gastrointestinal cancers. Developing a system-wide approach to cancer-microbiome studies will be crucial in understanding how microbiome influences carcinogenesis, and may enable to employ microbiome-targeting approaches as part of cancer treatment.

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INTRODUCTION

Bacteria, viruses, archaea and fungi coevolved with the human body for thousands of years. This resulted in diverse and extensive host-microbiome interactions, which influence multiple host physiological processes, including metabolism and the function of the immune system[1]. Disruption of the microbial community, termed dysbiosis, is suggested to constitute a major risk factor for an increasing array of diseases including metabolic syndrome and immune disorders as well as several forms of cancer.

Carcinogenesis is a process inflicted and influenced by many mechanisms. However, up to 20% of the cancers worldwide are believed to be caused or modulated by a microbial agent[2,3]. Of the various involved microorganisms, viruses are best studied for their role in carcinogenesis. Therefore, multiple mechanisms through which viruses promote development of tumors have been deciphered. The roles of archaea and fungal members of microbiome in cancer formation are much less studied, while only recently studies emerged focusing on bacterial involvement in cancer formation and progression.

This review will provide some conceptual examples of how different organ-specific microbiomes may modulate the carcinogenic processes through involvement of specific members or, alternatively, through changes observed in the microbial community as a whole (summarized in Table 1). An early example of an individual bacterial member that contributes to carcinogenesis is *Helicobacter pylori* (*H. pylori*). *H. pylori* colonizes the gastric mucosa in 50% of humans and causes cancer in 1%-3% of colonized individuals. It thus is recognized by the International Agency for Research on Cancer as a bone-fide carcinogen[4]. However, further experiments in germ-free mice showed that infection by *H. pylori* alone was not sufficient to promote neoplastic transformation. Mice mono-associated with the bacteria developed gastritis and subsequent neoplasia at a much slower rate than their fully colonized counterparts, suggests that *H. pylori* may require cooperation by other commensal microbiota members. In other cases an entire dysbiotic microbiome community was suggested to drive tumor development. One such example is colorectal cancer (CRC) that is transmissible by dysbiotic microbiota[5,6]. As such, germ-free mice are partially protected from disease, and treatment with broad-spectrum antibiotics ameliorates cancer development. Identifying the pathogenic bacterial "drivers" of cancer in these cases and differentiating them from secondary microbial alterations remains a major challenge to the field.

Most studies have focused on the effect of the microbiota on gastrointestinal cancers and these are reviewed in detail elsewhere[7-11]. In this review, we will discuss research into the carcinogenic properties of microbial agents in the non-gastrointestinal organs. We review these associations per body-site, and highlight the substantial effect microbial involvement may have on all stages of cancer development in the skin, breast, urogenital tract, lung, liver and pancreas.

SKIN

The human skin is the largest organ in the body and hosts a complex and heterogeneous microbiota. Until recently, the studies of the skin microbiota focused on bacteria, using culture-based assays. However, it has recently been appreciated that the skin is inhabited by a massive, unculturable bacterial ecosystem, as well as by fungi and viruses[12-14]. Together, these comprise the skin microbiome, which may have diverse effects on a multitude of skin-specific physiological and pathophysiological processes, including ones that promote the development of skin cancer.

Viral involvement in skin cancer

The skin virome has rarely been investigated, in part because most skin-associated viruses are not culturable and do not display consensus sequences that can be used for high throughput next generation sequencing techniques[15]. Several viruses are known to inhabit the healthy skin, but can also induce malignant transformation.

The papilloma virus family: Papilloma viruses (PVs) infect undifferentiated keratinocytes in the basal layer of the stratified squamous epithelia, and in the cutaneous and the mucosal levels. Oncogenic PVs, including the human papilloma virus (HPV-16), are responsible for nearly all cases of cervical and anal cancer[16]. PVs are commonly part of the skin and mucosal microbiota of healthy individuals, suggesting commensalism or mutualism between PVs and their host cells[17]. Moreover, the majority of HPV infections are subclinical and do not cause any physical lesions[17]. However, in some cases chronic inoculation is established through immune escape mechanisms, and a low yet persistent amount of virions in produced. The oncogenic features that allow the virus to induce cell transformation are dependent on the virus's E5, E6 and E7 oncogenes, which are exclusively present in oncogenic PVs. The E6 protein in

Table 1 The role of the microbiota in non-gastric cancers

Cancer	Mechanism	Ref.
Protective role		
B16/F10 melanoma and LLC	Microbiota was required for the development of anti-cancer immunity Commensal microbiota was essential for the development and anti-cancer activity of γδ-Th17 cells	[87]
HCC	Microbiota was required for immune system development Commensal microbiota was needed for the development of the immune system in the liver, which enables mice to clear HBV. A chronic infection with HBV is a major risk factor for HCC	[111]
Tumor-promoting role		
Skin cancer	Dysbiosis causes a cancer-stimulating inflammatory response in the host Microbiota-derived Flagellin stimulates TLR5-MyD88 signaling which promotes skin cancer development	[27]
Breast cancer	Upon injection of a carcinogen, GF mice showed a lower cancer burden than SPF mice	[43]
Lung	Dysbiosis causes a cancer-stimulating inflammatory response in the host *E. coli*/LPS in the lungs promotes lung injury and inflammation, which lead to an enhanced metastasis from the primary tumor to the lung	[102]
Ovarian and breast cancer	Dysbiosis inhibits anti-tumor immunity: Gut microbiota of $TLR5^{-/-}$ mice promoted the accumulation of MDSCs at the site of breast and ovarian cancers. MDSCs in their turn suppressed anti-cancer immunity	[44]
Breast cancer	Infection with a gastric pathogen promoted cancer-stimulating inflammatory responses In mice, infection with the gastric bacteria *H. hepaticus* led to an influx of neutrophils in the mammary gland that then promoted cancer. Treatment with antibiotics or the depletion of neutrophils significantly halted cancer development	[30]
Liver	Infection of mice prone to liver cancer with *H. hepaticus* led to a significant enhancement of carcinogenesis	[114]

LLC: Lewis lung carcinoma; HCC: Hepatocellular carcinoma; HBV: Hepatitis B virus; TLR: Toll-like receptor; MDSCs: Myeloid-derived suppressor cells.

oncogenic PVs is able to induce degradation of the p53 cellular protein, thus promoting uncontrolled cell growth. The E5 protein allows for evasion of the host's immune surveillance and decreases the dependence of infected cells on growth factors. Finally, the E7 oncoprotein binds to the tumorsuppressive pRb, dissociating the transcription factor E2F from the pRb/E2F complex. The process induced by these three oncoproteins is slow, with progression from precursor lesions to invasive cancer usually requiring more than a decade[16].

More recently, another HPV member was found to be associated with an unusual form of skin cancer called Merkel cell carcinoma (MCC)[18], concisely reviewed in[19]. The Merkel cell polyomavirus (MCPyV) causes a rare but aggressive form of skin cancer and is present in about 80% of MCC tumor specimens. The MCPyV genome was shown to integrate into the cellular DNA of some MCC tumors and their metastases. A majority of MCC tumors also display constitutive expression of the MCPyV large T-antigen oncoprotein[20]. This suggests involvement of MCPyV in the oncogenesis of MCC.

Bacterial involvement in skin cancer

The skin microbiome contains an entrenched bacterial population, forming a microbiome that features a high spatial and temporal stability[21-24]. The skin contains different skin microenvironments, defined by sebaceous, moist and dry areas and by the different follicle densities[25]. The cutaneous microbiome consists predominantly of 4 bacterial phyla; actinobacteria, firmicutes, proteobacteria and bacteroidetes and six genera, propionibacterium, corynebacterium, staphylococcus, streptococcus and acinetobacter[23,26].

Several studies link commensal skin bacteria to malignant transformation. In one example, antibiotic-treated mice showed an increased susceptibility to B16/F10 melanoma, as well as lewis lung carcinoma, and exhibited a shortened mean survival time, suggesting a protective role of the skin microbiome in cancer development in these models. In contrast, another experimental setting suggested that an intact commensal bacterial population, and specifically flagellated bacteria, may be required for malignant transformation in the murine skin[27]. In this context, toll-like receptor (TLR) 5 and its ligand flagellin linked between chronic inflammation, tissue damage and skin cancer. In the model described, bone-marrow chimeras lacking MyD88 and TLR5 in the hematopoietic cells exhibited protection against a chemical model of wound-induced tumor formation. When mice were treated with a broad-spectrum antibiotic regimen, the skin bacterial load was decreased and wound-induced tumor formation and tumor size were substantially reduced. Topical application of flagellin onto wounds increased tumor incidence in a dose-dependent manner and delayed wound closure. This indicates that MyD88 and TLR-5 signaling on radiosensitive leukocytes is required for tumor formation. Together, these examples suggest that the skin bacterial microbiome can play either a protective or a harmful role in cancerogenesis, depending on the physiological context and microbial composition.

BREAST

Breast cancer is the second leading cause of cancer-related deaths in women: One in eight women develop the malignancy in their lifetime[28]. Despite considerable and significant progress has been achieved in breast cancer research, in most cases it's etiology remains unknown[29]. Mammary glands are colonized by a distinct microbiota[30,31], but the role of microbial involvement in

breast cancer remains at its infancy.

Viral involvement in breast cancer

Until recently, most studies looking into microbial modulation of breast cancer have been focused on specific viruses. The results, however, remain inconclusive. While HPV infection has been reported by some groups are associated with breast cancer development[32-34], others have failed to find such correlation[35,36]. Some groups have reported that up to 50% of breast tumors to be EBV-positive[37-40], while others have been unable to detect the virus in breast tumors altogether[41,42]. Therefore, additional studies are needed to clarify the potential contribution of viral infections in breast carcinogenesis, and its modulatory mechanisms of activity.

Bacterial involvement in breast cancer

In parallel to viral infections, a number of studies suggest a link between bacterial infections and breast cancer. Involvement of the commensal microbiome was first suggested in a study in which injections of a carcinogen (DMAB) in various body sites of germ free rats resulted in a significantly lower cancer burden in the breast tissue and colon, but not in the skin, as compared to conventionalized rats[43]. Of note, this study did not delineate whether the observed effects were linked to the local breast microbiome, or to distal microbial communities such as that of the gut. More recent studies have sought to clarify this issue. Xuan *et al*[31] surveyed the microbiota in tumors or normal adjacent tissues from 20 estrogen receptor (ER)-positive breast cancer patients as well as in tissue from healthy donors. This study indicated that there is a 10-fold decrease in the absolute numbers of bacteria between cancer and control tissues. Moreover, the authors observed changes in the compositional abundance of bacterial species in tumor compared to control tissues. While the genus *Sphingomonas* was found to be more abundant in normal tissues, the tumor tissue hosted *Sphingomonas yanoikuyae* in increased numbers. Other members of the skin microbiome, such *Staphylococcus* and *Corynebacterium*, did not vary significantly between normal and tumor tissues. Nonetheless, these data suggest that mammary tumors bear a different microbial composition than the normal tissue. Significant microbial-associated effects on tumor progression are supported by a recent study, which showed an accelerated mammary malignant progression in TLR5-responsive mice. In this model, malignant progression of mammary tissue in p53-ablated and oncogenic K-ras-activated mice was measured on the background of TLR5 deficient mice[44]. Absence of TLR5 signaling in these mice resulted in a divergent microbial composition and reduced tumor progression. In TLR5 proficient mice, on the other hand, microbial signaling through TLR5 increased IL-6 secretion and the number of gamma delta T cells as well as tumor growth. Thus, the commensal microbiome was suggested to be able to induce tumor-promoting inflammation in a TLR5 dependent manner.

While the above studies focus on the whole microbiome composition and not on specific microbial "drivers" or "modulators" of cancer, a study by Lakritz *et al*[30] implicated a specific bacterium, *Helicobacter hepaticus* (*H. hepaticus*), in the progression of mammary malignancy. In this report, mice with a predisposition for breast cancer were infected with *H. hepaticus*. Compared to non-infected controls, infected mice showed increased mammary tumor burden characterized by extensive neutrophil infiltration. Depletion of neutrophils entirely inhibited tumor development[30]. Together, these data suggest that both the whole microbiome composition as well as specific bacteria can contribute to breast tumor progression by promoting inflammation, and that they can do so *via* multiple pathways.

UROGENITAL TRACT

Urogenital cancers include cervical, renal, bladder and ovarian carcinomas. Very few studies focusing on the roles of the microbiota in urogenital tract tumors have been published to date[45]. Nonetheless, there is some emerging evidence towards the possibility that chronic viral infections may promote the development of renal cell carcinoma and bladder cancer.

Cervical cancer

The most frequently occurring and the best studied of cancers of the female urogenital tract is cervical cancer. The most highly associated risk factor for cervical cancer is viral infection by the HPV family. Mucosal HPV serotypes infect the basal epithelial cells of the anogenital mucosa *via* micro-abrasions in the epithelial lining[46]. Vulval, vaginal, penile, cervical, and anorectal areas are affected. Cervical and anal squamous cell carcinoma develop at sites of squamous metaplasia; cervicovaginal and anorectal squamous columnar junctions are therefore especially vulnerable to HPV infection leading to malignant transformation[47]. Although data are limited, antibodies developed during natural infection do not seem to offer full protection against reinfection, possibly because of low or waning titers of the virus[48,49]. By contrast, the available prophylactic HPV vaccines induce high concentrations of neutralizing antibodies - at least two-log scale higher as compared to natural infection-induced concentrations, leading to a better immune memory[50,51]. The efficacy of the anti-HPV vaccine in cervical intraepithelial neoplasia associated with HPV 16 and 18 in women naive for infection is high: 93% (95%CI: 79.9-98.3) for the bivalent vaccine (HPV 16/18) after 35 mo of follow-up and 98% (95%CI: 93.3-99.8) for the quadrivalent vaccine (HPV 6/11/16/18) after 42 mo of follow-up[52-54].

Vaginal cancer

The vagina harbors a unique microbiota that serves as an important line of defense against pathogens, including sexually transmitted infections[55]. The dominant

members of the vaginal microbiome *Lactobacillus spp*. were shown to provide broad-spectrum protection from pathogens through their production of lactic acid[56], bacteriocins (bactericidal proteinaceous molecules)[57], antagonistic bacteriocin-like substances[58], and biosurfactants[59], that can adhere to mucus, a component of the barriers against pathogens[60] and disrupt biofilms[61]. Disruption of the protective microbiota configuration, termed bacterial vaginosis (BV) was shown in numerous studies to correlate with cervical cancer-inducing HPV infections[62-69]. BV affects one in three United States women[70] and is characterized by decrease in protective *Lactobacillus spp*., increased specie richness, and elevated numbers of anaerobic bacteria, including species of *Gardnerella, Prevotella,* and *Clostridiales*[71].

While numerous association studies showed a strong association between dysbiotic disruption of vaginal microbiota (BV) and HPV infections, the mechanistic link between the two events is yet to be explored.

Ovarian cancer

Chronic inflammation was previously suggested to be involved with the pathogenesis of Ovarian carcinogenesis[72], yet this evidence remains sparse. Specific pathogens suggested to be indirectly associated with human ovarian cancer include Chlamydial HSP60-1 IgG and *M. genitalium*, with IgG antibodies specific to these bacteria suggested to be associated with epithelial ovarian tumors in some patient subsets[73]. Possible involvement of impaired host-microbiome interactions in ovarian cancer was suggested from a study utilizing TLR-5 deficient mice that feature a dysbiotic microbiome configuration. These mice showed increased survival rates compare to WT controls when injected with syngenic ID8 ovarian tumor cells[44]. In addition, ovarian tumors of patients who were heterozygous for the dominant TLR5^{R392X} polymorphism showed negligible induction of IL-8 transcript levels but significantly higher IL-17A transcript levels in response to flagellin as compared to control population. Furthermore, the proportion of long-term survivors was significantly higher among TLR5^{R392X} carriers, all suggesting that host TLR5 microbe interactions may play a role in ovarian tumor pathogenesis[44].

Bladder cancer

Until recently, the healthy urinary tract was considered sterile and bacterial presence in the urine of patients identified *via* culture-based methods was considered a sign of a urinary tract infection[45,74]. In recent years, however, the emergence of next generation sequencing of the microbiome has established the presence of a urinal microbiome in the healthy humans urinary tract[75-85]. Among them, several works describe the presence of a complex bacterial community with the predominant genera *Lactobacillus, Prevotella* and *Gardnerella*, with a considerable variation featured between individuals[79,83].

Bladder cancer is the most prevalent malignancy of the urinary system. In 2015, it is estimated that 75000 new cases will be diagnosed, and more then 15000 patients will die due from Bladder cancer in the United States[86]. The most important risk factors known for urothelial carcinoma are cigarette smoking and various occupational exposures. The nematode, *Schistosoma haematobium* infection was also associated with the development of squamous cell carcinoma of bladder due to chronic inflammation. Regarding microbiome involvement in urothelial carcinoma, a study comparing the microbiome of urine specimens from healthy individuals and urothelial carcinoma revealed that *Streptococcus* was nearly undetected in normal samples but significantly elevated in 5 out of the 8 cancer samples. *Pseudomonas* or *Anaerococcus* were the most abundant genus in 2 out of the 3 cancer samples where *Streptococcus* abundance was low[87]. While descriptional in nature, this study suggests that urothelial carcinoma may be associated with altered microbiota of the urinary tract. More studies are needed to establish whether microbiome composition plays a causative role in bladder cancer.

Renal cancer

The role of the healthy microbiome on kidney cancer has not been studied to date. However, a number of studies suggested an association between viral infections and risk of renal cancer, yet these remain controversial and at times contradictory to each other[72,88,89]. One virus that has been implicated in RCC pathology is HPV. One study reported that 7 out of 49 RCC samples to be HPV positive[90]. A second study, including histology samples of 122 patients, found 30.3% of RCC tumor tissues to be HPV positive. Of these, 45% were positive for high-risk (HR)-HPVs such as HPV-16 and HPV-18. Moreover, HR-HPV infection correlated with the expression of p16INK4a, a viral immunosuppressant. The authors hypothesized that HR-HPV infection may precede RCC and promote oncogenesis[91]. However, more research is required to test this hypothesis.

In contrast, Newcastle disease virus has been suggested in *in vitro* studies to play a therapeutic role in RCC. This virus preferentially infects cancer cells and, upon infection induces apoptosis *via* the p38 MAPK/NF-κB/IκBα pathway. Similar outcomes were obtained in other cancer types[92]. Future studies are needed to translate these *in vitro* findings to the clinical context.

LUNG

The human microbiota is the body's first interface with environmental exposures. In this sense, the lung microbiota may play an important role in the body's response to airborne carcinogens. The mechanisms of lung carcinogenesis are still not fully understood. The current most important risk factor for lung cancer is smoking. In non-smokers, suggested risk factors include environmental tobacco smoke, exposure to radon gas, cooking oil vapors, indoor coal and wood burning, asbestos, genetic factors, parasitic infections as well as viral and bacterial agents[93] that will be described below.

Viral involvement in lung cancer

As in skin carcinoma, HPV has been associated with lung cell malignancies[94]. Studies suggest a link between HPV infection and lung cancer in non-smoking patients; it has been shown that epithelial changes in bronchial carcinoma closely resembled HPV-induced genital lesions[95]. Similarly to the mechanisms by which HPV contributes to skin cancer susceptibility, it is suggested that the molecular mechanism of transformation by HPV is mediated by its oncoproteins E5, E6 and E7. In addition, *in vivo* data show HPV integration, E6/E7 expression and down regulation of p53 in lung cancers, further supporting this classical oncogenic mechanism[96].

Bacterial involvement in lung cancer

C. pneumoniae is a gram-negative obligatory intracellular bacterium and a common cause of pneumonia[69]. *C. pneumoniae* can also cause other conditions, such as sinusitis, bronchitis, rhinitis and worsening of chronic obstructive pulmonary disease (COPD). However, infection can also be asymptomatic. The involvement of *C. pneumoniae* infection in lung cancer development and risk has been suggested by several studies[97-99]. However, the mechanisms for this association remain unclear[100,101]. Pulmonary infections with gram-negative bacteria have also been suggested to contribute to lung metastasis. Acute lung infection models induced by either infection with *E. coli* or administration of LPS increased cancer cell homing to the lung and enhanced lung metastasis[102]. Moreover, the broncho-alveolar lavage fluid from LPS or *E. coli*-injected mice induced the migration of transformed cells *in vivo*. The tumor cells migratory activity was blocked by AMD3100, a chemokine receptor-4 inhibitor, as well as by amoxicillin, an antibacterial agent. In addition, tracking of the metastatic tumor cell line in the mouse showed that bacteria injection enhanced early localization of the tumor cells to the lung.

The bacterium *C. pneumoniae* is a gram-negative obligatory intracellular bacterium and a common cause of pneumonia[101]. In addition to pneumonia, *C. pneumoniae* can cause other conditions, such as sinusitis, bronchitis, rhinitis, exacerbation of COPD. However, infection can also be asymptomatic. The association of *C. pneumoniae* infection with lung cancer risk has been suggested by multiple studies[97-99], although the mechanisms for this association remain unclear[100,103].

Pulmonary infections with gram-negative bacteria have also been suggested to contribute to lung metastasis. Acute lung infection models induced by either infection with *E. coli* or administration of LPS increased cancer cell homing to the lung and enhanced lung metastasis[102]. Moreover, the broncho-alveolar lavage fluid from LPS or *E. coli*-injected mice stimulated migration of tumor cells *in vivo*. The tumor cells migratory activity could be blocked by AMD3100, a chemokine receptor-4 inhibitor, as well as by the antibacterial agent amoxicillin. In addition, *in vivo* tracking of the metastatic tumor cell line showed that bacterial injection enhanced early dissemination of the tumor cells to the lung.

In contrast, antibiotics-treated mice were shown to be more susceptible to tumor development in the lungs after inoculation with B16/F10 melanoma or a lung carcinoma cell-line. In this model, commensal bacteria were found to be essential for the function of γδ-Th17 cells in the lung, and the absence of these cells increased the susceptibility to lung carcinoma and B16/F10 melanoma development[87]. This indicates, that the antitumor defense of the host through γδ-Th17 cells is dependent on an intact microbiome composition. Therewith, lung cancer is an excellent example of the healthy microbiome playing a protective role in tumorigeneis, whereas when dysbiosis develops, pathogenic or pathobiont bacteria may promote, in certain contexts, cancer development.

LIVER

Primary liver cancer is the fifth most diagnosed form of cancer in males, and the second most frequent cause of cancer death worldwide. Seventy percent to ninety percent of the primary liver cancer cases can be classified as hepatocellular carcinoma (HCC)[104,105]. With mortality to incidence ratio of 0.95, the prognosis for patients with HCC is extremely poor[105]. In developed countries, chronic hepatitis B virus (HBV) and hepatitis C virus (HCV) infections account for approximately 43% of cases. However, the majority of patients develop HCC secondary to alcoholic liver disease (ALD) and non-alcoholic fatty liver disease (NAFLD)[104]. It has recently been suggested that the microbiota plays an important role in HCC development. Although the liver, under normal conditions, is considered sterile, its environment is greatly influenced by the nutrients, metabolites and also toxins and pathogens derived from the gut *via* the portal vein. Therefore, the composition in the gut microbiota can greatly influence the functioning of the liver, by its myriad metabolic activities regulating the gut liver axis. Indeed, recent studies have suggested that the composition of the gut microbiota can both influence the development of diseases predisposing to HCC such as chronic HBV and HCV infections, ALD and NAFLD, and the transition from these diseases into HCC[106,107], yet the mechanisms driving these effects remain elusive.

Viral contribution to HCC

The majority of HCC cases occur in patients previously suffering of chronic hepatotrophic viral infection, mainly HBV and HCV[108]. A unique feature of HBV infection is that while 95% of adults are able to spontaneously clear the virus, over 90% of neonates and approximately 30% of children aged 1-5 develop persistent infection[109,110]. Possible involvement of the microbiota in this phenomenon was suggested from a study in which mice treated with oral antibiotics for 6 wk prior to HBV infection were no longer able to rapidly clear the virus[111]. Further experiments indicate that the microbiota in

young mice may induce HBV tolerance in the liver *via* LPS-TLR4 mediated secretion of IL-10 by kupffer cells (KC), whereas the mature microbiota shifts this balance towards clearance of the virus by stimulating KC-dependent lymphoid organization and tissue priming in the liver[111,112]. Future studies are needed to further validate this interesting association, and to determine possible roles of the gut microbiota in the clearance of other hepatotrophic viruses such as HCV.

Both HBV and HCV infections contribute to the development of HCC by promoting a pro-inflammatory liver micro-environment, affecting cell cycle regulation and inducing ER stress, as has been extensively reviewed elsewhere[113]. However, a study by Fox *et al*[114] indicated that colonization with *H. hepaticus* in the gut was sufficient to promote HCC in HCV-transgenic mice, in the absence of either translocation of *H. hepaticus* to the liver or overt hepatitis. From its niche in the intestinal mucosa, *H. hepaticus* activated NF-κB dependent networks associated with innate and T-helper 1 (Th-1)-type adaptive immunity, both in the intestines and in the liver. The resultant transcriptional changes promoted the development of pre-neoplastic and neoplastic liver foci in mice bearing an HCV transgene, while neither factor by itself was sufficient to induce tumor formation[114]. This demonstrates that *H. hepaticus* can alter hepatic immune regulation from its intestinal niche, in a manner that synergizes with viral tumorigenic factors. More research is warranted to uncover whether other changes in the intestinal microbiota can induce similar effect.

Bacterial contribution to HCC

Chronic alcoholic consumption is considered a major risk factor for chronic liver disease and HCC. Already in 1991 it was noted that patients with alcoholic cirrhosis displayed far higher levels of serum endotoxin than those with non-alcoholic cirrhosis, suggesting that alcoholic cirrhosis is associated with impaired intestinal barrier function[115]. Likewise, treatment of rats with antibiotics targeting Gram-negative bacteria drastically decreased serum endotoxin levels and liver injury in ethanol-fed rats[116]. These suggested that some of the features of chronic alcohol toxicity may be mediated by gut-associated pathogen-induced molecular patterns released by the gut microbiota. These findings were further confirmed, with the finding that germ-free mice, which are devoid of a microbiota, are protected from ethanol-induced liver disease[117]. Moreover, transplanting microbiota from alcohol-fed mice into naive germ-free mice was sufficient to induce liver injury and inflammation. Furthermore, excessive alcohol intake lead to dysbiosis by an overgrowth of Gram-negative bacteria, that caused increased gut permeability. As leaky gut leads to increased availability of bacterial metabolites to the liver, as well as pro-inflammatory molecules such as bacterial toxins, LPS and even living microbes, this may explain how alcohol-induced dysbiosis could lead to ALD. Indeed, feeding mice a high-fiber diet partially prevented alcohol-induced dysbiosis, decreased gut permeability and mitigated the damage to the liver[117]. This model is supported by preliminary data from patients with alcoholic cirrhosis that show an increase in Gram-negative bacteria as well as an increased bacterial translocation to the liver[118], suggesting that microbiota-targeting interventions may potentially mitigate alcohol-induced liver damage.

Alcohol-induced liver cirrhosis is characterized by cellular injury, inflammation, and fibrosis coupled with compensatory cell growth and proliferation, conditions promoting tumor development[119]. Furthermore, ethanol can induce epigenetic changes in hepatocytes that lead to tumor formation[120]. There is evidence that ethanol-mediated TLR4 signaling is crucial in the dedifferentiation of hepatocytes seen in HBV/HCV- and ALD-associated HCC. Ethanol-induced hepatic translocation of LPS and gram-negative bacteria may further synergize with these direct effects in activating the innate immune response. In agreement, diethylnitrosamine-induced liver cirrhosis was accompanied by dysbiosis. When treated with probiotics, a reduction in gut permeability and intestinal inflammation was observed together with a reduced incidence of cirrhosis and HCC in this model[121]. Together, these studies suggest that ALD-associated dysbiosis may contribute to HCC susceptibility.

NAFLD, a component of the "metabolic syndrome", is rapidly becoming a common cause of chronic liver disease in both developed and developing countries[122]. While most patients with NAFLD feature isolated liver steatosis, in approximately 20% of cases NAFLD evolves into non-alcoholic steatohepatitis (NASH), a progressive liver disease involving a combination of steatosis, hepatocellular damage, inflammation and fibrosis. NASH has the potential to develop into cirrhosis, which is a major risk factor for HCC as described above. In addition, there has been also a rising incidence of NAFLD-associated HCC in the absence of cirrhosis[122,123]. It is unclear to what extent the pathophysiology for NAFLD-associated HCC in the absence of cirrhosis differs from that in a cirrhotic liver. The microbiota has been described to contribute to this *via* two distinct pathways, which may play a role in either situation.

Obesity has been associated with dysbiosis and increased gut permeability, allowing for more LPS to translocate into the liver, where it can trigger TLR signaling resulting in NF-κB-dependent transcription of TNF-α, which drives NAFLD and NASH progression[106,124]. This state is further aggravated as leptin-mediated up-regulation of CD14 leads to hypersensitivity to LPS-signals in obese patients[125]. Furthermore, microbiota-induced TLR9 signaling and dysbiosis-induced repression of inflammasome signaling can further promote the development of NAFLD and NASH[106]. In this context, increased activation of Kupffer cells *via* TLR4 further exacerbates steatohepatitis[126] and promotes the activation of hepatic stellate cells (HSCs). Activated HSCs, in turn, can contribute to liver fibrosis as well as secrete EGFR, which leads to increased proliferation of HSCs and may promote tumor formation[127]. Nonetheless, a study

by Dapito *et al*[128] suggested that the gut microbiota may not be required for HCC initiation. Instead, it plays a major role in the progression of the disease, as TLR4 signaling was able to increase the expression of the hepatomitogen epiregulin as well as to promote proliferation and prevent apoptosis. Both sterilization of the gut during late stages of HCC as well as using a TLR4$^{-/-}$ model greatly reduced the progression of HCC, suggesting new avenues of treatment[128].

Whereas mainly gram-negative bacterial LPS drives the pathways above, a second and independent process has been suggested to primarily depend on Gram-positive bacteria. Yoshimoto *et al*[129] showed that an HFD-induced overgrowth of Gram-positive bacteria with the ability to produce the secondary bile acid deoxycholic acid (DCA) *via* 7α-dehydroxylation of primary bile acid lead to a marked rise in serum DCA levels[129]. DCA is known to cause DNA damage through the production of reactive oxygen species, as well as to promote liver carcinogenesis[130,131]. Furthermore, DCA can induce a state of senescence accompanied by the secretion of specific chemokines, called the senescence-associated secretory phenotype (SASP)[132,133]. Indeed, the authors showed DCA is able to induce SASP in HSCs *in vivo*. This phenotype then promoted HCC development in mice treated with a chemical carcinogen. By blocking DCA production or treating mice with vancomycin, an antibiotic preferentially targeting Gram-positive bacteria, the induction of SASP and the progression of HCC could almost completely be blocked. When antibiotic treatment was supplemented with DCA, the beneficial effect was lost. It should be noted, however, that treating lean mice with a carcinogen and DCA was not sufficient to enhance HCC development. This suggests that additional, obesity-associated tumor-promoting factors may be required[129]. Nonetheless, some preclinical studies have showed that probiotic treatment can substantially alter bile acid levels by increasing fecal secretion and enhancing hepatic bile synthesis[134]. In humans, who unlike rodents cannot revert DCA into cholic acid, DCA can accumulate until it represents > 50% of the total bile pool[135]. Enhanced secretion of bile acids accompanied by hepatic bile synthesis might be a clean way to lower DCA levels, which in turn may substantially decrease the progression of HCC.

Increasing evidence suggests that the microbial composition plays a crucial role mediating liver damage in response to hepatitis infections, excessive alcohol intake or obesity. As HCC rarely occurs without previous liver disease, modulating the microbiota to prevent primary damage would be a potentially effective method of HCC prevention. However, even after liver disease has developed, the microbiota plays an important role in its progression and in creating a tumorigenic environment, through bacterial signaling *via* toxins, LPS and metabolites. Although the bile acid-driven pathophysiology seems specific to NAFLD-associated HCC, the LPS-TLR4 pathway appears common to all cirrhosis-associated HCC entities. While human microbiome-HCC clinical correlations remain preliminary, they represent a potential new avenue for HCC prevention and treatment, which merits further studies.

PANCREAS

Pancreatic cancer is associated with a poor outcome due to its rapid dissemination through the lymphatic system. This aggressive biology combined with a lack of biomarkers for early detection and resistance to conventional therapy results in a 5-year survival rate of only 5%[136].

Suggestions for possible microbial involvement in pancreatic cancer comes from studies that found an epidemiological association between periodontitis and tooth loss, and the risk for pancreatic cancer[137,138]. However, a study by Stolzenberg-Solomon *et al*[139] trying to correlate this with a specific bacterium known to play a role in tumor formation, *Helicobactor pylori*, was unable to validate the association. Unlike Stolzenberg, Farell *et al*[140] decided to study the entire composition of the oral microbiome in relation to pancreatic cancer. In this work, researchers identified a total of 56 clusters of bacterial species to be changed significantly between patients with pancreatic cancer and healthy controls. A combination of two bacteria, *N elongata* and *S mitisas*, was suggested as a possible biomarker for the detection of pancreatic cancer. Although these results may allow for a better detection of pancreatic cancer, further research is required to understand whether these changes in the oral microbiota are causative and contribute to the pathogenesis of pancreatic cancer.

Mitsuhashi *et al*[141] took an entirely different approach in studying the role of the local pancreatic microbiota in cancer development. From a large databank of pancreatic cancer tissue specimens, they tested samples for the presence of an oral microbe group, *Fusobacterium*, in the pancreatic tissue. Members of *Fusobacterium* have been implicated in periodontitis as well as pancreatic abscesses and CRC[142,143]. Mitsuhashi *et al*[141] detected *Fusobacterium* in 8.8% of the samples. Despite a lack of correlation between these taxa and the molecular characteristics of the tumor tissue, the *Fusobacterium*-positive patients featured a higher rate of cancer-associated mortality than those without detectable microbial inoculation[141].

While these two studies suggest there may be some role for the oral and/or local pancreatic microbiota in the pathology of pancreatic cancer, they remain purely correlative. Further research is required to determine the causative role and mechanisms of activity through which microbial infection influences the occurrence or progression of pancreatic cancer.

CONCLUSION

Among the microbes affecting cancer development and progression, viruses are a major pathogenic cause of carcinogenesis in non-gastrotintestinal tumors, through

some well-established molecular mechanisms[144]. In addition, specific bacterial pathogens have been described to induce or contribute to carcinogenesis in these entities. Bacterial mechanisms implicated in carcinogenesis include directly DNA-damaging toxin secretion, induction of chronic inflammation and suppression of immune cell activation[4,145,146]. However, the promotion of cancer formation can also result from compositional and functional changes in the microbiome configuration as a whole. When considering the role of the microbiome in cancer, a distinction should be made between local and systemic microbiome-associated effects. Currently, most studies focus on local effects of organ-specific microbiomes, such as those illustrated for the lung microbiome that may contribute to the development of lung cancer. Nonetheless, research into HCC revealed that the gut microbiota might influence carcinogenesis at distal organs, such as the sterile liver. In this particular case, the liver is anatomically linked to the gut *via* the portal vein, thereby efflux of gut microbiome-secreted or modulated metabolites may provide a mechanism linking gut microbes to hepatic carcinogenesis. However, multiple other studies[147,148] recently suggested that the gut microbiome is also able to induce systemic and long-term changes in the immune system, thereby providing possible mechanisms by which one microbiome may contribute to cancer pathogenesis even in anatomically distinct organs.

Another focus of intense research is aimed at deciphering the mechanisms governing microbiota composition, which are currently thought to be determined by a balance between the state of the host and particularly its immune system and the microbial configuration that inhabits it[106,107,149]. When one of these components is disturbed the altered "dysbiotic" microbiota may contribute to the emergence of multifactorial disease, yet the mechanisms regulating these alterations and their consequences remain elusive. Indeed, in this review, we show examples for how alterations in host microbiota interactions may be involved in cancer promotion and progression. For example, Host immune alterations, such as TLR-5 deficiency may lead to tumor progression by its altered microbiota components[128,150]. In other cases, a single microbial component, such as Hepatitis B or C virus, may directly promote carcinogenesis[108]. Finally, we provide examples suggesting that a pathogen or a pathobiont may alter the whole microbiota composition and function, thereby indirectly promoting cancer development. For instance, multiple independent studies showed that the composition of the vaginal microbiota differs between individuals infected or uninfected with HPV[65,112]. Future studies merit elucidation of causality of these associations in promoting carcinogenesis, as well as delineating the mechanisms driving these effects.

Another currently unanswered question relates to the nature of microbe-microbe interactions in driving homeostasis or cancer susceptibility. Until recently, involvement of microorganisms in cancer development was an area of research dominated by studies implicating viral agents. This has recently changed as studies focusing on bacterial composition suggested that the bacterial microbiome may be involved, at steady state, in prevention of tumor development and when altered may participate in carcinogenesis. Studies focusing on the roles of interactions between the viral and bacterial microbiome components (such as phages affecting the composition of the bacterial microbiome) will add yet another complexity to our understanding of host-microbe interactions in cancer and merit further studies.

In summary, the host and the microbiome are increasingly regarded as two integral components of the "holobiome", and extensively interact through a complex communication network. As such, the host and its microbiome continuously affect each other and cooperate in inducing and maintaining a healthy steady state homeostasis. Alterations of the host-microbiome communications results in breech of normal interactions, and when coupled to host germ-line encoded disease susceptibility risks, may lead to emergence of multifactorial diseases, such as cancer. A more thorough understanding of the underlying mechanisms that govern this balance of protective and cancer-promoting effects of the host and its microbiome will highlight new therapeutic targets, offering novel avenues of therapy. In years to come extensive research will likely focus on the roles of the tumor and organ-specific microbiome in cancer development and progression, effects of one microbiome (such as the gut microbiome) on tumoregenesis in other locations, the effects of microbiome alterations (dysbiosis) on immune function and hence tumor immunity, and the possible roles of other commensal microbial kingdoms, such as fungi, archaea and parasites, and of environmental triggers in cancer biology.

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MINIREVIEWS

Distance caregiving a family member with cancer: A review of the literature on distance caregiving and recommendations for future research

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Abstract

Distance caregivers (DCGs) are a growing phenomenon in the United States Family members are struggling to provide care to loved ones with chronic illnesses such as cancer, from a distance. Unlike local caregiving research, distance caregiving research is limited and inconsistent definitions of distance make it difficult to compare studies. To date, DCGs have not been afforded the opportunities for educational and emotional support that local caregivers have received from the health care teams. Because they are not usually present at medical appointments, DCGs do not receive first-hand information from the health care team about the patient's condition, disease progression, and/or treatment options. These caregivers report feeling left out of important family discussions. They experience anxiety related to the uncertainty of the family members' well-being and guilt related to not being available to help local caregivers more. The challenges of distance caregiving are especially evident when the distance caregiver has a parent with advanced cancer. Family-centered care, attending to the needs of the whole family regardless of their geographic location is critical for quality cancer care. In this manuscript, the sparse literature on distance caregiving is reviewed. Recommendations for future research and for the development of creative technologically advanced interventions for this underserved caregiving population are suggested.

Key words: Distance caregivers; Caregiving; Cancer caregivers; Long distance caregivers; Caregivers

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Core tip: Distance caregivers (DCGs) are an important subset of family caregivers who are understudied and receive little attention from clinical providers. The limited

research has identified that this growing population of caregivers experience anxiety, mood disturbances and distress, yet few, if any interventions for them exist. There is a need for more research to identify the benefits and burdens of DCGs and the impact of this burden on the caregivers, patient and family. Interventions providing support and education for this subset of caregivers must be designed and tested. Advanced technology offers unique strategies to deliver these interventions.

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INTRODUCTION

With the increased mobilization of our society, the number of distance caregivers (DCGs) has continued to grow in the United States to represent about 7 million[1], and is projected to double by 2020[2]. Approximately 15%-20% of all family caregivers are caring from a distance[3]. While much is known about local caregivers, research on distance caregiving is very limited. The negative physical and psychological impact of caregiving upon local family caregivers has been well established and various psycho-educational interventions have been tested to find meaningful approaches to minimizing these negative outcomes[4-6]. However, a subgroup of family caregivers - DCGs have been eliminated from most of the caregiving research and often find themselves marginalized in the clinical arena as well.

According to Bevan *et al*[7], family members are the most important and frequent providers of informal care, yet they are rarely taken seriously. They are treated as secondary or informal caregivers even though some DCGs are the patient's primary caregiver[7]. This lack of recognition is especially difficult when the DCG's recipient is a family member with cancer and the DCG is dealing with worry and uncertainty associated with a life-threatening illness[8].

What is known clinically is that DCGs are rarely able to attend medical visits regularly with their family member. Often DCGs are surprised at the physical and functional changes in their family member who is undergoing cancer treatment or struggling with advanced cancer. These changes can trigger DCG anxiety and distress which may be transformed into anger at the patient's care, questioning the current plan of care or demanding a second opinion[9]. DCG anger and distress can increase anxiety and distress in the patient, local caregivers, and health care team. In the clinical setting, some professionals dread the DCGs' visits and in the United States, professionals have labeled this distance caregiving phenomenon as "the daughter from California", or "the son from New York", depending on the geographic location of the distance caregiver[9].

DEFINITION OF DISTANCE CAREGIVING

There is no consensus on the definition of distance caregiving. The common perception of a distance caregiver is one who lives far away and occasionally communicates with family members to see how their loved one is doing; however, evidence contradicts this assumption.

Several large national surveys of DCGs have reported that while, on average, DCGs do live far away (300-450 miles)[1,10], over half of caregivers surveyed reported visiting the patient frequently (more than once/month)[11].

The early work on distance caregiving was done with caregiving elders and parents with dementia and researchers used mileage to define distance caregiving. In 1988, Schoonover *et al*[12] studied adult children of 100 elderly mothers and described these caregivers as living more than 50 miles away.

Travel time, rather than mileage, has also been used to operationalize distance caregiving in research studies. However, travel time has not specified the means of travel, which complicates the generalizability of the studies. In a secondary cross-sectional analysis of the Family Caregiving Study, Wagner[1] defined DCGs (n = 200) as those living at least 1 h away but found in their study that the DCGs lived an average of 304 miles from the care recipient. Random digit telephone dialing interviews conducted by the National Alliance for Caregiving and AARP in 2004[13], and again in 2009[14], uncovered that 15% and 13% of self-identified caregivers respectively were DCGs based on the definition of providing care and living more than one hour away.

Neuharth *et al*[15] used travel time to explore the role of adult children in caregiving decisions. They examined the association between caregiving responsibilities among siblings and travel time. They classified the distance as < 10 min, 11-30 min, 31-60 min, 61 min to 24 h, and 1 h or more. Not surprising, those requiring greater travel time to reach the parent provided less hands-on care. Similar to the Wagner[1] study definition of DCGs, Koerin *et al*[16] identified DCGs as those living more than 1 h away. However, they did report that their sample lived an average of 304 miles away from their ill loved one, which most likely exceeded the 1 h travel time if travel was by automobile.

A recent definition by Bledsoe *et al*[17] acknowledges that adding distance to caregiving increases the complexity of caregiving. The researchers operationalized the definition of DCG as: "Efforts made by family members to provide for the needs of elderly, often ailing relatives who reside at a location that is sufficiently geographically distant that the caregivers cannot have daily face to face contact with the relative" (page 295).

While this definition may have been helpful in 2010, with the advent of face to face smart phone contact, it may not be applicable in the age of advancing technology. Following an extensive and systematic review

of the literature on long-distance caregiving, Cagle *et al*[18] suggested that the definition by Parker *et al*[19] is most comprehensive. It appears to be appropriate today and could apply to DCGs caring for a loved one with cancer. Their definition is: "Anyone (1) who provides informal, unpaid care to a person experiencing some degree of physical, mental, emotional, or economic impairment that limits independence and necessitates assistance; and (2) who experiences caregiving complications because of geographic distances from the recipient, as determined by distance, travel time, travel cost, personal mobility problems, limited transportation, and other related factors that affect the caregiver's access to the care recipient (page 391)".

Mazanec[20] conducted a small study (n = 80) comparing anxiety and depressive symptoms in local and DCGs of parents with advanced cancer and initially defined distance caregiving by mileage, based on previous studies. However, DCGs and their care recipients self-reported that distance was not based on either mileage or travel time, rather on perception of being "at a distance". Those self-identified DCGs were living 1 h away or more, by automobile, making 2 h of travel time for a visit or medical appointment challenging. Many had to arrange child care or take time away from work, limiting their ability to visit as frequently as they would like to.

While there is not a consensus on how to define the distance in distance caregiving, it would seem that perception of distance by the caregiver or care recipient warrants the critical factor in defining distance. Based on the few studies that exist, it also seems that being 1 h or more away by any means of travel complicates the DCGs schedule and supports the perception that they are "long-distance" rather than local caregivers.

Demographics and role of DCGs

The limited research that has been done has identified that the typical DCG is middle-aged, married, and has children[10,16,20]. Most DCGs provide care to a relative, usually a parent. Some studies found the majority of DCGs were female[13,20]; however, others have found the majority to be male[16]. Additional demographics show that most DCGs are employed full-time, are highly educated (college degree or more), and affluent[8,13,16].

Because little is known about the distance caregiving experience, literature on local caregiving may help provide some insight as to the role of distance caregiving. A nonprofessional caregiver's role is to help someone with physical care or cope with disease[22]. The tasks associated with local caregiving are complex and include providing hands-on physical care, helping with daily and weekly household chores, transporting to medical appointments, managing financial affairs, and providing emotional support[23].

DCGs are unable to provide hands-on physical care on a regular basis. However, they may be able to help with daily and weekly household chores from a distance if they have the resources to coordinate help for these chores and/or financially support hired help to assist the patient. Findings indicate that a majority of DCGs are significantly involved in not only making care decisions, but in managing daily aspects of their family member's care, such as arranging transportation, assisting with care coordination, and providing respite for local caregivers[10,11,24,25]. Many DCGs report taking over the management of the patients' financial affairs[15,26]. This eases the burden on local caregivers and gives the DGC a sense of involvement.

Probably the one of the most important roles of DCGs is that of providing emotional support to both the care recipient and the local caregivers. Providing emotional support from a distance can be a challenging responsibility, especially when the patient has advanced cancer and the entire family is struggling with the diagnosis, treatment sequelae, and prognosis. Knowing how to be an effective listener, provide hope while maintaining trust, and empower the patient to manage care takes skill. It is important to note that although caregivers have described the burdens associated with providing hands-on physical care or tangible assistance, many caregivers have reported that the provision of emotional support can be more burdensome than physical care[4,27].

BURDENS OF DISTANCE CAREGIVING

Much of the local caregiving research has focused on caregiver stress and burden. Caregiver burden is known to negatively influence the physical and psychological well-being of the caregivers[4-6]. Psycho-educational interventions have been designed to assist the local caregiver in the role and to provide support.

Like local caregivers, DCGs experience poor physical and psychological wellbeing related to caregiving. In addition, DCGs experience the added stressors associated with caring from a distance. Uncertainty as to when to visit, especially if resources for travel are limited, causes worry and distress. Additional financial burdens occur because of travel expenses and time away from employment. Psycho-educational and supportive interventions for DCGs are limited to popular press materials and internet websites for caregivers.

Burden on DCG psychological well-being

Schoonover *et al*[12] reported that more than half of the 50 DCGs studied reported feeling helpless and anxious and 80% reported "at least some strain" caused by living away from the patient. Koerin *et al*[16] described that almost 80% of DCGs reported stress related to feelings of inadequacy regarding how to assess the status of their loved one and uncertainty regarding how their loved one is progressing in terms of their illness. These additional sources of stress are felt to relate to evidence that DCGs are at greater risk than local caregivers for unrelenting anxiety and mood disturbances[20].

Financial burdens of distance caregiving

While a majority of DCGs are employed, almost 50% report having to rearrange their work schedules to accommodate activities related to caring for their loved one from a distance. Some have reported having to turn down a promotion and give up work benefits[16]. More than one-third report consistently missing days of work for caregiving responsibilities and in addition to lost work, they spend an average of $400-$700/month on travel and out-of-pocket expenses, depending on how far away they lived[16]. In a telephone survey of local and DCGs, DCGs had annual expenses of $8728 compared to $4570[28].

Distance caregiving burdens and cancer caregiving

A diagnosis of cancer is a major life stressor for the person with cancer and the whole family, including those living far away[29,30]. With the advent of many new cancer treatment options, patients with advanced cancer and their families are struggling with the prolonged and difficult course of the disease. Caregiving burdens not only have increased in intensity, but are being experienced over longer periods of time. Depending on the type of cancer and expected disease trajectory, fears related to prognosis and treatment options are overwhelming. Caregivers worry that they may be unable to manage pain and symptoms[4-6]. While patients have reported fears of cancer recurrence and uncertainty about the future, so have their local family caregivers. DCGs of family members with cancer experience these same worries and fears but often with limited social support or support from the oncology team.

There is limited research available on DCG of patients with cancer. In the previously cited small study comparing local and DCGs, Mazanec[20] found that distance caregiving was statistically significantly associated with caregiver anxiety and total mood disturbance. In addition, the distress scores reported exceeded the threshold for requiring intervention as established by the National Comprehensive Cancer Network Distress Guidelines[21] and the distress scores of DCGs were higher than those of local caregivers.

In the qualitative component of this mixed-methods study, DCGs (n = 14) described stress, uncertainty, guilt, and anxiety specifically related to their distant geographic location and in addition to the known stress of having a family member with advanced cancer. DCGs felt disconnected from their family and the oncology team, and "out of the loop" because they were not able to come to the cancer center with their parents or visit as often as they would like. Many struggled with anxiety over when to plan a visit, especially if travel resources were limited, if they would be able to get home quickly enough in time of need, or how to be helpful to their parents and local family members[8,20]. They relied on information from the patient and/or local caregivers and many DCGs believed information was withheld from them because their parents didn't want them to worry[8].

BENEFITS OF PROVIDING DISTANCE CAREGIVING

Benefits of caregiving, in addition to the burdens, have been reported from research findings on local caregivers[5,6]. Like local caregivers, DCGs have reported finding meaning and purpose in providing care[1,8,10,24,25]. Some DCGs have described a sense of personal satisfaction from being a caregiver and others noted the commented on the rewards of fulfilling a responsibility to their family.

Interestingly, some DCGs of persons with cancer reported that there is a unique benefit to be a DCG in that they don't have to deal with the cancer on a daily basis like the local caregivers may have to. These DCGs remarked that their telephone conversations with their parent could be about "everyday" things like ball games and grandchildren's recitals[8]. They enjoyed having this long-distance relationship with their parents which offered them the opportunity to talk about life rather than illness.

INTERVENTIONS FOR DISTANCE CAREGIVING

Although a number of psychosocial and educational interventions have been developed to help local caregivers with their tasks and minimize their caregiver burden, little has been developed to assist the DCGs. The limited research has identified DCGs want to feel more connected to the patient, family, and health care team, have the same access to information about the patient's disease trajectory and treatment options that local caregivers have, and have a system of support for the unique needs of DCGs[8,20].

Advancing technology should be able to provide the needed support to DCGs. The world-wide internet offers the best alternative to in-person support. While no one has examined the use of telemedicine with the patient and caregiver population, there is literature addressing the use of this type of technology for physician-to-patient communication in various settings as for patient-to-family communication in the home and in long-term care facilities for patients with dementia[31,32]. The key benefits of the use of telemedicine in these situations were a sense of closeness[32].

While significant evidence exists that distance caregiving is a growing phenomenon-one that brings untoward negative effects-little empirical work exists that examines feasible approaches to minimizing these negative effects upon these vulnerable caregivers. A recent pilot study (n = 7) using video-conferencing, offered DCGs the opportunity to communicate with their loved one, the local caregiver, and members of the healthcare team-in "real time" during oncology office visits. The researchers examined the effect of this intervention upon patient and distance caregiver anxiety and distress while also determining if the intervention was feasible and acceptable within the healthcare system[33].

The pilot study utilized a convenience sample of patients with advanced brain or lung cancer from a large Midwestern National Comprehensive Cancer Center. All individuals enrolled in this pilot study received the videoconference link for smart phone or tablet and were connected electronically into a single office visit that included the patient, oncologist, nurse, and local and distance caregiver. The average age of the patients was 60 and DCGs was 42. Most of the patients were female and most of the DCGs were male. The majority of patients and caregivers were Caucasian (71.4%) and married (85.7%; 57.1% respectively). Although only 28.6% of patients were employed, 100% of DCGs were employed. The average distance between the patient and DCG was 1059 (819.7) miles.

Although the pilot sample size was small, significant reductions in DCG anxiety and distress from pre- to post-videoconference meeting were identified. The pilot demonstrated the feasibility as well as the efficacy of the intervention but did not examine whether efficacy was maintained over time. Oncologists' reports of acceptance were very high, with scores averaging 2.83 (0.41) on a 1-3 scale when asked if they would like to use this technology with more patients. Anecdotal comments from oncologists included these: (1) "This was extremely helpful as this patient has some cognitive and compliance issues and his wife works out of town, *etc*. and I could tell that she really appreciated this aid"; and (2) "I was afraid that this would take more time - having another person involved in the office visit, *etc*. I found that I spent less overall time since the DCG didn't call me after the meeting asking questions and asking for clarification[32].

FUTURE RESEARCH

Much work remains to be done to understand the benefits and burdens associated with distance caregiving and to develop interventions to address those burdens. As the phenomenon continues to grow, supports must be put in place to deal with the stressors of this new role and to meet the educational needs of DCGs as we have done for local caregivers.

Research that has been done has often been limited to surveys and secondary analyses of large data sets with the inconsistent operationalization of distance. Most of the work has been descriptive and has been done with DCGs of elderly parents or patients with dementia. Although there is still a need for descriptive studies with larger sample sizes from different populations, it is time to move beyond the descriptive. Research is needed to demonstrate the effects of the known DCG anxiety, mood disturbances and distress on the patient and local caregiver. Quality cancer care addresses the needs of the whole family, regardless of the geographic location of the family members.

Longitudinal research and mixed methods approaches will give us the information we need to prioritize DCG needs over the patient's disease trajectory. This is especially critical for DCGs of patients with cancer, who now are living longer and have the potential for more intense and burdensome caregiving needs. The findings from this work could lay the groundwork for interventional research for DCGs of patients with cancer and other life-limiting chronic illnesses.

Most importantly, intervention studies, using a randomized controlled design are needed to advance the caregiving science. Knowledge gained from psychoeducational interventions for local caregivers can be applied to DCGs. Advanced technology can provide the strategies for delivering these interventions. Development of evidence-based DCG internet services can provide educational and social networking resources to DCGS around the clock, every day of the week. Caring from Afar[2] is an example of a web-based connection, but few DCGs are aware of its availability and to date, no research has been done on its effects on caregiver psychological wellbeing.

The Douglas *et al*[33] pilot study demonstrated the feasibility of using advanced technology to connect DCGs with family and the health care team. Interventions using smart-phones and/or tablets have the potential to decrease anxiety and distress for not only the DCGs but also for the patients and local caregivers. Larger randomized controlled trials comparing usual care for DCGs with a psychoeducational program including web-based resources and smart-phone/tablet connections into the health care system are needed. Although there are challenges with privacy and institutional firewalls, the benefits of integrating this level of family care far outweigh the challenges. The potential for providing family-centered care through the use of technology is within our reach.

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Symptom management during and after treatment with concurrent chemoradiotherapy for oropharyngeal cancer: A review of the literature and areas for future research

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Abstract

Patients with locally advanced oropharyngeal cancer are at risk for poor outcomes due to the multi-modal nature of treatment and the potential for treatment-related toxicity. Although treatment with concurrent chemotherapy and radiotherapy has drastically reduced the need for a debilitating and disfiguring surgery, treatment related toxicities are often difficult to control. Acute toxicities include mucositis, skin desquamation, depression, cachexia, fatigue and nausea and vomiting. Failure to control these symptoms can adversely affect the patient's ability to complete their treatment regimen. Although there are many promising new treatments in the area of symptom management for this patient population, a review of the literature reflects the need for more research.

Key words: Head and neck cancer; Chemoradiotherapy; Depression; Fatigue; Cachexia; Nausea; Desquamation; Mucositis; Vomiting

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Core tip: Patients receiving chemoradiotherpay for head and neck cancer have significant side effects which can interfere with treatment and negatively affect quality of life. While narcotics are often required to treat mucositis pain, adjuvant medication and preventative measures are necessary. Further research in this area is imperative. Dietitians should be readily available for consultation as proper nutrition is crucial. Treatment for nausea and vomiting in the delayed setting as well as for patients who are more sensitive to chemotherapy, needs further attention. Exercise can successfully treat and prevent depression and fatigue. Close monitoring by a multidisciplinary team can ensure adequate symptom management.

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INTRODUCTION

Patients with locally advanced oropharyngeal cancer are at risk for poor outcomes due to the multi-modal nature of treatment and the potential for treatment-related toxicity. The primary treatment of patients with locally advanced oropharyngeal cancer no longer includes the debilitating and disfiguring surgery of the past. Currently, the primary treatment is a non-surgical, organ preservation approach that avoids permanent alteration of the patient's ability to speak and swallow. Chemoradiotherapy - concurrent chemotherapy with radiation therapy-for 7 wk has replaced surgery as the standard of care[1]. Although organ preservation is possible in many of these patients, both the short- and long-term side effects of chemoradiotheraphy can also be debilitating. Common acute toxicities include mucositis, skin desquamation, depression and anxiety, cachexia, fatigue, nausea and vomiting[2]. When these toxicities are not properly managed they can lead to treatment delays, chemotherapy dose deviations, hospitalizations and poor quality of life[3]. Increased severity of side effects including mucositis, dermatitis and hematological toxicities can have a negative impact on survival rates and quality of life, as well as decrease treatment efficacy secondary to delays in treatment[4]. Research findings suggest that treatment with chemoradiotheraphy has dramatically increased the supportive care needs of the patient with advanced oropharyngeal cancer[5].

Both emotional and physical functioning may be affected in this patient population[6]. One third of patients have a high symptom burden prior to beginning treatment secondary to their cancer. These pre-treatment symptoms include pain, fatigue, distress and disturbed sleep, which have a negative impact on the patient's nutritional status[7]. This necessitates a unique individual symptom profile to be developed for personalized management of each patient[8].

Although treatment with chemoradiotherapy, rather than surgery, has improved the lives of these patients in many ways, it has also dramatically increased their supportive care needs. Managing these symptoms is key to avoiding treatment delays, chemotherapy dose deviations and hospitalizations[9]. Although there are many promising new treatments in this area, a review of the literature reflects the need for more research.

A REVIEW OF TREATMENT AND SUPPORTIVE CARE

Mucositis

Mucositis is a major concern during chemoradiotherapy and can cause pain, poor nutrition and decreased quality of life. In patients receiving aggressive tumor treatment, incidence rates of mucositis as high as 80% have been reported[10]. Mucositis is known to be more severe and last longer in patients receiving chemoradiation compared to radiation alone. The risk of oral mucositis is higher for smokers, the elderly, those with a high alcohol intake and patients with a lower body mass index[11]. The standard of care continues to be good oral hygiene, dietary adjustments (such as avoiding spicy foods) and medication[12].

The pain of mucositis is often described as a burning sensation. Narcotics can help ease the pain, but does not completely eliminate the discomfort, especially during eating. This will often lead to difficulty maintaining adequate caloric intake, leading to subsequent weight loss. Prophylatic placement of a feeding tube is controversial, secondary to the increased risk of esophageal stenosis during radiation if the patient does not continue to swallow or perform swallowing exercises during therapy. Guidelines from The National Comprehensive Cancer Network (NCCN) only support prophylatic placement in patients with significant weight loss, dysphaqia, aspiration, dehydration or comorbiditles that impact the ability to eat or drink[12]. The placement of feeding tubes is not without risk. Complications include bleeding, bowel perforation, infection, potential seeding of tumors and swallowing resistance, all leading to a greater risk of fibrosis and long-term dependence. One randomized study conducted in a curative setting, however, demonstrated that patients who had a feeding tube placed prior to the start of treatment reported higher qualities of life (QoL) following treatment[13]. Another randomized clinical trial in a palliative setting using the prophylactic placement of feeding tubes demonstrated higher post-treatment quality of life in this patient population[14].

Even with high-dose narcotics, patients receiving concurrent chemoradiotherapy for head and neck cancer are still not able to achieve adequate pain control from oral mucositis[15]. Hence, the development of pharmacological treatments for oral mucositis is urgent[15]. Since narcotics are not as effective in controlling burning pain, further research in this area is crucial.

Some treatments, however, have been shown to be effective. The European Society for Medical Oncology guidelines recommend the prophylactic use of Benzydamine, which has been shown to lower the severity of pain associated with mucositis[16]. Additionally, lactobacillus lozenges were also found to reduce the incidence of oral mucositis[17]. Low-level laser therapy (LLLT), which has been used for over a decade in Europe and South America to prophylactically treat mucositis in head and neck cancer patients during radiation therapy, has demonstrated positive results. LLLT, administered three times per week after radiation therapy, is thought to work on the mitochondria to displace the nitric oxide that is generated from radiation therapy. It is now being studied in the United States. The procedure is not presently

reimbursed, therefore, further research is still necessary in this area[18].

Neuropathic pain due to oral mucositis is common[10]. Use of gabapentin or pregabalin has been found to be useful in controlling pain and limiting the use of other narcotics[10]. A study of 155 patients discovered that using a doxepin rinse (25 mg in 5 mL of water) improved mucositis pain when compared to a placebo (pc 0.001). Doxepin, a tricyclic antidepressant, most likely functions locally on nerves in the oral cavity[19]. Based on current literature, it is clear that more research in the area of neuropathic pain and mucositis is warranted.

Currently, there is not a FDA approved cytoprotective agent that reliably reduces or prevents radiation indiced mucositis. There are clinical trials investigating the use of innate immune defense regulators (IDR). Mucositis has been linked to the dysregulation of innate defense mechanisms. This can lead to a cascade of inflammatory action, causing further damange to the mucosal lining[20]. This is still under investigation, but looks to be promising.

The standard of care for oral mucositis continues to be excellent oral hygiene. NCCN guidelines for oral hygiene recommend that patients brush their teeth with a soft toothbrush twice per day, floss once per day and rinse with a bland rinse. A simple bland rinse is saline, sodium bicarbonate and water[3]. Moreover, patient education is an important component. In one study, patients who received an oral care protocol suffered less pain from oral mucositis and reported improved QoL compared to patients in the control group[21].

In general, development of pharmacological treatments for oral mucositis is urgent, in addition to further research for LLLT and IDRs.

Skin desquamation

Skin desquamation is also a major source of discomfort for this patient population. Radiation causes direct tissue injury and inflammation. This leads to an increased expression of epithelial growth factor receptors in keratinocytes, which assist in repopulation[22]. Grade 1 skin desquamation is mild erythema or dry desquamation. Grade 2 desquamation involves erythema and moist desquamation in the skin folds. Grade 3 desquamation demonstrates erythema and more wide-spread moist desquamation[23].

The skin should be dry and clean prior to the patient's radiation treatment. Patients should be counseled to avoid sunlight and skin irritants. Aquaphor, aloe vera, biafine or non-perfumed moisturizers are recommended for dry desquamation. Patients should avoid clothes that rub. Moreover, they should use mild soaps and detergents. Drying gels, zinc oxide past, silver sulfadizine or a chlorhexidine-based solution without alcohol are suggest for moist desquamation. It is important to watch for infections[24].

Depression and fatigue

Current literature supports that depression is a significant problem for patients receiving treatment for head and neck cancer[25-29]. Depression is experienced by 22%-57% of these patients[30]. The incidence of suicide is four times higher in head and neck cancer patients than in the general population[31]. This also contributes to greater difficulty with smoking cessation among smokers, which can indirectly compromise treatment outcomes. Although depression appears to be highest at diagnosis and during active treatment, it may continue for up to 6 mo following treatment, leading to frustration and further isolation since patients are unable to return to their normal activities of daily living[32-35]. One study found a direct correlation between overall symptom severity and the patient's radiation dosage and depression level[36]. The severity of symptoms and depression increased as radiation treatments progressed, peaking around 2 mo from the start of therapy.

Anti-depressants and anti-anxiety medications are often necessary during treatment. Since there are higher rates of mental health issues and elevated risks of suicide in this population, anti-depressants have a positive effect on patients. After assessing a patient's perception of the benefit of including a psychologist in their care, one trial found that integrated psychological care improves care for head and neck cancer patients[37]. Assessment for the presence of psychosocial distress has led to early intervention for patients and their loved ones. Use of The NCCN's Emotional Distress Thermometer assessment tool demonstrated patient distress - worry, fatigue, pain, nervousness and depression[38]. The use of the thermometer prompted the providers to refer their patients with high distress levels to an onsite social worker.

Fatigue is a common treatment-related side effect of chemoradiotherapy and can be more pronounced when patients experience pain. Fatigue tends to peak within the first 1-2 wk following the completion of radiation, and these symptoms can remain an issue for up to 2 years post radiation therapy[39]. It often leads to a sedentary lifestyle during the course of treatment, despite substantial evidence demonstrates that QoL can be enhanced with the use of moderate physical activity[40,41].

Although little research has been done specifically with head and neck cancer patients, the literature supports the use of exercise to improve quality of life in the domain of emotional well-being, self-esteem, social functioning, pain and anxiety in cancer patients during and after treatment[42-50]. Physical activity was shown to be beneficial with depression and fatigue in men receiving androgen deprivation therapy for prostate cancer[51]. Another study also found exercise intervention to be beneficial on psychological distress from cancer patients receiving chemotherapy with low to moderate levels of baseline psychomorbidity[51]. Yet another study demonstrates the positive effects of moderate activity in improving depression and fatigue in cancer patients[52]. A prospective randomized clinical trial compared resistance exercise, walking and a home program with a trainer *via* phone to usual care. Signs of improvement ($P < 0.05$) were seen in mental health, night time sleeping and

strength[53]. Capozzi *et al*[54] concluded that progressive strength training programs are feasible for head and neck cancer survivors during and following treatment. Such programs are associated with improved acute and chronic fitness outcomes and symptom management.

More research is needed in the area of exercise and depression, as well as the use of exercise as a tool to prevent fatigue and depression in head and neck cancer patients[55].

Cachexia and sarcopenia

Head and neck cancer patients experience one of the highest rates of malnutrition, with 25%-50% being classified as nutritionally compromised prior to initiation of treatment[56]. A study comparing elderly patients (over 65) with those under 65 found that the elderly patients under intensive nutritional support were able to tolerate aggressive therapy as well as the group under 65[57].

The literature supports that early and intensive nutrition intervention can minimize weight loss and physical functioning while improving overall QoL[58,59]. Another study found that nutritional counseling had an equal or greater benefit than oral supplementation, thereby highlighting the importance of dietitians on the multidisciplinary team[60].

Ensuring adequate nutritional support through regular nutrition therapy in combination with oral supplements has been shown to decrease the incidence of mucositis, dysphaqia and skin desquamation[61].

Sarcopenia (the isolated loss of lean body mass) has been shown to negatively affect locoregional control, overall survival and disease specific survival[62].

Nausea and vomiting

Nausea and vomiting can affect a patient's ability to tolerate and complete treatment. The risk of post-treatment nausea and vomiting is higher in patients under 40, those with a prior history of chemotherapy induced nausea and vomiting (CINV), high pre-treatment expectations and those with weight loss of greater than 5% from their baseline. Alternatively, patients with a history of alcohol abuse have a lower incidence of CINV[63]. Our own clinical practice has demonstrated that a history of motion sickness and nausea during pregnancy can also be predictors of difficulty in controlling chemotherapy induced nausea. Pre-treatment assessment for the presence of such risk factors is as vital as prophylactic treatment of nausea and can help increase the likelihood of treating nausea while preventing anticipatory nausea[64].

Since more intensive treatment regimens integrate concurrent chemotherapy along with radiation, nausea and vomiting associated with these treatments have become a greater concern in recent years. Following standard prevention guidelines based on emetogenicity can help reduce CIVN, but complete prevention remains challenging because of individual patient factors[65]. NCCN guidelines recommend that chemotherapeutic agents deemed as moderate to highly emetogenic, such as platinum based agents, should be administered with prophylactic antiemetics from different drug classes. These should include a 5-HT3 receptor antagonist (that inhibits serotonin), a substance P and Neurokinin 1 receptor antagonist and dexamethasone[66]. Adding olanzaphine to moderate or highly emetogenic chemotheraputic regimen can increase the complete control rate of nausea and vomiting[67]. A newer agent, sustained-release Granisetron (a 5-HT3 receptor antagonism), has been shown to prevent acute and delayed nausea and vomiting over multiple cycles of chemotherapy regimen that are moderately to highly emetogenic. The transdermal administration of this drug makes it ideal for patients having difficulty swallowing secondary to pain, tumor or nausea.

Furthermore, non-pharmacological approaches to treatment of nausea are important. Ettinger *et al*[68] suggests preventing dyspepsia, eating small frequent meals at room temperature, keeping well hydrated and maintaining electrolyte balance. The use of ginger as an effective antiemetic is also a promising treatment during the acute phase of nausea and vomiting. It appears to work through 5-HT3 receptor antagonism[69]. It has not, however, proven beneficial in the delayed phases of nausea and vomiting[70,71].

Additionally, there are some alternative therapies, such as oral marijuana, that have shown to be effective both alone and in combination with other traditional anti-emetics[72,73]. More research in the prevention of nausea and vomiting is needed, especially in the treatment of delayed nausea and vomiting. Delayed nausea and vomiting is a multifactorial problem and may not be triggered by serotonin alone, hence, cannabis may be an effective alternative. However, toxicities, such as delirium, disturbance in coordination, memory and perception loss, tachycardia and hyperemesis, make cannabis as the first line of therapy less appealing[74,75].

COMPREHENSIVE SYMPTOM MANAGEMENT

Patients receiving concurrent chemoradiotherapy for head and neck cancer have significant side effects which threaten to limit their ability to complete their treatment course without interruptions or dose reductions. Mason *et al*[9] found that head and neck cancer patients receiving concurrent chemoradiotherapy benefit from close monitoring for toxicities by nurse practitioners. In a retrospective trial, patients seen weekly in a nurse practitioner symptom management clinic had less hospitalizations, dose reductions and treatment deviations. This illustrates the importance of having providers, trained in symptom management, follow these patients closely during and immediately after treatment. Mucositis and adequate pain control remain areas of major concern. Insufficient treatment of these symptoms leads to malnutrition, dehydration and increased fatigue. While narcotics are

often necessary, the use of adjunctive medications needs to be further explored. Although several promising treatments to avoid mucositis are being analyzed, further research is warranted.

Vigilant monitoring with a multidisciplinary team is essential. Registered dietitians, social workers, physicians and nurse practitioners are crucial members of the head and neck cancer team and should be readily available for consultation during clinic. Treatment for nausea and vomiting, in the delayed setting and with patients deemed to be more sensitive to the emetic effects of chemotherapy, needs further attention. The literature supports the importance of following established guidelines in treating nausea and vomiting, yet individualized assessment and recommendations are vital since personal characteristics play an important role in a patient's ability to tolerate chemotherapy. Exercise, depression and fatigue are all inter-related, and there is ample research available regarding the importance of exercise. More specific research relating to patient's with head and neck cancer receiving concurrent chemoratiotherapy is needed, especially since the rate of suicide is substantially higher in this population.

Finally, patients receiving concurrent chemoradiotherapy for head and neck cancer require close monitoring by a multi-disciplinary health care team to ensure adequate symptom management. Such a multidisciplinary in the optimal setting provides the expertise to promote the best possible care of these complex patients.

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Integrating palliative care in oncologic emergency departments: Challenges and opportunities

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Abstract

Although visiting the emergency departments (EDs) is considered poor quality of cancer care, there are indications these visits are increasing. Similarly, there is growing interest in providing palliative care (PC) to cancer patients in EDs. However, this integration is not without major challenges. In this article, we review the literature on why cancer patients visit EDs, the rates of hospitalization and mortality for these patients, and the models for integrating PC in EDs. We discuss opportunities such integration will bring to the quality of cancer care, and resource utilization of resources. We also discuss barriers faced by this integration. We found that the most common reasons for ED visits by cancer patients are pain, fever, shortness of breath, and gastrointestinal symptoms. The majority of the patients are admitted to hospitals, about 13% of the admitted patients die during hospitalization, and some patients die in ED. Patients who receive PC at an ED have shorter hospitalization and lower resource utilization. Models based solely on increasing PC provision in EDs by PC specialists have had modest success, while very limited ED-based PC provision has had slightly higher impact. However, details of these programs are lacking, and coordination between ED based PC and hospital-wide PC is not clear. In some studies, the objectives were to improve care in the communities and reduce ED visits and hospitalizations. We conclude that as more patients receive cancer therapy late in their disease trajectory, more cancer patients will visit EDs. Integration of PC with emergency medicine will require active participation of ED physicians in providing PC to cancer patients. PC specialist should play an active role in educating ED physicians about PC, and provide timely consultations. The impact of integrating PC in EDs on quality and cost of cancer care should be studied.

Key words: Emergency department; Cancer; Palliative care; Integration; Quality; Cost

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Core tip: Understandably, visiting the emergency department (ED) could be a difficult experience for the many cancer patients especially in the late stages. However, these visits are increasing, and it mirrors the increased

in cancer therapies particularly in the last two decades. In this article; we discuss why cancer patients visit EDs, the outcome of these visits, models to help cancer patients avoid ED visits, the benefits of integrating palliative care in ED, and the challenges facing such integration.

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INTRODUCTION

Although palliative care (PC) and emergency medicine are viewed as two extremes of care, there is growing interest in providing PC to patients visiting emergency departments (EDs)[1]. Emergency medicine represents the gateway of care and focuses on curing disease and preventing poor outcomes such as death, while PC represents the end of care and focuses on comfort and support. A visit to the ED by a cancer patient is viewed as an indicator of poor cancer care[1-3]. However, as more patients continue to receive cancer therapy late during the cancer trajectory, many of them will end up in an ED either because of treatment-associated complications or due to the cancer itself[4].

Referrals to PC programs continue to occur late and in many cases in the last few days of life[5]. In view of the problem of late referral after hospital admission, PC specialists became interested in providing PC in the ED[6,7]. In parallel, ED clinicians facing an increasing number of very sick cancer patients with multiple symptoms and end-of-life needs became interested in PC[6,8,9]. The purpose of this article is to review the current status of PC in EDs and to highlight the challenges and opportunities faced by the integration of PC and emergency medicine.

HISTORY OF PC

PC originated in the United States in the 1970's in the form of hospice care[10]. At that time, cancer patients accounted for the majority of hospice admissions. Over the years, the number of hospices grew progressively, and a demonstration project in 1979 showed hospice to be a cost-effective model of care for patients with terminal conditions[10]. In 1982, the United States Congress enacted the Medicare Hospice Benefit[11]. According to this law, to be eligible for hospice, the patient should have a life expectancy of less than 6 mo and the type of care is mainly palliative. This law has resulted in an increased number of hospices and the accreditation of hospices by multiple organizations[12,13], including the National Hospice and Palliative Care Organization[10].

Due to the introduction of new cancer therapies and advances in medical oncology during the last 2 decades, many cancer patients continue to receive cancer care late in their disease trajectory[14]. As a result, many cancer patients with distressing symptoms are admitted to hospitals instead of receiving hospice care at home. This development prompted the introduction of hospital-based PC services to support these patients and their families. Patients with advanced cancer are usually admitted through EDs.

In 2002, the World Health Organization defined PC as "an approach that improves the quality of life of patients and their families facing the problems associated with life threatening illness, through the prevention and relief of suffering by means of early identification and impeccable assessment and treatment of pain and other problems, physical, psychological, and spiritual"[15].

The number of PC programs has increased significantly over the last 2 decades, and currently almost all large hospitals and comprehensive cancer centers have PC programs[16]. Today, cancer patients account for less than half of all hospice admissions, and the length of service provided to cancer patients is decreasing, with a median hospice stay of 18 d[1]. In view of these data, many PC programs became interested in promoting early referral. In the outpatient setting, initiation of PC soon after cancer diagnosis was associated with improved quality of life and less aggressive care[17,18]. In parallel, emergency medicine specialists facing the growing challenges associated with increasing numbers of advanced cancer patients presenting to EDs became interested in integrating palliative into emergency medicine[6,9,19].

REASONS FOR ED VISITS BY CANCER PATIENTS

Patients with cancer come to EDs because of symptoms related to the cancer itself, complications associated with cancer treatment, or other reasons, such as complications associated with chronic comorbidities (*e.g.*, exacerbation of chronic obstructive pulmonary disease) or acute problems similar to those that occur in non-cancer populations. In a large population study, Mayer *et al*[4] identified 37760 cancer-related ED visits by 27644 patients in the state of North Carolina in the year 2008. The most common presenting symptoms were pain, respiratory distress, and gastrointestinal symptoms. The most common cancers associated with these symptoms were lung, breast, prostate, and colorectal cancers (Table 1).

In a Canadian study, Barbera *et al*[20] reviewed ED visits by cancer patients in the province of Ontario in the period 2002 through 2005. The researchers found that 194017 ED visits were made by 76759 patients during the last 6 mo of life, with 31076 of those patients making 36600 ED visits during the last 2 wk of life. Approximately 25%, 16%, and 10% of patients with lung cancer, gastrointestinal cancer, and leukemia or lymphoma had more than one ED visit, respectively. The

Table 1 Symptoms of cancer patients visiting emergency departments

Ref.	Country	Sample size	Study design	Most common symptoms (*n*)	Most common cancers
Mayer *et al*[4]	United States	27644	Retrospective	Pain (9000) Respiratory (5856) Gastrointestinal (3280)	Lung Breast Colon Prostate
Barbera *et al*[20]	Canada	76759	Retrospective	Abdominal pain (9224) Dyspnea (6171) Malaise (4972) Chest pain (4463)	Lung Pancreatic Breast
Yildirim *et al*[22]	Turkey	107	Retrospective	Dyspnea Pain	Lung
Vandyk *et al*[23]	Canada	18 studies with a median sample size of 143	Meta-analysis	Febrile neutropenia Infection Pain Fever Dyspnea	Multiple

Table 2 Hospital admissions and outcomes for cancer patients visiting emergency departments

Ref.	Country	Sample size	Hospital admission rate	Deaths
Vandyk *et al*[23]	Canada	16 studies	58%	13% (hospital)
Mayer *et al*[4]	United States	37760	63%	283 (ED)
Yildirim *et al*[22]	Turkey	107	60%	9% (ED)

ED: Emergency department.

most common reasons for ED visits by cancer patients were pain, respiratory distress, and gastrointestinal symptoms (Table 1). The most common cancers were lung, pancreatic, and breast cancers.

Ncer-associated pain was reported to be the most common presenting symptom for ED visits in over 27% of cancer patients in Taiwan[21]. Interestingly, 8.2% of the patients in that study returned to the ED within 72 h for the same symptom.

Yildirim *et al*[22] conducted study on cancer patients who visited an ED in Turkey. They found pain and dyspnea to be the most common reasons for visits, 60% of patients were admitted to the hospital, and 9% died during hospitalization.

In 2012, Vandyk *et al*[23] published a systematic review of 18 studies (6 prospective and 12 retrospective; median sample size, 143) on symptoms associated with ED visits by cancer patients. Ten of the studies focused on a specific symptom (such as dyspnea) or medical complication (*e.g.*, febrile neutropenia or pulmonary embolism). The authors concluded that the most common presenting symptoms were febrile neutropenia, infection, fever, pain, and dyspnea (however, five of the studies focused specifically on febrile neutropenia) (Table 1).

OUTCOMES OF ED VISITS BY CANCER PATIENTS

The majority of cancer patients who visit EDs are admitted to the hospital[4,20,24]. Table 2 shows the frequencies of hospital admissions reported in two studies and a systematic review. In the North Carolina study[4], about 23800 (63%) of the 37760 patients were admitted to the hospital. A total of 283 of patients died in the ED; 104 of those patients had lung cancer, and the most common presenting symptom was dyspnea[25]. In the Canadian study[3], 72% of the cancer patients who presented to the ED in the last 2 wk of life were admitted to hospitals. Of those patients, 77% died in the hospital, 5% died in the ED, and 8% died in a chronic care facility.

Sixteen of the 18 studies in the systematic review[19] provided data on hospital admissions. In the nine studies focused on a specific symptom or condition, all cancer patients presenting at an ED were admitted to the hospital. Seven studies examining multiple symptoms reported a 58% rate of hospital admissions for cancer patients presenting at an ED. Mortality rates were reported in 10 of the studies. The mean mortality rate for five studies focused on multiple symptoms was 13%, and the mean mortality rate for five studies focused on a specific symptom was 20%[23].

Two conditions known to be associated with increased mortality in patients with advanced cancer are dyspnea and delirium[26,27]. In our own ED, dyspnea, particularly in lung cancer patients, was found to be associated with increased overall and 2-wk mortality[24,28]. Delirium is also known to be associated with increased mortality in advanced cancer and elderly patients[29,30]. However, this condition is underdiagnosed and frequently missed. Studies are under way to predict the frequency of delirium and altered mental status in advanced cancer patients presenting to our own ED.

Table 3 Benefits and challenges associated with integration of palliative care into emergency medicine

Benefits	Challenges
Control pain and other symptoms early	ED culture of fast pace, timely intervention, and save life
Address emotional distress in patients and families early	Time constraints
Address goals of care and resuscitation preferences	Overcrowding
Prevent unnecessary hospitalization	Limited resources
Reduce admissions to ICUs	Delays in palliative care consultations
Reduce length of hospital stay	Patient's and family's expectations
Reduce costs	

ICUs: Intensive care units; ED: Emergency department.

BENEFITS OF AND BARRIERS TO PC IN EDs

Although the majority of advanced cancer patients are admitted to hospitals through EDs, referrals to PC often occur late after hospital admission and in many cases close to the patient's death[5]. This lateness of referral deprives patients and their family members from the full benefit of PC. Moreover, since many important decision processes, such as those involving cardiopulmonary resuscitation and admission to an intensive care unit, are frequently initiated in EDs, integration of PC with emergency medicine would likely result in improved quality of life for these patients and prevent heroic interventions, most likely reducing the cost of cancer care. Table 3 highlights some of the benefits of and barriers to integration of PC into emergency medicine. One of the major barriers is the time required for an effective PC consultation. PC specialists are more likely to allocate sufficient time for such consultations than ED physicians, who are generally pressed for time.

To study some of the barriers to provision of PC in EDs as perceived by ED physicians, Lamba *et al*[31] conducted a survey in a large urban hospital and ranked physician responses on a five-point Likert scale (1 = strongly disagree, 5 = strongly agree). The barriers with the highest scores were lack of 24-h PC service (score, 4.4), lack of access to medical records (score, 4.2), communication-related issues such as availability of time and emotional distress associated with the goal-of-care discussion (score, 3.3), and the ED environment (score, 2.8).

Grudzen *et al*[32] studied delays in PC consultations from the time of an ED visit in two periods 4 years apart (2005 and 2009) and the impact of educating ED physicians on PC after the first period. Only 3% of PC consultations were initiated in the ED in 2005, and the rate increased to 6% in 2009. However, the mean time from the ED visit to a PC consultation increased from 6 to 9 d.

Delgado-Guay *et al*[33] studied ED visits in a random sample of 200 patients already receiving outpatient PC at our cancer center, to determine whether any of the ED visits were avoidable. The authors determined that for 154 (77%) patients the ED visit was unavoidable. Uncontrolled pain was the major reason for both avoidable and unavoidable ED visits. Other symptoms associated with unavoidable ED visits included delirium, dyspnea, fever, and bleeding. The findings of that study highlight the need to improve PC services in the ED for cancer patients[33].

OTHER MODELS OF EMERGENCY CARE FOR CANCER PATIENTS

A few initiatives have been developed outside of the United States and Canada to improve emergency care for cancer patients[34]. Some of these initiatives are provided in the community to reduce the need for ED visits.

In Seoul, South Korea, Asan Medical Center has established an ED cancer unit to manage oncologic emergencies[34]. In 2010, this unit provided care to 5502 patients, 55% of whom had disease progression. Gastrointestinal, lung, and hepatobiliary cancers were the most common. Of all patients; 2902 (53%) were discharged with planned outpatient follow-up, 2310 were admitted to the hospital, and 248 (4.5%) were discharged to hospice. The authors reported reductions in the cost of care in both the ED and inpatient units as compared to the year 2008. Integrating PC into this model will likely improve the quality of care for patients with disease progression.

In a multicenter cross-sectional survey, Le Conte *et al*[35] analyzed withholding and withdrawal of life support in patients who died in 174 EDs in France and Belgium. Of a total of 1970 decedents, 81% had chronic diseases including cancer. The main presenting conditions were cardiovascular, neurological, and respiratory problems. Life support was initiated in 74% of the patients, and PC was provided to 57% of the patients. The option to withhold or withdraw life support was provided mainly to elderly patients with metastatic cancer. The authors recommended training of ED physicians on the principles of PC to improve communication and care provided to dying patients.

A few out-of-hospital initiatives; mainly in Europe; have been established to improve care for patients with advanced cancer in the community to reduce the need for ED visits and hospitalizations[36-41]. Table 4 shows examples of these programs. Interventions such as palliative emergency care at home, early discharge

Table 4 Programs to reduce Emergency visits and hospitalization for patients receiving palliative care

Ref./country	Study	Objective	Intervention/observation	Outcome
Prudy *et al*[36] United Kingdom	Marie curie cancer care DCP	Help PC patients die at home and avoid emergency department visits	(1) Expedited hospital discharges for terminal patients; (2) After hour specialist PC nurses to respond to patients, families, and clinicians	Patients who used DCP are 30% less likely to die in hospital
Wiese *et al*[40] Germany	Quality of out-of-hospital emergency medical team. Prospective Multicenter Analysis	To evaluate the impact of physician's expertise in PC and emergency care on the outcome of emergency call for PC patients in the community	Number of ICU admissions, PC unit admission, general ward admission, and discharge after ambulatory care	Physicians with expertise in PC provided a better quality end of life care with less ICU admissions and more PC unit admission
Mercadante *et al*[39] Italy	Emergencies in patients with advanced cancer followed at home	Assess the frequency and reasons of emergency calls by patients receiving palliative care at home	Characteristic and outcome of consecutive emergency calls	Of 689 patients; 17% made emergency calls. Main reasons were dyspnea, pain, and delirium. Family initiated most calls
Porzio *et al*[37] Italy	Integrating oncology and palliative home care in Italy	Evaluate efficacy of home care program integrated with a medical oncology unit	Compare outcome of patients from the integrated oncology program to other patients coming from other hospitals	Patients in the integrated program had longer length of stay at home, less emergency calls, less hospitalization, and more death at home
Alonso-Babarro *et al*[38] Spain	Association between inpatient death, utilization of hospital resources and availability of PC	Evaluate the impact of community PC service on use of hospital resources in 2 areas with and without PC	(1) Use of emergency calls; (2) Hospital death; (3) Emergency visit and hospitalization	Community with PC service had less emergency calls, less hospitalization and inpatient death

ICU: Intensive care unit; PC: Palliative care; DCP: Delivering choice program.

planning of patients with terminal cancer, and hotline phone calls to support patients and their families in the community were examples of these models. The details of these programs are beyond the scope of this review.

OPPORTUNITIES AND FUTURE DIRECTIONS

A few programs have been developed to integrate PC and emergency medicine[42,43]. These programs were developed to educate ED physicians on basic PC, including symptom management and end-of-life discussion. At Wayne State University, a division of PC was developed within the department of emergency medicine.

Mark Rosenberg initiated an ED-based PC program in a large hospital in New Jersey, and he was able to build a PC team[6]. The majority of patients seen by this team had goals-of-care discussions, and 56% of 131 total consults in the period between March 2010 and July 2011 resulted in a do-not-resuscitate order initiated in the ED. The authors reported that the program resulted in improved symptom control, reduced hospital stay, improved satisfaction, and reduced cost. However, the details of how these outcomes were measured were not provided[8]. The above findings suggest that PC in the emergency setting should be initiated by ED physicians prior to a PC consultation. However, many ED physicians will need training in providing PC, breaking bad news, and discussing goals of care.

Quill *et al*[44] suggested categorizing provision of PC into the primary and specialist roles. In that framework, providing end-of-life discussion and simple management of symptoms for cancer patients should be part of the primary PC provided by ED physicians. Moreover, PC is usually provided by a team consisting of social workers, psychology counselors, chaplains, and case managers, and these providers should be readily available to help with PC in the ED. These ED-based services should be coordinated with existing hospital-based PC services for continuity of care. Some hospitals have started developing PC divisions within EDs, and this is a promising direction for integration of PC and emergency medicine.

CONCLUSION

As the number of cancer patients receiving various cancer therapies continues to increase, so will the number of cancer patients presenting with multiple distressing symptoms at EDs. The majority of these patients will be admitted to hospitals, and many of them will die in hospitals. Integrating PC in EDs will require coordination between ED physicians, PC specialists, and hospital administrators to improve the quality of cancer care and reduce costs. More research is needed to study the impact of this integration on quality of cancer care, satisfaction of patients and their families, and resource utilization.

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Is breast conservative surgery a reasonable option in multifocal or multicentric tumors?

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Abstract

The incidence of multifocal (MF) and multicentric (MC) carcinomas varies widely among clinical studies, depending on definitions and methods for pathological sampling. Magnetic resonance imaging is increasingly used because it can help identify additional and conventionally occult tumors with high sensitivity. However, false positive lesions might incorrectly influence treatment decisions. Therefore, preoperative biopsies must be performed to avoid unnecessary surgery. Most studies have shown higher lymph node involvement rates in MF/MC tumors than in unifocal tumors. However, the rate of local recurrences is usually low after breast conservative treatment (BCT) of MC/MF tumors. It has been suggested that BCT is a reasonable option for MC/MF tumors in women aged 50-69 years, with small tumors and absence of extensive ductal carcinoma *in situ*. A meta-analysis showed an apparent decreased overall survival in MC/MF tumors but data are controversial. Surgery should achieve both acceptable cosmetic results and negative margins, which requires thorough preoperative radiological workup and localization of lesions. Boost radiotherapy techniques must be evaluated since double boosts might result in increased toxicity, namely fibrosis. In conclusion, BCT is feasible in selected patients with MC/MF but the choice of surgery must be discussed in a multidisciplinary team comprising at least radiologists, surgeons and radiotherapists.

Key words: Mastectomy; Breast conservative surgery; Multifocal tumors; Multicentric tumors; Radiotherapy; Local recurrence; Breast cancer; Survival

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Core tip: Multicentric and multifocal breast tumors should be identified preoperatively in order to adapt surgical treatment. They might be associated with more frequent lymph node involvement and worse prognosis but in most studies, the rates of local recurrence are low and similar to those of unifocal tumors. Breast conservative treatment is a reasonable option in selected patients

(age 50-69 years, small tumors and absence of extensive ductal carcinoma *in situ*). Postoperative radiotherapy, and especially boost radiotherapy must be discussed and evaluated due to the risk of increased toxicity in case of double boost.

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INTRODUCTION

Multicentric (MC: At least 2 invasive tumors in 2 different quadrants) or multifocal (MF: At least 2 invasive tumors in the same quadrant) carcinoma can be diagnosed preoperatively or in resected specimens[1,2]. The frequency of these tumors ranges from 4% to 75%[3-10]. This large variability results from differences in the definitions used and the methods of pathologic sampling[11,12]. With continuous advances in preoperative imaging, the rate of MF and MC tumors is increasing[13-15].

Conservative surgery with radiotherapy has been widely accepted as an alternative to mastectomy in the management of early stage breast cancer[16,17], with a long-term local recurrence rate of approximately 15%-20%[16-28]. The diagnosis of multifocality may influence breast cancer management, particularly with regard to the choice of surgery. Conservative treatment as an alternative to mastectomy in patients with synchronous ipsilateral breast cancer is controversial and no consensus exists. MF/MC breast cancer is generally considered as a contraindication for conservative surgery because of concerns about an increased risk of local recurrence[29-32]. According to some reports, the local recurrence rate in MF/MC breast cancer after breast conservative therapy (BCT) is higher than that of unifocal tumors[31,33]. This is the main reason for excluding BCT for MF/MC breast cancer[34]. Moreover, poor cosmetic results, due to large resections, are also evoked. Therefore, many surgeons continue to perform mastectomy in patients with MF/MC breast cancer.

In contrast, extensive data have confirmed an excellent local control after BCT for unifocal breast cancer[16,17,35-43]. At the 12-year follow-up of the National Surgical Adjuvant Breast and Bowel Project B-06 trial, the cumulative incidence of ipsilateral breast recurrence was only 10% in the group treated by lumpectomy and breast irradiation[36].

The effectiveness of boost radiation treatment to decrease local recurrence has been established in a randomized trial by Bartelink *et al*[44]. However, in MF/MC breast cancer, the influence of boost radiation has been poorly reported. From a surgical point of view, BCT with negative margins and acceptable aesthetic outcome can be achieved if tumour foci are close enough to be resected as a single specimen[45].

One study has shown a significant association between positive surgical margins and failure of attempted BCT in the case of MF tumors[46].

This review will focus on the issue of conservative surgery with radiotherapy in the management of patients with MF/MC breast cancer.

DEFINITION OF MF AND MC TUMORS

MC carcinomas are defined by the presence of at least two invasive tumors in two different quadrants of the breast or in the same quadrant but at least 5 cm apart[1]. MF carcinomas are defined by the presence of several invasive tumors in the same quadrant of the breast or in different quadrants if the distance between foci is below 5 cm.

Multiple tumors are defined by the presence of synchronous, distinct, invasive tumors in the same breast, and comprise MC and MF carcinomas. They can be discovered in two different settings: (1) Preoperative diagnosis of at least 2 different invasive tumors, based on clinical and/or radiological findings; and (2) Histological diagnosis when pathological examination of surgical specimens shows several foci, while the tumor was considered as unifocal based on preoperative workup.

However, various situations must be considered according to the localization of multiple tumors in the different quadrants of the breast and to the distance from the nipple-areola complex[47].

FREQUENCY OF MC AND MF TUMORS

In the meta-analysis published by Vera-Badillo *et al*[3], including 67557 patients, the rate of MC/MF tumors was 9.5% (6434 patients). In the EORTC 10981-22023 AMAROS trial, MF tumors of the same quadrant were included after 2008 and represented 33% of cases (342/1026)[4].

However, the prevalence of MC/MF tumors varies from 5% to 44% in published series[4-8], depending on the definition used, the method of histological examination of mastectomy specimens and the type of imaging used for diagnosis (Table 1).

Imaging

Mammography and ultrasound are the standard imaging tests for the diagnosis of breast cancer, and are also used to determine the extent of the disease within the affected breast. Because of its high sensitivity in breast cancer diagnosis and screening, magnetic resonance imaging (MRI) is being increasingly evaluated and used for preoperative local staging of breast cancer. Several multicenter trials showed that, in women with newly diagnosed breast cancer, MRI helped identify additional, conventionally occult lesions in 15%-27% of cases[48-51]. In addition, MR helped identify unsuspected synchronous cancer in the opposite breast in 3%-6%

Table 1 Incidence of multifocal/multicentric tumors in the literature

Ref.	Yr	MF/MC (*n*)	MF/MC (%)
NIH *et al*[81]	1986	342	9
Vlastos *et al*[82]	2000	60	21
[1]Katz *et al*[83]	2001	149	14
Andea *et al*[62]	2002	101	18
[1]Pedersen *et al*[84]	2004	158	17
EBCTCG[85]	2005	1187	6
Coombs *et al*[8]	2005	94	11
[1]Litton *et al*[86]	2007	58	19
[1]Joergensen *et al*[87]	2008	945	13
[1]Cabioglu *et al*[88]	2009	147	11
[1]Yerushalmi *et al*[11]	2009	1554	6.1
[1]Weissenbacher *et al*[65]	2010	288	5
[1]Tot *et al*[89]	2011	148	30
Tot *et al*[90]	2011	225	44
Rezo *et al*[91]	2011	141	17
[1]Ustaalioglu *et al*[2]	2012	107	15.4
[1]Lynch *et al*[63]	2012	942	24
[1]Yerushalmi *et al*[5]	2012	1187	6
[1]Chung *et al*[66]	2012	164	14
Meretoja *et al*[92]	2012	206	20.6
[1]Pekar *et al*[93]	2013	153	34
Wolters *et al*[64]	2013	1862	20.8
Lynch *et al*[63]	2013	906	24
Hilton *et al*[94]	2013	202	15
van der Heiden-van der Loo *et al*[95]	2013	1729	13.1
Vera-Badillo *et al*[3]	2014	6565	9.7

[1]Included in the meta-analysis of Vera-Badillo *et al*[3]. MF/MC: Multifocal/multicentric.

of women with a recent diagnosis of unilateral breast cancer[51]. However, the impact of breast MRI on breast cancer management is debated, due to a large number of additional benign lesions that could be detected and incorrectly influence clinical decisions[52,53]. Indeed, one of the major limitations of breast MRI is that false-positive enhancement may appear in benign lesions, resulting in a relatively low specificity[49]. If additional suspicious findings are identified, preoperative biopsies must be performed to limit the number of unnecessary wider excisions or mastectomies[54].

INCIDENCE OF LYMPH NODE INVOLVEMENT IN MC/MF TUMORS

Although the meta-analysis of Vera-Bardillo *et al*[3] did not show differences in the rate of lymph node involvement, all the other studies demonstrated a higher rate in MC/MF tumors compared to unifocal tumors, with a mean difference of 10% to 20%.

The studies that reported lymph node detection showed the positivity of sentinel nodes in 42% to 59% of cases[4,55-62]. The main hypothesis to explain this higher rate is that the global tumor volume, that includes all MC/MF tumors, is usually more important than that of unifocal tumors. However, in MC/MF carcinomas, tumor size is determined by the largest index lesion regardless of the number and size of other lesions, which does not take into account the cumulative tumor volume.

In the EORTC 10981-22023 AMAROS trial, sentinel node involvement for MF and unifocal tumors respectively was the following: Macrometastases 61% (105/171) and 57% (109/192) (NS), micrometastases 30% (52/171) and 29% (55/192) (NS), isolated tumor cells 8% (13/171) and 14% (27/192) ($P = 0.05$)[4].

LOCAL RECURRENCE RATE OF MC/MF TUMORS COMPARED WITH UNIFOCAL TUMORS

The rate of local recurrences for MC/MF tumors in case of conservative treatment is low, except in the 3 oldest studies (Table 2), and similar to that observed after conservative treatment of unifocal tumors. As for unifocal tumors, this rate depends on selection criteria, particularly resection in negative margins, age over 35 or 40 years and tumor phenotype (hormone receptors and HER2 status).

LOCAL RECURRENCE RATES AND SURVIVAL IN MC/MF TUMORS COMPARED TO UNIFOCAL TUMORS BY TREATMENT STRATEGY (CONSERVATIVE OR NOT)

In the study by Lynch *et al*[63], published in 2013, the rate of local recurrences was determined for unifocal tumors ($n = 2816$) and for MC ($n = 233$) or MF ($n = 673$) tumors according to treatment, namely 256 BCT, 466 mastectomies without radiotherapy and 184 mastectomies followed by radiotherapy (PMRT). After a median follow-up of 52 mo, the rate of locoregional control was 99%, 96% and 98% for MF, MC and unifocal tumors respectively ($P = 0.44$). Subgroup analyses showed similar results for the three treatment strategies (BCT, mastectomy without radiotherapy or PMRT). In multivariate analysis, multicentricity/multifocality was not associated with decreased locoregional control. The authors concluded that BCT was a valid option for MC/MF carcinomas of the breast and that the presence of MC/MF alone is not an indication of PMRT.

In the study by Yerushalmi *et al*[5], local recurrence rate was determined after a median follow-up of 7.9 years and the authors compared the outcome of 11983 BCT (11683 unifocal tumors and 300 MC/MF tumors), and 7771 mastectomies (6884 unifocal tumors and 887 MC/MF tumors)[5]. One fourth of MC/MF patients had BCT (300/1187). MC/MF patients who benefited from BCT were aged 50 to 69 years, they had no extensive ductal carcinoma *in situ* (DCIS) and they had smaller tumors. Cumulative local recurrence rate at 10 years was 1) for BCT 4.6% (95%CI: 4.1, 5) in unifocal tumors *vs* 5.5% (95%CI: 2.6, 9.9) in MC/MF tumors, $P = 0.76$, 2) for mastectomies 5.8% (95%CI: 5.2, 6.5) for unifocal tumors *vs* 6.5% (95%CI: 4.7, 8.7) in MC/MF tumors,

Table 2 Rates of local recurrence in multifocal/multicentric breast cancer[5,31-33,63,66,68-73,93-96]

Ref.	Yr	Patients	MF or MC	Median follow-up (mo)	Local recurrences, %
Leopold *et al*[32]	1989	10	MF/MC	64	40
Kurtz *et al*[31]	1990	61	MF/MC	71	25
Wilson *et al*[33]	1993	13	MF	72	25
Hartsell *et al*[71]	1994	27	MC	53	3.7
Nos *et al*[72]	1999	56	MF	60	11
Cho *et al*[73]	2002	15	MF/MC	76	0
Kaplan *et al*[74]	2003	36	MF/MC	45	3
Okumura *et al*[75]	2004	34	MF/MC	58	0
Oh *et al*[96]	2006	97	MF/MC	66	6
Gentilini *et al*[76]	2008	476	MF/MC	73	5
Lim *et al*[97]	2009	147	MF	59	2
Bauman *et al*[98]	2010	22	MF/MC	42	4.5
Chung *et al*[66]	2012	164	MF	112	6.1
Yerushalmi *et al*[5]	2012	300	MF/MC	95	5.51
Lynch *et al*[63]	2013	256	MF	52	1.95
Kadioğlu *et al*[99]	2014	237	MF	46	5.2
Kadioğlu *et al*[99]	2014	36	MC	46	2

MF/MC: Multifocal/multicentric.

P = 0.77. In multivariate analysis, MC/MF was not significantly associated with recurrence or poor survival. In an additional matched analysis, recurrence rates were similar for MC/MF and unifocal tumors (P = 0.6). The authors concluded that BCT is a reasonable option in selected cases of MC/MF tumors, in particular in women aged 50-69 years, with small size tumors (< 1 cm) without extensive DCIS.

Wolters *et al*[64] compared recurrence free survival and overall survival in 8935 patients with 7073 unifocal tumors (79.2%), 1398 MF tumors (15.6%) and 464 MC tumors (5.2%). They did not show any difference in RFS (1) in MF tumors (T1/T2 treated according to guidelines) for BCT (n = 623) *vs* mastectomy (n = 319): HR = 1.25, (95%CI: 0.83-1.88), P = 0.284, and (2) in MC tumors after adjustment on tumor size in case of negative margins, for BCT (n = 60) *vs* mastectomy (n = 217): HR = 1.19, (95%CI: 0.48-2.97), P = 0.7 and *vs* mastectomy + PMRT, HR = 1.23, (95%CI: 0.51-3.00), P = 0.64.

IMPACT OF MC/MF TUMORS ON SURVIVAL AND SYSTEMIC RISK COMPARED TO UNIFOCAL TUMORS, REGARDLESS OF TREATMENT (BCT OR MASTECTOMY)

In a study on 288 unifocal tumors matched with 288 MC/MF tumors the presence of MC/MF was significantly associated with decreased OS (P = 0.016), increased local recurrences (P = 0.001) and development of metastases (P = 0.038)[65].

In the study by Wolters *et al*[64], after adjustment on age, tumor size, grade and nodal status, no difference was shown in RFS or OS in patients who received adjuvant therapy according to guidelines in MC or MF tumors compared to unifocal tumors: (1) For MC carcinomas, no difference in RFS [HR = 0.88, (95%CI: 0.67-1.16), P = 0.35] and in OS [HR = 1.08, (95%CI: 0.85-1.36), P = 0.54]; and (2) for MF carcinomas, no difference in RFS [HR = 1.05, (95%CI: 0.89-1.24), P = 0.597] and in OS [HR = 0.92, (95%CI: 0.78-1.08), P = 0.28].

In the meta-analysis of Vera-Badillo *et al*[3], the impact on survival of MF/MC tumors was compared to that of unifocal tumors from 22 studies and 67557 patients (6565 MF/MC et 62326 unifocal tumors. In multivariate analysis, MC/MF tumors were associated with decreased OS (HR = 1.65, 95%CI: 1.07-2.52; P = 0.02), but the difference was not statistically significant in RFS (HR = 1.96, 95%CI: 0.94-4.12; P = 0.07). The authors concluded that MC/MF tumors seem to be associated with worse prognosis; however, the heterogeneity between studies did not allow an accurate determination of the real risk (one study alone, that differs from other studies, determined the shorter OS[66]).

SURGICAL PROCEDURES AND COSMETIC RESULTS

In MC/MF carcinomas, the localization of tumors is of utmost importance to determine the type of resection allowing both favorable cosmetic results and negative margins. Types of incision and resections are determined according to the localization of tumors, the breast size, the degree of ptosis, the areola size and the distance from areola. In the last decade with the introduction of oncoplastic techniques, the surgical approach of MC tumors have changed. Oncoplastic techniques are therefore particularly adapted and valuable in this situation, achieving negative margins and a good cosmetic results better than conventional BCS; a schematic cartography of various possible situations and resection techniques[47] and a classification quadrant per quadrant atlas for many oncoplastic surgical procedures were proposed[67]. This strategy was applied to a consecutive series of 175

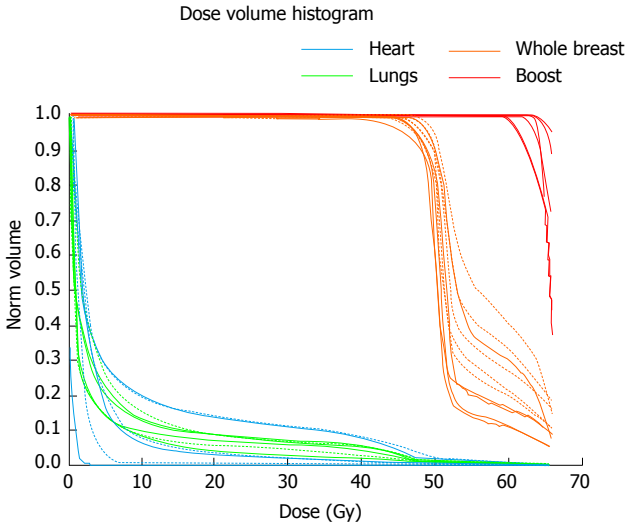


Figure 1 Dose-volume histograms for patients treated with a single boost (continuous lines) and with a double boost (dashed lines). The percentage of breast receiving 60 Gy or more with a single boost varies from 10% to 29% (median 15%, mean 16%) and with a double boost from 19% to 39% (median 25%, mean 27%).

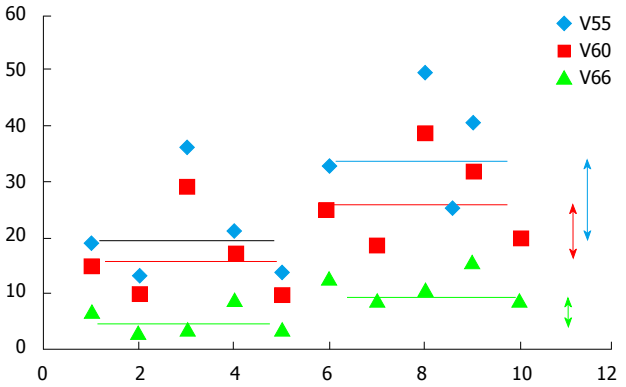


Figure 2 Volume of whole breast receiving an increased dose according to the dose-level. On the left side of the figure are reported the amount of breast receiving 66 Gy (green), 60 Gy (red) or 55 Gy (blue), in patients treated with a single boost (first 5 patients); on the right side of the figure are reported the amount of breast receiving 66 Gy, 60 Gy or 55 Gy, in patients treated with a double boost. The double arrows show the magnitude of increasing dose from single to double boost, according to dose level.

women with breast cancer who required mammoplasty, including 27 patients (15.4%) with MF tumors[68]. This study has confirmed that oncoplastic surgery techniques for breast cancer are associated with a low reoperation rate, a low risk of delay to adjuvant therapy and good cosmetic results. In an another study, Clough *et al*[69] reported 17.2% (10/58) of positive margins after oncoplastic surgery for MF breast cancer, without significant difference with positive margins rate after oncoplastic surgery for unifocal tumor (10.6%: 23/217).

Radiological workup and preoperative tracking are essential to perform appropriate resection with negative margins. The orientation and the identification of resection margins on surgical specimens, that sometimes have complex shapes and localizations, must be accurate and requires the collaboration of surgeons and pathologists. A completion resection might be necessary.

BOOST RADIOTHERAPY IN MF/MC TUMORS

The benefits of a boost to the tumor bed have been demonstrated for invasive breast cancer treated with conservative surgery. However, extended boost, and more specifically boost fields for two locations in the breast, should be thoroughly evaluated because of possible toxicity and side effects, particularly fibrosis[70]. A preoperative consultation with radiotherapists should be proposed, if not recommended, when different boost fields are considered.

Fifteen studies have reported the outcomes of patients with multiple ipsilateral synchronous breast cancer treated with BCT followed by whole-breast irradiation (WBI)[11,31-33,66,71-79]. Most of these studies included patients treated for MF disease rather than MC disease and the patients were mostly operated with a single incision and therefore a single field designed for the boost[31-33,71,72]. BCT through double lumpectomy for MC disease raises the question of the safety of a double boost, regarding particularly the cosmetic result. Adding a boost after 50 Gy WBI increases the 10-year rate of severe fibrosis from 1.6% to 4.4% and of moderate fibrosis from 13% to 26%[80]. Increasing the volume of the boost may increase this risk resulting in a poor cosmetic outcome, which is however the goal of BCT. This is the reason why we conducted a dosimetric study to assess the volume of breast receiving an increased dose, in patients treated in a classical manner (50 Gy-whole-breast + 16 Gy-single boost) and in patients treated with a double boost. The dose levels investigated were 110% and 120% of the prescribed dose (V55 and V60), and V66 as 66 Gy was the dose prescribed to each boost volume. Adding a second boost resulted in a 14%-increase of the volume of breast receiving more than 55 Gy, (from 19% to 33%), a 10%-increase of the volume of breast receiving more than 60 Gy, (from 15% to 25%) and a 2 Gy-increase in the mean dose received by the ipsilateral whole breast (Figures 1 and 2). The clinical significance of this increased dose is unknown but is expected to be real and deserves evaluation. An alternative could be an intraoperative boost, which would allow the preservation of the surrounding structures (normal tissues).

CONCLUSION

Conservative treatment is a reasonable option in selected cases of MF or MC tumors. Radiological workup and preoperative evaluation of all tumor sites are essential. A multidisciplinary discussion should be mandatory, especially for distant localizations, involving above all surgeons, radiologists and radiotherapists.

The selection of patients with low risk of recurrence

might be determined on the following criteria[11,47,76]: (1) Technical feasibility, acceptable planed cosmetic result; (2) patient's choice after information about the risk of a new resection or mastectomy in cases of positive margins; (3) age > 40 years or > 50 years, absence of DCIS; (4) size of the largest lesion < 20 mm; and (5) feasibility of radiotherapy, including boost.

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MINIREVIEWS

Locoregional treatment of early breast cancer with isolated tumor cells or micrometastases on sentinel lymph node biopsy

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Abstract

The advent of sentinel lymph-node technique has led to a shift in lymph-node staging, due to the emergence of new entities namely micrometastases (pN1mi) and isolated tumor cells [pN0(i+)]. The prognostic significance of this low positivity in axillary lymph nodes is currently debated, as is, therefore its management. This article provides updates evidence-based medicine data to take into account for treatment decision-making in this setting, discussing the locoregional treatment in pN0(i+) and pN1mi patients (completion axillary dissection, axillary irradiation with or without regional nodes irradiation, or observation), according to systemic treatment, with the goal to help physicians in their daily practice.

Key words: Breast cancer; Micrometastases; Axillary lymph node dissection; Radiotherapy; Isolated tumor cells

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Core tip: Sentinel lymph-node biopsy has led to a shift in lymph-node staging, due to the emergence of new entities namely micro-metastases and isolated tumor cells. The prognostic significance of this low positivity in axillary lymph nodes is currently debated, as is, therefore its management. This review provides updates evidence-based medicine data to take into account for treatment decision-making in this setting, discussing several loco-regional therapeutic strategies based on recent clinical trials results, particularly completion axillary dissection, axillary irradiation, regional nodes

irradiation, with according to systemic treatment, with the goal to help physicians in daily practice.

Tallet A, Lambaudie E, Cohen M, Minsat M, Bannier M, Resbeut M, Houvenaeghel G. Locoregional treatment of early breast cancer with isolated tumor cells or micrometastases on sentinel lymph node biopsy. *World J Clin Oncol* 2016; 7(2): 243-252 Available from: URL: http://www.wjgnet.com/2218-4333/full/v7/i2/243.htm DOI: http://dx.doi.org/10.5306/wjco.v7.i2.243

INTRODUCTION

Although adjuvant systemic treatments are mainly based upon biological tumor features, lymph-nodes status remains an important prognostic factor that influences the adjuvant treatment decision[1]. The sentinel lymph-node (SLN) technique in breast cancer (BC) (surgical technique as well as bio-pathological analyses) has altered the lymph-node status assessment, with the emergence of new entities formerly ignored such as isolated tumor cells (ITCs) [pN0(i+), metastasis size 0.2 mm or less] or micrometastases (MMs) (pN1mi, metastasis size above 0.2 mm up to 2 mm), that have been introduced in the sixth TNM classification[2]. In the latest case (MMs), two subpopulations with different prognosis have been described according to the detection mode (hematoxylin-eosin staining and/or immuno-histochemistry on fine serial slices)[3,4].

The aim of this article is to determine what is the current evidence for the locoregional treatment of early BC patients, with limited axillary lymph-node metastases [pN0(i+) and pN1mi], discovered on SLN biopsy (SLNB).

LYMPH-NODES MMS FREQUENCY

In patients with small tumors suitable for a SLNB procedure, approximately one third present with a lymph node involvement[5-7], and 25% to 46% of positive SLNs are MMs[5,8,9] (Table 1); in other words, MMs are present in 10% to 15% of SLNB[5,10]. Noteworthy, this rate is closely dependent of the technique used for biopathological analyses, and discrimination between ITCs and MMs is fluctuant, even with trained pathologists[5,7,11-14] (Table 2).

RATE OF NON-SENTINEL LYMPH-NODES INVOLVED IN AXILLARY LYMPH-NODE DISSECTION SUBSEQUENT TO POSITIVE SLN

The rate of non-sentinel lymph-nodes (NSLN) involved is grossly from 40% to 50%: About 50% if positive SLN are macrometastases, 16% if positive SLN are MMs and 11% if ITCs were found in SLN[15-24] (Table 3). Once again, this rate depends on the method of detection of SLN involvement[15,18], the higher is the SLN metastasis size, higher is the rate of NSLN involvement. Some studies have reported that the risk of 3 or more positive NSLNs in patients with microscopically positive SLN, ranged from 1.5% to 5%. In the study reported by van Rijk *et al*[7], 5.6% patients (6/106) had 3 or more positive NSLNs. Rivers *et al*[25] reported the risk of 4 or more positive NSLNs at less than 1.5% in the group of patients with MMs in the SLN. In the study from Houvenaeghel *et al*[15], the risk of 3 or more positive NSLNs was 2.1% (15/700 patients), and the risk of 4 or more positive NSLNs was 1.4% (10/700 patients). Zhou *et al*[26] reported a 3.4% risk (3/87 patients) of 4 or more positive NSLNs. In the IBCSG 23-01 trial[23], among 447 patients having had a complementary axillary lymph node dissection (cALND), 59 (13%) harbored at least one positive NSLN among whom one fourth had 2 or more positive NSLN.

The rate of NSLN involvement is also correlated with the tumor characteristics[18]. This has led some authors to seek for prognostic factors for NSLN involvement and predictive models guiding the axillary treatment decision.

PREDICTIVE MODELS OF POSITIVE NSLN RISK

Several predictive models of the risk of NSLN involvement have been developed in order to determine a low-risk group of patients (at low risk of harboring positive-NSLN, less than 10%)[16,17,22], and conversely a high-risk group of patients (at high risk of harboring positive-NSLN, more than 30%)[20,21]. Few of them were focused on patients with ITCs or MMs in the SLNs[16,17,20-22,27]. The risk of positive-NSLNs in patients with MMs or ITCs in the SLN is higher than that of patients with negative SLN (whose risk ranges from 7% to 8% related to the false negative rate), and lower than that of patients with macrometastases in the SLNs (whose risk ranges from 30% to 50%). Many clinical parameters were reported as risk factors of positive NSLNs, including size of the primary tumor, presence of lymphovascular invasion, the molecular subtype, the SLN metastasis detection mode or size of the SLN metastasis, tumor histologic type, number of positive SLNs or proportion of positive SLNs, and multicentric tumors (Table 4).

PROGNOSTIC IMPACT OF MMS

The clinical significance and the therapeutic implication of this weak positivity in the axillary lymph nodes remain controversial, although, there is growing evidence to suggest that micrometastatic disease in the SLN is associated with worse outcomes (Table 5). It seems that the metastatic tumor burden in axillary lymph nodes acts as a continuous variable, prognostic of locoregional recurrence (LRR) and of survival[4,28-33].

In the study from Weaver *et al*[4], investigating the prognostic impact of occult metastases in the SLNs, in

Table 1 Incidence of micrometastatic lymph-nodes among positive sentinel lymph nodes

Ref.	Population	*n*	ITCs (%)	MMs (%)	ITCs + MMs	Macrometastases
Houvenaeghel *et al*[5]	cT0-2N0	2413	13	33	46	54
Yi *et al*[8]	Positive SLNB	26986	-	25	-	75
Madsen *et al*[9]	cT1-2N0	517	10	24	34	66

SEER: Surveillance, epidemiology and end results; SLNB: Sentinel lymph node biopsy; ITC: Isolated tumor cells; MMs: Micrometastases.

Table 2 Distribution between pN0(i+) and pN1mi

Ref.	*n*	ITCs (%)	MMs (%)
Houvenaeghel *et al*[5]	1099	28	72
van Rijk *et al*[7]	253	42	58
Meretoja *et al*[13]	484	43	57
Tvedskov *et al*[14]	1881	16	84

ITC: Isolated tumor cells; MMs: Micrometastases.

Table 3 Rate of non-sentinel lymph node involved

Ref.	*n*	Rate of positive-NSLN			
		IHC (%)	ITC (%)	pN1mi (%)	pN1a (%)
Houvenaeghel *et al*[15]	700	10.8	12.6	12	-
Meretoja *et al*[16]	1000	13.9	5.8	12.2	42.6
Viale *et al*[19]	1228	-	14.6	21.3	50.2
Tvedskov *et al*[20]	1881	-	9	18	-
Tvedskov *et al*[21]	900	-	13	17	-
Calhoun *et al*[24]	61	-	4.9	-	-

NSLN: Non-sentinel lymph node; IHC: Immunohistochemistry; ITC: Isolated tumor cells.

patients included in the NSABP-B32 trial[6] (for whom no treatment has been planned for this minimal nodal involvement since physicians were not aware of it at the time of the treatment decision), a significant difference was found in the 5-year overall survival (OS) (absolute value: 1.2%) and in the 5-year recurrence-free survival (RFS) (absolute value: 2.8%), between pN0 patients and patients with occult metastases in the SLN, as detected by additional tissue section levels and the use of Immunohistochemistry. This difference was all the greater than the size of the lymph node metastasis increased. The occult metastases incidence was correlated to other prognostic factors (age and tumor size). In a surveillance, epidemiology and end results population-based analysis including 209720 patients treated between 1992 and 2003, a micrometastatic node has been shown to carry a prognosis intermediate to pN0 (HR = 1.35) and pN1 disease (HR = 0.82)[28]. In a systematic review assessing the outcomes of patients with minimal nodal positivity compared to those without disease in the axillary lymph nodes, categorized by pathologic assessment of the excised lymph nodes (SLNB or ALND), de Boer *et al*[29] found that micrometastatic disease in the lymph nodes, as detected by staining with hematoxylin-eosin in one section of each axillary lymph node, was associated with decreased DFS and OS (HR = 1.44, 95%CI: 1.29-1.62). Nonetheless, when considering studies in which axillary lymph nodes were obtained by SLNB, definite conclusions could not be drawn due to poor sample sizes and short follow-up. Lupe *et al*[30] have focused on long-term LRR outcomes in 9616 patients, according to their nodal status (pN0:7977, pN1mi:490, pN1:1149). This study was carried out in a population of patients treated before the era of SLNB. Women with pN1mi disease were found to be at greater risk of LRR than those with pN0 disease (HR = 1.6; *P* = 0.002). In the study from Andersson *et al*[31], the presence of a MM in the axillary lymph nodes was also associated with a worse outcome, since both the 5-year specific survival and the 5-year RFS were significantly lower in pN1mi disease than in pN0 disease (94.1% *vs* 96.9% and 79.6% *vs* 87.1%, respectively), but without significant difference in OS. The prognosis of patients with pN0- and pN0(i+)-disease was similar. On the other hand, a dutch study (MIRROR study: Micrometastases and ITC: Relevant and Robust or Rubbish?), on 856 patients with small tumor sizes, found statistically significant differences in 5-year RFS between pN0(i+) and pN0 as well as between pN1mi and pN0, without significant difference between pN1mi and pN0(i+)[32]. Moreover, systemic treatment (hormonal therapy and/or chemotherapy) was able to improve RFS both in pN0(i+)- and pN1mi-disease. In the same way, several retrospective studies comparing survival outcomes of patients with pN0-, pN0(i+)-, pN1mi -and pN1-disease have found a similar prognosis of pN0-, pN0(i+-) and pN1mi-patients, emphasizing that the patients with nodal involvement had much higher frequently received adjuvant treatments such as systemic treatments and radiotherapy[5,34]. In the study from Cox *et al*[33], including 2381 patients having had a SLNB, with 2108 pN0(i-), 151 pN0(i+), and 122 pN1mi patients, and a median follow-up of 1.5 to 2 years, OS and RFS were significantly worse in pN1mi patients than in pN0(i-) patients ($P < 0.001$ and < 0.006, respectively), whereas they were similar between patients pN0(i+) or pN0(i-). In a cohort study of 18370 patients, [16011 pN0, 703 pN0(i+), and 1656 pN1mi], with a median follow-up of 5 years, after adjusting for prognostic factors, patients with ITCs in the SLN had a LRR risk and a metastatic recurrence risk similar to those without disease in the SLN (pN0) (HR = 1.2), whereas patients with a micrometastatic SLN had a 38%-50% higher risk (HR = 1.50; *P* = 0.001). Similar results were obtained in a subgroup analysis of patients without any adjuvant

Table 4 Studies of prognostic factors for positive-non-sentinel lymph nodes in patients with micrometastasis or isolated tumor cells in sentinel lymph nodes

Ref.	*n*	Prognostic factors for positive NSLNs											
		Tumor grade	Size of positive SLN	Proportion of positive SLN	Molecular subtype	Tumor size	Age	Nb of SLN examined	Presence of LVI	Histologic type	Multicentric tumor	ECE	Tumor location
Zhou *et al*[26]	130 (pN1mi 25%)	+	+	+	+	+	-	-	NR	NE	NE	NE	NE
Meretoja *et al*[16]	1000 (pN1mi 28%)	-	+	+	+	+	NR	NR	+	-	+	+	NE
Houvenaeghel *et al*[17]	909 (pN1mi 100%)	-	+	NR	NR	+	NR	NR	+	+	NE	NE	NE
Tvedskov *et al*[20]	1881 (pN1mi 100%)	-	NR	+	+	+	-	-	+	-	-	-	+

NSLN: Non-sentinel lymph node; SLN: Sentinel lymph node; LVI: Lymphovascular invasion; ECE: Evaluable capsular effraction; NR: Not reported; NE: Non evaluable.

Table 5 Recurrence-free survival, disease-specific survival and overall survival according to the burden of axillary positivity

LN status		LRR	RFS	DSS	OS
pN0/pN0(i+)	=		[3,34,36,39]		[3,32,34,36,38]
	>		[4,33]		[4]
pN0/pN1mi	=		[38]		[32,37,38]
	>	[30]	[4,29,32,33,34,36]	[32]	[4,29,30,34]
pN0(i+)/pN1mi	=		[33]		
	>		[4]		[4]
pN1mi/pN1	=				
	>				[30]

LN: Lymph node; LRR: Locoregional recurrence; RFS: Recurrence-free survival; DSS: Disease-specific survival; OS: Overall survival.

treatment[35].

In the same time, several studies didn't find any significant difference in outcomes between patients with minimal axillary nodal involvement and patients without axillary nodal disease[3,36-38]. The ACOSOG Z10 trial did not find any impact on survival of occult axillary metastases as detected by IHC exclusively[3]. In this study, physicians were not aware of IHC results at the time of treatment decision, but patients with occult axillary metastases received systemic treatment significantly more often due to the correlation between occult axillary metastases and other unfavorable prognostic factors (such as age and tumor size). Imoto *et al*[38] also found that ITCs in SLNs detected by immunohistochemical staining had no impact on RFS. In the dutch study from Maaskant-Braat *et al*[36], with a median follow-up of 50 mo, no significant difference of survival was found between patients with a MM (n = 128) or with ITCs (n = 53) in the SLN and patients without disease in the SLN (n = 3285), even after adjusting for tumor- and patient-related factors (age, grade, tumor size, systemic treatment or not). A lack of significant difference in OS or RFS has also been reported in other series[37].

To summarize, ITCs as well as MMs in the SLNs have been correlated with an increased recurrence risk, and were inconstantly related to a poorer survival. The prognostic significance of minimal nodal involvement could be different according to the molecular subtype.

Finally, the presence of a minimal nodal involvement after a SLNB procedure raises two questions: Is a complementary axillary treatment mandatory? And, is this minimal nodal involvement prognostic enough to prompt an adjuvant systemic treatment?

IS A CALND MANDATORY?

This question could be otherwise formulated: What is the risk to leave disease in the NSLNs? Is the axillary recurrence (AR) risk increased? Is there an increased risk of under-treatment (particularly for adjuvant chemotherapy and regional radiotherapy)?

AR

The AR rates observed in patients with involved SLNs spared of cALND, are extremely low (0%-2%), and widely lesser than the rates of positive NSLNs observed in cALND performed for positive SLNs. This fact could be due to the contribution of tangential fields of breast external beam radiation therapy, and also due to the efficacy of adjuvant systemic treatments such as hormonal therapy, chemotherapy and targeted therapies.

In the above-mentioned MIRROR trial, the 5-year rate of AR in patients with pN1mi-disease without cALND was 5%[32]. In the ACOSOG Z0011 trial randomizing patients with positive SLNs to either cALND or observation, half of the population had a minimal SLN involvement [pN0(i+) or pN1mi]; no significant difference in AR rates was observed between treatment groups (0.9% and 0.5% in the SLND alone group and in the ALND group, respectively, at 6.3 years of follow-up); all patients enrolled in this trial underwent a breast-conserving therapy with adjuvant whole breast irradiation; most of them (> 96%) received an adjuvant systemic treatment[39]. The IBCSG trial was designed to determine whether no axillary dissection was non-inferior to axillary dissection in patients with one or more micrometastatic ($\leqslant$ 2 mm) sentinel nodes[23]. In this trial, patients have had either a conservative surgery or a mastectomy, and no significant difference in AR rate was observed between the 2 groups of patients (with cALND: 1AR/465 patients, without cALND: 4AR/464 patients). In the Spanish randomized trial, which assessed cALND *vs*

clinical follow-up, in patients with SLN MM, 233 patients have been analysed (112 in the cALND group, 121 in the observation group), only one AR has been reported in the "observation" group[40].

In the Netherlands Cancer Registry study, Pepels *et al*[41] have found a higher 5-year regional recurrence rate in patients with MMs in the SLN who were not submitted to cALND, compared to those in whom cALND was performed (5.6% *vs* 2.3%, with an adjusted HR of 4.39, 95%CI: 1.46-13.24). The authors also showed that the omission of adjuvant systemic treatment and of breast irradiation was significantly associated with a higher AR rate and that these adjuvant treatments significantly lowered the risk.

In the meta-analysis from Francissen *et al*[42], AR rates ranged from 0% to 0.9% in patients with SLN MMs, and from 0.2% to 1.2% in patients with SLN macrometastses. These rates compare favorably with those (0.2% to 1%) of patients with positive SLNs who have had a cALND[39], and those (0% to 1.4%) of patients with negative SLN without cALND[43,44].

Actually, minimal nodal involvement seems to confer a worse outcome if ignored, reversed by the adjuvant treatments, and ARs are not of concern, likely due to the efficacy of adjuvant treatments. Therefore ARs are probably not an adapted end-point to judge the importance of axillary treatment.

Breast external beam radiotherapy by tangential fields and its impact on axillary nodes control

The role of external beam radiotherapy in the axillary area control, in patients with positive SLN without cALND has been widely commented in literature[45]. In the ACOZOG Z0011 trial, despite 27% of positive NSLNs, only 1% of AR rate has been observed in the SLN alone group[46]. The authors explained this unexpected low rate of AR, by the use of adjuvant systemic treatments combined with the use of external beam radiation therapy through tangential fields encompassing the vast majority of axillary levels Ⅰ and Ⅱ, although exact radiotherapy data were known in only one third of cases[47]. Therefore, the authors suggest limiting the ALND omission in patients meeting strictly the inclusion criteria of the ACOSOG Z0011 trial and suggest a radiotherapy scheme adapted to several tumor characteristics such as histologic type, the tumor grade, hormonal status, LVI presence, the size of node metastasis and number of positive nodes. Nonetheless, it has been recently reported that standard tangential fields used for breast irradiation do not allow optimal coverage and dose distribution in axillary levels Ⅰ-Ⅱ and sentinel node area[48,49].

On the other hand, when adapted tangential fields targeting axillary area, are used, it has also been shown in the AMAROS randomized trial, that, in the T1-2 BC patients with positive SLNs, axillary surgery or radiotherapy provide excellent and comparable axillary control[50]. However, AR rate was far less common than what was hypothesized, making the trial's primary test underpowered. With this reserve in mind, axillary radiotherapy would seem equivalent to cALND in positive SLNs, but with less 5-years lymphedema rate, without any difference in quality of life[50].

Systemic chemotherapy and its impact on axillary nodes control

The positive impact of systemic treatments on loco-regional control has been already documented. In the early 1990s, the NSABP B13 trial, which randomized node-negative, estrogen receptor-negative women, to chemotherapy or no-treatment control group, reported an 8-year LRR of 2.6% and 13.4% in the chemotherapy group and in the no-treatment control group, respectively[51]. More recently, a chemo-induced downstaging was observed in the SENTINA trial: Among 474 patients with a documented axillary lymph node involvement, 248 patients (52.3%) were free of disease after neoadjuvant chemotherapy[52]. Among the 1023 evaluable patients from NSABP B-14, the 10-year Kaplan-Meier estimate of the proportion of patients with LRR was 14.9% (95%CI: 10.7%-19.1%) for patients treated with placebo and 7.7% (95%CI: 5.7%-10.2%) for those treated with tamoxifen[53]. The addition of trastuzumab to chemotherapy also has resulted in a reduction in LRR (4% *vs* 6%, with and without trastuzumab)[54].

Impact of omission of cALND on OS and RFS in patients with micrometastatic lymph node involvement

Three phase Ⅲ, randomized controlled trials addressed the question of the impact on survival of cALND in patients with minimal SLN involvement[23,40,46]; all of them were in favor of cALND omission, but all of them with limitations and shortcomings precluding any definitive conclusion.

Criticisms and shortcomings of these trials related to statistical methods as well as lack of radiation data. Relative to the statistical methods, all of the 3 trials have been criticized due to: (1) A lack of accrual (70% of the planned sample size has been enrolled in the spanish trial, less than half of the required sample size to verify the non-inferiority hypothesis has been enrolled both in the IBCSG 23-01 and the ACOSOG Z0011 trials); (2) the expected number of events was always superior to the number observed (5 times superior both in the IBCSG 23-01 and the ACOSOG Z0011 trials); and (3) a 5-years OS or a 5-years RFS (used for the sample size calculation) underestimated in all the trials (IBCSG 23-01: Expected RFS: 70%, observed RFS: > 87%; ACOSOG Z0011: Expected OS: 80%, observed OS: > 91%; spanish trial: Expected RFS: 48%, observed RFS: > 97%). Relative to the radiation data, no information was provided in the IBCSG 23-01 trial as well as in the ACOSOG Z0011 trial, and it has been suggested, particularly in the IBCSG 23-01 trial, that radiation beams have been modified in patients without cALND in this non-blinded study. This is all the more important that some authors explained the difference

between the positive-NSLN rate after cALND (13%) and the low AR rate in the "observation" arm (< 1%) through the efficacy of systemic treatments and breast irradiation and its axillary contribution[23]. Furthermore, surgery performed in all these 3 randomized trials was mainly conservative (mastectomy rate 0%, 9%, and 7.7% in the ACOSOG Z0011, IBCSG 23-01, and Spanish trial, respectively[23,40,46]), therefore precluding recommendation of cALND omission in patients with MM in the SLN treated by mastectomy (without adjuvant irradiation). A recent meta-analysis, including the above-mentioned randomized trials found no difference in RFS according to the performance of cALND or not, with a HR of 0.94 (95%CI: 0.79-1.13), however emphasizing on important shortcomings in these trials[55]. A French randomized trial (SERC trial, Clinicaltrials.gov NCT01717131) assessing the impact of cALND in patients with positive (MM or macrometastases) SLNs, is ongoing[56]. This trial was designed to determine the tangential field contribution to the radiation of each levels of the axilla.

Completion ALND omission has never been assessed in a phase Ⅲ trial in patients without adjuvant treatments. This issue has already been discussed in the subsection "Prognostic impact of MMs".

IS CALND A COMPONENT OF ADJUVANT TREATMENT DECISION-MAKING?

Since tumor biological criteria (tumor size, grade, LVI, hormonal receptors status, HER2 status) are commonly used for adjuvant treatment decision-making, NSLN status is nowadays of lesser importance in this regard. The majority of studies that have reported the rate of patients receiving adjuvant systemic treatment according to axillary staging (cALND or SLNB alone), concluded that the absence of knowledge regarding the extent of nodal involvement seemed to have no major impact on the administration of adjuvant systemic treatments[23,40,57-61] (Table 6). The proportion of additional patients being considered for adjuvant chemotherapy upon cALND information ranges from 2% to 10% (median 4%). Only 2 authors out of 6 concluded that this difference was relevant[58,61]. Aigner *et al*[58] have also found a 4.6% increase in adjuvant chemotherapy administration taking into account cALND information, but also studied the type of chemotherapy related to the number positive nodes. Twelve percent of patients would be offered a more aggressive chemotherapy regimen upon the knowledge of more than 3 positive axillary nodes. This was the reason why the authors concluded to the relevance of cALND information. In the study from Montemurro *et al*[61], 16% more patients would have receive an adjuvant chemotherapy based on cALND information. Nonetheless, this study raised some criticisms[62]. The main concern was related to the study design. The authors have selected from their institutional database, patients meeting the ACOSOG Z0011 criteria (having had a cALND), and their breast team have blindly reviewed these cases in two rounds, and the total number of positive lymph nodes was disclosed only in the second. At each round was discussed the recommendation of chemotherapy (mandatory, discussed, or not required). The "chemotherapy discussed" group brings somewhat confusional because chemotherapy would have probably been considered in these patients. Indeed, if the 2 groups "chemotherapy mandatory" and "chemotherapy discussed" had been combined, then the absolute difference of chemotherapy administration between the 2 rounds would have been 3%.

Moreover, in the multivariate analysis from AMAROS trial, the patient age, tumor grade, size of SLN metastasis (ITCs, HR = 1.9; MMS, HR = 4.1; macrometastases, HR = 10.8), and multifocality were all significantly associated to chemotherapy administration, whereas number of positive nodes were not[60]. Mazouni *et al*[57] also showed the low impact of NSLN status in adjuvant treatment decision-making. Indeed, tumor grade was the major factor considered for adjuvant systemic treatment, followed by HER2 status, and then NSLNs positivity for low grade, HER2-negative tumors.

To summarize, the need for further axillary treatment (cALND or axillary radiation) in pN0(i+)- and pN1mi-positive SLN remains uncertain. It seems that usual adjuvant treatment, combining systemic treatment and classic radiation therapy (usual tangential fields) leads to a comparable survival to completion axillary treatment, without that we could assign this equivalence to either of the adjuvant treatments (radiation therapy, systemic therapy or both)[45].

The risk of positive NSLNs in pN0(i+) SLNs is quite similar to those of pN0 (5% and 4% respectively), the prognostic impact of ITCs seems negligible in the above-mentioned studies, all which lead to consider pN0(i+) as pN0. Lastly, the risk of positive NSLNs is also correlated to other patient- and tumor-related prognostic factors[3,4], that can be taken into account in the decision for further axillary treatment, particularly for patients treated with mastectomy without adjuvant irradiation, and for adjuvant systemic therapy consideration.

REGIONAL NODE IRRADIATION (RNI) IN PN1MI SLNS PATIENTS

The objective of RNI is to eradicate micrometastatic disease, which could lead to LRR and also, and above all, to distant recurrence, if we trust the Halsted's theory (secondary diffusion hypothesis)[63], which do not preclude the systemic theory from Fisher (hematogenous diffusion)[64], both phenomenons probably coexisting, the preponderance of one or the other being related to tumoral characteristics, particularly molecular subtype. The present subject is not to discuss these two hypotheses, but just to remember that recent studies support the Halsted's hypothesis (sanctuary role of

Table 6 Impact of completion axillary lymph node dissection on adjuvant therapy decision-making

Ref.	*n*	Variable analysed	Results						Conclusion
			Treatment arm	CT	HT	CT + HT	Any	Type	
Montemurro *et al*[61]	566 pN0(i+)	Systemic treatments[1]	cALND	58	78	-	-	-	No difference
	pN1mi		ART	61	76	-	-	-	
	pN1a		Absolute difference	3	2	-	-	-	
Galimberti *et al*[23]	931 pN0(i+)	Systemic therapy	cALND	9	63	23	95	-	No difference
	pN1mi		SLNB	7	67	22	97	-	
			Absolute difference	2	4	1	2	-	
Aigner *et al*[58]	132 pN0(i+)	Administration of CT and type of CT	cALND	72.7	-	-	-	-	Relevant difference (type of CT)
	pN1mi		SLNB	77.3	-	-	-	-	
	pN1a		Absolute difference	4.6	-	-	-	12.1	
Pepels *et al*[41]	233 pN1mi	Systemic therapy	cALND	36.8	6.2	57	-	-	No difference
			SLNB	40.2	9.8	51	-	-	
			Absolute difference	3.4	3.6	6	-	-	
Sávolt *et al*[59]	474 pN0(i+)	Systemic therapy	cALND	78	87	65	-		No difference
	pN1mi		ART	69	89	58	-		
	pN1a		Absolute difference	9	2	7	-		
Montemurro *et al*[61]	321 pN0(i+)	Adjuvant chemotherapy	cALND	62	-	-	-	-	Relevant difference
	pN1mi		SLNB	52	-	-	-	-	
	pN1a		Absolute difference	10	-	-	-	-	

[1]Proportion of patients treated with adjuvant systemic therapy, based on Adjuvant. Absolute differences in proportion of prescriptions appear in grey. cALND: Completion axillary lymph node dissection; CT: Chemotherapy; HT: Hormonal therapy; SLNB: Sentinel lymph node biopsy; ART: Axillary radiation therapy.

lymph nodes areas), justifying RNI. The NCIC CTG MA20 randomized trial have compared, in patients with "high risk of LRR" BC, after systemic treatment, breast and RNI to breast irradiation only[65]. RNI was associated with a significantly improved LRR-free survival, but also, and above all, an improved distant DFS. There was a trend to better OS (P = 0.07), and survival curves diverging after 5 years ("carry-over effect"), it could result in a larger difference with time and a significant impact on survival. The assessment of the risk of positive regional nodes has mainly been studied for internal mammary chain, in relation to tumor characteristics. As expected the first risk factor for accessory nodal involvement is the macroscopic axillary nodal involvement[66-69]. These studies have not assessed the impact of ITCs or micrometastatic disease in the axillary lymph nodes, because they were conducted long before the advent of SLNB.

With the lack of focused studies, and in sofar as pN0(i+) patients are assumed to have a comparable prognosis to pN0 patients, it seems reasonable not to consider RNI only on the basis of ITCs in the SLN. In patients with micrometastatic disease in the SLN (pN1mi), no recommendation can be drawn, since several studies have shown its unfavorable impact. It seems reasonable to consider that if tumor characteristics ask for adjuvant chemotherapy (due to the risk of systemic diffusion), these same characteristics must be considered for a RNI, regional nodes that have no reason to be spared of metastatic diffusion even if mechanisms could be different.

The putative positive impact on survival of RNI in this setting must be weighted against the risk of adverse events. It has, for example, been suggested that RNI increased the dose delivered to the lung, resulting in a significant increase in lung cancer incidence[70].

CONCLUSION

The AR rate has been proved to be very low (< 2%), even without cALND, despite a NSLN-positivity proved to range from 10% to 18%, likely in relation to adjuvant treatments such as chemotherapy, hormonal therapy and radiotherapy. In the setting of BCS and MMs in the SLN, the literature data favor the omission of cALND but with a low level of evidence, precluding any definitive conclusion. Axillary irradiation in positive-SLN patients is an alternative to cALND. In pN1mi patients, treated with mastectomy without adjuvant radiotherapy, current data are insufficient to support the omission of cALND.

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Off-label use of targeted therapies in oncology

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Abstract

Off-label use is defined by the prescription of a marketed drug outside the conditions described in the summary of product characteristics. In oncology, off-label prescribing of targeted therapies may occur in patients with other tumor types expressing the same target. Agents associated to phenotypic approaches such as therapies against the tumoral vasculature (anti-angiogenic drugs) and new immunotherapies (checkpoint inhibitors) also carry the potential of alternative indications or combinations. Off-label use of targeted therapies is little documented and appears to be in the same range than that regarding older drugs with wide variations among agents. When compared with older agents, off-label use of targeted therapies is probably more rational through tumoral genotyping but is faced with a limited clinical support, reimbursement challenges related to the very high pricing and the cost of genotyping or molecular profiling, when applicable.

Key words: Targeted therapy; Monoclonal antibody; Off-label anticancer drug use; Reimbursement; Enzyme inhibitor

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Core tip: Off-label use is defined by the prescription of a marketed drug outside the conditions described in the summary of product characteristics. This review is the first one focussing on the off-label use of targeted therapies in oncology. When compared with older agents, off-label use of targeted therapies is probably more rational through tumoral genotyping but is faced with a limited clinical support, reimbursement challenges related to the very high pricing and the cost of genotyping or molecular profiling, when applicable.

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INTRODUCTION

Off-label use is defined by the prescription of a marketed drug outside the conditions described in the summary of product characteristics (also referred as the official labeling or the package insert). Off-label drug use covers many aspects such as the targeted population, the indication, the dosing regimen, the duration of treatment.

The goal of off-label prescribing is to offer a patient an alternative treatment in the absence of a licensed therapy or a lack of clinical trial access[1]. In rare cases, off-label therapy may be given instead of the approved treatment for efficacy reason (oxaliplatin and irinotecan combined with fluorouracile in metastatic pancreas cancer) or can constitute a less toxic alternative (carboplatin in stage I seminoma)[2,3].

Generally, off-label prescribing does not beneficiate from the expertise of a drug regulatory agency and its rationale is based and supported on an analysis of published data of good clinical evidence or compendia. Regarding manufacturers, they profit from off-label use because it permits the increase of sales without undergoing costly clinical trials. Ultimately, off-label prescribing has to bring an acceptable clinical response and safety profile to the patient.

Off-label use raises numerous questions of legality, responsibility, frequency, clinical evidence and reimbursement. It may be analyzed globally or more specifically from the point of view of a type of cancer, a drug or a country. Indeed, differences in labeling exist between Europe and the United States. Trotta *et al*[4] reported that for the 42 anticancer agents approved in Europe (European Medicines Agency) between 1995 and 2008, a difference of labeling with the United States (Food and Drug Administration) was identified for 47 of the 100 indications. So, rates of off-label use varies with the country of labeling, the type of tumor, the evolution of the disease, the availability of effective marketed treatments and the anticancer agent. For instance, malignancies with limited active treatments or orphan cancers are subject to off-label prescribing. In addition, drugs with few indications and possessing a non specific mechanism of action ("wide spectrum") are prone to off-label use (*i.e.*, oxaliplatine)[1].

TARGETED THERAPIES

In oncology, "targeted therapies" may be arbitrarily defined as drugs which development is based on a predetermined tumoral or endogenous target. They are generally opposed to cytotoxics even if methotrexate (amethopterine, a methyl derivative of aminopterine) could also be considered as a targeted antifolate agent[5]. These drugs (around 50 approved agents since the marketing of rituximab in 1997) are either monoclonal antibodies/fusion proteins that interact with cell membrane receptors or circulating ligands or protein/enzyme inhibitors that interfere with various tumoral signaling pathways. They mainly have narrow indications in relation with the expression of the target in a particular type of cancer (often a rare or an orphan indication) and regarding enzyme inhibitors they are mostly used orally as a single agent-therapy. Targeted therapies are very expensive (around 120000$/year in the United States)[6] when compared with previous agents or medications of others therapeutic classes and according to the country, their access to patients may be hindered by funding difficulties or partial covering.

OFF-LABEL USE OF TARGETED THERAPIES

Prevalence and clinical evidence

Off-label prescribing of targeted therapies may occur in patients with other tumor types expressing the same target (referred as precision medicine)[7]. For instance, vemurafenib, a kinase inhibitor indicated in the treatment of metastatic melanoma with activating *BRAF V600E* mutation has been used off-label in refractory *BRAF V600E* mutation positive-hairy cell leukemia[8]. Furthermore, most of kinase inhibitors are not selective meaning that they display activity against other kinases not associated with approved indications[9]. For example, sorafenib is used off-label as a FMS-like receptor tyrosine kinase-3 inhibitor in relapsed acute myeloid leukemia[10]. Agents associated to phenotypic approaches, that is to say therapies against the tumoral vasculature (anti-angiogenic drugs such as bevacizumab) and new immunotherapies (checkpoint inhibitors such as ipilimumab) also carry the potential of alternative indications or combinations.

The prevalence of off-label use focusing on targeted therapies has not been investigated in detail. These studies are not easy to perform because these agents are numerous and the class is rapidly growing (more than 30 enzyme inhibitors approved worldwide since imatinib in 2001). In addition, they are both used in the in- and outpatient settings.

A swiss study has reported a low frequence of unsupported off-label use (7.8%) for 8 recent agents in a cohort of 985 consecutive patients under systemic anticancer treatment in 2012[11]. Variations were observed among these agents with almost no off-label use for pazopanib and a high level of unsupported use for bevacizumab (29.6%)[11]. The global prevalence (supported by the European Society of Medical Oncology and unsupported) was not reported for these 8 agents.

A similar study has been conducted in the United States in 2010 using patient database and focusing on the off-label use of some expensive intravenous agents (including the monoclonal antibodies cetuximab, rituximab, trastuzumab, bevacizumab)[12]. The frequence of off-label use was 30% of that half was clinically supported by the National Comprehensive Care Network (NCCN). Among agents, the rate of off-label utilization also varied considerably between trastuzumab (1%) and rituximab (67%)[12].

Another American study based on insurance administrative database found a rate of off-label use of rituximab of 25.3% during the period 2001-2007[13]. Around 50% of off-label use was evidence-based. Among targeted therapies, rituximab is the agent that probably carries the greatest potential for off-label indications mostly beyond oncology. Indeed, a Spanish prospective investigation reported that rituximab was the most frequently used agent off-label (21.1%) among 232

drugs (considering all therapeutic classes) in 5 tertiary hospitals during one year (2011-2012)[14]. In addition, a prospective Australian national study found that off-label use of rituximab covers 63 different diagnosis with 89% of off-label use outside oncology in the year 2012[15]. This is not surprising because rituximab is a non specific lymphocytical agent having potential numerous applications in the treatment of corticosteroid-refractory autoimmune diseases.

An Italian investigation described the off-label utilization of bevacizumab during the period 2006-2007 using patient database in the region of Lombardy[16]. The anti-angiogenic monoclonal antibody was mostly used (81.7%) in patients with metastatic colorectal cancer. On-label prescribing (according to the Italian Medicines Agency) represented only 241 (30%) of the 780 patients (*i.e.*, first line treatment of metastatic colorectal cancer with fluorouracil-based chemotherapy). Off-label use concerned the timing of treatment in metastatic colorectal cancer (40%) and the use outside oncology in age-related macular degeneration (10%)[16].

More specifically, off-label use of anticancer agents has been investigated over a 10-years period (2000-2009) in a population of 2663 patients with breast cancer in the United States using an administrative data base[17]. A proportion of 13% of the patients were treated off-label mainly with cytotoxic agents. Regarding targeted therapies, off-label use of kinase inhibitors was anecdotal (0.4% of the patients). Off-label prescription of monoclonal antibodies was more prevalent (8% of the patients), particularly the agent anti-angiogenic bevacizumab (before the FDA cancelled the approval in breast cancer in 2011)[17].

Unsupported off-label use of the monoclonal antibodies panitumumab and bevacizumab has been retrospectively studied in a population of privatized insurance patients with metastatic colorectal cancer on progression, in the United States[18]. Between 2007 and 2010, off-label prescribing non-supported by the NCCN concerned 10% of the patients under bevacizumab and 16% of those under panitumumab.

Some studies have reported off-label use in populations of selected cancer patients with late-stage disease following tumor genomic testing. Preliminary experiences have described the opportunity of using tumor genomic information to guide a specific treatment in certain patients through so-called molecular tumor boards[19]. In a prospective study including 250 adult patients mostly with colorectal, breast, lung and pancreas cancers, only 10% of the patients tested could be treated mainly through a clinical trial[20]. Overall, following tumor profiling, off-label use only represented 2.8% of the patients[20]. Le Tourneau *et al*[21] investigated molecular tumor profiling representing 3 pathways (hormone receptor, PI3K/AKT/mTOR and RAF/MEK) in 741 patients with refractory metastatic disease. Around 40% of the patients were eligible to a panel of 11 off-label targeted therapies including imatinib, dasatinib, vemurafenib, sorafenib, erlotinib, lapatinib, trastuzumab and everolimus. Currently, these new strategies of treatment are not expected to bring extended off-label use because a minority of genomic alterations (10%-40%) are targetable or "druggable". Furthermore, the delay of treatment may impede access for patients whose disease progresses and in some countries like the United States, patients could be denied from treatment for covering reasons.

Overall and based on these preliminary data, off-label use of targeted therapies appears to be in the same range as that regarding older drugs (6.7%-33%) with wide variations among agents[1].

Clinical impact

As seen above, Le Tourneau *et al*[21] evaluated in a randomized phase 2 trial the clinical impact of selected molecular tumor profiling. Unfortunately, among the treated and randomized patients with "druggable tumors" (n = 195), the off-label use of targeted therapies did not improve the progression free survival (primary endpoint) when compared with those treated by chemotherapy according to the oncologist choice (around 2 mo in both arms).

A French registry has collected the off-label use of kinase inhibitors in 249 patients with sarcomas mainly pretreated (89%)[22]. Sarcoma is a very heterogeneous disease with little therapeutic options. Decision of off-label treatment was made following discussion with experts, based on a scientific rationale (96%). Sorafenib (45%), sunitinib (25%), sirolimus (9%) and imatinib (8%) were mostly used. Toxicities above or equal to grade 3 were observed in 32% of the patients. The median progression-free survival was 4.1 mo (Interval of confidence or 95%CI: 3.2-4.8) and overall, the results were judged similar to those of published trials[22].

In 2010, off-label use of the multi-kinase inhibitors sunitinib and sorafenib has been reported in 15 patients with follicular/papillary radioactive iodine refractory cancer[23]. The progression free survival was 19 mo. Since then, sorafenib gained its approval in 2013 based on a phase 3 randomized study which showed a significant improvement in the progression free survival (10.8 mo *vs* 5.8 mo with placebo)[24].

Limits

Clinically, the limits of off-label use are a lack of activity and/or the appearance of serious side effects. Tumor types carrying the same mutation and potentially eligible to off-label treatment do not respond uniformly to the targeted therapy as it has been shown in cancers with *BRAF* V600 mutations[25]. Furthermore, some off-label combinations may be detrimental. Ipilimumab and vemurafenib are both targeted therapies used as a single agent-treatment in patients with metastatic melanoma. The trial evaluating the association has been closed for safety reasons (hepatotoxicity) underscoring the risk of co-administering off-label recent agents with new mechanisms of action[26].

Regarding the re-birth of immunotherapy, this phenotypic approach that induces a T-cell response against

tumour is susceptible to be used in any kind of cancer. However, the reality is more complex and the responses vary among patients and cancers. So, the exploration of new indications should be devoted to clinical trials. Otherwise, even if in case of positive and promising results (associations of immunotherapies in metastatic melanoma), enthusiasm should be tempered[27]. Indeed, ipilimumab with nivolumab has been shown to be superior in terms of median progression free survival than the immunotherapies given alone (11.5 mo *vs* 6.9 mo for nivolumab and 2.9 mo for ipilimumab). The association was also more toxic[27]. Ipilimumab and nivolumab are currently approved as a single agent-therapy in metastatic melanoma. The off-label use of combinations of these checkpoint inhibitors is premature and is not sustainable for financial reasons.

Covering

Due to their astronomical pricing, covering recent anticancer agents in their labeled indication is a major concern in most health systems. So, off-label prescribing adds reimbursement difficulties. Covering of off-label use depends on the country, the level of clinical evidence and can constitute a barrier for certain patients. Loss of patent of monoclonal antibodies and kinase inhibitors and the forthcoming arrival of less costly biosimilars (rituximab) as well as generics may improve access in well supported clinical situations.

CONCLUSION

The prevalence of off-label use of targeted therapies in oncology is little documented but appears to be in the same range as that of cytotoxics. When compared with old agents, off-label use of targeted therapies is probably more rational through tumoral genotyping but is faced with a limited clinical support, reimbursement challenges related to the very high pricing and the cost of genotyping or molecular profiling, when applicable. Beyond positive results published through anecdotal case reports, proposals have been made to gather clinical data relative to off-label use in the United States to get better evidence[28]. Furthermore, regarding enzyme inhibitors, their activity is generally characterized by a short duration of response due to the rapid development of resistance. Sometimes, as seen with old agents, off-label use preceded a labeling (sorafenib in differentiated thyroid cancer). Some of these agents also carry a significant potential of off-label use outside oncology such as bevacizumab in ophthalmology (for economic reasons)[16,29] and rituximab in refractory autoimmune diseases.

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ORIGINAL ARTICLE

Retrospective Study

Sentinel lymph node biopsy in clinically detected ductal carcinoma *in situ*

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Informed consent statement: Patients were not required to give informed consent for the study because the analysis used anonymous clinical data that were obtained after each patient agreed to treatment by written consent. For full disclosure, the details of the study are published on the home page of Fukushima Medical University.

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Abstract

AIM: To study the indications for sentinel lymph node biopsy (SLNB) in clinically-detected ductal carcinoma *in situ* (CD-DCIS).

METHODS: A retrospective analysis of 20 patients with an initial diagnosis of pure DCIS by an image-guided core needle biopsy (CNB) between June 2006 and June 2012 was conducted at King Faisal Specialist Hospital. The accuracy of performing SLNB in CD-DCIS, the rate

of sentinel and non-sentinel nodal metastasis, and the histologic underestimation rate of invasive cancer at initial diagnosis were analyzed. The inclusion criteria were a preoperative diagnosis of pure DCIS with no evidence of invasion. We excluded any patient with evidence of microinvasion or invasion. There were two cases of mammographically detected DCIS and 18 cases of CD-DCIS. All our patients were diagnosed by an image-guided CNB except two patients who were diagnosed by fine needle aspiration (FNA). All patients underwent breast surgery, SLNB, and axillary lymph node dissection (ALND) if the SLN was positive.

RESULTS: Twenty patients with an initial diagnosis of pure DCIS underwent SLNB, 2 of whom had an ALND. The mean age of the patients was 49.7 years (range, 35-70). Twelve patients (60%) were premenopausal and 8 (40%) were postmenopausal. CNB was the diagnostic procedure for 18 patients, and 2 who were diagnosed by FNA were excluded from the calculation of the underestimation rate. Two out of 20 had a positive SLNB and underwent an ALND and neither had additional non sentinel lymph node metastasis. Both the sentinel visualization rate and the intraoperative sentinel identification rate were 100%. The false negative rate was 0%. Only 2 patients had a positive SLNB (10%) and neither had additional metastasis following an ALND. After definitive surgery, 3 patients were upstaged to invasive ductal carcinoma (3/18 = 16.6%) and 3 other patients were upstaged to DCIS with microinvasion (3/18 = 16.6%). Therefore the histologic underestimation rate of invasive disease was 33%.

CONCLUSION: SLNB in CD-DCIS is technically feasible and highly accurate. We recommend limiting SLNB to patients undergoing a mastectomy.

Key words: Non-invasive tumor; Sentinel lymph node biopsy; Ductal carcinoma *in situ*; Diagnosis; Breast cancer

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Core tip: While most ductal carcinoma *in situ* (DCIS) cases present with a radiologically detected abnormality, our sample represented a rare group of ductal carcinoma which was detected clinically. This study had a specific objective to determine the indications for sentinel lymph node in clinically detected DCIS. There are very few studies worldwide tracking this specific group, and there is no screening program in our community for breast cancer. This study will help communities who have no screening program to put protocols in place for such a specific group of patients.

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INTRODUCTION

Ductal carcinoma *in situ* (DCIS) is a non-invasive disease that does not have the metastatic potential to spread to the axillary lymph nodes. However, DCIS commonly coexists with microinvasion and invasive disease[1,2].

The preoperative diagnosis of pure DCIS can be achieved either by an image-guided core needle biopsy (CNB) or by a surgical biopsy. Image-guided CNB carries a risk of sampling error and underestimation of the presence of occult invasive disease. However, patients diagnosed with pure DCIS by a surgical biopsy show fewer sampling errors and underestimation because the whole lesion is examined to exclude the presence of invasive disease. Therefore, the diagnosis is more definitive. About 8.8%-51.5% of pure DCIS diagnosed by CNB are upstaged to DCIS with microinvasion (DCIS-MI) or invasive disease on final pathology[3]. Thus, it is of value to stage the axilla in patients diagnosed with pure DCIS by a CNB.

The simplicity of performing sentinel lymph node biopsy (SLNB), the positive SLN metastasis rate (0.39%-20%), and the high underestimation rate (8.8%-51.5%) associated with CNB has encouraged surgeons to perform SLNB in patients diagnosed preoperatively with pure DCIS[4,5]. Another argument in favor of this approach is that a positive SLN is an indirect means of diagnosing occult microinvasion or invasive disease in the breast specimen, since DCIS can coexist with microinvasion or invasive disease. Although SLNB is a less invasive procedure compared with axillary lymph node dissection (ALND), it is associated with a certain degree of morbidity and may lead to overdiagnosis and overtreatment[6]. Paresthesias, lymphedemas, and seromas have also been reported[7,8]. Therefore, the role of SLNB in DCIS is controversial and the indications for its use are not clear. In this study, we aimed to evaluate the indications for the use of SLNB in clinically-detected DCIS (CD-DCIS) with a preoperative image-guided CNB diagnosis of pure DCIS by measuring the following parameters: (1) the accuracy of performing SLNB in CD-DCIS; (2) sentinel and non-sentinel nodal positivity rate; and (3) the underestimation rate.

MATERIALS AND METHODS

We describe a single surgeon's experience with SLNB in 20 cases of CD-DCIS treated between June 2006 and June 2012 at King Faisal Specialist Hospital (KFSH) and Research Centre (RC). All data were collected prospectively and analyzed retrospectively. The inclusion criteria were a preoperative diagnosis of pure DCIS and no evidence of invasion. We excluded any patient with evidence of microinvasion or invasion. There were two

cases of mammographically detected DCIS and 18 cases of CD-DCIS. All patients were diagnosed by an image-guided CNB except for 2 patients who were diagnosed by fine needle aspiration (FNA). All patients underwent breast surgery, SLNB, and ALND if the SLN was positive.

The following clinical and pathological data were collected from the medical records: Age at diagnosis; menopausal status; initial diagnostic method; type of surgery; whether they presented mammographically or clinically; type of clinical presentation; the presence of multicentricity and multifocality; tumor size; nuclear grade; hormone receptor status; type of histology; and initial and final pathological status. Patient, clinical, treatment, and pathological characteristics are outlined in Table 1.

Preoperative lymphatic mapping

Lymphatic mapping was performed by a peri-areolar intradermal injection of 4 deposits of 0.1 mL each with 10 MBq technetium-labeled (Tc-99m) nano-colloid in each quadrant of the areola. This was performed in the nuclear medicine suite 1-4 h before surgery. Static lymphoscintigraphy was performed to visualize and localize the sentinel node.

Surgery SLNB

A hand-held gamma detector probe was used during surgery to identify the sentinel node/s. The highest radioactive node count relative to the background was regarded as the sentinel node. The SLN identification procedure at KFSH involves the sole use of Tc-99m injection and no blue dye is used during surgery.

Pathological assessment

The entire lymph node/s was submitted for intraoperative pathological examination. The node/s was sliced into 2 mm thick sections along its longitudinal axis while nodes less than 5 mm were processed uncut. Three levels were obtained from the frozen section and stained by hematoxylin and eosin (H and E). Three additional H and E-stained slices at 200 μm intervals were made on formalin-fixed, paraffin-embedded leftover frozen section tissue. Immunohistochemical stain for cytokeratin (AE1/AE3) was performed on equivocal cases. Pathological assessment for the presence of metastasis was categorized as macrometastasis (size > 2.0 mm), micrometastasis (> 0.2 mm but no larger than 2.0 mm), or isolated tumor cells (< 0.2 mm).

RESULTS

The mean age of the 20 female patients with an initial diagnosis of pure DCIS was 49.7 years (range: 35-70), 12 (60%) were premenopausal and 8 (40%) were postmenopausal. Of these patients, 18 (90%) were clinically detected and 2 (10%) were mammographically detected. Among the CD-DCIS, 15 (83.3%) presented with a palpable mass, and one with nipple discharge and 2 with Paget's disease. Eighteen (90%) patients had their initial diagnosis by CNB and 2 by FNA (10%).

The final histopathology was pure DCIS in 14 (70%) cases, DCIS with microinvasion in 3 (15%) cases, and DCIS with invasive disease in 3 (15%) cases. High nuclear grade and central necrosis was present in 11 (55%) cases and intermediate nuclear grade without central necrosis was present in 9 (45%) cases. Eleven (55%) patients had high nuclear grade and 9 (45%) patients had intermediate grade. Five (25%) patients had multicentricity or multifocality, and 17 (85%) patients had tumors larger than 3 cm.

Of the 20 patients studied, 15 (75%) had either a simple mastectomy or skin sparing mastectomy, and 5 (25%) had a lumpectomy. Postoperative radiotherapy was offered to patients who underwent a lumpectomy. Hormonal therapy was given only to patients with DCIS with microinvasion or invasive carcinoma if they were hormone receptor positive. The mastectomy rate was high because patients either had Paget's disease, multifocality, multicentricity, or extensive disease. All had a SLNB and only 2 had an ALND.

Accuracy

The accuracy of performing SLNB in DCIS was as follows (Table 2): The mean number of SLN's removed was 2 (range: 1-3); the SLN visualization rate was 100%; the intraoperative SLN identification rate was 100%; and the false negative rate was zero. All 18 cases of negative sentinels were also negative on final pathology.

Sentinel and non-sentinel nodal positivity rate

There were 2 positive SLNs and 18 negative SLNs (Table 3). One positive SLN was detected in a pure DCIS case (1/14) and the other in a patient with invasive disease (1/3). Two ALNDs were performed for the 2 positive SLNs, and in both cases the SLN was the only positive node. Therefore, the SLN positivity rate was 10%, and the non-SLN positivity rate was zero.

Underestimation rate

The underestimation rate of microinvasion and occult invasive foci is outlined in Table 4. Eighteen patients had their initial diagnosis achieved by CNB and 2 by FNA. After definitive surgery, 3 patients were upstaged to invasive ductal carcinoma (3/18 = 16.6%) and 3 to DCIS with microinvasion (3/18 = 16.6%). The 2 cases diagnosed by FNA were excluded from the calculation of the underestimation rate. Therefore image-guided CNB was associated with a 33% (6/18) underestimation rate in 18 cases with an initial diagnosis of pure DCIS.

DISCUSSION

DCIS in Saudi Arabia differs from that in Western societies in several aspects. Our patient population of DCIS present clinically, whereas DCIS in the West present with mammographically detected disease as a

Table 1 Patient characteristics

Character	NO.	%
Age		
Mean	49.7	
Range	35-70	
Menopausal status		
Pre	12	60
Post	8	40
Clinical presentation		
Mammographically detected	2	10
Clinically detected	18	90
Palpable mass	15	83.30
Nipple discharge	1	5.50
Paget's	2	11.10
Multicentricity/mulifocality	5	25
Initial diagnostic tool		
FNA	2	10
CNB	18	90
Type of surgery		
Lumpectomy	5	25
Simple mastectomy	10	50
Skin sparing mastectomy	5	25
Tumor size		
< 3 cm	3	15
> 3 cm < 6 cm	12	60
> 6 cm	5	25
Nuclear grade		
Low	0	
Intermediate	9	45
High	11	55
Histology		
With central necrosis	11	55
Without central necrosis	9	45
Final histology		
Pure DCIS	14	70
DCIS + MIC	3	15
DCIS + IDC	3	15
Hormonal receptors		
ER+ PR+	6	30
ER- PR-	10	50
Unknown	4	1
Her2/neu		
Her2/neu+	9	45
Her2/neu-	4	1
Unknown	7	35
Adjuvant radiotherapy		
Yes	5	25
No	15	75

FNA: Fine needle aspiration; CNB: Core needle biopsy; DCIS: Ductal carcinoma *in situ*; IDC: Invasive ductal carcinoma; MIC: Microinvasion; ER: Estrogen receptor; PR: Progesterone receptor.

Table 2 Accuracy of sentinel lymph node

Accuracy	NO.	%
Total number	20	
SLN removed		
Range	1-3	
Mean	2	
SLN positive	2-20	10
Non-SLN positive	0/2	0
SLN visualization rate	20	100
SLN identification rate	20	100
False negative rate	0 out of 18	0

SLN: Sentinel lymph node.

Table 3 Pathology of sentinel lymph node and non-sentinel lymph node

Final diagnosis	*n*	%	SLN positive		Non-SLN positive	
			n	%	*n*	%
Pure DCIS	14	70	1/14	7.1	None	0
DCIS/MIC	3	15	0/3	0		
DCIS/IDC	3	15	1/3	33.3	None	0
Total	20		2/20	10	0/2	0

DCIS: Ductal carcinoma in situ; MIC: Microinvasion; IDC: Invasive ductal carcinoma; SLN: Sentinel lymph node.

Table 4 Comparison between initial and final pathology "underestimation rate" *n* (%)

Initial diagnosis	Final diagnosis	Underestimation rate	
		NO.	%
Pure DCIS	Pure DCIS 14 (70) DCIS/MIC 3 (15) DCIS/IDC 3 (15)	6/18	33%

DCIS: Ductal carcinoma in situ; MIC: Microinvasion; IDC: Invasive ductal carcinoma.

result of the widespread implementation of screening programs. Paget's disease, bloody nipple discharge, and extensive and palpable disease formed a large proportion of our study population.

Saudi Arabia does not have a population-based screening program, therefore, the incidence is low (2.6%)[9] compared with the incidence of DCIS in Western societies (20%-30%)[10-12]. In Western societies during the pre-screening era, the incidence of DCIS did not exceed 5%, and patients would present with CD-DCIS similar to the current situation in Saudi Arabia[11].

The incidence and clinical significance of nodal metastasis in DCIS has been evaluated in both the pre- and the post-screening era. In the pre-screening era, ALND was not performed in DCIS because of the low incidence of axillary metastasis (1%-2%), and the high morbidity associated with ALND[12,13]. This low rate of axillary lymph node involvement in pure DCIS was attributed to missed diagnosis of invasion in the final pathology of the breast specimen[2,12]. Currently, the reported high incidence (0.39%-20%) of SLN metastasis in pure DCIS is different from that reported historically (1%-2%)[4,5,12]. The extent of the disease and the methods used for the diagnosis of DCIS are different between the 2 periods.

In the pre-screening era, a surgical biopsy was used to achieve a diagnosis, and it is a more definitive method of diagnosing pure DCIS because the whole specimen is examined to exclude the presence of invasive disease. However a CNB is currently used for the diagnosis of DCIS and is associated with sampling error and histologic underestimation of the presence of invasive disease

that varies between 8.8% and 51%[3]. There are two problems associated with the diagnosis of pure DCIS. First, a definitive preoperative diagnosis of pure DCIS cannot be achieved with an image-guided CNB. Second, even a postoperative diagnosis of pure DCIS is difficult, especially when the lesion is large and extensive[4]. The presence of occult microinvasion and invasive disease cannot be ruled out without complete tissue processing. Hence, the method of tissue diagnosis plays a central role in deciding whether or not to perform a SLNB in DCIS.

The reported high underestimation rate of (8.8%-51.5%) associated with image-guided CNB diagnosis of pure DCIS is the main factor that explains the large difference between the historically reported incidence of lymph node metastasis during the pre-screening era (1%-2%) and the currently reported incidence of SLN metastasis (0.39%-20%). To our knowledge, there is no published data on the use of SLNB in CD-DCIS. Our patient population is unique, characterized by having a large palpable DCIS with extensive disease. Despite the presence of extensive DCIS, our study has shown that the use of SLNB in CD-DCIS is highly accurate and technically feasible. The false negative rate was zero and both the SLN intraoperative identification rate and SLN visualization rate were 100%.

The SLN identification procedure at KFSH and RC involves the sole use of Tc-99m injection. We have achieved high identification rates with the use of the gamma probe technique and we believe like others (European Institute of Oncology in Milan) that the combination approach (blue dye and Tc-99m) is not worthwhile[14]. The success of this approach is obvious with the rates outlined in (Table 2).

In our study, the incidence of SLN metastasis was 10% and the incidence of non-SLN metastasis was zero. Neither of our 2 patients with a positive SLN had additional disease in the axillary nodes following complete axillary dissection. Therefore, the risk of additional metastasis after a positive SLNB in pure DCIS was nil in our experience. Also, none of our patients with DCIS developed an axillary recurrence. We had one case of pure DCIS with a positive SLNB. The only explanation would be a missed undetected occult invasive focus in the breast specimen. This reflects that even the most meticulous complete tissue processing with serial sectioning can miss the an invasive component responsible for the sentinel node metastasis[15,16]. This is a very rare event that does not justify performing routinely SLNB routinely in pure DCIS. We had one additional case of DCIS with a positive SLN who had an occult invasive focus.

The low positive rate of SLN metastasis in this study (10%) is in line with that reported in the literature and is consistent with other published reports[17], suggesting the safety of delaying a SLNB until a definitive diagnosis of invasive disease is achieved. The single largest institutional study of 854 patients with DCIS treated with SLNB at the European Institute of Oncology had an incidence of SLN metastasis of only 1.4% and when patients with micrometastasis were excluded the incidence was only 0.6%[14]. A meta-analysis conducted by Ansari *et al*[14] has shown that the incidence of SLN metastasis was 7.4% in patients with a preoperative CNB diagnosis of DCIS, compared to 3.7% in patients with a definitive postoperative diagnosis of DCIS.

The reported rate of underestimation of invasive disease following a CNB diagnosis of pure DCIS varies among different institutions (8.8%-51.5%)[3]. This wide range reflects the different methods used for core biopsy, pathological assessment, and different patient populations. Image-guided CNB in our institution was associated with a high rate of underestimation (33%). After definitive surgery, 3 patients were upstaged to invasive ductal carcinoma (16.6%) and 3 to DCIS with microinvasion (16.6%). Therefore the total histologic underestimation rate of invasive disease was 33%. This is similar to other published studies (8.8%-51.5%)[3]. However, this high rate should not be used as an argument favoring the routine use of SLNB because it will expose about 67% of our patients to an unnecessary SLNB. CD-DCIS and widespread DCIS are expected to be associated with a higher risk of occult microinvasion and invasive disease than localized screening detected DCIS[5,12].

The risk of microinvasion and occult invasive ductal carcinoma correlate with the extent and size of DCIS, nuclear grade, and histologic type, and our results support this as the majority of our patients had large volume tumors and a 33% probability of containing either microinvasion or occult invasive disease. Therefore the high underestimation rate encountered in this study may be explained by the larger tumor size and greater extent of disease.

Advocates of the routine use of SLNB in pure DCIS diagnosed by image-guided CNB base their rationale on 4 main facts. First, the reported high incidence of SLN metastasis (0.39%-20%) in DCIS[4,5]. Second, the reported high rate of underestimation (8.8%-51.5%) associated with CNB[3]. Third, SLN metastasis is an indirect method of diagnosing occult invasive disease that may be missed on routine postoperative examination of the final specimen[17]. Fourth, an immediate SLNB will spare patients a second surgery if they are found to have invasive disease on final pathology[17,18]. Opponents of the routine use SLNB in DCIS summarize their reasoning according to the following 5 reasons. First, SLNB should not be performed for patients with pure DCIS because of its non-invasive biological behavior[1]. Second, a SLNB will be unnecessary in the majority of patients with a preoperative image-guided CNB diagnosis of DCIS because the final postoperative histology will only contain pure DCIS and will not contain any invasive disease[2,19]. Third, although SLNB is a less morbid surgical procedure compared with ALND, it is also associated with complications[7,8]. Fourth, SLNB can also lead to excessive and unnecessary treatment by providing misleading information about the axillary status. The disease detected in the SLN may not be clinically significant and

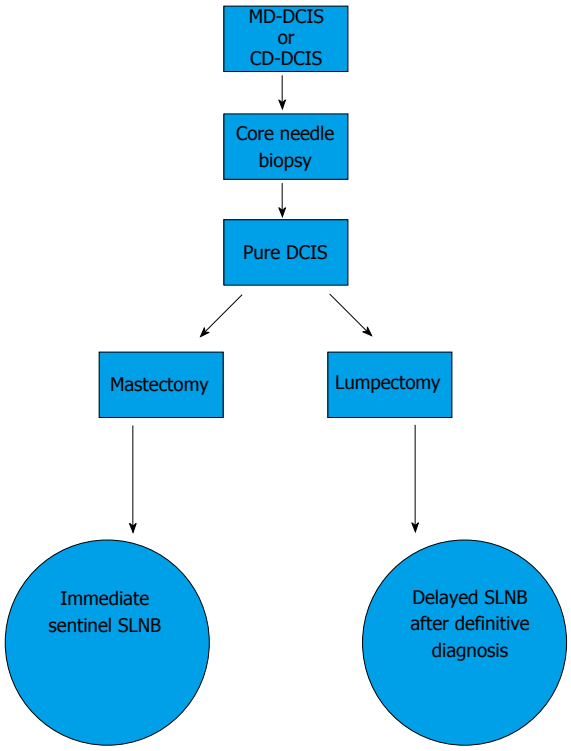


Figure 1 Ductal carcinoma *in situ* algorithm at King Faisal Specialist Hospital and Research Centre. MD-DCIS: Mammographically detected ductal carcinoma *in situ*; CD-DCIS: Clinically-detected ductal carcinoma *in situ*; SLNB: Sentinel lymph node biopsy.

may lead to unnecessary axillary dissection and adjuvant chemotherapy[6]. Finally a redo SLNB may be inaccurate and less successful in patients who later develop invasive disease[17].

The following is our suggested algorithm for SLNB in DCIS (Figure 1). The aim is to minimize the morbidity as much as possible and to limit SLNB to only those who will benefit from the procedure. We believe that the method of diagnosis (CNB and surgical biopsy), the risk of invasion, and the type of surgery (lumpectomy and mastectomy) are the 3 major determinants for the need for SLNB in DCIS. The high underestimation rate (33%), the low positive SLN rate (10%), and the morbidity associated with SLNB has encouraged us to limit the procedure to those who will undergo a mastectomy or immediate reconstruction or a wide local excision involving the upper outer quadrant. This is because of the difficulty of performing a SLN procedure after these surgical procedures as they may disrupt the lymphatic pathways toward the axilla. All patients presenting with DCIS and microinvasion or occult invasive disease should have a SLNB because of the risk of SLN metastasis. Patients with a diagnosis of pure DCIS following an excisional biopsy do not need a SLNB because the diagnosis of pure DCIS is definitive.

This approach of limiting SLNB to these patients is consistent with our understanding of the natural history of pure DCIS, *i.e.*, its inability to metastasize to the lymph nodes. Patients with a diagnosis of pure DCIS following a CNB have a high rate of underestimation (8.8%-51.5%), as in our patient population (33%), and may require a SLNB because the diagnosis of pure DCIS is not definitive. They can either have an immediate SLNB at the same setting of their conservative surgery, or they can have a delayed SLNB after a definitive diagnosis of invasive disease is made following a diagnostic and therapeutic lumpectomy. Both options are valid, but we recommend the delayed SLNB approach as this will limit axillary staging to only those who have an invasive component and will spare patients with pure DCIS an unnecessary SLNB. The main disadvantage of an immediate SLNB is unnecessarily subjecting the majority of our patients with pure DCIS to SLNB. This corresponds to 67% of our DCIS population. The main advantage of an immediate SLNB is in patients with DCIS with microinvasion and DCIS with occult invasive foci, hence avoiding a revisit to the operating theatre. This corresponds to (33%) in our patient population of DCIS.

Delayed SLNB has the advantage of avoiding an unnecessary SLNB in pure DCIS (67% in our population), thus limiting SLNB to DCIS with microinvasion or occult invasive disease. Also, it will not interfere with a future SLNB if needed in case of invasive recurrence. The only disadvantage is a second visit to the operating room in cases of upstaging to occult invasive disease.

Theoretically, there is concern that a wide local excision in the upper outer quadrant will disrupt the lymphatic drainage into the sentinel lymph node and therefore will negatively affect the accuracy of performing a SLNB after such a surgical procedure[19,20]. Unfortunately, the literature in this area is limited and controversial[20]. Therefore, we would recommend performing an immediate SLNB for patients having a wide local excision in the upper outer quadrant, in order to minimize the risk of an unsuccessful delayed SLNB. For patients undergoing a limited small lumpectomy in the upper outer quadrant that will not interfere with the lymphatic drainage of the SLN, a delayed SLNB is appropriate.

In conclusion, SLNB in CD-DCIS is technically feasible and highly accurate. Although the majority of our patients presented with extensive DCIS and had a high rate of underestimation of invasive cancer (33%), the overall rate of SLN positivity was only 10%. Therefore, we recommend limiting SLNB to patients with pure DCIS undergoing a mastectomy or a wide local excision in the upper outer quadrant.

COMMENTS

Background

Ductal carcinoma *in situ* (DCIS) is a non-invasive disease that does not have the metastatic potential to spread to the axillary lymph nodes. DCIS commonly coexists with microinvasion and invasive disease. The main diagnostic tool for DCIS is core needle biopsy (CNB) which is associated with a high underestimation rate, which indicates the coexistence of invasive disease. The simplicity of the sentinel lymph node biopsy (SLNB) encourages surgeons to perform SLNB. SLNB is a surgical procedure not devoid of complications. Hence, the use of SLNB in DCIS is controversial. The authors aimed to evaluate the indications of SLNB in clinically detected (CD) DCIS by measuring accuracy (100%), nodal positivity (10%), and underestimation rate (33%).

Research frontiers

The use of SLNB in CD-DCIS which is diagnosed by CNB prior to excision in this study is indicated in patients undergoing mastectomy or wide local excision in the upper outer quadrant.

Innovations and breakthroughs

The results of this study regarding accuracy, nodal positivity, and underestimation rate are similar to other reported studies. The authors had a high identification rate for SLN with use of Tc-99m without a dye like some other centers in the world, and the authors believe the combined technique (radioisotope and dye) is not worthwhile.

Applications

The use of SLNB in CD-DCIS is feasible but should not be indicated for all patients with such a diagnosis. The criteria are patients who are undergoing mastectomy or a wide local excision in the upper outer quadrant.

Terminology

DCIS is a noninvasive form of ductal carcinoma, limited to the confines of the basement membrane of the duct (also referred to as intraductal carcinoma). CD-DCIS is DCIS which is palpable or has specific signs which can be detected clinically. SLN is the first node on the lymphatic drainage pathway from a primary. SLNB is a technique using a detector material (dye, radioisotope material) to identify the SLN.

Peer-review

This is an interesting small clinical study on the use of SLNB for carcinoma *in situ* of the breast. The study is of particular interest for settings where screening programs are not in place and DCIS is diagnosed clinically.

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CASE REPORT

Gallbladder adenocarcinoma and paraneoplastic parathyroid hormone mediated hypercalcemia

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Author contributions: Yogarajah M, Sivasambu B and Shiferaw-Deribe Z made substantial contributions to the conception, drafting and revision of the manuscript; Yogarajah M, Sivasambu B and Shiferaw-Deribe Z approve the version submitted and are in agreement to be accountable for all aspects of the work.

Institutional review board statement: This case report was exempt from the standards of Interfaith Medical center Institutional review board.

Informed consent statement: The patient involved in this study gave her written informed consent authorizing use and disclosure of her protected health information.

Conflict-of-interest statement: None of the authors has any conflicts of interest to declare.

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Abstract

Parathyroid hormone mediated hypercalcemia is not always exclusively primary hyperparathyroidism and rarely could be due to ectopic parathyroid hormone secretion from tumor cells. We present a case of 86-year-old female with metastatic gall bladder adenocarcinoma diagnosed eight months back who presented with generalized fatigue and poor oral intake and was found to be hypercalcemic with elevated parathyroid hormone levels. Imaging with technetium 99 m sestamibi scintigraphy with dual phase, subtraction thyroid scan (dual isotope scintigraphy), magnetic resonance imaging and ultrasonography did not demonstrate any parathyroid lesion in normal or ectopic sites. We believe that the tumor cells were the source of ectopic parathyroid hormone secretion as we had excluded all the other possibilities with extensive combined imaging thereby increasing the sensitivity of our testing. We report the first case of metastatic gall bladder adenocarcinoma with paraneoplastic ectopic parathyroid hormone secretion.

Key words: Paraneoplastic hypercalcemia; Gallbladder adenocarcinoma; Hypercalcemia; Hyperparathyroidism; Ectopic parathyroid secretion

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Core tip: Gallbladder carcinoma rarely can cause hypercalcemia with very few reported cases but they were all due to parathyroid hormone (PTH) related peptide. We present the first case of metastatic gallbladder adenocarcinoma with hypercalcemia due to ectopic PTH secretion from tumor cells. PTH mediated hypercalcemia is not exclusively primary hyperparathyroidism and awareness of this rare paraneoplastic presentation will prevent unnecessary parathyroid surgeries. Moreover this case prompts physicians to look for another

ectopic source in the absence of parathyroid lesion which would facilitate early diagnosis of an underlying malignancy.

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INTRODUCTION

Hypercalcemia is a commonly encountered metabolic disorder which can range from mild asymptomatic hypercalcemia to severe symptomatic hypercalcemia. The imbalance between calcium absorption, urine excretion and bone resorption leads to elevated levels of calcium levels as seen in excessive bone resorption, increased gastro intestinal absorption of calcium and impaired urinary excretion of calcium. The most common etiologies are primary hyperparathyroidism and malignancy which constitute more than 90% of the hypercalcemia cases. 20%-30% of patients with malignancy develop hypercalcemia and is considered to be a poor prognostic factor[1]. Hypercalcemia of malignancy has been described since early 1920 and has been evolving with the humoral factor involvement first proposed in 1941 followed by discovery of parathyroid hormone related peptide (PTHrP) in 1987. Humoral hypercalcemia of malignancy due to ectopic parathyroid hormone (PTH) secretion by tumor cells is a very rare entity with only few reported cases. We report the first case of metastatic gallbladder adenocarcinoma presenting with hypercalcemia secondary to ectopic PTH secretion.

CASE REPORT

An 86-year-old female was brought to the emergency department due to generalized weakness, loss of appetite and poor oral intake for 5 d. She denied any fever, increased urine output or constipation. Review of the systems was unrevealing. Her granddaughter whom the patient lives with stated that the patient was lethargic and was occasionally confused for the last 5 d. Eight months back patient was diagnosed to have gall bladder adenocarcinoma with hepatic and peritoneal metastasis and underwent cholecystectomy, however decided not to continue any further treatment with chemotherapy. She had no other medical problems and was not on any medications. Physical examination revealed evidence of dehydration with dry mucous membranes but she was hemodynamically stable. Patient was found to be lethargic however was arousable and oriented to time, place and person. All other systems examination was negative.

Initial labs revealed mild macrocytic anemia with a hemoglobin of 11.4 g/dL. The leucocyte count and platelet count was normal. There was evidence of mild dehydration with mildly increased blood urea nitrogen at 29 mg/dL (NR 8-20) and normal creatinine. She had significantly elevated corrected total calcium of 15.5 mg/dL with albumin of 3 g/dL. Phosphorous was 2 mg/dL (NR = 2.4-4.7). The liver enzymes showed an alkaline phosphatase of 142 IU/L (NR = 32-91) and other liver enzymes and bilirubin were normal.

Patient was hydrated and was started on calcitonin for immediate control of calcium levels and subsequently was given intravenous pamidronate of 90 mg. Hypercalcemia resolved by day 3 of admission and she became more awake and alert.

Further evaluation to determine the etiology of hypercalcemia showed a significantly elevated PTH of 162 pg/mL (normal range 15-65) and normal PTHrP, 25-hydroxyvitamin D and 1,25 dihydroxyvitamin D levels suggestive of a biochemical diagnosis of primary hyperparathyroidism. To localise the parathyroid lesion imaging with technetium-99 m (Tc-99 m) sestamibi scintigraphy with dual phase (Figure 1) and subtraction thyroid scan (dual isotope scintigraphy) with technetium pertechnetate (Figure 2) were done and reviewed by experienced nuclear medicine radiologist however did not reveal any parathyroid lesion.

PTH was repeated again and was persistently high at 185 pg/mL with normal PTHrP. Additional imaging with magnetic resonance imaging and ultrasonography was obtained to increase the sensitivity of the testing however was negative for parathyroid lesion in anatomical and ectopic sites but did show a 1.1 cm nodule in the left lobe of the thyroid gland. The possibility of ectopic parathyroid tissue was considered and ultrasound guided fine needle aspiration of thyroid gland performed did not demonstrate any parathyroid tissue and cytology revealed benign follicular adenoma. Thyroid function test was normal. A bone scan was negative for metastasis.

Patient had biochemical evidence of PTH mediated hypercalcemia but had no parathyroid lesions in anatomical and ectopic sites after extensive multiple imaging confirming the ectopic nature of the PTH secretion. The calcium levels prior to this admission was reviewed and was always normal with sudden dramatic elevation during this admission which is usually unlikely with primary hyperparathyroidism in which gradual increase of calcium is noted. This elevated PTH likely could be explained by ectopic PTH secretion by tumor cells in a patient with history of metastatic gallbladder cancer. We reviewed the previous histological slides of the primary tumor (Figure 3) and did immunohistochemical staining for PTH (Figure 4) but it was negative, as at the time of diagnosis patient was not hypercalcemic and likely tumor cells did not demonstrate PTH.

Patient developed another episode of hypercalcemia after 18 d of pamidronate administration and was started on cinacalcet after which her calcium levels remained controlled. Patient did not want to pursue with chemotherapy which would have been the best option to control the hypercalcemia and preferred to be in comfort

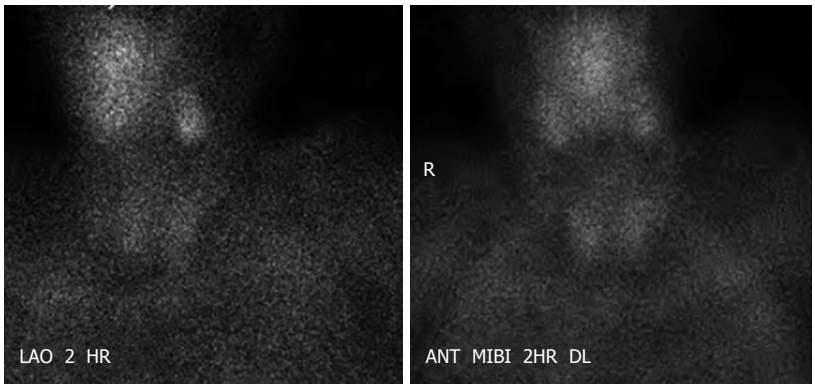


Figure 1 Technetium-99 m sestamibi scintigraphy with dual phase.

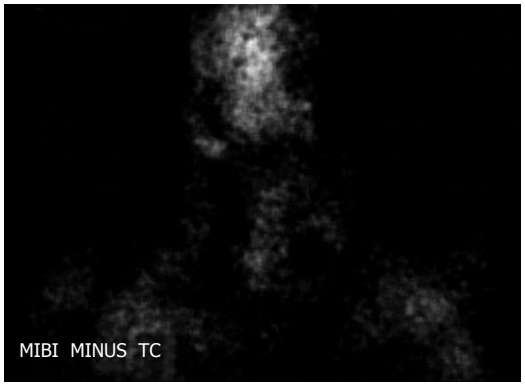


Figure 2 Subtraction thyroid scan (dual isotope scintigraphy) with technetium pertechnetate.

care and was placed on home hospice.

DISCUSSION

Hypercalcemia could be a paraneoplastic manifestation that could occur due to multiple mechanisms. The commonest cancers associated with hypercalcemia are breast cancer, lung cancer and multiple myeloma[1]. Hypercalcemia of malignancy could result due to bone metastasis and induction of osteoclasts *via* cytokines or could occur in the absence of bone metastases due to humoral factors such as PTHrP or calcitriol secreted by the tumor. In 1941 Fuller Albright first described humoral hypercalcemia of malignancy which he postulated is occurring due to PTH or a similar peptide[2]. In 1987 PTHrP was discovered and attributed to the development of humoral hypercalcemia of malignancy. PTHrP is encoded in a different gene to that of PTH and therefore the amino acids sequence and immunoreactivity are distinct though both have a common receptor.

Hypercalcemia is classified based on PTH levels with a normal or high value pointing towards PTH mediated hypercalcemia as seen in primary hyperparathyroidism and suppressed levels favoring the diagnosis PTH independent hypercalcemia as seen in humoral hypercalcemia. Elevated PTHrP will lead to suppressed PTH levels and presence of elevated PTH levels highly suggests concurrent primary hyperparathyroidism. However there are reported cases of humoral hypercalcemia in which the tumor cells secreted both PTH and PTHrP[3].

Our patient had significantly elevated PTH and normal PTHrP suggesting a diagnosis of primary hyperparathyroidism. However with combined imaging of technetium 99 m sestamibi scintigraphy with dual phase, subtraction thyroid scan (dual isotope scintigraphy), ultrasonography and magnetic resonance imaging with T1 and T2 weighted images we were unable to demonstrate any parathyroid lesion in anatomical or ectopic sites excluding the possibility of primary hyperparathyroidism. The sensitivity of sestamibi scan combined with ultrasonography is 95% in detecting parathyroid lesions[4]. When magnetic resonance imaging is added, the sensitivity to detect parathyroid lesions increases to 96.2% and sensitivity to detect ectopic parathyroid lesions increases to 100%[5].

Ectopic secretion of PTH by tumor cells as a mechanism of hypercalcemia of malignancy has been reported very rarely with various malignancies. Doyle *et al*[6] reviewed the cases of malignancies associated with ectopic PTH secretion since the discovery of PTHrP, and the techniques used to demonstrate PTH and found 17 reported cases so far with their case being the 18th case.

Gall bladder adenocarcinoma is not commonly associated with paraneoplastic hypercalcemia and has been scarcely described in literature[7-10]. However the hypercalcemia was associated with elevated PTHrP[7] and this is the first case of Gall bladder adenocarcinoma with ectopic PTH secretion.

Various techniques has been used in demonstrating ectopic PTH secretion. Immunohistochemical staining for PTH, PTH mRNA synthesis of tumor cells similar to that of parathyroid cells identified by Northern Blot, PTH gene expression, venous sampling with high level of PTH in the draining vein and sestamibi scan were the methods used to confirm ectopic PTH secretion.

Unfortunately we were unable to demonstrate PTH in the tumor cells. Initial primary tumor cells did not show PTH on immunohistochemical staining as likely at that point there was no PTH expression and patient also didn't have hypercalcemia. She developed hyper-

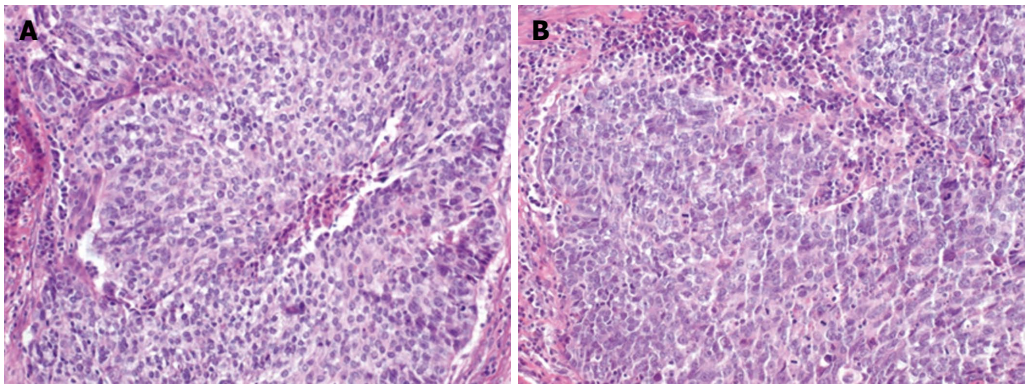


Figure 3 Gallbladder H and E stain. High power (40 ×) view of the nested tumor shows a relatively monotonous cell population with medium-sized nuclei and a moderate amount of cytoplasm. Central coagulative necrosis is evident on (A) while (B) has frequent mitotic figures.

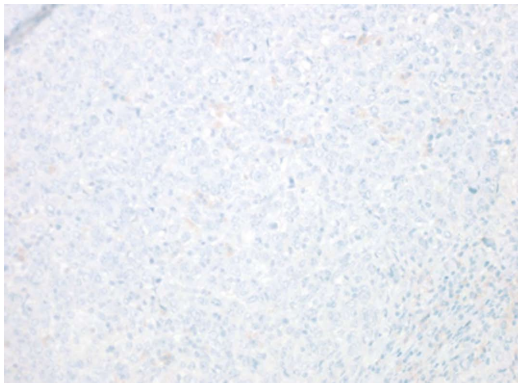


Figure 4 Gallbladder Immunohistochemical stain is negative for parathyroid hormone (a positive stain would have clear and sharp membranous expression).

calcemia after 8 mo of diagnosis of metastatic gall bladder adenocarcinoma and repeating the biopsy to demonstrate PTH in the tumor cell at the hypercalcemic phase was not at the best interest of the patient who was not willing for any further intervention. The only explanation for the significantly elevated PTH in the absence of a parathyroid lesion and ectopic parathyroid could only be ectopic PTH secretion by the tumor cells.

Treatment of ectopic PTH mediated hypercalcemia of malignancy is challenging with no definitive management. In most cases treatment of the underlying malignancy helped in controlling the hypercalcemia. Transcatheter arterial embolization has been utilized in treating hepatocellular carcinoma with PTH secretion[11]. Hypercalcemia of malignancy is treated with bisphosphonates and in refractory cases denusomab has been approved by Food and Drug Administration. However ectopic PTH secretion biochemically mimic's primary hyperparathyroidism. In patients with primary hyperparathyroidism who are not candidates for surgery bisphosphonates are preferred when there is evidence of osteoporosis or osteopenia. In the absence bone loss cinacalcet is the choice. Cinacalcet lowers PTH and calcium by increasing sensitivity of calcium-sensing receptor (CaSR). Under and over expression of CaSR in various malignancies has been found and the role of cinacalcet in treating cancer needs further studies and therefore the role in treating ectopic PTH induced hypercalcemia of malignancy is still obscure[12].

We started our patient on pamidronate and though she initially had a response she relapsed later with recurrent hypercalcemia. There was no option for chemotherapy to control the hypercalcemia as patient refused. She was started on cinacalcet to lower her calcium and responded well.

We present the first case of hypercalcemia due to ectopic PTH secretion of metastatic gallbladder adenocarcinoma. Awareness of this rare presentation will prevent unnecessary parathyroid surgeries. Moreover this case prompts physicians to look for another ectopic source in the absence of parathyroid lesion which would facilitate early diagnosis of an underlying malignancy.

COMMENTS

Case characteristics

An 86-year-old female was brought to the emergency department due to generalized weakness, loss of appetite and poor oral intake for 5 d and a history of metastatic gallbladder carcinoma.

Clinical diagnosis

She had evidence of dehydration with dry mucous membranes. She was found to be lethargic however was arousable and oriented to time, place and person.

Differential diagnosis

Dehydration due to poor oral intake, sepsis.

Laboratory diagnosis

Corrected total calcium was 15.5 mg/dL. Further workup showed parathyroid hormone of 162 pg/mL (normal range 15-65).

Imaging diagnosis

Technetium-99 m sestamibi scintigraphy with dual phase, subtraction thyroid scan (dual isotope scintigraphy) with technetium pertechnetate, magnetic resonance imaging and ultrasonography did not show and parathyroid lesion.

Pathological diagnosis

Biopsy of the thyroid nodule showed benign follicular adenoma and no ectopic parathyroid gland.

Treatment

IV pamidronate followed by Cinnacalcet to control Ca as patient refused chemotherapy.

Related reports

Gallbladder carcinoma rarely can cause hypercalcemia with very few reported cases but they were all due parathyroid hormone (PTH) related peptide. The authors present the first case of metastatic gallbladder adenocarcinoma with hypercalcemia due to ectopic PTH secretion from tumor cells.

Experiences and lessons

The authors present the first case of hypercalcemia due to ectopic PTH secretion of metastatic gallbladder adenocarcinoma. Awareness of this rare presentation will prevent unnecessary parathyroid surgeries. Moreover this case prompts physicians to look for another ectopic source in the absence of parathyroid lesion which would facilitate early diagnosis of an underlying malignancy.

Peer-review

The paper is well written.

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CASE REPORT

Isolated subcutaneous implantation of a borderline ovarian tumor: A case report and review of the literature

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Author contributions: Banys-Paluchowski M and Yeganeh B designed the report; Luettges J and Maibach A performed the histopathological analyses; Langenberg R and Maul H collected the patient's clinical data; Banys-Paluchowski M, Krawczyk N, Paluchowski P and Gebauer G analyzed the data and wrote the paper.

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Abstract

Laparoscopy-related tumor implantations of gynecological malignancies into the subcutaneous tissue are rarely diagnosed. We report an interesting case of a 46-year-old female who presented with an abdominal subcutaneous metastasis of a borderline ovarian tumor. The patient received a laparoscopic unilateral adnexectomy for a solid-cystic tumor of the right ovary. Histopathological workup showed a papillary borderline tumor of mucinous type. Nine days later she underwent a hysterectomy, left adnexectomy, appendectomy and omentectomy. Exploration of the peritoneum revealed no intraperitoneal implants. Further exploration showed a non-invasive implant of a borderline tumor in the subcutaneous tissue above the fascia that had no contact to the peritoneum. It is hypothesized that tumor cells may have been implanted during a previous laparoscopy, the most recent of which had been fourteen years prior to her current presentation. Various risk factors for port-site malignancies have been identified. Tumor manipulation and extraction of tumor tissue without a protective bag may contribute to development of trocar-site metastasis.

Key words: Laparoscopy; Subcutaneous metastasis; Tumor implantation; Borderline tumor of the ovary

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Core tip: Trocar-site subcutaneous metastasis rarely develops after laparoscopic surgery. Avoidance of over-manipulation of the tumor and use of a protective bag for removal of tumor tissue may minimize the risk of this complication.

Banys-Paluchowski M, Yeganeh B, Luettges J, Maibach A, Langenberg R, Krawczyk N, Paluchowski P, Maul H, Gebauer G. Isolated subcutaneous implantation of a borderline ovarian tumor: A case report and review of the literature. *World J Clin Oncol* 2016; 7(2): 270-274 Available from: URL: http://www.wjgnet.com/2218-4333/full/v7/i2/270.htm DOI: http://dx.doi.org/10.5306/wjco.v7.i2.270

INTRODUCTION

Minimally invasive surgery is a standard procedure to clarify undefined intraabdominal masses and its use is increasing in gastroenterologic surgery, gynecology and general surgery. However, tumor seeding into abdominal wall tissues may occur during laparoscopic removal of the mass[1]. We report an interesting case of subcutaneous implantation of a borderline ovarian tumor (BOT) and hypothesize that the implantation occurred during last laparoscopy conducted 14 years prior to presentation.

CASE REPORT

A 46-year-old female presented at our Gynecological Cancer Center (certified by the German Cancer Society) with a cyst with intracystic papillary projections in the right ovary, measuring 2.5 cm × 2.8 cm (Figure 1). Her left ovary and uterus were sonographically normal and she had no free intraabdominal fluid. The patient was asymptomatic. She reported no abdominal discomfort or changes in her bowel or bladder habit. CA125 was 38.7 U/mL. Her concurrent medical history included obesity, latent hypothyroidism and World Health Organization (WHO) grade I arterial hypertension. She had undergone two previous laparoscopies, one for an ectopic pregnancy in 1990, and another for a sterilization procedure six years later, and had had one vaginal birth. Her family history was unremarkable.

We recommended one-sided laparoscopic adnexectomy. Laparoscopy revealed an enlarged right ovary with smooth surface. The patient's uterus and left ovary were macroscopically unremarkable and she had no ascites. A right adnexectomy was performed, with a protective bag used for retrieval of the intact tumor. Histopathological analysis revealed a mucinous papillary borderline tumor (Figure 2). After discussion in the interdisciplinary tumor board meeting, explorative laparotomy with adequate surgical staging was recommended according to national treatment guidelines[2]. Nine days after the laparoscopy, the patient received median exploratory laparotomy with hysterectomy, left adnexectomy, multiple peritoneal biopsies, appendectomy and omentectomy. Pelvic and para-aortic lymph nodes were clinically unsuspicious. Systematic staging revealed no intraperitoneal implants. During further exploration, a cystic-solid structure (5 cm × 5 cm) was found in the subcutaneous fat tissue above the fascia with no contact with the peritoneal cavity. This tumor was located far from the trocar sites used during laparoscopy nine days prior to laparotomy. The tumor was completely excised; histological analysis showed a non-invasive implant of a papillary borderline tumor (Figure 3). The patient had an uneventful recovery and received no further therapy. After five years of follow-up she is alive and disease-free.

DISCUSSION

Tumor implantation in the surgical wound is a rare complication of oncological surgery (Table 1). In the present report, the patient was diagnosed with a synchronous abdominal and subcutaneous manifestation of a BOT. This heterogeneous group of tumors with characteristic histological features comprises 15%-20% of all epithelial ovarian malignancies with an incidence of 1.8-4.8 per 100000 women per year[3]. In contrast to ovarian cancer, BOTs occur at a younger age and have excellent prognosis. However, recurrences are observed in one-tenth of patients; tumor stage, postoperative macroscopic disease and the presence of invasive and noninvasive implants are predictors of relapse risk and survival. In a large meta-analysis of more than 4000 patients, the most reliable prognostic indicator for advanced stage tumors was the type of peritoneal implant; the survival of patients with noninvasive implants was 95%, compared to only 66% for invasive implants[4]. With regard to histological subtype, the majority of borderline tumors are serous, followed by mucinous subtype, whereas most ovarian cancers are serous but the mucinous type is rare. According to the WHO definition, the principal diagnostic feature of a borderline tumor is the absence of obvious stromal invasion; their mitotic activity and nuclear abnormalities are between clearly benign and unquestionably malignant tumours of a similar cell type. In many cases, extensive specimen sampling is necessary to establish the diagnosis; a histological review by a reference pathologist may be helpful to adequately classify the tumor.

The use of laparoscopy in BOTs and ovarian cancer remains controversial. Heitz *et al*[5] retrospectively analyzed a cohort of ovarian cancer patients who had laparoscopy before definite cytoreductive surgery. In 66 patients, port sites were excised and histopathologically examined: Abdominal wall metastasis was revealed in 47% of cases. However, this finding did not influence clinical outcome. The authors concluded that patients with highly suspected ovarian malignancy and ascites were the most likely to benefit from a direct referral to a

Table 1 Literature review of studies focusing on laparoscopy-related port-site metastasis

Ref.	No. of patients	Cohort	Type of surgery	Results
Chua *et al*[9], 2011	11027 from 17 studies	Various cancers (colon, rectal, gynecological, gastric, liver, urological)	Laparoscopic surgery or diagnostic surgery for malignant abdominal disease	The incidence of PSM is low (< 2%); in eight randomized trials comparing laparoscopy to open surgery for cancer, there was no statistical difference in the development of PSM or wound metastasis (none of these eight trials included gynaecological cancer patients)
Zivanovic *et al*[1], 2008	1694 (retrospective analysis of all laparoscopies in a 16-yr period)	Various gynecological cancers (ovarian, perotineal, uterine, cervical)	Laparoscopy in women with malignant disease	Laparoscopy-related subcutaneous tumor implantation occurred in 20 cases (1.18%); only one case of "isolated" PSM was reported; subcutaneous tumor implantations usually occurred with carcinomatosis, with synchronous metastases to other sites; patients whose preceding laparoscopy was performed for advanced or recurrent abdominopelvic disease were more at risk for developing PSM; patients who developed PSM within 7 mo after laparoscopy had a median survival of 12 mo compared to 37 mo in patients who developed PSM > 7 mo after laparoscopy
Martínez *et al*[10], 2010	1216 (retrospective analysis of all laparoscopies)	Uterine (921) and cervical (295) cancer	Laparoscopy in women with malignant disease	The overall incidence of PSM was very low (0.4% per procedure) after a median follow-up of 49 mo; the median time to the development of PSM was 8 mo; patients with peritoneal carcinomatosis were most at risk for developing PSM
Ndofor *et al*[8], 2011	181	Various gynecological cancers (uterine, cervical, ovarian)	Robotic surgery (DaVinci Surgical system) in women with malignant disease	PSM occurred in two cases (1.1%); there were no cases of isolated PSM
Heitz *et al*[5], 2009	66	Ovarian cancer	Laparoscopy followed by cytoreductive laparotomy with resection of port sites	Port site metastasis were present in 47% of patients; patients with ascites, higher tumor stage and peritoneal carcinomatosis were more at risk of developing PSM

PSM: Port site metastases.

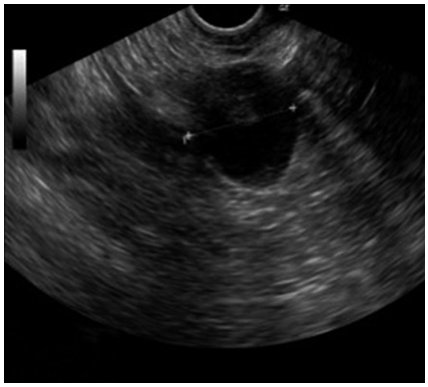


Figure 1 Ultrasound of the right ovary showing a smooth cyst with intracystic papillary structures. There was no ascites.

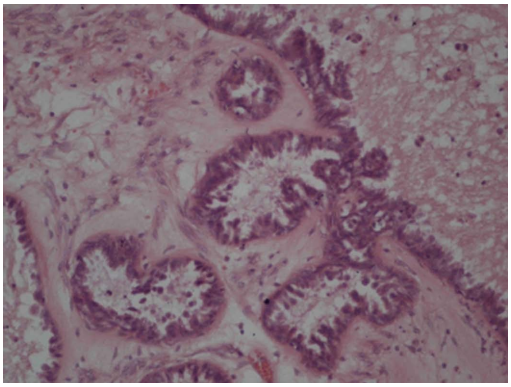


Figure 2 Borderline tumor of the right ovary. Hyperchromatic nuclei with large nucleoli; tumorous structures with hypervascularized papillary stroma without invasion into the center of the cyst.

cancer center. In their review, Wang *et al*[6] reported that the risk of an early occurrence of port site metastases (PSM) was the highest in patients with ovarian cancer, ascites, and diagnostic or palliative procedures for malignancy. A more recent review by Ramirez *et al*[7] showed that port site recurrences may occur in patients with various gynecological malignancies, such as ovarian, cervical, uterine, vaginal and fallopian tube carcinoma and are isolated to the tissue-manipulating port in most patients. Ndofor *et al*[8] evaluated the rate of PSM after robotic endoscopic surgery for gynecological cancer. In a cohort of 181 patients undergoing robotic oncological operations, two (1.1%) developed trocar site metastases. Both patients were diagnosed with other metastases as well; there were no cases of isolated port site recurrences. In the present report, since the subcutaneous tumor was localized far from the trocar

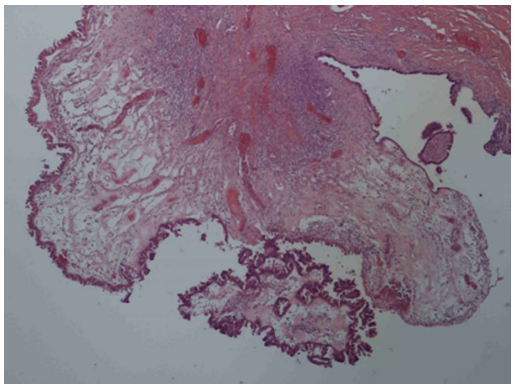


Figure 3 Epifascial non-invasive implant of a papillary borderline tumor.

incisions used during the laparoscopy nine days before laparotomy and had no contact to the intraperitoneal cavity, it may be hypothesized that tumor cells may have been implanted during one of the previous laparoscopies, the most recent of which had been fourteen years prior to her current presentation.

It is not yet clear which mechanisms play a role during trocar site implantation of tumor cells. Direct implantation may occur through forced retrieval of the mass or by instruments that had contact with tumor cells during dissection. One possible way to minimize the risk of tumor seeding is the use of a protective bag for removal of all tissue. Tumor seeding may occur during excision of the diseased organ in case of over-manipulation leading to tumor spillage, and dissemination in the peritoneal cavity[8]. Since a higher rate of trocar site recurrences has been reported in patients with advanced ovarian cancer, ascites and omental cake, the risk may be reduced by avoidance of laparoscopic surgery in patients with advanced malignancy. In case of gynecological cancer suspicion, patients benefit from a referral to a gynecological oncologist[11]. In case of laparoscopy for advanced ovarian cancer, careful closure of the peritoneum, rectus sheath, and skin followed by chemotherapy or cytoreductive surgery with excision of the trocar sites within seven days has been recommended as well[12]. Among other factors, the effects of gas turbulence and high pressure peritoneum during laparoscopy have been discussed as a potential mechanism of the development of PSM. Since carbon dioxide exposure stimulates the growth of ovarian cancer cells *in vitro*, it has been speculated that CO_2 used during laparoscopy may increase the rate of wound metastasis[13]. Probably, a multifactorial mechanism may be responsible, in which the main parameters are tumor manipulation, forced removal of the unprotected tissue and high pressure pneumoperitoneum.

The estimated incidence of port site metastasis following laparoscopic surgery is 1%-2%. Several risk factors, such as tumor manipulation and extraction of tumor tissue without a protective bag, have been identified. In patients with advanced malignancy and ascites, the possibility of this complication should be kept in mind when choosing laparoscopic surgery.

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COMMENTS

Case characteristics

A 46-year-old female presented with an asymptomatic cyst.

Clinical diagnosis

A cyst with intracystic papillary projections in the right ovary, measuring 2.5 cm × 2.8 cm, no abdominal pain.

Differential diagnosis

Cyst, benign ovarian tumor, borderline ovarian tumor, ovarian cancer.

Laboratory diagnosis

CA125: 38.7 U/mL.

Imaging diagnosis

Ultrasound: An ovarian cyst with intracystic papillary projections, no ascites.

Pathological diagnosis

Mucinous papillary borderline tumor of the ovary with a non-invasive subcutaneous implant.

Treatment

Laparoscopic right adnexectomy followed by median exploratory laparotomy with hysterectomy, left adnexectomy, multiple peritoneal biopsies, appendectomy and omentectomy.

Term explanation

Port site metastasis (or trocar site metastasis) is the development of a metastasis in a port site (usually following laparoscopy) or in a drain site.

Experiences and lessons

The incidence of trocar site metastasis following laparoscopic surgery is low (1%-2%). Tumor manipulation and extraction of tumor tissue without a protective bag have been identified as risk factors.

Peer-review

This is an interesting case report and review of the literature of port site implants in ovarian malignancy.

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REVIEW

Relationship and interactions of curcumin with radiation therapy

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Abstract

Curcumin is widely reported to have remarkable medicinal - and antineoplastic - properties. This review details curcumin's relationship with radiotherapy (RT), principally as a radiosensitizer for various malignancies and a radioprotector for normal tissues. First, examples of radiosensitization are provided for various cancers: Pediatric, lymphoma, sarcoma, prostate, gynecologic, pancreas, liver, colorectal, breast, lung, head/neck, and glioma. It is not the purpose of this article to comprehensively review all radiosensitization data; however, high-quality studies are discussed in relationship to currently-controversial RT questions for many cancers, and thus the importance of developing a natural radiosensitizer. Attention is then shifted to radioprotection, for which supporting research is discussed for the following RT toxicities: Dermatitis, pneumonitis, cataractogenesis, neurocognition, myelosuppression, secondary malignancies, and mucositis/enteritis. Though there is fewer data for radioprotection, the overall quality of clinical evidence is higher, and small clinical trials implicating the efficacy of curcumin for RT toxicities (*vs* placebo/current therapies) are also detailed. Though the overall level of evidence for curcumin as a radiosensitizer and radioprotector is low, it must be recognized that risks of adverse effects are exceedingly low, and clinicians may need to judge the yet-unproven rewards with low toxicity risks.

Key words: Curcumin; Turmeric; Radiation therapy; Cancer; Radioprotection; Radiosensitization

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Core tip: The Indian spice curcumin (turmeric) has been widely reported, largely in the preclinical realm, to offer many health - including antineoplastic - benefits. Though this article is not meant as a summative review of all studies of curcumin and radiotherapy, selected studies will be discussed that demonstrate curcumin to be a radiosensitizer of many types of tumor cells. Furthermore, data illustrating curcumin as a radioprotector of normal organs - including clinical studies - are also described. It is a sincere hope that these promising results can lead to curcumin use in cancer patients, either on or off a clinical protocol.

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INTRODUCTION

The Indian spice curcumin (also known as diferuloylmethane), extracted from the turmeric plant, has long held a role in Indian/Hindu rituals, traditions, customs, and cuisines. More recently, scientific evidence is mounting that curcumin offers innumerable health benefits (reviewed in multiple sources[1-4]), all stemming from the fundamental property of decreasing inflammatory mediators[5]. This leads hope to curb the unchecked progression of fundamentally inflammatory diseases[6], many of which are considered the scourge of medicine in the present day and age. Moreover, curcumin is a completely natural compound with essentially no side effects; tolerance in phase I clinical trials have shown no medically adverse effects for doses up to 8-12 g orally per day[7].

Cancer is a common conglomeration of diseases that can be termed as a "bane of healthcare" throughout the world, and affects hundreds of millions of persons throughout the world per year. Extensive work has been performed on curcumin's immense anti-cancer potential, which have been grossly underappreciated, largely owing to the notable roadblock of few clinical studies to date[8-13]. The phase I-II clinical trials that have been performed, however, have done nothing to dissuade further clinical study of this compound[14-16].

Primary management of cancer centers on various combinations of surgery, chemotherapy, and radiation therapy (RT). A comprehensive discussion of curcumin's effects on chemotherapy and surgical intervention is extremely broad and clearly beyond the scope of this article; rather, curcumin's interactions with RT will be evaluated. Additionally, though it is not the goal of this article to comprehensively and systematically detail all data of the curcumin-RT relationship[17,18], selected examples of curcumin's (1) radiosensitization ability and (2) radioprotective ability, will be enumerated in order to characterize the sheer breadth of curcumin's actions along with RT on cancer. The goal of this article, in turn, is to encourage clinicians to (1) commence clinical trials related to curcumin; and/or more importantly; (2) encourage their patients to routinely take curcumin for cancer therapy (despite a general dearth of solid data).

RADIOSENSITIZATION BY CURCUMIN

There is a well-charted history of radiosensitizers, defined as molecular compounds that act to functionally amplify radiation-induced DNA and cellular damage, regardless of whether the compounds cause damage individually[19]. Though several radiosensitizers are used in cancer care today, such as platinum-based chemotherapeutic agents, the focus of this section is to describe many examples of curcumin as a radiosensitizer. The reader is first cautioned that nearly all evidence of radiosensitization comes from laboratory data, and clinically-apparent benefits of curcumin as a radiosensitizer are yet to be determined.

First, attention will be paid to pediatric, lymphoma, and musculoskeletal cancers. Why are these important? Clinically speaking, the fields of pediatric and lymphoma RT have undergone - and are undergoing - dramatic decreases in RT doses, so as to minimize secondary malignancy risk and ancillary procedures in the younger population[20-22]. The presence of a radiosensitizing agent, if proven clinically efficacious, would certainly aid the movement to de-escalate RT doses in this population. Sarcomas (many of which occur in children) are a logical extension for curcumin therapy, given its success in musculoskeletal inflammatory-based disorders[23]. As previously mentioned, inhibition of the transcription factor NF-κB is a primary mode of action of curcumin, which act to mediate various anti-inflammatory effects for various diseases[24]. However, what is often an overlooked fact between inflammatory diseases and cancer is that NF-κB has been widely implicated in both tumorigenesis and radioresistance[24]. Hence, results of pre-RT curcumin intake leading to radiosensitization in murine rhabdomyosarcoma models are not surprising in light of suppressing NF-κB[25]. These results have been echoed in neuroblastoma cells in a high-quality study by Aravindan *et al*[26]. However, the diverse pathways of curcumin's actions are not limited to this transcription factor; the same group studied mutant p53 Ewing's sarcoma cells, and radiosensitivity was found to be associated with other p53-response genes (despite the p53-mutated nature of the studied cells)[27]. There are also data to support the NF-κB suppression theory as means for radiosensitization in lymphomas, which are important in light of resistance to biologic therapies for some types of lymphomas[28]. Though RT is not the centerpiece of therapy for Burkitt's lymphoma, there are data supporting radiosensitization in this otherwise aggressive lymphoma[29]. The same group did demonstrate another interesting mechanism of radiosensitization in non-Hodgkin's lymphoma (which constitute large proportions of lymphomas treated with RT)[30]. The authors found that cell cycle arrest in the G2-M checkpoint was associated with curcumin administration, which is a normal effect of irradiating tumor cells and is hence presumably augmented by curcumin.

Shifting to genitourinary cancers, dose-escalation for prostate cancer (the most common genitourinary malignancy) is strongly proven to associate with improved outcomes[31], and hence great emphasis is placed on using high-fidelity imaging technology to guide RT planning/delivery[32,33]. Radiosensitization for these tumors could thus allow for "functional dose escalation", providing even greater tumor doses while

keeping a constant prescribed RT dose. Two convincing preclinical studies demonstrated the radiosensitizing effects of curcumin on the human prostate cancer cell line PC3. Chendil *et al*[34] postulated the mechanism to be related to NF-κB and found threefold fewer surviving PC3 cells when treated with both RT and curcumin. However, another report found another novel pathway of action, downregulation of the MDM2 oncogene (a p53-independent pathway), which provide encouragement that spontaneous mutagenesis in cancer cells could be less likely to cause multi-drug resistance affecting curcumin[35].

Next, data is not limited to the male genitourinary system, with one report demonstrating increased reactive oxygen species formation in cervical cancer cells with the addition of curcumin[36]. Similar to the aforementioned report on cell cycle arrest[30], this is a normal effect of RT that curcumin seems to augment. Lastly, though RT is not routinely utilized for ovarian neoplasms, a group at the University of South Dakota conjugated curcumin nanoparticles to an ovarian cancer-specific antibody and elicited both chemo- and radiosensitization phenomena[37]. Though the issue of curcumin delivery is beyond the scope of this review, it will briefly be addressed in the final section, and this study's use of nanoparticles is hence quite noteworthy.

Though gastrointestinal tumors are inherently very heterogeneous and diverse, brief examples for several tumor types are united by the overarching theme of NF-κB suppression by curcumin, despite any rises that could occur after a RT fraction. Again, this transcription factor is widely purported to relate to radioresistance, and the studies discussed hereafter in this paragraph will demonstrate sustained cellular killing, potentially as a result of decreased radioresistance. First, though pancreatic cancer is one of the deadliest known neoplasms, data have shown enhanced cell killing with five-fraction RT[38] especially as delivered by Veeraraghavan *et al*[39]. However, it is important to be skeptical of results insofar as questioning whether curcumin administration could be a panacea for a disease with dismal prognosis from aggressive tumor biology and high metastatic proclivity. The same criticism is true for similar results recently published on hepatocellular carcinoma, which also demonstrated NF-κB downregulation as a putative mechanism[40]. Lastly, curcumin may be a relatively good candidate to clinically sensitize colorectal cancer (the most common gastrointestinal malignancy) to RT. Two high-impact publications from M.D. Anderson Cancer Center also implicated NF-κB modulation - although its expression rises after RT - as an effector of curcumin[41,42]. There were several additional effects of note as measured by the authors. First, not only were proliferation markers downregulated, angiogenesis was decreased as well. Though this effect could result in decreased nutrients feeding the tumor (thus augmenting cell killing), potential decreases in tumor oxygenation could be problematic, as this is strongly related to tumor radioresistance. Importantly, this study also demonstrated decrease in matrix metalloproteinase (MMP) expression. This enzyme is thought to be a gateway for metastasis, by dissolving bonds to extracellular matrix (an "anchor" preventing dissemination) as well as promoting an overall microenvironment for growth and spread[43]. Hence, after RT the upregulation (presumably nonsustained) in NF-κB and MMP lead to some degree of increased risk for radioresistance (persistent growth) and spread[44]. Curcumin may hence act to decrease this risk, and it would certainly be helpful to examine tumor growth and metastasis from a clinical perspective to examine whether decreased NF-κB and MMP expression translate into "clinical gains".

Moving to neoplasms of the thorax, breast cancer is the most common noncutaneous cancer in the United States; RT is a major part of management, including several different techniques and RT modalities[45-49]. One example of breast cancer radiosensitization with curcumin was shown by Calaf *et al*[50]. The most important observation of this study was increased amounts of cleaved (inactive) PARP-1, a protein known to repair DNA after RT damage and thus attenuate RT damage[51]. There is an enormous amount of current research being done on PARP inhibitors, including multiple phase Ⅱ and Ⅲ clinical trials. If further results can corroborate the association between cleavage/inactivation of PARP by curcumin, these could have substantial implications on this burgeoning field.

Lung cancer, most commonly non-small cell lung cancer (NSCLC), is another common and deadly tumor[52,53] for which screening has recently been instituted[54-56]. A radiosensitizer would therefore be a welcome addition to recently-developed and cutting-edge RT technologies used for treatment of some NSCLCs[57]. Two important studies in NSCLC will be highlighted. A group from the University of Pennsylvania claimed a survival improvement with dietary curcumin administration along with RT, although there are several methodological flaws precluding reliability of these data[58]. More importantly, however, that dietary curcumin was able to cause clinical effect in the murine model is encouraging, because bioavailability remains a challenge of curcumin (further discussed in a subsequent section). In light of this fact, another research group utilizing liposomal curcumin was able to demonstrate potentiated NSCLC cell apoptosis with the presence of curcumin, and additionally found greater evidence of post-RT microvascular change, which (though uncorroborated) could be a surrogate marker for greater tumoral RT damage[59].

Regarding the diverse head and neck cancers, more common in Southern and Eastern Asia than the United States, treatment centers are on RT for the vast majority. Furthermore, cisplatin (administered concurrently with RT in select patients) has proven to be a radiosensitizer, increasing local tumor control in large randomized trials[60,61]. However, cisplatin's amplification of adverse RT toxicities beckons whether

the lack thereof with curcumin could prove to be a helpful utility[62]. Since an initial publication describing curcumin's ability to radiosensitize head and neck tumor cells *in vitro* and *in vivo*[63], another demonstrated the mechanism to be NF-κB - consistent with mechanistic relationships of curcumin on multiple aforementioned neoplasms[64]. Next, it is also worth mentioning another study of curcumin in nasopharyngeal carcinoma, in which greater amounts of cleaved PARP were discovered[65]. This is consistent with results for malignant breast carcinoma cells in a previously discussed study[50]. However, the most thought-provoking results were published by Tuttle *et al*[66] who illustrated that curcumin offers radiosensitization to head and neck malignancies that were human papillomavirus (HPV)-negative but not HPV^+. Ever since it was published in 2010 that HPV^+ oropharyngeal cancers had substantially better prognoses[67], a major focus of upcoming trials has been to determine whether de-escalation of therapy is feasible for HPV^+ tumors[68]. Though it is counterintuitive that curcumin did not radiosensitize HPV^+ tumors - they are vastly more sensitive to RT - it is in fact important that the HPV-negative neoplasms (worse prognosis) could be favorably addressed by curcumin and RT, if proven clinically efficacious.

Lastly, application of curcumin radiosensitization in gliomas will be briefly touched upon. Although a report posited G2-M cell cycle arrest as a mechanism[69], other data has displayed synergism of curcumin with an anti-glioblastoma antibody, including sustained NF-κB suppression[70]. This is noteworthy because biologics are at the forefront of oncologic therapy, and are already approved for relapsed glioblastoma[71]. Though it may be unlikely that simple administration of curcumin could curb the aggressive spread of glioblastoma, it rather provides hope that a clinical difference could be gleaned with curcumin for less aggressive neoplasms.

In summary, there is a great breadth of corroboratory data for many different tumor types available that demonstrate the radiosensitizing potential of curcumin. It is likely that other untested tumor types could likely show similar radiosensitization in laboratory models[72-77]. Though there has been no documentation to date in patients, encouragement does exist that there could be small observed differences in outcomes (with appropriate sample sizes), and even if there are no changes in survival parameters, recurrence rates and local control (a prime marker of radiosensitization) could be affected if eventually tested in the clinic.

RADIOPROTECTION BY CURCUMIN

Though radiosensitization is important to enhance tumor death, equally important is toxicity minimization of normal tissues, the pursuit of which is one of the most prime goals of radiation oncology. Though the evidence for curcumin's radioprotection is less diverse/broad as compared to radiosensitization, the overall quality and applicability of data to human patients is noticeably greater. In this section, focus will be on curcumin's benefits against the following common RT toxicities: Dermatitis, pneumonitis, cataractogenesis, neurocognition, myelosuppression, secondary tumors, and mucositis/enteritis. Many of these toxicities are inflammatory in nature, so it intuitively follows that curcumin's potent anti-inflammatory effects[1,3,4,6] could lessen these inflammatory toxicities, likely through decreased inflammatory molecule production[5] as well as increasing the balance of antioxidants to oxidants[78].

RT dermatitis is one of the most common adverse effects of RT regardless of anatomic area, and two high-quality studies are as follows. Okunieff *et al*[79] documented reduction in both acute and chronic RT dermatitis in mice. This correlated with decreased levels of proinflammatory cytokines as well as subsequently-released fibrogenic cytokines such as TGF-β. Though a criticism of the study is the utilization of a single 50 Gray RT dose (extremely rare in humans), the radioprotection was consistent with another study that showed improved irradiated wound healing with curcumin[80]. The second major piece of evidence is a randomized and double-blinded trial of oral (2.0 g thrice daily) curcumin tablets (n = 14) *vs* placebo (n = 16) in breast cancer RT[81]. Patients were equal in terms of demographics, receipt of chemotherapy, surgery type, stage, RT dose, and baseline skin and pain assessment. A RT dermatitis standardized scale was the primary endpoint and favored curcumin (P = 0.008) along with decreased moist desquamation in the curcumin group (P = 0.002). There were largely no differences in patient-reported pain scores. This trial provides the highest level of evidence offering real hope that curcumin can have clinically significant impact on radiotoxicity, and it should secondarily not be discounted that the study was able to obtain statistically significant differences between groups despite randomizing only thirty patients.

Radiation pneumonitis has been extensively studied and well-validated to several RT dose-volume parameters; hence, it is a major focus of RT treatment planning especially because severe RT pneumonitis can be fatal[82-84]. Two studies demonstrating radioprotection against RT pneumonitis and its delayed sequela - pulmonary fibrosis - have already been discussed in the radiosensitization section[60,61] and corroborated by another report[85]. All three studies have shown, mechanistically, that curcumin's action is due to decreasing oxidative stress, proinflammatory cytokines, NF-κB expression, and fibrogenic cytokines - all of which tend to occur both simultaneously and sequentially. Undoubtedly, the presence of a lung radioprotector, if clinically proven, would be of great use to the ubiquitous NSCLC patients, many of which have risk factors for RT pneumonitis such as baseline lung disease and receipt of concurrent carboplatin-paclitaxel[86].

Two small studies examining central nervous system adverse effects of RT will now be addressed. Ozgen *et al*[87] examined cataractogenesis, a late toxicity that was hastened in the study by giving high single-

fraction doses to the lens (a relatively uncommon clinical scenario). Irradiation with curcumin lowered the cataract rate from 100% to 40%, correlating with lower levels of oxidative stress. Next, substantial ongoing research (and clinical trials) in radiation oncology relates to whether patients with primary or secondary brain tumors that undergo brain irradiation could be spared of its resulting memory/cognitive decline[88,89]. Curcumin is widely thought to be neuroprotective; its high consumption is associated with minimal rates of several neurodegenerative diseases in India, which is backed by convincing experimental evidence of such[90]. Pre-RT administration of curcumin was able to improve results in post-RT spatial/memory functional tests (Morris water maze) in mice administered carbon ion RT (high biologically effective dose owing to the heavy particle size)[91]. Furthermore, histologically-apparent neuropathological changes were also present between both groups. Hence, if other research can confirm these results, it will not be difficult to design clinical trials examining learning/memory tests in patients undergoing whole-brain RT with or without curcumin.

Curcumin can also protect lymphocytes, the most RT-susceptible blood cell, especially when radiating bony lesions (marrow) in patients[92]. The authors postulated that curcumin's actions could consist of radiosensitization or radioprotection, with the latter observed in non-cycling cells (in G0 phase) and the former in cycling cells (G2 transitioning to M phase), which is a theory that could sum up all the radioprotective and radiosensitizing data in this entire review.

Japanese researchers published an impactful article in 2002 demonstrating that rats undergoing whole body irradiation (dose of 9.6 Gray) - simulating a natural disaster such as Chernobyl - produced lower levels of an oxidant metabolite if fed curcumin pre- and post-RT, as compared to control rats[93]. Furthermore, post-exposure implantation of the carcinogen diethylstilbestrol led to significantly more secondary tumors in rats not having been administered curcumin. The results lead to query as to whether curcumin could directly prevent further mutations, but data for this is scant at best. Nevertheless, there is more evidence to support that curcumin lowers circulating reactive oxygen species (oxidative stress), which are normally known to cause DNA mutations (*i.e.,* how ionizing radiation causes DNA damage in cancer cells).

Lastly, owing to the relatively high proliferative index of mucosal cells, any mucosal surface is particularly sensitive and susceptible to acute and chronic RT-induced damage[94-96]. A Turkish study demonstrated that intestinal mucosa was protected to a greater degree in rats fed curcumin, as detected histopathologically[97]. These results, especially if validated, are important for three major reasons: (1) bowel toxicity is relatively common and may occur at any point of the RT course (even well-below the bowel tolerance dose); (2) parts of the bowel receive RT dose for several common (*e.g.,* prostate, gastrointestinal, gynecologic, and some palliative/pediatric) cancers; and (3) because curcumin is poorly absorbed in the gastrointestinal tract, it remains in direct contact with intestinal mucosa and hence could directly act on mucosal cells.

Next, another large area of morbidity in irradiated patients is mucositis of the soft tissues of the head and neck, some of which can be so severe that it necessitates feeding tube placement due to lack of oral feeding[98]. In 2004, a publication demonstrated a clinically evident decrease in oral mucosal ulceration in rats fed curcumin[99]. However, this issue remained untranslated into the clinical realm until Indian researchers published a study in 2013[100]. In this single-blinded and randomized trial, patients with mostly oral cavity/pharyngeal neoplasms undergoing RT (with or without surgery and chemotherapy) were given oral rinses of turmeric (n = 40) or povidone-iodine (n = 39) to take six and two times per day respectively. Tumor characteristics and treatment interventions (including RT dose and chemotherapy receipt) were balanced between groups. The group receiving curcumin was less likely to receive treatment breaks in the initial (< 4 wk) period (P < 0.01) and displayed decreased weight loss (P < 0.001). Though incidence of overall mucositis did not differ between groups, the curcumin group experienced lesser intolerable mucositis (P < 0.0001) as well as decreased severity of overall mucositis as per Radiation Therapy Oncology Group criteria (P < 0.003). Though povidone-iodine is uncommonly used for RT mucositis, similar agents (*e.g.,* lidocaine, chlorhexidine) used more often are likely no different because they are designed to treat symptoms rather than causative molecular inflammatory agents as curcumin does.

Another recent clinical protocol by Patil *et al*[101] will now be expounded upon. Twenty patients, mostly with oral cavity/pharyngeal cancer that received concurrent chemoradiation, received either chlorhexidine (n = 10) or 0.004% curcumin oral rinse (n = 10) thrice daily during RT. Patient and tumor characteristics, including chemotherapy receipt and RT dose, were underreported but equivalent between groups. Outcomes included a prespecified numerical pain score, oral mucositis assessment scales for erythema and ulceration, and the World Health Organization (WHO) mucositis scale. Most of these parameters were favorable for the curcumin group (P < 0.001 for pain, P = 0.05 for erythema, P < 0.001 for ulceration, and P = 0.003 for WHO mucositis). Though the methodology of this trial was less sound as compared to the aforementioned dermatitis trial[81], it should again be mentioned that a statistically significant difference was found despite the comparison of only ten patients in each group.

Taken together, there are greater clinical data available to support the use of curcumin as a radioprotector of normal tissues, especially epithelial tissues, potentially owing to direct contact with at-risk surfaces. Curcumin's mechanisms seem associated with decreased

oxidative stress in normal tissues.

CONCLUSION

A substantial volume of evidence exists that curcumin is a radiosensitizer of multiple cancers as well as a radioprotector of several normal tissues. However, the overall quality of evidence is low; there is no clinical evidence of radiosensitization and a few low-volume clinical trials of radioprotection published thus far. However, there is certainly something to be said about the sheer volume of corroborative positive data, particularly in radioprotection.

What do the aforementioned litany of laboratory and clinical studies mean for the clinician? On one hand, clinical evidence is weak, and there is no guarantee curcumin would provide a clinical difference in outcomes (*e.g.,* survival, local/regional recurrence). On the other hand, as previously discussed, curcumin administration has exceedingly low chances to produce adverse effects; empiric administration without solid clinical evidence will likely not harm the patient whatsoever.

Further research is greatly needed to strengthen curcumin's major weakness - poor gastrointestinal absorption leading to low oral bioavailability. After absorption in the gastrointestinal tract *via* a liposomal mechanism, four double bonds are reduced, followed by glucuronidation/sulfation and excretion through bile[102-104]. Several discussed studies[37,59], as well as undiscussed studies in other diseases[23], have used special formulations (*e.g.,* liposomal, intravenous, molecular analogs, and conjugated forms such as Meriva® and Theracurmin®) which could become more mainstream in the future with more research.

A subsequent question, then, is whether to administer curcumin therapeutically as in these studies, or preventatively - long prior to any therapy - or even prophylactically prior to any disease onset. These questions should likely be addressed after basic clinical efficacy/utility issues, so as to provide more solid footing on curcumin use. Although not the purpose of this article to provide recommendations of curcumin administration to clinicians, it is certainly encouraged to consider that in light of immature but still broadly corroborative data (including clinical studies), curcumin is extremely safe and not harmful to the cancer patient undergoing radio(chemo)therapy.

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MINIREVIEWS

Neoadjuvant therapy for gastroesophageal adenocarcinoma

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Abstract

Gastric and esophageal adenocarcinomas are one of the main causes of cancer-related death worldwide. While the incidence of gastric adenocarcinoma is decreasing, the incidence of gastroesophageal junction adenocarcinoma is rising rapidly in Western countries. Considering that surgical resection is currently the major curative treatment, and that the 5-year survival rate highly depends on the pTNM stage at diagnosis, gastroesophageal adenocarcinoma management is very challenging for oncologists. Several treatment strategies are being evaluated, and among them systemic chemotherapy, to decrease recurrences and improve overall survival. The MAGIC and FNCLCC-FFCD trials showed a survival benefit of perioperative chemotherapy in patients with operable gastric and lower esophageal cancer, and these results had an impact on the European clinical practice. New strategies, including induction chemotherapy followed by preoperative chemoradiotherapy, targeted therapies in combination with perioperative chemotherapy and the new cytotoxic regimens, are currently assessed to improve current standards and help developing patient-tailored therapeutic interventions.

Key words: Gastric adenocarcinoma; Lower esophagus adenocarcinoma; Gastroesophageal junction adenocarcinoma; Preoperative treatment; Neoadjuvant treatment

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Core tip: Gastric and esophageal adenocarcinomas are one of the main causes of cancer-related death worldwide. The incidence of gastroesophageal junction adenocarcinoma is rapidly rising in Western countries. Surgical resection is currently the major curative treatment. As the 5-year survival rate highly depends on the pTNM stage, the treatment strategy is very challenging for oncologists. Several treatments, including systemic chemotherapy, are being assessed to prevent recurrences and improve overall survival. New strategies, such as induction chemotherapy followed by preoperative chemoradiotherapy, targeted therapies and new cytotoxic regimens in perioperative chemotherapy, are currently assessed to improve current standards and develop more tailored therapeutic interventions.

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INTRODUCTION

Survival of patients with esophageal, gastric or gastro-esophageal junction adenocarcinoma is poor because they are frequently locally-advanced or with distant metastases at diagnosis. Even if the incidence of gastric adenocarcinoma is decreasing, it stays the second most frequent cause of cancer-related death worldwide. In 2012, 952000 new cases of gastric cancer were diagnosed with 723000 estimated deaths worldwide[1]. In Western countries, a faster increase of the incidence of gastroesophageal junction adenocarcinoma compared to that of other gastrointestinal adenocarcinomas has been reported over the last 25 years[2,3]. Surgical resection of the primary tumor is the major curative treatment for these upper gastrointestinal cancers. Esophageal cancers can be treated with exclusive radiochemotherapy[4], but this review will focus on (neo)adjuvant therapies. The 5-year survival rate is correlated with the pTNM stage, lymph node metastases being the major poor prognostic factor. A 5-year survival of 20% to 30% is reported in localized tumors, which extend beyond the submucosa[5-7]. Also, gastroesophageal adenocarcinomas (GEA) are often detected at an already advanced stage, in Western countries, about 30% of resectable patients were not identified at an early stage. For this group, the rate of recurrence following resection of a gastric cancer is high. Currently, various strategies (including nutritional management) are developed and tested to reduce this risk and improve patient's survival[8].

ADJUVANT THERAPIES FOR GASTROESOPHAGEAL ADENOCARCINOMA

Adjuvant chemotherapy

Like in colorectal cancer, adjuvant systemic therapy in patients with GEA is used to treat post-resection occult residual micro-metastatic disease and to increase survival. Many clinical studies have investigated the possible positive impact of adjuvant therapy on the patients' outcomes. Between 1994 and 2002, meta-analyses of the data from these trials suggested that adjuvant chemotherapy slightly increases overall survival, with a reduction of the risk of death between 12% and 18%[9-12]. However, no recommendation for the treatment of resectable gastric cancer could be proposed because of the heterogeneity of the methodology used in these meta-analyses including studies with a small number of patients, and because old chemotherapy regimens were often followed in these trials. Several clinical studies have focused on chemotherapy drugs that are currently used in the clinical practice. For instance, in France, the randomized phase Ⅲ trial of the French Federation of Digestive Oncology [Fédération Française de Cancérologie Digestive (FFCD)] on adjuvant chemotherapy combined with 5-fluorouracil (5-FU) and cisplatin (CDDP) after curative resection of gastric cancer showed no benefit on survival compared with surgery alone[13]. In Japan, a randomized phase Ⅲ trial on adjuvant chemotherapy with the S-1 fluoropyrimidin derivative in patients with stage Ⅱ-Ⅲ GEA found that the 5-year overall survival (OS) rate was 71.7% in the S-1 arm compared with 61.1% in the surgery alone arm (HR = 0.68, 95%CI: 0.52-0.87, P = 0.003)[14]. More recently, the CLASSIC study reported a higher 5-year OS in patients with stage Ⅱ or Ⅲ gastric cancer who received adjuvant chemotherapy treatment with capecitabine plus oxaliplatin compared to patients who underwent only D2 gastrectomy (5-year OS rate: 78% *vs* 69%; HR = 0.58, 95%CI: 0.47-0.72, P < 0.0001)[15].

Finally, a meta-analysis by the Global Advanced/Adjuvant Stomach Tumor RESEARCH International Collaboration (GASTRIC) Group that combined data from 3838 patients (17 trials) showed a benefit of 5-FU-based adjuvant chemotherapy *vs* surgery alone on OS (55.3% *vs* 49.6%, respectively; HR = 0.82, 95%CI: 0.76-0.90, P < 0.001) with stable results at 10 years (48% *vs* 40%, respectively)[16]. For this reason, 5-FU-based adjuvant chemotherapy is now considered as a therapeutic option and has been included in the French National Thesaurus of Digestive Oncology (www.snfge.asso.fr) for patients with resected GEA.

Adjuvant chemoradiotherapy

The efficacy of adjuvant chemoradiotherapy after a R0 resection in GEA patients (stage Ⅰb to Ⅳ M0) was studied with the SWOG 9008/INT 0116 phase Ⅲ trial in 603 patients[17,18]. They were randomized in two therapeutic arms: Surgery alone *vs* surgery combined with adjuvant chemoradiotherapy. The patients' characteristics, including the tumor stage, were similar in the two groups: 65% of patients had pT3/T4 stage tumors and 85% N^+ stage tumors. Treatment started with chemotherapy (1 cycle of the FUFOL Mayo Clinic regimen) followed with chemoradiotherapy after 1 mo. Radiotherapy was delivered in 25 fractions (45 Gy each) and FUFOL was administered during the first 4 d and the last 3 d of irradiation. Two additional FUFOL cycles were then given 1 mo after the end of chemoradiotherapy. In this study, 64% of patients completed the therapeutic protocol. The digestive and hematological toxicity rates were respectively of 33% and 54%. The administration of adjuvant chemoradiotherapy resulted in an improvement of the 5-year OS rate compared with surgery alone (40% *vs* 26%; HR = 1.31, 95%CI: 1.09-1.39, P = 0.005) and of the median disease-free survival (27 mo *vs* 19 mo; HR

= 1.52, 95%CI: 1.25-1.53, $P < 0.0001$), with a median follow-up of more than 10 years. The risk of death was reduced by 31%, and relapses were decreased by 52%. However, D2 lymph node dissection was reported in 10% of patients (36% had a D1 resection, and 54% a < D1 resection). In conclusion, adjuvant chemoradiotherapy appears to be a reasonable option for the treatment of resectable GEA, for patients with inadequate lymph node dissection and/or at high risk of recurrence (pT3/T4 and/or N^+ cancer).

Recently, the ARTIST study reported a benefit of adjuvant chemoradiotherapy with capecitabine and cisplatin on disease-free survival of patients with node-positive and intestinal-type gastric adenocarcinoma compared with patients treated with adjuvant chemotherapy alone[19].

In all cases, the nutritional status of patients should systematically and safely be evaluated before initiating adjuvant therapy at 6 wk post-surgery[20].

NEOADJUVANT SYSTEMIC THERAPIES IN GEA

The choice of administering systemic chemotherapy to the patients before surgery of resectable GEA is mainly based on the possibility of improvement of the R0 resections and primary tumor downstaging/downsizing. Systemic neoadjuvant therapy may also remove occult micro-metastatic disease and facilitate the preoperative chemo-sensitivity assessment. Possible disadvantages of neoadjuvant systemic therapies include the risk of disease progression until surgery, the increase in secondary morbidity and chemotherapy-related toxicity and the difficulty of assessing the preoperative treatment response.

In all clinical trials, patients with gastric, gastro-esophageal junction and lower esophagus adenocarcinomas were considered together as a single population. In this review, we will distinguish the different tumor sites, and, concerning esophageal cancer, we will focus on the results of neoadjuvant chemoradiotherapy.

Neoadjuvant chemotherapy

The feasibility of neoadjuvant chemotherapy as treatment of resectable gastric cancers was initially shown in phase Ⅱ studies[21-23], which reported an acceptable toxicity and no increase of the surgical mortality and morbidity rates using this therapeutic approach. The R0 resection rates following neoadjuvant chemotherapy were of 61% and 77%, with or without intra-peritoneal chemotherapy, respectively.

A randomized clinical trial coordinated by the Dutch Gastric Cancer Group, compared two groups of patients who underwent surgery alone ($n = 30$) or received neoadjuvant chemotherapy after surgery ($n = 29$), *i.e.*, 4 cycles of the FAMTX regimen (5-FU, doxorubicin and methotrexate)[24,25]. The R0 resection rates were 62% *vs* 56% in the surgery alone arm and in the neoadjuvant chemotherapy plus surgery arm, respectively. Although the trial was stopped early due to the small number of patients included ($n = 59$), the authors concluded to the poor efficacy of the FAMTX regimen in the treatment of resectable gastric cancer.

A randomized phase Ⅲ trial compared in 503 patients with resectable stomach, lower esophagus or esophageal-gastric junction adenocarcinomas, surgery alone ($n = 253$) with perioperative chemotherapy ($n = 250$)[26]. This Medical Research Council Adjuvant Gastric Infusional Chemotherapy (MAGIC) trial comprised the ECF regimen (50 mg/m² epirubicin on day 1, 60 mg/m² CDDP on day 1 and continuous venous infusion of 5-FU, 200 mg/m² per day, for 3 wk). Three cycles were administered pre- and post-surgery, which took place within the 6 wk after randomization for patients in the surgery group, or 3 to 6 wk after the third chemotherapy cycle for the perioperative chemotherapy group. The surgical procedure, which was left to the surgeon's discretion, included D1 or D2 lymphadenectomy. The patients' characteristics were similar in both arms; 26% of patients had a lower esophageal or gastroesophageal junction tumor. In the chemotherapy arm, 86% of patients completed the neoadjuvant treatment, 55% initiated postoperative treatment and 42% completed the chemotherapy protocol. Concerning surgery, 88% and 95% patients underwent the intervention in the chemotherapy and surgery alone arms. The pathological analysis of the resected specimens showed significant tumor downsizing in the chemotherapy group compared to the surgery alone arm (mean tumor size: 3 cm *vs* 5 cm; $P < 0.001$). An improvement of T stage ($P = 0.009$) and N stage ($P = 0.01$) was also reported in the chemotherapy group. The 79% R0 resection rate was significantly higher in the chemotherapy arm than in the surgery alone group (70%; $P = 0.03$). Grade 3-4 hematological toxicities during pre- and postoperative chemotherapy were not significantly different in the two groups, with 24% and 28% of neutropenia, respectively. With a median follow-up of 3 years, the median OS was 24 mo *vs* 20 mo in the chemotherapy and surgery alone groups, respectively (HR = 0.75, 95%CI: 0.60-0.93, $P = 0.009$) and the 5-year survival rates were 36% and 23%, respectively. Progression-free survival was significantly longer in the chemotherapy than in the surgery alone group (HR = 0.66, 95%CI: 0.53-0.81, $P = 0.0001$) (Table 1). The therapy efficacy was independent of the tumor site. The ECF chemotherapy regimen was chosen based on the results of a phase Ⅲ trial that compared the ECF and FAMTX regimens in patients with advanced GEA. In this study, ECF was associated with better response rate (45% *vs* 21%; $P < 0.001$), better median OS (8.7 mo *vs* 6.1 mo) and acceptable hematological toxicity (grade 3-4 neutropenia: 36% *vs* 58%) compared to FAMTX[27]. Recently, a meta-analysis showed that addition of an anthracycline to the CDDP and 5-FU regimens increased the OS of patients with advanced disease, but this

Table 1 Two neoadjuvant chemotherapy schedules offered for the treatment of resectable gastroesophageal adenocarcinomas

	MAGIC[24]			FNCLCC-FFCD[27]		
	ECF *n* = 250	Surgery *n* = 253	*P*	FP *n* = 113	Surgery *n* = 111	*P*
Median age, yr (range)	62 (29-85)	62 (23-81)		63 (36-75)	63 (38-75)	
Sex, male (%)	82%	76%		85%	82%	
Performance status, 0/1	68/32	68/32		74/26	75/25	
Gastric ADK (%)	74%	74%		25%	24%	
GOJ ADK (%)	26%	26%		75%	76%	
Downstaging/downsizing (%)						
T1/T2	52%	38%	0.09	42%	32%	0.16
T3/T4	48%	62%		58%	68%	
N0/N1	84%	76%	0.01	(N0) 33%	20%	0.54
N2/N3	16%	29%		(N+) 67%	80%	
R0 resection rate (%)	79%	70%	0.03	84%	73%	0.04
5-yr overall survival rate (%)	36%	23%	0.009 (HR = 0.75)	38%	24%	0.002 (HR = 0.69)

ADK: Adenocarcinomas; GOJ: Gastro-esophageal junction; ECF: Epirubicin, cisplatin and 5-FU; FP: 5-fluorouracil and cisplatin; MAGIC: Medical research council; FNCLCC-FFCD: Fédération nationale des centres de lutte contre le cancer - fédération française de cancérologie digestive.

advantage was not reported in the GASTRIC meta-analysis that combined data from 3226 patients[16,28].

A recently published French trial (FNCLCC 94012-FFCD 9703) evaluated another perioperative (two neoadjuvant cycles and four postoperative cycles) chemotherapy regimen (continuous protracted intravenous infusion of 5-FU 800 mg/m² per day from days 1 to 4 and 100 mg/m² CDDP on day 1 or 2 every 4 wk)[29]. It included 224 patients who were randomized between perioperative chemotherapy (*n* = 113) and surgery alone (*n* = 111) Patients underwent surgery 4 to 6 wk after neoadjuvant chemotherapy, and postoperative chemotherapy started 4 to 6 wk after surgery. The patients' characteristics were similar in the two groups. The originality in this study was the high percentage (75%) of patients with tumor in the cardia and in the lower esophagus. Preoperative staging was evaluated by endoscopic ultrasound examination and CT scan. In the perioperative chemotherapy arm, 96% underwent surgery compared with 99% in the surgery alone arm, and 87% of patients completed neoadjuvant chemotherapy. The pathological assessment of the resected specimens was similar in the two groups in terms of pT, whereas slightly less tumors were classified as N+ in the chemotherapy arm than in the surgery alone arm (67% *vs* 80%; not significant difference). The R0 resection rate was of 87% in the chemotherapy group, higher than the 74% rate in the surgery alone group (*P* = 0.004). The 5-year disease-free survival rates were 34% (95%CI: 26%-44%) *vs* 19% (95%CI: 13%-28%), and the 5-year OS rates were 38% (95%CI: 29%-47%) *vs* 24% (95%CI: 17%-33%) (HR = 0.69, 95%CI: 0.50-0.95, *P* = 0.02).

The results of the EORTC study showed an improvement of the R0 resection rate in patients treated with neoadjuvant chemotherapy (two courses of 50 mg/m² CDDP, Ⅳ on days 1, 15 and 29 followed by 500 mg/m² folinic acid, IV and 2000 mg/m² 5-FU by continuous infusion for 24 h on days 1, 8, 15, 22, 29 and 36; day 1 = day 48) compared to patients treated with surgery alone (81.9% *vs* 66.7%; *P* = 0.036). OS was comparable between the two arms, but this study lacked statistical power due to recruitment failure (expected patients per arm = 180; patients included in each arm = 72)[30].

The MAGIC and the FNCLCC 94012-FFCD 9703 studies reported similar benefits as those of the MRC OE02 trial that assessed the effect of CDDP (80 mg/m² administered by intravenous infusion for 4 h on day 1, day 1 = day 21) and 5-FU (1000 mg/m² by continuous protracted venous infusion from days 1 to 4, day 1 = day 21) in patients with resectable esophageal cancer[31]. In this study, 802 patients (66% had tumors located in the lower portion of the esophagus or in the cardia) were randomized in two arms, surgery alone (*n* = 402) *vs* preoperative chemotherapy (*n* = 400). The median OS was significantly higher in the chemotherapy group (17 mo *vs* 13 mo; 95%CI: 30-196 d) as well as the 5-year OS (23% *vs* 17%; HR = 0.84, 95%CI: 0.72-0.98, *P* = 0.03) according to the updated results[32].

At the 2015 ASCO annual meeting, Alderson *et al*[33] reported the results of the OEO5 trial that compared the ECX (4 cycles, *n* = 446) and 5-FU with CDDP (2 cycles, *n* = 451) regimens as neoadjuvant chemotherapy in patients with resectable adenocarcinoma of the esophagus or of gastroesophageal junction (Siewert type Ⅰ and Ⅱ). No significantly survival difference was observed between treatments even when an anthracycline was added to the CDDP and 5-FU regimen [3-year OS: 42% (95%CI: 37-46) in the 5-FU/CDDP arm *vs* 39% (95%CI: 35-44) in the ECX arm; HR = 0.92, 95%CI: 0.79-1.08, *P* = 0.8]. However, grade 3-4 toxicities were more frequent in patients treated with ECX (diarrhea, neutropenia, hand-foot syndrome and mucitis).

These two trials (MAGIC and FNCLCC 94012-FFCD 9703) were the first studies to demonstrate better survival rates with a perioperative systemic approach for the treatment of localized GEA (Table 1). These results

Table 2 Neoadjuvant chemoradiotherapy schedule offered in the treatment of resectable gastroesophageal junction and lower esophagus adenocarcinomas

Trials	Patients (*n*)	Pathology	CT-RT	5-yr OS rate (%)
Walsh 1996	CT-RT = 58	ADK (100%)	CDDP-5FU	CT-RT 32%
	S = 55		40 Gy	S 6% (3-yr OS) (P = 0.01)
Urba 2001	CT-RT = 50	ADK (75%)	CDDP-VLB-5FU	CT-RT 30%
	S = 50	SCC	45 Gy	S 16% (3-yr S) (P = 0.15)
Burmeister 2005	CT-RT = 128	ADK (62%)	CDDP-5FU	CT-RT 22 mo
	S = 128	SCC	35 Gy	S 19 mo (P = 0.38)
Tepper 2008	CT-RT = 30	ADK (75%)	CDDP-5FU	CT-RT 39%
	S = 26	SCC	50.4 Gy	S 16% (P < 0.008)
CROSS 2010	CT-RT = 180	ADK (75%)	Paclitaxel-carboplatin	CT-RT 47%
	S = 188	SCC	41.4 Gy	S 34% (P = 0.03)

CT-RT: Chemoradiotherapy; OS: Overall survival; S: Surgery; ADK: Adenocarcinoma; SCC: Squamous cell carcinoma; VLB: Vinblastin.

have been confirmed in the meta-analysis by Li *et al*[34], and neoadjuvant chemotherapy is now considered as standard treatment for GEA in Europe. Moreover, based on the first OEO5 trial results, neoadjuvant chemotherapy with 5-FU and CDDP seems to be the best option in this setting.

Neoadjuvant chemoradiotherapy

Considering the improvement brought by systemic neoadjuvant chemotherapy for the management of patients with gastroesophageal junction and lower esophagus adenocarcinoma, several randomized phase Ⅲ study assessed the benefit of neoadjuvant chemoradiotherapy compared to surgery alone. The clinical trials by Urba *et al*[35] and Burmeister *et al*[36] did not show any benefit of chemoradiotherapy compared with surgery concerning the 3-year OS rate (30% *vs* 16%; P = 0.16) and the median OS (21.7 mo *vs* 18.5 mo; P = 0.38), in a population of patients among whom 75% had a gastroesophageal junction or lower esophagus adenocarcinoma (Table 2).

Only one phase Ⅲ trial found that chemoradiotherapy before surgery improved survival[37]. In this study, 113 patients with gastroesophageal junction and lower esophagus adenocarcinoma were randomized in two groups, PReoperative chemoradiotherapy plus surgery or surgery alone. Two cycles of 5-FU and CDDP were administered during radiotherapy, followed by surgery (multiple procedures) 8 wk after the beginning of the combined treatment. After a median follow-up of 10 mo, the median OS (16 mo *vs* 11 mo, P = 0.01) and the 3-year OS rates (32% *vs* 6%, P = 0.01) were significantly higher in the combined treatment arm than in the surgery alone arm, and the pathologic complete response rate was 25% in the chemoradiotherapy plus surgery arm. However, this monocentric study was closed prematurely after an intermediate analysis and with an unusually low survival rate in the surgery alone arm. Therefore, it is unclear how its results could be generalized.

The meta-analysis of 10 randomized trials (1209 patients) by Gebski *et al*[38] found an OS benefit of 13% at two years in patients treated with neoadjuvant chemoradiotherapy plus surgery *vs* surgery alone (HR = 0.81, 95%CI: 0.70-0.93, P = 0.002 and HR = 0.75, 95%CI: 0.59-0.95, P = 0.05) for the adenocarcinoma type in esophageal carcinoma. More recently, the CALGB Group reported a benefit of neoadjuvant chemoradiotherapy with a 5-FU-CDDP regimen in 56 patients (75% with a gastroesophageal or lower esophagus cancer)[39]. The 5-year OS rate was 39% in patients treated with neoadjuvant chemoradiotherapy compared with 16% (P < 0.008) in patients treated with surgery alone (median follow-up of 6 years). The complete pathologic response rate was 40%. These results are controversial due to the recruitment failure relative to the number of expected patients (n = 500).

Finally, the CROSS trial (n = 368 with esophageal or gastroesophageal junction cancer) clearly reported an improvement of the R0 resection rate (92% *vs* 69%; P < 0.0010) in patients treated with neoadjuvant chemoradiotherapy (paclitaxel-carboplatin regimen) plus surgery (n = 178) compared with patients who had only surgery (n = 188), with a complete pathologic response of 29%. The median OS was also significantly higher in the combined treatment arm than in the surgery arm (49 mo *vs* 24 mo; HR = 0.66, 95%CI: 0.49, 0.87, P = 0.003)[40].

COMBINATION OF NEOADJUVANT INDUCTION CHEMOTHERAPY AND CHEMORADIOTHERAPY

Ajani *et al*[41] proposed a three-step strategy combining induction chemotherapy (two cycles of 5-FU, levofolinate and cisplatin) with preoperative chemoradiotherapy (45 Gy of radiation concomitantly with 5-FU) and surgery. Their phase Ⅱ trial assessed response and survival in 33 patients with resectable gastric cancer. They showed a R0 resection rate of 70% and a 54% pathological response rate and a complete pathologic response of 30%. The median OS was 34 mo with a median follow-up of 5 years. The same authors assessed in another phase Ⅱ trial the same three-step strategy adding paclitaxel to 5-FU during chemoradiotherapy[42]. The trial

included 49 patients with resectable gastric carcinoma and showed a 77% R0 resection rate and a complete pathologic response rate of 26%. A median OS of 23 mo was reported, with a median follow-up of 22 mo.

A similar strategy (neoadjuvant chemoradiotherapy after two or three cycles of 5-FU-cisplatin induction chemotherapy followed by surgery) was also evaluated in patients with T3/T4 GEA who were randomized in the three-step protocol group or in the neoadjuvant chemotherapy alone plus surgery group. Although the trial was stopped early due to poor accrual, there was a trend towards a higher efficacy in the three-step protocol arm than in the neoadjuvant chemotherapy arm (3-year disease-free survival rate: 47.7% *vs* 27.7%; HR = 0.67; 95%CI: 0.41-1.07; $P = 0.07$)[43]. An Australian randomized phase Ⅱ study reported a significant reduction of the R1 resection rate in a similar population of patients treated with neoadjuvant chemoradiotherapy *vs* neoadjuvant chemotherapy alone (0% *vs* 11%, $P = 0.04$; pathological complete response rates of 31% *vs* 8%, respectively, $P = 0.01$)[44].

PERSPECTIVES IN THE GEA MANAGEMENT: UNANSWERED QUESTIONS

We described different treatment modalities and therapeutic strategies in the management of resectable GEA. However, some questions remain unresolved.

First, there is no strong evidence of the benefit of preoperative chemoradiotherapy over perioperative chemotherapy in patients with gastroesophageal junction adenocarcinoma. As surgical findings showed a higher incomplete tumor resection in locally-advanced T3/T4 gastroesophageal junction adenocarcinomas, the evaluation of the effects of preoperative chemoradiotherapy is urgently needed in this setting[45]. One ongoing phase Ⅲ clinical trial is focusing on this question. The all-Ireland Cooperative Oncology Research Group trial is currently comparing preoperative chemoradiotherapy (as it was done in the CROSS study) and perioperative chemotherapy with the epirubicin, cisplatin and 5-FU (ECF) regimen. The Dutch Colorectal Cancer Group is currently assessing postoperative chemoradiotherapy, comparing perioperative epirubicin, cisplatin, capecitabin (ECC) chemotherapy and preoperative ECC chemotherapy combined with postoperative chemoradiotherapy (the CRITICS study) in GEA.

Second, during chemotherapy/chemoradiotherapy, many patients experience life-threating effects and cannot complete the treatment. Conroy *et al*[46] showed that in patients with non-resectable esophageal carcinoma treated only with chemoradiotherapy, using oxaliplatin instead of CDDP (the FOLFOX4 regimen), reduced toxicities and toxic deaths compared with the standard 5-FU-CDDP regimen. A neoadjuvant FOLFOX6 chemotherapy regimen could be substituted to 5-FU-CDDP and proposed as ambulatory treatment.

In patients with metastatic GEA, the intensification of chemotherapy with docetaxel, 5-FU and cisplatin (TCF) is more effective than with 5-FU plus cisplatin alone, but with a significantly higher level of hematologic toxicities. The FLOT regimen (50 mg/m² docetaxel and infusion of 5-FU, leucovorin and oxaliplatin (TEF regimen) was compared with 5-FU plus oxaliplatin (the FLO regimen) in a randomized phase Ⅱ clinical trial. This study included patients older than 65 years and showed an improvement of the response rate and the progression-free survival in patients with locally-advanced cancer[47]. The combination of docetaxel, 5-FU, leucovorin and oxaliplatin as first-line treatment was effective with an intention-to-treat objective response rate of 66% (95%CI: 50.55-78.44) and two confirmed complete responses, progression-free survival of 6.3 mo (95%CI: 4.5-7.3) and OS of 12.1 mo (95%CI: 6.5-15.3)[48]. At the 2015 ASCO meeting, the authors presented preliminary results on the pathologic response in patients with resectable lower esophagus or gastroesophageal junction adenocarcinomas treated with FLOT or ECF. The complete pathologic response rate was significantly higher in patients who received FLOT than in those treated with ECF (15.6% *vs* 5.8%, $P = 0.015$)[49].

For T3/T4 and or N+ GEA, using hyperthermic intraperitoneal chemotherapy (HIPEC) could reduce the frequent peritoneal recurrences in this setting. The PRODIGE French scientific group study is currently assessing HIPEC as adjuvant treatment.

Concerning targeted therapies, the use of trastuzumab combined with neoadjuvant chemotherapy is not recommended in patients with resectable GEA that overexpress HER2. Also, the MAGIC group is currently assessing the association of bevacizumab and the ECC (capecitabine instead of 5-FU) chemotherapy regimen in a perioperative setting compared to chemotherapy with ECF alone.

Finally we need predictive markers of neoadjuvant treatment response as early PET-scan metabolic response or assessment of biomarkers using systematic pre-therapeutic or liquid biopsies.

CONCLUSION

Currently, two main therapeutic options can be proposed for the treatment of resectable GEA: (1) adjuvant chemoradiotherapy (Macdonald *et al*[17]) and (2) perioperative chemotherapy with 5-FU and platin salts-based regimens (Cunningham *et al*[26] or Ychou *et al*[29]). But what is best for our patients? Comparing the two strategies is not possible because the patients' profiles are too different. In both the MAGIC and FNCLCC 94012-FFCD 9703 trials, patients were identified at diagnosis, and not after surgery as it was done in the INT-0116 trial regarding the pT3/T4 or pN stage, the OMS (0 or 1) and the nutritional status.

Therefore, the treatment choice could be summarized

between two unsatisfactory options: A preoperative approach in which patients with a good-prognosis tumor may be over-treated, or a postoperative approach in which patients with high risk of recurrence and poor nutritional status after surgery might be under-treated. We recommend deciding the therapeutic management of each individual patient in a multidisciplinary committee, before the primary tumor surgery. Future applications of cytotoxic therapies, *e.g.*, oxaliplatin, capecitabine or docetaxel, or targeted therapies may help improving resectable GEA management.

For patients with gastroesophageal junction or lower esophagus adenocarcinomas, neoadjuvant chemoradiotherapy could be a viable option, but needs to be compared with perioperative chemotherapy.

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ORIGINAL ARTICLE

Basic Study

Association of colorectal cancer with pathogenic *Escherichia coli*: Focus on mechanisms using optical imaging

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Abstract

AIM: To investigate the molecular or cellular mechanisms related to the infection of epithelial colonic mucosa by *pks*-positive *Escherichia coli* (*E. coli*) using optical imaging.

METHODS: We choose to evaluate the tumor metabolic activity using a fluorodeoxyglucose analogue as 2-deoxyglucosone fluorescent probes and to correlate it with tumoral volume (mm^3). Inflammation measuring myeloperoxidase (MPO) activity and reactive oxygen species production was monitored by a bioluminescent (BLI) inflammation probe and related to histological examination and MPO levels by enzyme-linked immunosorbent assay (ELISA) on tumor specimens. The detection and quantitation of these two signals were

validated on a xenograft model of human colon adenocarcinoma epithelial cells (HCT116) in nude mice infected with a *pks*-positive *E. coli*. The inflammatory BLI signal was validated intra-digestively in the colitis-CEABAC10 DSS models, which mimicked Crohn's disease.

RESULTS: Using a 2-deoxyglucosone fluorescent probe, we observed a high and specific HCT116 tumor uptake in correlation with tumoral volume (P = 0.0036). Using the inflammation probe targeting MPO, we detected a rapid systemic elimination and a significant increase of the BLI signal in the *pks*-positive *E. coli*-infected HCT116 xenograft group (P < 0.005). ELISA confirmed that MPO levels were significantly higher (1556 ± 313.6 *vs* 234.6 ± 121.6 ng/mL P = 0.001) in xenografts infected with the pathogenic *E. coli* strain. Moreover, histological examination of tumor samples confirmed massive infiltration of *pks*-positive *E. coli*-infected HCT116 tumors by inflammatory cells compared to the uninfected group. These data showed that infection with the pathogenic *E. coli* strain enhanced inflammation and ROS production in tumors before tumor growth. Moreover, we demonstrated that the intra-digestive monitoring of inflammation is feasible in a reference colitis murine model (CEABAC10/DSS).

CONCLUSION: Using BLI and fluorescence optical imaging, we provided tools to better understand host-pathogen interactions at the early stage of disease, such as inflammatory bowel disease and colorectal cancer.

Key words: Colorectal carcinoma; *Escherichia coli*; Colibactin; Myeloperoxidase; *In vivo* optical imaging

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Core tip: Approximately 15% of cancers are related to infectious agents. Colorectal cancer (CRC) is thus a complex association of non-neoplastic and tumoral cells and a large amount of microorganisms. Recent studies reported that *pks*-positive *Escherichia coli* (*E. coli*) strains are more frequently detected in CRC, suggesting their possible role in tumor development. Optical imaging has emerged as a powerful tool in translational cancer research, providing new possibilities for the spatiotemporal monitoring of carcinogenesis in mouse models. It may be particularly helpful in better understanding the *in vivo* host-pathogen-interactions in tumor development. This is the first study to use optical imaging to explore CRC carcinogenesis and associated pathogenic *E. coli*.

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INTRODUCTION

Colorectal cancer (CRC) is the third most frequently diagnosed cancer worldwide[1]. Despite recent advances in therapeutic care, CRC remains the second cause of cancer-related death after lung neoplasia and is responsible for over 600000 deaths annually[1,2]. It is a multifactorial disease, strongly associated with genetic and environmental factors that favor tumor development[3]. Approximately 15% of cancers can be related to infectious agents[4,5], such as *Helicobacter pylori* (*H. pylori*) and gastric cancer[6]. Colorectal cancer thus involves a complex association of non-neoplastic and tumoral cells and a large amount of microorganisms. Gut microbiota, a bacterial community of over 100 trillion microbial cells, plays a major role in colorectal carcinogenesis. Indeed, high bacterial density in the colon (10^{12} commensal bacteria/g of intestinal contents) compared to the small intestine (10^{2} commensal bacteria/g of intestinal contents) is correlated with a higher risk of cancer development[7]. Gut microbiota dysbiosis has recently been linked to CRC[8-13], and several bacteria are involved in colorectal carcinogenesis, such as *Streptococcus bovis*[14,15], *Enterococcus* spp.[16], *H. pylori*[17-19], *Bacteroides fragilis*[20,21], *Clostridium septicum*[22], *Fusobacterium* spp.[23,24] and *Escherichia coli* (*E. coli*)[25,26].

E. coli is a commensal bacteria of the human gut microbiota that plays a major role in maintaining intestinal homeostasis[27]. Some strains became pathogenic, carrying virulence factors and producing toxins, such as cyclomodulins. These toxins can affect differentiation, apoptosis, and cell proliferation by interfering with the eukaryotic cell cycle and/or inducing DNA damage. Particularly, one of these toxins, the colibactin, is encoded by the *pks* genomic island and can lead to the creation of double-strand DNA breaks and thus induce the chromosomal instability involved in CRC[28,29]. Recent studies reported that *pks*-positive *E. coli* is more frequently detected in CRC patients, suggesting a possible role in tumor development[30-32]. Various independent studies showed that *pks*-positive-*E. coli* exhibit procarcinogenic properties in murine models, such as the multiple intestinal neoplasia (Min) mice model[33], azoxymethane (AOM)-treated *Il10*$^{-/-}$ mice[34] and AOM/DSS models[26]. Thereby, some pathogenic *E. coli* strains involved in colon carcinogenesis are now emerging. Nevertheless, mechanisms of action remain to be clarified, particularly in *in vivo* models.

The aim of the present study was to investigate *in vivo* the molecular or cellular mechanisms related to the infection of epithelial mucosa by *pks*-positive *E. coli* using 2D optical imaging. Indeed, optical imaging is emerging as a new powerful sensitive technology for the non-invasive spatiotemporal visualization of carcinogenesis in mice models, and it may help to better understand the host-pathogen interactions in colorectal tumor development[35-37]. Because chronic inflammation and reactive oxygen species (ROS) production are

key factors in bacteria and CRC interactions, we chose to evaluate *pks*-positive *E. coli* infection on these mechanisms using commercial, available and validated probes[31,38-40]. Indeed, inflammation could play a key role in the development of dysbiosis related to CRC[40]. *E. coli* is also the most characterized bacteria associated with inflammatory bowel disease, which is a known risk factor for CRC[41,42]. Moreover, Raisch *et al*[38] demonstrated that *E. coli* in colon cancer induces a significant increase in COX-2 expression in macrophages, the predominant type of immune cell that infiltrates tumors. Moreover, macrophages and other immune cells infiltrate the tumors, release myeloperoxidase (MPO), and produce ROS by several chemical reactions. Arthur *et al*[31,39] investigated *in vivo* the complex interplay between inflammation, bacteria and carcinogenesis and suggested that chronic inflammation is essential for tumor development by maintaining the expression of *pks* island genes. ROS production has also been reported in many suspected mechanisms related to CRC development. Neutrophils and macrophages, which are present in inflamed tissues such as colon tumors, are major providers of ROS. Maddocks *et al*[40,43] described a possible interaction between *E. coli* and the DNA repair system with elevated ROS levels. Because ROS oxidizes the luminescent probe and thus produces proportional light that is detectable *in vivo* with an optical imager[44,45], we choose to monitor the inflammatory pathway and ROS production using on a bioluminescent (BLI) approach. The monitoring of inflammation was first performed and validated on a colitis murine model (CEABAC10/DSS mice). Then, by this approach, we showed, on a xenograft murine model, that *pks*-positive-*E. coli* significantly induces oxidative stress and inflammation before stimulating HCT116-tumor growth. While monitoring longitudinal inflammation, we choose to assess tumor growth by determining tumor metabolic activity with a fluorescent tool based on the fluorodeoxyglucose analogue 2-deoxyglucosone.

MATERIALS AND METHODS

Animal models

Studies were performed in accordance with the French Regional Ethical Animal Use Committee (No. CEEA-02). All mouse models were housed in specific pathogen-free conditions (22 ± 2 ℃, 50% humidity, 12 h light/12 h dark) in the animal care facility of the Université d'Auvergne, Clermont-Ferrand, France.

HCT116 xenograft models

The HCT116 colorectal cancer cells were maintained as monolayers in culture flasks using culture medium consisting of McCoy's 5a Medium (Modified) supplemented with 10% FCS (Biowest, Nuaillé, France), 2 mmol/L glutamine and 1% antibiotics. All the cells were grown at 37 ℃ in a humidified incubator containing 5% CO_2.

Xenografts of human CRC were induced in male nude mice (Swiss nu/nu), weighting 26-33 g at the time of injection (7 wk old, Charles Rivers). We excluded female nude mice in order to avoid a possible hormonal influence. A total of 10 male nude mice were divided into two groups: Non-infected control xenograft (n = 5) and *pks*-positive *E. coli*-infected xenograft (n = 5). According to the infected xenograft, HCT116 cells were mixed with *pks*-positive *E. coli* as previously described by Cougnoux *et al*[34]. Beforehand, bacteria were grown at 37 ℃ in Luria-Bertani medium. *Pks*-positive *E. coli* is an ampicillin- and kanamycin-resistant *E. coli* strain named 11G5, isolated from a patient presenting with colon cancer and previously presented by Bonnet *et al*[33]. We used human colon adenocarcinoma epithelial cells (HCT116) to establish the xenograft models.

Then, animals anesthetized by isoflurane inhalation were inoculated with 2×10^6 HCT116 cells embedded in growth factor-reduced Matrigel (Becton Dickinson) by dorsal subcutaneous injection at day 0 of the experiment.

Tumor size was assessed two times per week and tumor volume was obtained according to the following formula: $(\text{width}^2 \times \text{length})/2 = V\ (\text{mm}^3)$. The longest diameter (L) and maximum diameter (W) perpendicular to the direction of the longest diameter were determined using a caliper. Mice were sacrificed at 35 d post-injection, and the xenograft was collected from all animals and subjected to histologic examination and enzyme-linked immunosorbent assay (ELISA).

Colitis-CEABAC10 DSS models

Six CEABAC10 transgenic mice in an *in vivo* model mimicking colitis and Crohn's disease[46] were used to monitor intra-digestive inflammation. They were divided into two groups. Mice from the same generation were used for experimentation. One group (n = 3) received one cycle of dextran sodium sulfate (DSS) in drinking water for 6 d at 1% (DSS-treated mice group) as described previously in Denizot *et al*[46]. The other group received only drinking water (n = 3; DSS-).

Optical imaging

For both BLI and fluorescence imaging acquisition, all animals were imaged using a dedicated high-sensitivity peltier-cooled (-90 ℃) backlit charge-coupled device camera (IVIS Spectrum®, Perkin Elmer, United States). All acquisitions were performed under the same exposure conditions according to fluorescence or BLI imaging, with acquisition settings (binning and duration) set up depending upon the signal at the time of acquisition.

Prior to imaging, animals were anesthetized with 2%-3% isoflurane in an induction chamber; then, 2% isoflurane in air/O_2 was continuously delivered *via* a nose cone system in the dark box of the imaging system (delivered gas to up to 5 mice). To limit auto-

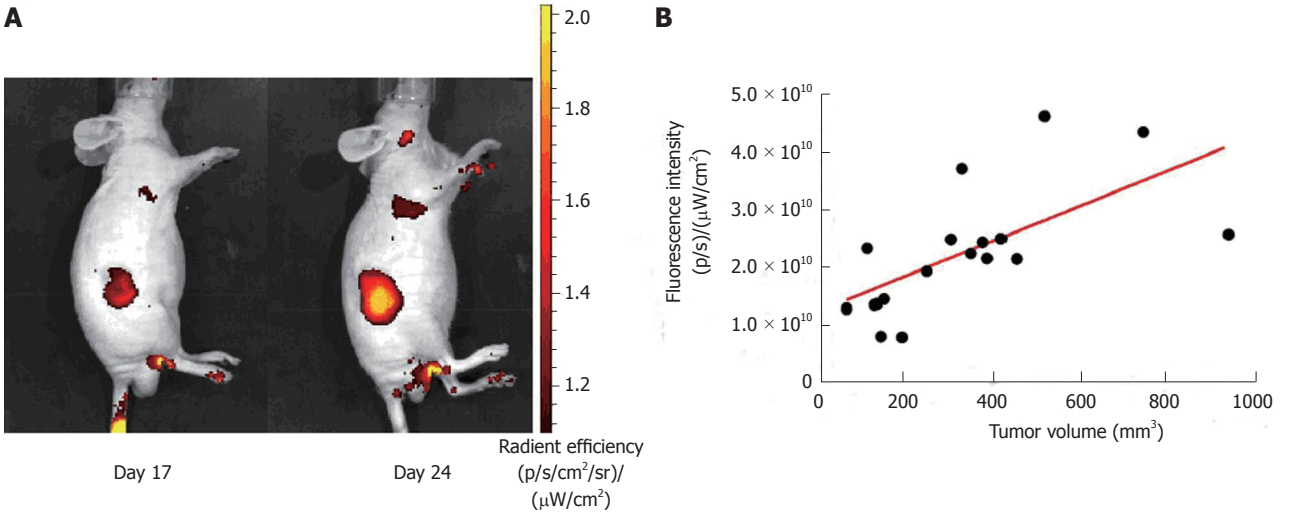


Figure 1 Correlation of tumor volume (HCT116 cells xenograft) and 2-DG-750 fluorescence signal uptake. A: Representative animal images showing the increase in fluorescence signal intensity with tumor development (day 17 and day 24 post-xenograft); B: Correlation between tumor development determined using a caliper and increase in fluorescence signal intensity (Pearson's correlation factor $r = 0.4197$; $P = 0.0036$).

fluorescence related to melanin, mice were shaved before all imaging procedures (except nude mice, which are hairless).

We used the XénoLight Rediject 2-DG-750 fluorescent probe or the XénoLight Rediject Inflammation chemiluminescent probe (Perkin Elmer, United States) to monitor metabolic activity or inflammation (MPO and ROS detection), respectively.

To monitor inflammation in HCT116-grafted nude mice at 20 d and 34 d post-xenograft, we administered by intraperitoneal (i.p.) injection of 150 μL of the XenoLight Rediject Inflammation probe per mouse. Mice were then imaged 10 min post-injection (exposure time of 5 min). In the colitis CEABAC10 model, imaging was performed 6 d after the DSS cycle. To monitor inflammation at depth and limit the decrease of the BLI signal intensity in this model, we administered by an intravenous (i.v.) injection of 150 μL/mouse. With i.v. injection, the best time to image the animal was immediately post-injection (exposure time of 5 min).

For tumor metabolic activity, we administered by an intravenous injection of 100 μL/mouse and imaged them 3 h after 2-DG-750 probe injection using one filter set (excitation: 745 nm, emission: 820 nm) and a high-throughput epi-illumination acquisition mode. All nude mice were imaged individually at 17 d and 24 d post-xenograft.

Quantitative analysis of imaging was performed using Living Image® Software (Caliper Life Science, United States) with the region of interest delineated manually over organs exhibiting probe accumulation. Every image series had the same scales, set manually, to facilitate the visual comparison of signal intensity at each time point. For the BLI signal, photon flux was expressed as the average radiance in p/s/cm²/sr. Fluorescence emission was also normalized to photons per second per centimeter squared per steradian (p/s/cm²/sr).

Histological analysis

After mouse sacrifice, tumor pieces were fixed in formol solution. Paraffin-embedded sections were cut into 5-μm slices, and tissue sections were prepared for hematoxylin-eosin-safran staining and routine pathological analysis with focus on the mitotic index, infiltrating cells and tumor necrosis. Sample preparations and observations were made in the Centre Imagerie Cellulaire Santé platform (Clermont-Ferrand).

MPO activity determination

After mouse sacrifice, tumor pieces were frozen in liquid nitrogen and stored at -80 ℃ until used. We performed an enzyme-linked immunosorbent assay to determine the levels of MPO (ng/mL) in all the tumors according to the manufacturer's instructions (R and D systems). Data were standardized on whole protein extracts stained with Coomassie blue.

Statistical analysis

Graph Pad Prism 5 STATA (StataCorp) was used for all statistical analysis. Unpaired Student's *t* test was used for the comparisons of the 2 groups. We determined the Pearson's correlation coefficient *r* to assess the degree of correlation. We considered *P* values of < 0.05 to be statistically significant.

RESULTS

Metabolic activity of the CRC xenograft model

In HCT116-grafted nude models, we confirmed a rapid systemic elimination of the 2-deoxyglucosone fluorescent probe with a very weak whole body uptake 180 min after probe-injection (Figure 1A). Moreover, a high and specific HCT116 tumor uptake was evidenced for each tumor 17 and 34 d post-graft. We demonstrated an increase of signal intensity over time, reflecting tumor growth. The tumor uptake of the

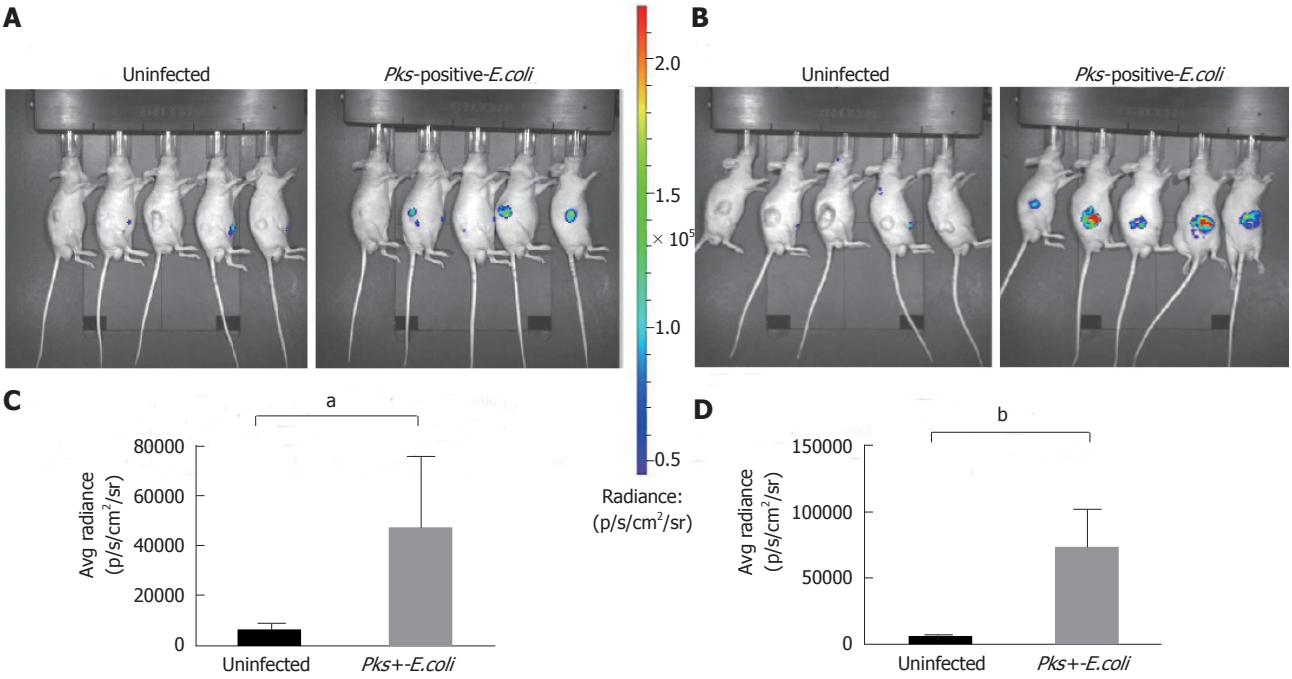


Figure 2 Significant increase of inflammation in HCT116 tumors infected with *Escherichia coli* strains measured by optical imaging using the inflammation probe. *In vivo* BLI imaging was performed at day 20 (A) and day 34 (B) post-HCT116 xenograft in nude mice (n = 5 animals with uninfected xenograft; n = 5 animals in *pks*-positive *E. coli*-infected xenograft). BLI images comparing uninfected to *pks*-positive *E. coli*-infected xenografts are shown on day 20 (A) and day 34 (B) post-xenograft. Intensity of emission is represented as the pseudocolor image. A sharp increase in the BLI signal was seen in the *pks*-positive *E. coli*-infected xenograft group at each time point. The BLI signal detected 10 min after i.p. probe injection was quantified from the ROI drawn manually. Photon flux was expressed as the average radiance in p/s/cm^2/sr. Graphs reveal a statistically significant increase in the BLI signal in the *pks*-positive *E. coli*-infected tumors: day 20, ^aP = 0.0132 (C) and day 34, ^bP = 0.0006 (D). BLI: Bioluminescent; *E. coli*: *Escherichia coli*; i.p.: Intraperitoneal.

2-deoxyglucosone fluorescent signal was significantly (P = 0.0036) correlated with tumor volume, as determined using a caliper (Figure 1B). The *in vivo* monitoring of HCT116 tumor growth by the 2-deoxyglucosone fluorescent probe was efficient. No difference in tumor uptake was observed between uninfected and *pks*-positive *E. coli*-infected HCT116 cells (data not shown), as previously described with caliper determination by Cougnoux *et al*[34] at 34 d post-xenograft.

We tested 2-deoxyglucosone fluorescent imaging on Colitis-CEABAC10 DSS models. We did not observe any fluorescent signal *in vivo* in mice, reflecting that the targeting probe is specific for tumor cells (data not shown).

Pks-positive *E. coli in vivo* induces inflammation in the CRC xenograft model

Using the inflammation probe, all nude mice were imaged 20 and 34 d post-xenograft. We detected a rapid systemic elimination in all mice and a strong BLI signal in HCT116 tumors in the infected group 10 min after probe injection (Figure 2A and B). Figure 2A and B clearly show that the intensity of the BLI signal (average radiance in p/s/cm^2) was stronger in xenografts infected with the pathogenic *pks*-positive *E. coli* strain compared to uninfected ones at each time point investigated (20 and 34 d post-xenograft). Quantitation confirmed a significant increase of the BLI signal in the infected tumors 20 d (P = 0.0132, Figure 2C) and 34 d (P = 0.0006, Figure 2D) after the xenograft.

Monitoring intra-digestive BLI signals in Colitis-CEABAC10 DSS models

Then, we analyzed the monitoring of intra-digestive BLI signals using the inflammation probe in the CEABAC10 colitis mouse model. We induced intra-digestive inflammation using DSS in the first group, while control mice received only drinking water. To visualize BLI imaging in deep tissues *in vivo* in mice, the probe was injected intravenously. DSS group imaging showed a high BLI signal in DSS animals, reflecting severe intra-digestive inflammation (DSS+) relative to the untreated group (DSS-) (Figure 3). We demonstrated that monitoring intradigestive inflammation with the BLI signal is feasible and consistent.

Histological characterization

The histologic analysis of tumor samples indicated and confirmed that tumor cells in *pks*-positive *E. coli*-infected xenografts were surrounded by a remarkable infiltration of activated phagocytes (Figure 4C and D) compared to the uninfected group (Figure 4A and B). In addition, tumor necrosis was observed, especially in the *pks*-positive *E. coli*-infected group (Figure 4C). Moreover, HCT116 are characterized by megalocytosis and the progressive enlargement of the cell body and nucleus in *pks*-positive *E. coli*-infected xenografts. Histological examination confirmed the data from BLI

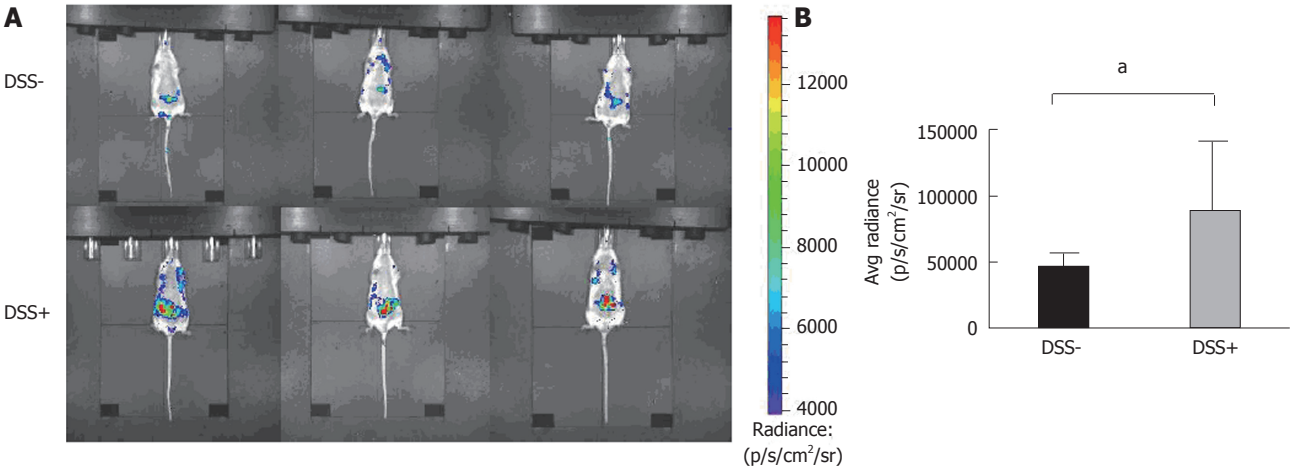


Figure 3 Monitoring of intra-digestive bioluminescent signals using the inflammation probe in the CEABAC10 mouse model. One mouse group was treated with 1% dextran sodium sulfate (DSS-treated mice group) in drinking water for 6 d (n = 3), while the other group received only drinking water (n = 3; DSS-). Mice were subjected to one cycle of DSS and then imaging with the inflammation probe before sacrifice. Mice were intravenously injected with the inflammation probe, and the BLI signal was detected immediately after injection. DSS-treated mouse group acquisition (A) showed severe intra-digestive inflammation was correlated with a significant increase in the BLI signal on graphs (B) compared to the DSS group (^aP = 0.03). BLI: Bioluminescent; DSS: Dextran sodium sulfate.

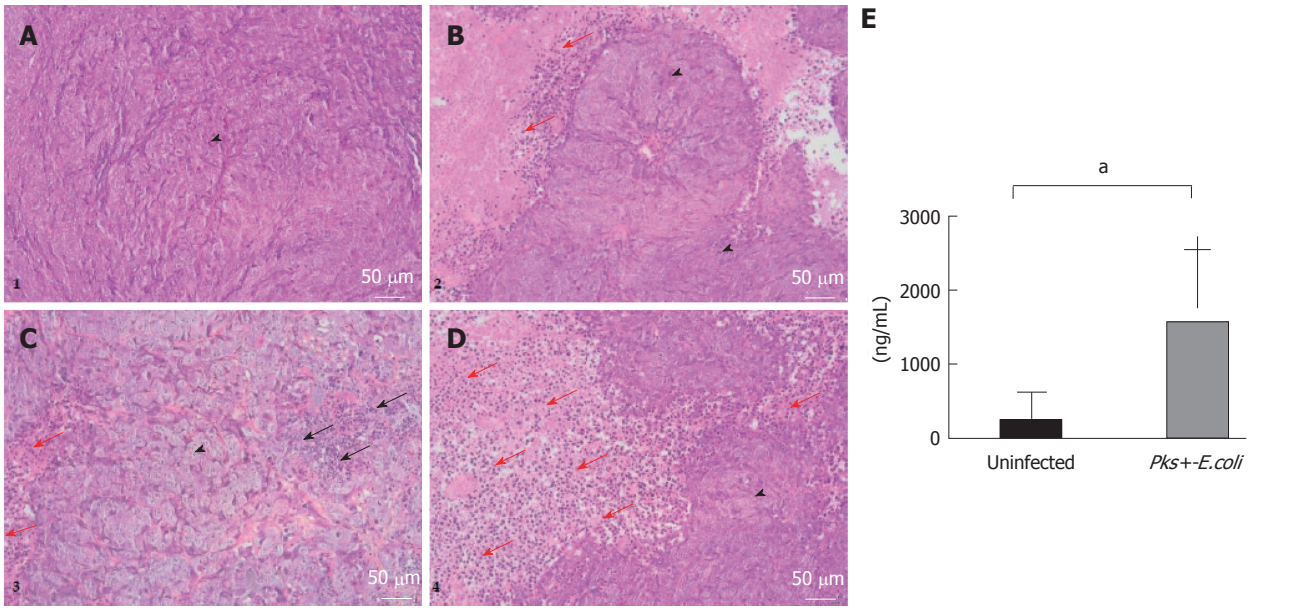


Figure 4 Histological and molecular analyses of HCT116 tumor samples. A-D: Histological examination of representative HCT116 tumor samples. Xenografts were harvested, paraffin embedded and processed for hematoxylin/eosin/safran. A and B are representative histological examinations from the uninfected xenograft group. C and D are representative histological examinations from the *pks*-positive-*E. coli* infected xenograft group. We noted that HCT116 tumor cells (arrowheads) infected with pathogenic *E. coli* strains are characterized by megalocytosis and progressive enlargement of the cell body and nucleus. Tumor cells in the *pks*-positive *E. coli*-infected xenograft group were surrounded by a remarkable infiltration of inflammatory cells (red arrow) compared to the uninfected xenograft group. Tumor necrosis was observed, especially in the infected xenograft group (black arrow). (Scale bars: 50 μm = × 20); E: MPO levels by enzyme-linked immunosorbent assay (ELISA) on HCT116 tumor specimens. An ELISA test was performed on tumor specimens after mouse sacrifice (day 34 post-xenograft). MPO standardized levels were significantly higher (1556 ± 313.6 *vs* 234.6 ± 121.6, ^aP = 0.001) in xenografts infected with pathogenic *pks*-positive *E. coli* strains. *E. coli*: *Escherichia coli*; MPO: Myeloperoxidase.

imaging (inflammation probe).

MPO levels by ELISA

To confirm the quantitation of inflammation imaging, we assessed MPO activity on HCT116-xenograft-tumor specimens by performing an ELISA. MPO levels (ng/mL) were significantly higher (1556 ± 313.6 *vs* 234 ± 121.6, P = 0.001) in xenografts infected with the *pks*-positive *E. coli* strain. These results showed that the pathogenic *E. coli* strain enhanced MPO release compared to uninfected xenografts and confirmed the data from BLI imaging.

DISCUSSION

Optical imaging appears to be a powerful, highly sensitive tool in translational cancer research, providing new possibilities for *in vivo* molecular imaging and

allowing a better understanding of host-pathogen interactions in several tumor processes. Some studies have reported the pro-carcinogenic activities of *pks*-positive *E. coli* in murine models[26,33,34]. Most of these studies required animal sacrifice and did not allow longitudinal investigation. Ideally, it would be useful to non-invasively, longitudinally monitor these procarcinogenic processes. Here, we report the first study that utilized optical imaging in these settings. More precisely, we focused on CRC carcinogenesis and pathogenic *E. coli* association.

We described a specific accumulation of the 2-deoxyglucosone fluorescent probe to the tumor site (CRC xenograft model), thus establishing a correlation between tumor volume and fluorescent signal intensity. We showed that this method provides an effective tool to assess longitudinal data on CRC tumor growth *in vivo*. Moreover, in our experimental conditions, xenografts infected with *pks*-positive *E. coli* exhibited comparable development to uninfected ones, confirming results reported for the same experimental conditions by Cougnoux *et al*[34]. However, they observed a significant increase in tumor volume induced by colibactin, starting from day 44 after the xenograft. In the present study, we chose to evaluate inflammation and ROS production at an early stage, before the effect of colibactin on tumor cells proliferation. Indeed, Arthur *et al*[39] suggested that inflammation is necessary for *E. coli*'s cancer-promoting activity, probably through the enhancement of its resilience among gut microbiota in the intestine. Our results showed that the BLI signal significantly increases with bacterial infection. Pathogenic *E. coli* seemed to enhance inflammation and ROS production, which could participate in carcinogenesis. Using luminol-based BLI, we showed that *pks*-positive *E. coli* induced oxidative stress, which is involved in carcinogenesis process. We confirmed this observation on histological examination, which showed that inflammatory cells were mostly recruited in infected xenografts. Tumor necrosis also appeared in the *pks*-positive *E. coli* group. Moreover, a significant increase of MPO activity, which led to ROS production by infiltrating immune cells, was confirmed with an ELISA on HCT116-cells tumor specimens. Finally, these data showed that *pks*-positive-*E. coli* induced inflammation and ROS production at an early stage after infection, and could thus be an important mechanism involved in pro-carcinogenic activity.

To validate the use of luminol-based BLI imaging to monitor inflammation and oxidative stress, we used an *in vivo* colitis model (CEABAC10/DSS). We proved that monitoring inflammation in deep tissues is feasible and effective. This suggests that optical imaging should be tested on other murine models ($APC^{min/+}$, AOM-$IL10^{-/-}$, AOM-DSS) used to determine the pro-carcinogenic proprieties of *pks*-positive *E. coli* strains, and it may facilitate a better understanding of how pathogenic bacteria impact the carcinogenesis process by various mechanisms. Using CEABAC10 models mimicking Crohn's disease, we suggest that optical imaging is an effective method in inflammatory bowel disease research.

In conclusion, by using optical imaging, particularly the BLI approach, we provided additional tools to better understand host-pathogen interactions in digestive pathology, including CRC and inflammatory bowel disease.

COMMENTS

Background

Colorectal carcinoma (CRC) is a complex association of non-neoplastic and tumoral cells and a large amount of microorganisms. *Escherichia coli* (*E. coli*) is a consistent commensal of the human gut microbiota but some pathogenic strains have acquired the ability to produce toxins as cyclomodulins that can interfere with eukaryotic cell cycle or directly induce DNA damages. It was observed that cyclomudulin-producing-*E. coli* are more frequently detected on CRC patients and exhibit procarcinogenic properties on murine models.

Research frontiers

Novel imaging techniques like optical imaging could be a powerful tool in translational cancer. Particularly, *in vivo* optical imaging is an innovative tool for non-invasive, spatiotemporal and quantitative monitoring of carcinogenesis process in murine models. It may help to better understand the host-pathogen interactions in colorectal tumor development.

Innovations and breakthroughs

This study investigates the *in vivo* mechanisms of epithelial colonic mucosa infection by cyclomodulin-positive-*E. coli*. Because chronic inflammation and reactive oxygen species (ROS) production are key factors in bacteria and CRC interactions, the authors choose to evaluate cyclomodulin-positive-*E. coli* infection on these mechanisms using optical imaging and commercial, available and validated probes. By using this technique, the authors provided tools to better understand host-pathogen interactions on murine models at the early stage of disease, such as inflammatory bowel disease and CRC.

Applications

By using optical imaging, particularly the bioluminescent approach, the authors provided additional tools to better understand host-pathogen interactions in digestive pathology, including CRC and inflammatory bowel disease. The data suggest that cyclomodulin-positive-*E. coli* induced inflammation and ROS production at an early stage after infection, and could thus be an important mechanism involved in pro-carcinogenic activity of these bacteria.

Terminology

Oxidative stress reflects an imbalance between the systemic manifestation of ROS and the cellular biological system's ability to readily detoxify the reactive intermediates or to repair the resulting damage. Disturbances in the normal redox state of cells can cause toxic effects through the production of peroxides and free radicals that damage all components of the cell, including proteins, lipids, and DNA. In humans, oxidative stress is thought to be involved in the development of several cancers.

Peer-review

Good interesting novel study. The experiments were well designed.

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ORIGINAL ARTICLE

Retrospective Cohort Study

Contralateral prophylactic mastectomy rate stable at major Canadian breast cancer center

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Author contributions: Roberts A completed the data collection and prepared the manuscript; Sandhu L analyzed the data; Roberts A, Cil TD and Zhong T developed the research question and concept; Sandhu L, Cil TD, Hofer SOP and Zhong T provided feedback on research and critically appraised manuscript.

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Informed consent statement: Not applicable.

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Abstract

AIM: To examine trends of contralateral prophylactic mastectomy (CPM) rates at a Canadian academic breast cancer center.

METHODS: A single-institution retrospective cohort study was completed. Women of any age who underwent at least a unilateral mastectomy (UM) for primary unilateral breast carcinoma between January 1, 2004 and December 31, 2010 were included. Patients who underwent CPM on the same day as UM were isolated to create two distinct cohorts. Patient and procedure characteristics were compared across groups using R software (version 3.1.0). The percentage of CPMs per year was determined. The Cochrane-Armitage test was used to assess the trend of CPMs over time. A P value of < 0.05 was considered significant.

RESULTS: A total of 811 women met the inclusions/exclusion criteria; 759 (93.6%) underwent UM alone and 52 (6.4%) underwent UM with immediate CPM. The absolute number of procedures (UM and UM + CPM) increased over time, from 83 in 2004 to 147 in 2010 reflecting an increase in mastectomy volume. Annual CPM rates did not increase over time ($P = 0.7$) and varied between 2.6% to 10.7%. Family history of breast cancer [OR 3.6 (1.8-7.3)] and immediate reconstruction [10.0 (5.2-19.3)] were both significantly associated

with CPM. Women who underwent CPM were younger (median age CPM 49 years *vs* UM 52 years, $P < 0.0001$) but age less than 50 years was not statistically associated with increased rates of CPM.

CONCLUSION: CPM rates from 2004 to 2010 at a high-volume Canadian breast cancer center did not increase over time, in contrast to trends observed in the United States.

Key words: Breast; Oncology; Prophylactic; Mastectomy; Surgery

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Core tip: Contralateral prophylactic mastectomy rates from 2004 to 2010 at a high-volume Canadian breast cancer center do not demonstrate the same rising trend observed in the United States.

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INTRODUCTION

During the past decade, the percentage of women with unilateral breast carcinomas undergoing immediate contralateral prophylactic mastectomy (CPM) has steadily increased over time[1-3] despite minimal evidence supporting a survival benefit[4-7]. These increased CPM rates have mainly been described in literature from the United States. CPM trends of similar magnitude and scope have not been quantitatively documented in literature from countries outside of the United States. Switzerland and England have reported much lower CPM rates during a similar time period as the United States data[8,9] - with no significant increase over time noted in Switzerland. It is possible that in these countries, as well as in Canada, where the health care system is based on a socialized medicine model, and surgeons routinely practice under budgetary restraints; a nonlife-saving surgical service such as CPM may not be as readily available as in the United States.

In Canada, lower CPM rates have been noted on a national level compared to the United States[10], but these rates were obtained through administrative databases and no patient level, surgical procedure detail, or outcomes data were reported[10]. Since CPM rates are known to be influenced by resource factors, such as access to genetic testing and immediate breast reconstruction, and other patient factors such as age[1-3,7,8,11-14], examining CPM trends with patient and procedure level data will provide more detailed information on the CPM landscape in Canada.

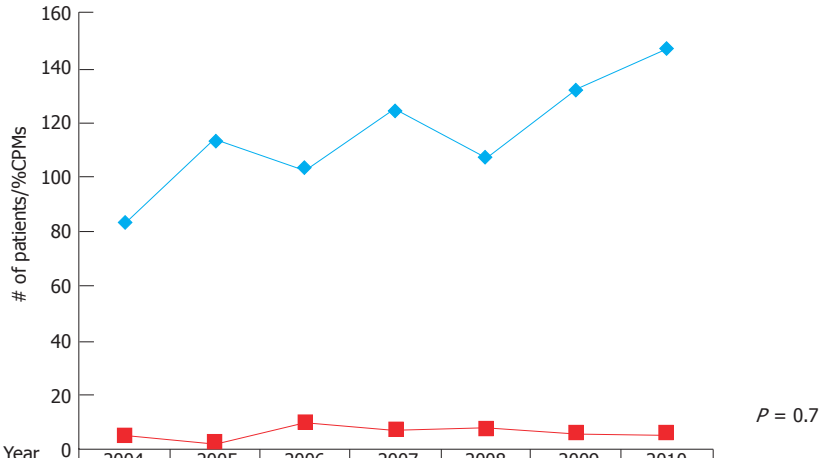
Therefore, our main objective was to examine the rates and trends of immediate CPM and to compare outcomes of patients undergoing CPM to patients having unilateral mastectomy (UM) alone at a Canadian academic breast cancer center.

MATERIALS AND METHODS

We conducted a single institution retrospective cohort study using a prospectively maintained database. The Princess Margaret Breast Disease Database contains information on all patients who undergo any form of breast disease evaluation or treatment at the Princess Margaret Hospital in Toronto, Ontario, Canada. There is an opt-out policy for patients who do not wish to have their medical information collected and used for research purposes. Research Ethics Board approval was acquired and maintained throughout the study. The population of interest included women of any age who underwent at least a UM for primary unilateral invasive or *in situ* breast carcinoma between January 1, 2004 and December 31, 2010. This time period was chosen to be as contemporary as possible while still coinciding with the literature from the United States and Switzerland. Patients who underwent CPM on the same day as the UM were isolated to create two distinct cohorts: Women who underwent UM alone and women who underwent UM with immediate CPM. Patients with known bilateral disease at the time of their primary surgery were excluded. A diagnosis of atypical hyperplasia, lobular carcinoma *in situ*, ductal carcinoma *in situ* or invasive breast cancer in the contralateral breast was considered as bilateral disease. These exclusions were applied in order to isolate, as accurately as possible, CPMs of truly benign breasts.

Patient and procedure variables were extracted from the database and included: Age, history of benign disease, family history of breast cancer, pre-operative BRCA status, pre-operative diagnosis, pre-operative systemic therapy, receipt of radiation, year of surgery, axillary evaluation (sentinel lymph node biopsy or axillary lymph node dissection) and receipt of reconstruction. The incidence of ipsilateral locoregional recurrence and development of a new contralateral breast cancer were also extracted.

Patient and procedure characteristics were compared across groups using R software (version 3.1.0). Mean values and standard deviations were calculated for normally distributed continuous variables and median values with interquartile range (IQR) were calculated for variables with a non-normal distribution. For categorical variables, results were expressed as counts and percentages. The Cochrane-Armitage test was used to assess the trend of CPMs over time. A P value of < 0.05 was considered significant. A multivariable logistic regression model was generated with receipt of CPM as the main outcome variable. Covariates used in this model included age, family history of breast cancer, personal history of benign breast disease and receipt



Year	2004	2005	2006	2007	2008	2009	2010
Total # patients	83	114	103	125	107	132	147
%CPM	4.8	2.6	10.7	7.2	8.4	6.1	5.4

Figure 1 Contralateral prophylactic mastectomy trends. CPM: Contralateral prophylactic mastectomies.

of immediate reconstruction. Age was dichotomized to a binary variable (< 50 years or ⩾ 50 years old). The remaining covariates were also binary. Covariates were chosen based on clinical significance. Model fit took into consideration any interactions and assessments of collinearity. The statistical review of the study was performed by a biomedical statistician.

RESULTS

A total of 811 women met the inclusions/exclusion criteria; 759 (93.6%) underwent UM alone and 52 (6.4%) underwent UM with immediate CPM. The characteristics of each group are summarized in Table 1. The absolute number of procedures (UM and UM + CPM) increased over time, from 83 in 2004 to 147 in 2010 reflecting an increase in mastectomy volume (Figure 1). CPMs per year did not increase over the study time period (P = 0.7) and varied between 2.6% to 10.7% (Figure 1).

Women who had a CPM were more likely to be younger [median age CPM = 49 years (range 29-67); UM = 52 years (range 28-88)] and to have a breast cancer family history (CPM 67% *vs* UM 37%, P < 0.001). Receipt of neoadjuvant chemotherapy was not significantly different between the two groups (CPM 31% *vs* UM 23%, P = 0.23), yet there were significantly more patients in the CPM group who underwent an ALND at the time of mastectomy (CPM 81% *vs* UM 49%, P < 0.001). Significantly more patients having CPM underwent immediate reconstruction (CPM 54% *vs* UM 8%, P < 0.001).

On the multivariable analysis, CPM patients were almost four times as likely to have a family history of breast cancer compared to those patients who opted for UM alone [OR = 3.6, (1.8-7.3) P < 0.001]. Patients who had a CPM were also 10 times more likely to receive immediate reconstruction when compared to patients who underwent UM alone [OR = 10.0 (5.2-19.3), P < 0.001] (Table 2).

Locoregional recurrence rates were no different between the two groups (CPM 4% *vs* UM 3%, P = 0.68). Over the same follow-up period, a new contralateral breast cancer was identified in 13 (2.0%) of patients who underwent UM alone. No new contralateral breast cancers were identified in the CPM group, although three (6.0%) of the patients who underwent CPM were found to have incidental invasive disease in the contralateral breast on final pathology.

DISCUSSION

Between 2004 and 2010, CPM rates at our single Canadian institution did not show the same increasing trend observed in the United States. During this time period, our CPM rate was 6.4% (range: 2.6% to 10.7%) with no increase over time, while rates in the United States were noted to increase between 2003 and 2010 from 4.1% to 9.7% for all ages, and 9.3% to 24.1% for patients aged 45 years or less[1]. In contrast, during a similar time period in Switzerland, CPM rates remained stable at 7%[8]. In Canada, the only reported data examined national CPM rates between 2007 and 2010 and demonstrated an overall CPM rate of 6% (5% in 2007/2008 to 7% in 2009/2010) - consistent with our institutional results and the rates from Switzerland[8,10].

Interestingly, the factors associated with increased rates of immediate CPM at our institution are similar to those found in the United States literature - suggesting that the patient factors influencing the decision to undergo a CPM are possibly the same between the two countries. Within our population, younger patients and those with family history were more likely to undergo CPM, similar to patients in the numerous United States studies[1-3,7,8,12-14]. Furthermore, the use of immediate reconstruction has been associated with increased CPM rates[11,13], and our study results support this finding.

As noted, the lower CPM rates reported from

Table 1 Patient characteristics

	UM	CPM	*P* value
	n = 759 (94%)	*n* = 52 (6%)	
Age (median years, range)	52 (28-88)	49 (29-67)	< 0.001
Pre-operative diagnosis (%)			0.74
Invasive carcinoma only	585 (77.1)	38 (73.1)	
DCIS only	144 (19.0)	12 (23.1)	
Invasive + DCIS	30 (4.0)	2 (3.8)	
Previous benign disease (%)			0.26
No	566 (74.6)	34 (65.4)	
Yes	164 (21.6)	15 (28.8)	
Unknown	29 (3.8)	3 (5.8)	
Pre-op BRCA1/BRCA2 mutation status (%)			< 0.001
Negative	43 (6)	9 (17)	
Positive	6 (1)	11 (21)	
Both unknown	710 (94)	32 (62)	
Family history (%)			< 0.001
Positive	283 (37.3)	35 (67.3)	
Negative	440 (58.0)	13 (25.0)	
Unknown	36 (4.7)	4 (7.7)	
Pre-op systemic therapy (%)			0.23
No	588 (77)	36 (69)	
Yes	171 (23)	16 (31)	
Any radiation treatment (%)			0.008
No	435 (57)	40 (77)	
Yes	324 (43)	12 (23)	
SLNB (%)			0.76
No	316 (42)	20 (38)	
Yes	443 (58)	32 (62)	
ALND (%)			< 0.001
No	387 (51)	10 (19)	
Yes	372 (49)	42 (81)	
Reconstruction (%)			< 0.001
No	695 (92)	24 (46)	
Yes	64 (8)	28 (54)	
Local/regional recurrence (%)			0.68
No	734 (97)	50 (96)	
Yes	25 (3)	2 (4)	
New contralateral breast cancer (%)			0.05
No	746 (98)	49 (94)	
Yes	13 (2)	3 (6)	
Follow-up (median years, range)	2.32 (0.04-7.15)	2.54 (0.13-6.91)	0.56

UM: Unilateral mastectomy; CPM: Contralateral prophylactic mastectomies; BRCA: Breast cancer; SLNB: Sentinel lymph node biopsy; ALND: Axillary lymph node dissection; DCIS: Ductal carcinoma *in situ*.

Table 2 Multivariable analysis

Predictor	OR	95%CI	*P* value
Age			0.99
< 50	1	0.52-1.90	
⩾ 50	Ref	Ref	
Family history			< 0.001
Yes	3.56	1.83-7.30	
No	Ref	Ref	
History of benign disease			0.51
Yes	1.26	0.61-2.49	
No	Ref	Ref	
Immediate reconstruction			< 0.001
Yes	10.01	5.22-19.32	
No	Ref	Ref	

our single institution are consistent with the national Canadian administrative data yet are considerably lower than those reported in the United States during a similar time period. Despite this lower rate, our data highlight similar patient factors influencing the receipt of CPM. Given these similar patient factors, it may be the health system, or practice environment, of Canadian breast cancer surgeons and patients that is contributing to the discrepancy in CPM rates between Canada and the United States.

To address the possible influence of the surgeon and their practice environment on mastectomy and CPM rates, Covelli *et al*[15] obtained qualitative data from surgeons in both Canada and the United States in 2012 and 2013. Anecdotally, surgeons in both countries endorsed an increasing CPM rate and highlighted incidental findings on pre-operative MRI, access to reconstruction and the surgeon's initial discussion of treatment options as possible factors influencing these trends. Differences between the surgeons' practices were noted mainly by the availability of immediate breast reconstruction - a product of the health system. The majority of United States surgeons had ready access to immediate breast reconstruction whereas this was less commonly available to Canadian breast surgeons[15-17]. Barriers to access immediate breast reconstruction may contribute to more women in Canada seeking out a delayed CPM with bilateral reconstruction years after their initial unilateral therapeutic mastectomy. This model of care has several advantages in our healthcare system, as it obviates any cancer surgery wait-time constraints, delay in adjuvant cancer therapies and allows patients time to seek out an institution where specialized breast reconstruction surgeons are available. In addition to immediate breast reconstruction availability, legislation and guidelines within each country were also felt to influence surgical decisions. Legislation in various United States require the discussion of all treatment options available (including reconstruction) at initial consultation whereas, in Canada, no such legislation exists. This examination at the surgeon level provides evidence that the medico-social context of the patient's treatment environment, or health system, influences the decision to undergo mastectomy with CPM. These results also lead one to consider if the stable CPM rates in Canada are actually lower overall than in the United States due to these possible practice or health system differences, or if the trends in Canada will eventually mimic those of the United States over time given the surgeons' anecdotal observations, as a similar "lag" was observed regarding IBR trends in Canada[16,17].

Within our single institution study, we were able to evaluate CPM trends among a large sample of patients undergoing mastectomy for unilateral breast carcinoma at a high-volume academic breast center. The prospectively-collected patient level data provided important information regarding factors that may influence a patient's decision to undergo a CPM. This patient level data allowed us to not only evaluate the CPM rates, but to also investigate possible influencing factors for CPM and to provide insight on the demogra-

phics of CPM patients in Canada.

While our study provides patient level data related to CPM trends, the overall low number of patients who underwent CPM (n = 52) should be considered a limitation when interpreting the results as we did not observe a trend over time in regards to CPM rates. While our overall rates are consistent with the national level data, at this point in time it is challenging to draw accurate conclusions related to the influence of the health system on Canadian CPM rates. Our results suggest that health system factors such as access to immediate breast reconstruction and patient factors such as a family history of breast cancer, both influence Canadian CPM rates. In addition, it should be noted that this study only evaluated immediate CPM and did not identify the number of delayed CPMs that occurred at our institution.

Overall, the immediate CPM rates between 2004 and 2010 at a high-volume Canadian breast cancer center did not demonstrate the same increasing trend documented in the United States. However, in keeping with the United States findings, our analysis showed that Canadian patients who had CPM were generally younger, more often had a breast cancer family history and also had immediate breast reconstruction.

COMMENTS

Background

Rates of contralateral prophylactic mastectomies (CPM) in women with early stage unilateral invasive breast cancer have been increasing, despite a lack of survival advantage. These rates have mainly been described in literature from the United States. Rates of CPM, and the factors influencing these rates, may be different in the Canadian healthcare system.

Research frontiers

CPM is often performed for younger patients and associated with immediate breast reconstruction. With increasing interest in healthcare costs, plus patient satisfaction and outcomes, CPM is an important topic of investigation.

Innovations and breakthroughs

CPM rates at a single Canadian institution do not show the same increasing trend as reported in the United States. Similar to United States literature, CPM is associated with immediate reconstruction and patients with a family history.

Applications

By identifying findings in contrast to those reported in the United States, further investigation into potential reasons (*i.e*., lack of hospital resources, lack of patient desire, *etc*.) for the difference in CPM rates should be undertaken.

Terminology

CPM is the removal of a healthy breast for breast cancer prevention or for cosmetic balancing requirements.

Peer-review

The proffered paper is thoughtful, timely and publishable.

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Extrapulmonary small cell carcinoma of lymph node: Pooled analysis of all reported cases

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Author contributions: Sehgal IS collected patient data, systematic review, drafted and revised the manuscript; Kaur H collected patient data, drafted and revised the manuscript; Dhooria S drafted and revised the manuscript; Bal A and Gupta N interpreted and reported pathological specimens of index cases, drafted and revised the manuscript; Behera D managed index cases, revised the manuscript; Singh N managed index cases, conceived the idea, systematic review, drafted and revised the manuscript.

Conflict-of-interest statement: None.

Data sharing statement: The technical appendix, statistical code, and dataset are available from the corresponding author at navneetchd@yahoo.com.

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Abstract

AIM: To study clinical outcomes and management of lymph nodes extrapulmonary small cell carcinoma (LNEPSCC).

METHODS: Herein, we perform a systematic search of published literature in the PubMed and EMBASE databases for studies describing LNEPSCC. For uniformity of reporting, LNEPSCC was staged as limited if it involved either single lymph node station or if surgery with curative intent had been undertaken. The disease was staged extensive if it involved two or more lymph node regions.

RESULTS: The systematic literature review yielded eight descriptions (n = 14) involving cervical, submandibular and inguinal lymph nodes. Eleven (64.7%) patients had limited disease (LD) and six (35.3%) had extensive disease (ED) at presentation. Chemotherapy (n = 6, 35.3%) or surgery (n = 4, 23.5%) were the most common form of treatment given to these patients. Complete response was achieved in 12 (70.6%) of the patients. Median (interquartile range) progression free survival and overall survival was 15 (7-42) mo and 22 (12.75-42) mo respectively. Of the three illustrative cases, two patients each had ED at presentation and achieved complete remission with platinum based combination chemotherapy.

CONCLUSION: LNEPSCC is a rare disease with less than 15 reported cases in world literature. Surgical resection with curative intent is feasible in those with LD while platinum based combination chemoradiation is associated with favorable outcomes in patients with ED. Prognosis of LNEPSCC is better than that of small cell lung cancer in general.

Key words: Extrapulmonary; Small cell; Carcinoma; Lymph node; Small cell lung cancer

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Core tip: Extrapulmonary small cell cancer confined to lymph nodes (LNEPSCC) is extremely rare. A systematic literature review yielded 3 index and 14 previous case descriptions. Chemotherapy or surgery was most common treatments given with complete response achieved in 70% of the cases. Surgical resection with curative intent is feasible in those with limited disease. Prognosis of LNEPSCC is better than that of small cell lung cancer in general.

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INTRODUCTION

Extrapulmonary small cell carcinoma (EPSCC) is a rare disorder that is characterized by histological evidence of small cell carcinoma (SCC) from body organs other than the lungs[1]. First described in 1930[2], much needs to be determined about natural history and clinical behaviour of EPSCC. EPSCC is known to involve gastrointestinal tract (esophagus, stomach, liver, bile ducts, intestines and pancreas)[3-5], genitourinary tract (kidney, ureter, pelvis, bladder)[6-10], head and neck region (tonsils, larynx, nasopharynx, and paranasal sinuses)[11-14], bones[15] and lymph nodes (locoregional or distant)[16-23]. Although, EPSCC has histological similarity with small cell lung cancer, it has a different biological behaviour[1,24,25]. Also, the biological behaviour varies with the site of origin and the extent of the cancer. The disease limited to the site of origin and female genital tract is associated with a better survival[26,27]. Amongst the various types of EPSCC, patients with EPSCC of the lymph nodes have an even better overall survival and is considered to be a separate subgroup amongst patients with EPSCC[28]. Due to the paucity of evidence not much is known regarding the optimal schema for staging and classification of lymph node EPSCC (LNEPSCC). Further, the best treatment modality and the diagnostic approach remains to be determined. Herein, we describe three cases of LNEPSCC involving the cervical lymph nodes. We also perform a systematic review of the literature describing LNEPSCC.

MATERIALS AND METHODS

Search strategy

We searched the PubMed and EMBASE databases for articles published until August 15, 2015 using the free text terms: ("extra pulmonary small cell cancer" or "extra pulmonary small cell carcinoma" or "extra pulmonary small cell malignancy" or "extra pulmonary small cell tumor" or "extra thoracic small cell cancer" or "extra thoracic small cell carcinoma" or "extra thoracic small cell tumor" or "extra thoracic small cell malignancy"). We reviewed the reference list of all the included articles and previous review articles.

Inclusion criteria

We included full-text, peer-reviewed, cross-sectional studies, cohort studies and case-reports that described SCC of the lymph node (LN). We excluded the following studies: (1) abstracts, comments, editorials, and reviews; (2) studies published in non-English language; (3) studies done in pediatric age group; and (4) animal studies. We also excluded the studies describing LNSCC involving the mediastinal LNs and studies or case reports that did not provide details about the site of LN station involved or follow up.

Initial review of studies

The database thus created from the electronic searches was assimilated in the reference manager package Endnote (version X7.4; Thomson Reuters) and all duplicate citations were discarded. Two authors (Sehgal IS and Singh N) screened these citations by review of the title and abstract to identify the relevant studies. Any disagreement was resolved by discussion between the authors. The full text of each of these studies was obtained and reviewed in detail.

Study selection and data abstraction

Two authors (Sehgal IS and Singh N) independently assessed all the articles for inclusion in the systematic review and extracted the data; the data was entered into a standard data extraction form. The following items were extracted: (1) publication details (authors, year of publication); (2) study design (prospective, retrospective or case-report); (3) number of patients (including the demographic profile) and inclusion criteria; (4) details such as LN region involved, size of the LN, number of LNs involved; (5) stage of the disease; (6) details of the treatment given (surgical or chemotherapy); (7) response to treatment (complication of chemotherapy progression free survival, overall survival, site of relapse, second line treatment given); and (8) final outcome. Any differences in the study selection and data extraction process between the two authors were resolved by discussion. For uniformity of

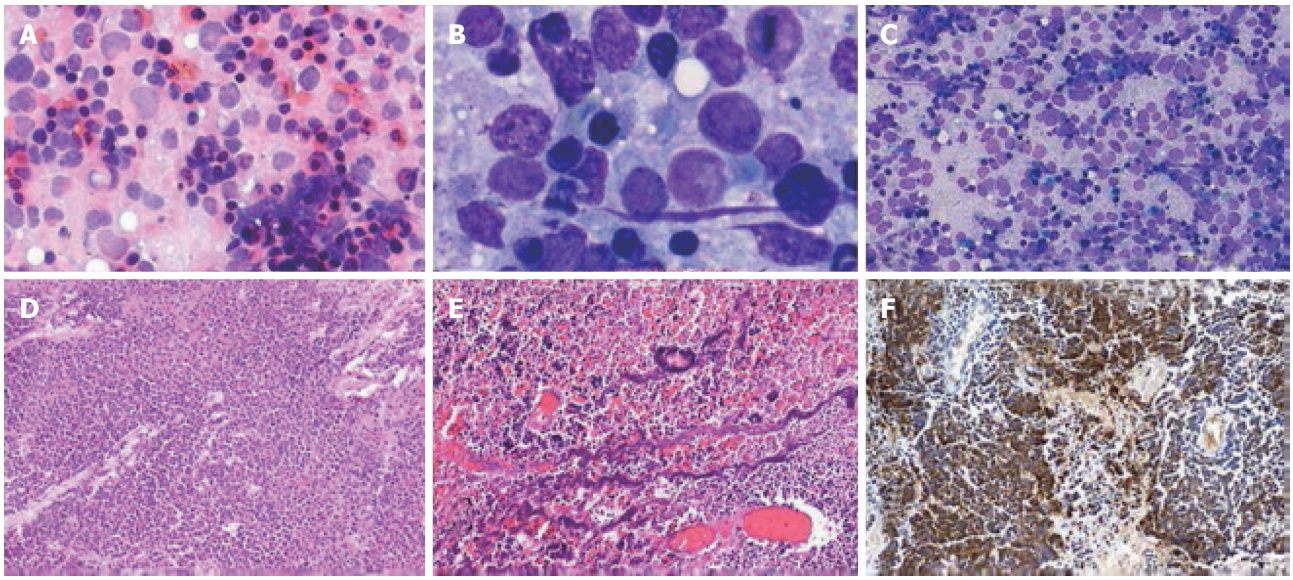


Figure 1 Histopathology and cytology of lymph node samples of illustrative case 1. A: Microphotograph showing predominantly dispersed population of tumor cells (MGG × 20 ×); B: Microphotograph showing tumor cells with very high nuclear cytoplasmic ratio, scant cytoplasm, round nuclei and fine granular chromatin (MGG × 100 ×); C: Microphotograph showing tumor cells with many apoptotic bodies (HE × 40 ×); D: Photomicrographs showing clusters of tumour cells with small hyperchromatic nuclei, scanty cytoplasm and apoptosis; E: Photomicrographs showing Azzopardi phenomena (basophilic nuclear chromatin spreading to wall of blood vessels); F: Photomicrographs showing synaptophysin immunostain showing intense cytoplasmic positivity.

reporting, LNEPSCC was staged as limited if it involved either single LN station or if surgical resection with curative intent had been undertaken. The disease was staged extensive if it involved two or more LN regions and/or other body organs.

Statistical analysis

Data from all individual patients (case reports or case series) were entered into a spreadsheet (Microsoft Excel 2016). Data was analyzed using the commercial statistical package SPSS (version 22, IBM Inc.) and is presented in a descriptive fashion as proportions, mean (95%CI) or median (IQR). χ^2 and Mann Whitney *U* tests were used to compare the categorical and numerical data, respectively.

RESULTS

Illustrative cases

Case 1: A 59-year-male, a known case of chronic obstructive pulmonary disease, presented with progressively increasing swelling in the left cervical region of 9-mo duration. He denied any history of fever or night sweats. On examination a 3 cm × 3 cm hard mass was identified in the left cervical region. FNAC and LN biopsy revealed clusters of tumour cells with hyperchromatic nuclei, nuclear molding and scanty cytoplasm (Figure 1). The pancytokeratin staining showed patchy dot like positivity and synaptophysin immunostain had an intense cytoplasmic positivity with an overall morphology suggestive of small cell carcinoma. Contrast enhanced computed tomography of the neck revealed a conglomerate mass of left cervical LN of size 2 cm × 1.2 cm abutting the left sternocleidomastoid muscle (Figure 2). Further evaluation with CECT thorax and abdomen did not show a primary anywhere and a diagnosis of limited disease (LD) LNEPSCC involving the left cervical LN was considered. Patient was unwilling for radical neck dissection and hence was started on platinum based doublet chemotherapy regimen. He was started on a combination of irinotecan (100 mg/m^2) and cisplatin (60 mg/m^2) each on D_1 of three weekly cycle for six cycles. After third cycle of chemotherapy patient developed grade Ⅱ hematological toxicity. A repeat CT of the neck revealed complete resolution of the LN mass. He achieved complete remission and was kept on follow up. Nine months after chemotherapy he again had a locoregional relapse of his disease and presented with a LN swelling of 5 cm × 4 cm. He was reinitiated on the same chemotherapy regimen (sensitive disease) to which he had responded and is currently doing well on follow up with no evidence of metastasis elsewhere in the body.

Case 2: A 65-year-old female with no previous comorbid illness presented with a progressively increasing mass over the right side of the neck. She also complained of loss of weight and appetite. She denied any history of cough, hemoptysis, hoarseness of voice, and fever. On examination there was a 6.8 cm × 4.2 cm hard mass in the right cervical and submandibular region that was fixed to the underlying structures. FNAC and a subsequent biopsy from the LN mass was suggestive of SCC morphology (Figure 3). Further evaluation with CECT thorax, paranasal sinuses, and abdomen did not reveal any primary in the lung, sinuses, or the abdomen. CECT of the neck revealed a right LN

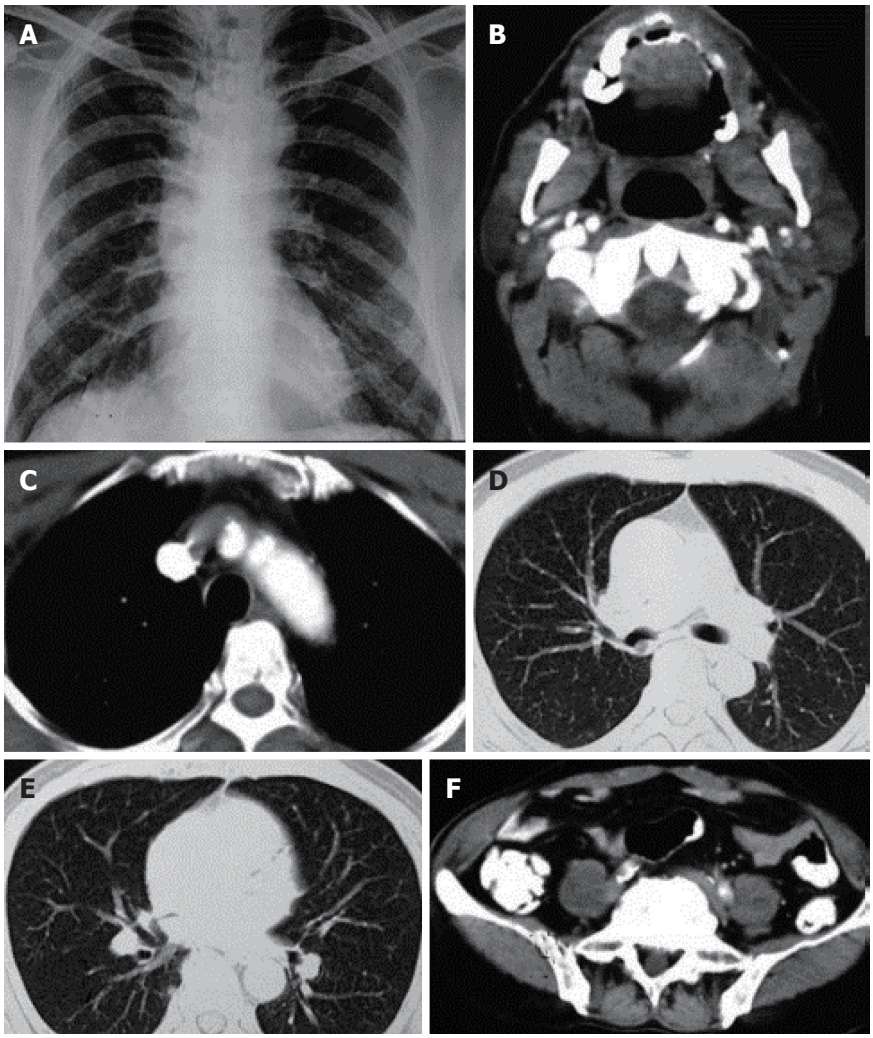


Figure 2 Thoracic imaging at baseline and after treatment of illustrative case 1. A: Chest radiograph revealing hyperinflated lung fields with no evidence of any parenchymal abnormality; B: Contrast enhanced computed tomography (CECT) of the neck revealing enlarged right sided cervical group of lymph nodes; C: Mediastinal window of CECT of the thorax with no evidence of mediastinal lymph node enlargement; D and E: Lung window of CECT thorax with no evidence of primary in the lung; F: CECT of the abdomen with no evidence of any abnormality in the abdomen.

mass extending from the submandibular region to the supra sternal region (Figure 4). Patient was diagnosed with extensive disease (ED) EPSCC of lymph node (submandibular and cervical) and was initiated on platinum based combination chemotherapy regimen [intravenous irinotecan (65 mg/m^2) and intravenous cisplatin (30 mg/m^2) each on D_1 and D_8 of three weekly cycle for six cycles]. The patient developed grade Ⅱ constipation, and hematological complication (anaemia and leukopenia) that responded to conservative treatment with hematinics and stool softening agent. A partial response was achieved with the chemotherapy. Patient was advised radiotherapy but was unwilling for any further treatment. Three months after the last cycle of chemotherapy she presented with an increase in the size of submandibular lymph node mass and was restarted on same regimen (sensitive disease) to which she had initially responded. The submandibular mass reduced in size clinically. After third cycle of chemotherapy she presented in the emergency department with generalized tonic-clonic seizures and altered sensorium. CECT head revealed bony metastasis to the skull bone invading the underlying brain parenchyma. She was given the option of cranial irradiation but she denied the same and succumbed to her illness 13 mo after her initial presentation.

Case 3: A 38-year-old previously healthy male presented with history of gradually increasing swelling in the neck region and loss of weight of nine-month duration. He also complained of fever of three-month duration. He denied any history of cough, hemoptysis, night sweats or hoarseness of voice. On physical examination he had bilateral enlarged cervical LNs (3 cm × 3 cm; 3 cm × 2 cm) that were firm to feel and were freely mobile. Fine-needle cytological examination was performed and he was diagnosed as non-Hodgkin's lymphoma and was referred to our center for further management. At presentation to our center (six months after initial presentation), he had jaundice and the LN size had increased (5 cm × 4 cm; 4 cm × 4 cm). A repeat FNAC was performed from the

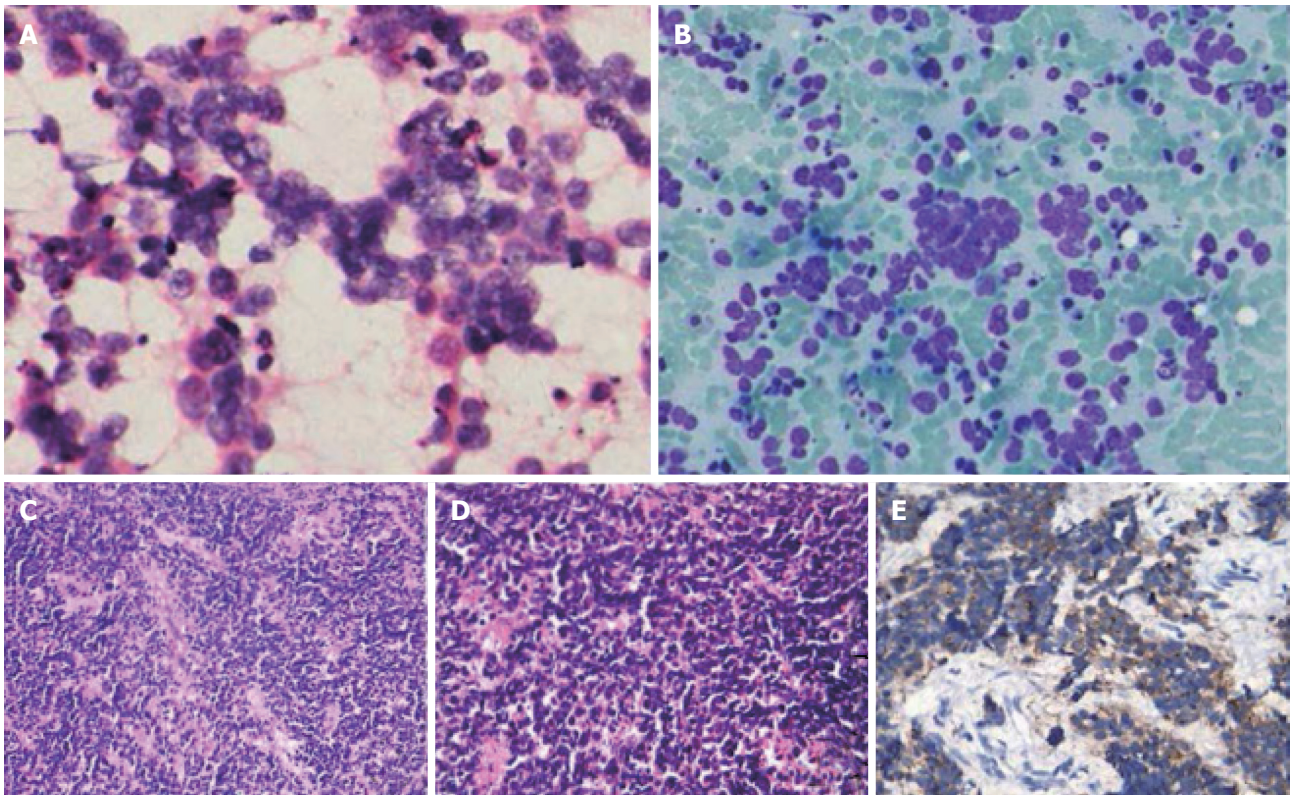


Figure 3 Histopathology and cytology of lymph node samples of illustrative case 2. A: Microphotograph showing dispersed population of tumor cells along with few loose clusters. The tumor cells with high nuclear cytoplasmic ratio and showing nuclear moulding (MGG × 20 ×); B: Microphotograph showing small tumor cells with high N:C ratio, salt and pepper type chromatin and inconspicuous nucleoli (HE × 40 ×); C: Photomicrographs showing tumour with extensive crushing artefact; D: Tumour cells having hyperchromatic nuclei, scanty cytoplasm and apoptosis; E: Synaptophysin immunostain showing cytoplasmic positivity.

right cervical LN that revealed small sized tumor cells that were positive for cytokeratin, and synaptophysin and negative for CD-3, CD-20 suggestive of SCC (Figure 5). A whole body 18F-fluorodeoxyglucose positron emission tomography (FDG-PET CT) was performed that revealed FDG avid LNs in bilateral cervical region with diffuse FDG uptake over background uptake in liver and entire skeleton. A diagnosis of ED LNEPSCC (primary in cervical LN with metastasis to liver and skeletal system) was made. The patient was initiated on palliative platinum based chemotherapy [irinotecan (65 mg/m^2) and cisplatin (30 mg/m^2) on D_1 and D_8 of 3 weekly cycle]. He developed grade Ⅳ hematological toxicity (neutropenia and anemia) and deranged liver function test (more than four times the baseline) due to which further chemotherapy was deferred. He succumbed to his illness four week after his presentation at our centre due to disease progression.

Systematic review

Our initial search of the PubMed and EMBASE databases yielded 1189 citations of which 954 were excluded after initial review. Eight studies (n = 14) were included in the current analysis (Figure 6)[16-23]. Studies that did not provide separate information for patients with LNEPSCC[29-41], and the treatment given or follow up were not included in the current review[42-50]. A total of 17 patients including the three index cases (mean ± SD, 59.5 ± 10.8 years; 81.8% males) with LNEPSCC were included in the current analysis (Table 1). There was no difference in the age and gender distribution based on the stage of the disease at presentation (61 ± 10.3 years in LD *vs* 57.6 ± 12.3 years in ED; P = 0.931). Cervical group (9, 52.9%) of LN region followed by submandibular LNs (4, 23.5%) was the most common site of primary disease. Two patients had inguinal LN enlargement while in two patients both submandibular and cervical LNs were involved. Eleven (64.7%) patients had LD as the disease involved only single LN region whereas 6 (35.3%) had ED at presentation. One patient had evidence of involvement of liver and skeletal system, and in two patients' central nervous system was involved at presentation, while three patients were labelled ED as they involved two LN regions (bilateral cervical LN; submandibular and cervical LNs). To rule out primary at other sites, chest radiograph, flexible bronchoscopy, CECT thorax and abdomen was performed in all patients. Whole body PET-CT was done in only one patient (index case 2). Chemotherapy (n = 6, 35.3%) or surgery (n = 4, 23.5%) were the most common form of treatment given to the patients. A combination of chemotherapy with radiation (n = 2, 11.8%) and surgery with radiation (n = 2, 11.8%) were the other forms of treatment. Five patients received radiation (one only radiotherapy, two each combined radiotherapy and chemotherapy, and combined

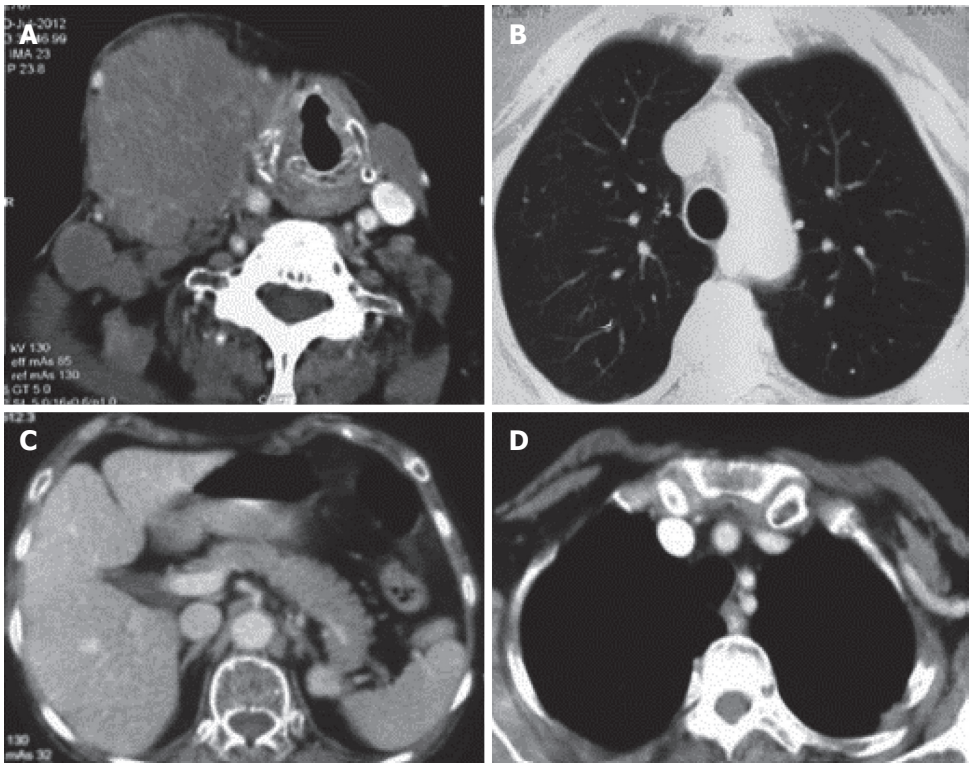


Figure 4 Thoracic imaging at baseline and after treatment of case 2. A: Contrast enhanced computed tomography of the neck revealing a conglomerate lymph node mass of size 7 cm × 4 cm involving the right submandibular region. The mass is pushing the larynx towards the left side; B: Contrast enhanced computed tomography of the thorax (lung window) with no evidence of primary in the lung; C: Contrast enhanced computed tomography of the abdomen with normal abdominal organs and no evidence of any primary in the abdomen; D: Mediastinal window of CECT thorax revealing no enlarged lymph node stations in the mediastinum. CECT: Contrast enhanced computed tomography.

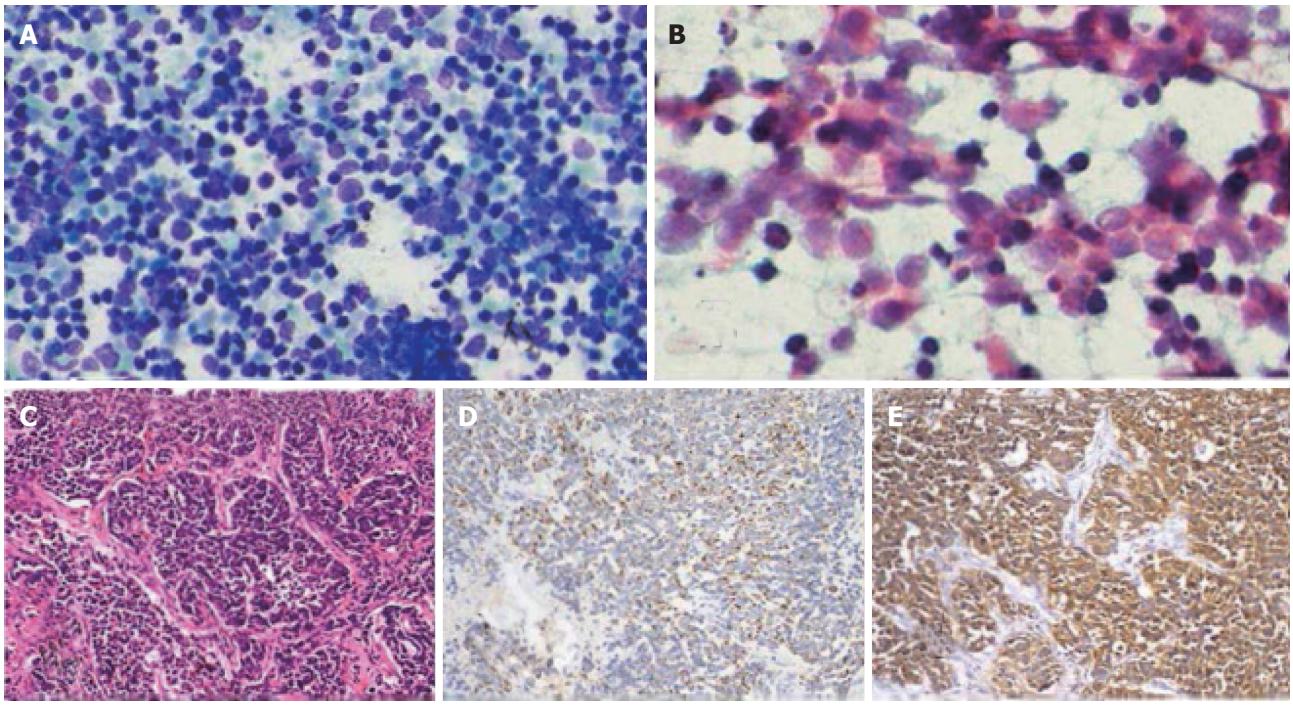


Figure 5 Histopathology and cytology of lymph node samples of case 3. A: Microphotograph showing dispersed population of small sized tumor cells with high nuclear cytoplasmic ratio along with many degenerated cells (MGG × 20 ×); B: Microphotograph showing nuclear threading and crushing along with tumor cells with round nuclei, high N:C ratio, salt and pepper type chromatin and inconspicuous nucleoli (Pap × 40 ×); C: Photomicrographs showing clusters of tumour cells with hyperchromatic nuclei, nuclear molding and scanty cytoplasm; D: Pancytokeratin staining showing patchy dot like positivity; E: Synaptophysin immunostain showing intense cytoplasmic positivity.

Table 1 Details of studies included in the review

Ref.	No. of patients	Gender	Age	Stage	Lymph node area involved	Investigations performed to rule out primary elsewhere	Treatment regimen used	No. of cycles received	Response	Complication of treatment	PFS, in months	Overall survival, in months	Site of relapse	Final outcome
Levenson *et al*[23]	1	Male	49	LD	Cervical	CXR, FB, radionuclide scan of liver, bone, spleen and brain	CMC-VAP + local RT	NA	CR	NA	15+	15+	None	Alive with disease
Kasimis *et al*[22]	2	Male	60	ED	Right submandibular	CXR, panendoscopy of larynx, pharynx, bronchial tree and esophagus	Surgery (radical neck dissection) + RT (5860 rads); cyclophosphamide and doxorubicin + cranial irradiation	NA	SD	None	24	36	Left cervical LN, left scapular region, left parotid gland, left submandibular LN, right tonsil, right testis	Died of disease
		Male	70	ED	Right submandibular and posterior cervical	CXR, sputum cytology, bone and liver scan, panendoscopy of larynx, pharynx, bronchial tree and esophagus	None (patient refused treatment)	-	SD	None	18+	40+	Locoregional enlargement of submandibular LN at 18 mo	Alive with disease
Remick *et al*[21]	1	Male	52	LD	Left cervical LN	CXR, CECT thorax and abdomen, bone scan	RT (46 Gray)	NA	CR	None	NA	84+	None	Alive with disease
Hainsworth *et al*[20]	2	Female	77	LD	Inguinal	CXR, CECT thorax and abdomen	Surgery	-	CR	None	12+	12+	None	Alive with disease
		Male	61	LD	Cervical	CXR, CECT thorax and abdomen, bone scan	CAV + RT	NA	CR	NA	100+	100+	None	Alive with disease
Van Der Gaast *et al*[19]	2	NA	55 (26-72)	LD	Cervical LN	CXR, CECT thorax and abdomen, bone scan	CDE (*iv* cyclophosphamide 1 g/sq.m on day 1, *iv* doxorubicin 45 mg/sq.m on day 1 and *iv* etoposide 100 mg/sq.m on days 1, 3 and 5)	Five	CR	Hematological (grade 3-4 leukopenia, neutropenia, thrombocytopenia), nausea and vomiting, alopecia	NA	22+	Locoregional	Alive with disease
		NA	55 (26-72)	LD	Cervical LN	CXR, CECT thorax and abdomen, bone scan	CDE (*iv* cyclophosphamide 1 g/sq.m on day 1, *iv* doxorubicin 45 mg/sq.m on day 1 and *iv* etoposide 100 mg/sq.m on days 1, 3 and 5) + RT (6000 cGy)	Five	CR	Hematological (grade 3-4 leukopenia, neutropenia, thrombocytopenia), nausea and vomiting, alopecia	12+	12+	None	Alive with disease
Galanis *et al*[18]	4	NA	NA	LD	Submandibular LN	CXR, CECT thorax and abdomen, bone scan	Surgery	Nil	CR	None	42+	42+	None	Alive at with disease
		NA	NA	LD	Submandibular LN	CXR, CECT thorax and abdomen, bone scan	Surgery	Nil	CR	None	42+	42+	None	Alive with disease

		NA	NA	LD	Inguinal	CXR, CECT thorax and abdomen, bone scan	Surgery	Nil	CR	None	42+	42+	None	Alive with disease
		NA	NA	ED	Submandibular	CXR, CECT thorax and abdomen, bone scan	NA	NA	NA	None	NA	42+	None	Alive with disease
Orhan *et al*[17]	1	Male	55	ED	Right cervical LN and multiple cranial metastasis	CXR, CT (thorax and abdomen), FB	Etoposide (100 mg/sq.m on days 1, 3, 5) and cisplatin (80 mg/sq.m on day 1)	Six	CR	None	7	7+	None	Alive with disease
Ochsenreither *et al*[16]	1	Male	68	LD	Cervical LN	CECT thorax and FB	Surgery and RT	-	CR	None	5	22	NA	Died of disease
Current report	3	Male	59	LD	Unilateral LN (left cervical LN)	CXR, CECT, neck, thorax and abdomen, FB	Irinotecan (100 mg/sq.m) and cisplatin (60 mg/sq.m) each on D1 of 3 weekly cycle	6 (3 weekly)	CR	None	9	16+	Locoregional	Alive with disease
		Male	38	ED	Bilateral cervical LNs	CXR, CECT thorax and abdomen and whole body PET CT	Irinotecan (65 mg/sq.m) and cisplatin (30 mg/sq.m) each on D1 and D8 of 3 weekly cycle	1 (C1D1)	NR	Grade IV hematological (pancytopenia), deranged liver enzymes	Not assessable	1	Systemic	Died of disease
		Female	65	ED	Right sided submandibular and cervical LNs	CXR, CECT neck, thorax, abdomen and; CT head	Irinotecan (65 mg/sq.m) and cisplatin (30 mg/sq.m) each on D1 and D8 of 3 weekly cycle	4 (twice weekly; C1D1 and C1D8)	PR	Nausea and vomiting, constipation (grade II) and grade II hematological (anaemia and leucopenia)	7	13	Increase in cervical LN size and skull bone metastasis with invading the bone	Died of disease

CAV: Cyclophosphamide, doxorubicin and vincristine; CDE: Cyclophosphamide, doxorubicin, etoposide; CMC-VAP: Cytoxan, methotrexate, CCNU [1-(2-chlorethyl)-3-cyclohexyl-1-nitrosourea] alternating with vincristine, adriamycin, and procarbazine; CECT: Contrast enhanced computed tomography; CR: Complete response; CXR: Chest radiograph; FB: Flexible bronchoscopy; ED: Extensive disease; LD: Limited disease; LN: Lymph node; NA: Not available; PR: Partial response; RT: Radiotherapy; SD: Stable disease.

surgery and radiotherapy). All patients who had ED at presentation received platinum based chemotherapy except one who refused any form of treatment and was kept on follow up. A complete response (CR) was achieved in 12 (70.6%) of the patients whereas one had partial response, and in two patients the disease remained stable. Eleven patients with LD achieved CR whereas only one with ED had CR (P = 0.008). Median (IQR) PFS and overall survival (OS) was 15 (7-42) mo and 22 (12.25-42) mo respectively. In patients with LD the median (IQR) overall and PFS was not significantly different from those with ED LNEPSCC at presentation [22 (15-42) and 28.5 (11.3-52.5) mo *vs* 13 (4-38) and 7 (3.5-21) mo respectively; P value 0.145 and 0.129]. Overall 4 (23.5%) patient died while 13 (76.5%) subjects were labelled as alive with disease at the time of reporting of their individual publications. One patient with LD and three patients in ED died (P = 0.057). Adverse events due to chemotherapy were reported in 4 (23.5%) patients and included grade 2 hematological complications, alopecia, constipation, nausea and vomiting.

DISCUSSION

The results of the systematic review and the illustrative cases highlight that LNEPSCC is a distinct clinical entity. Patients with disease limited to only one LN region responded well to surgical management consisting of radical neck dissection or regional LN dissection. Patients with ED at presentation responded favorably to

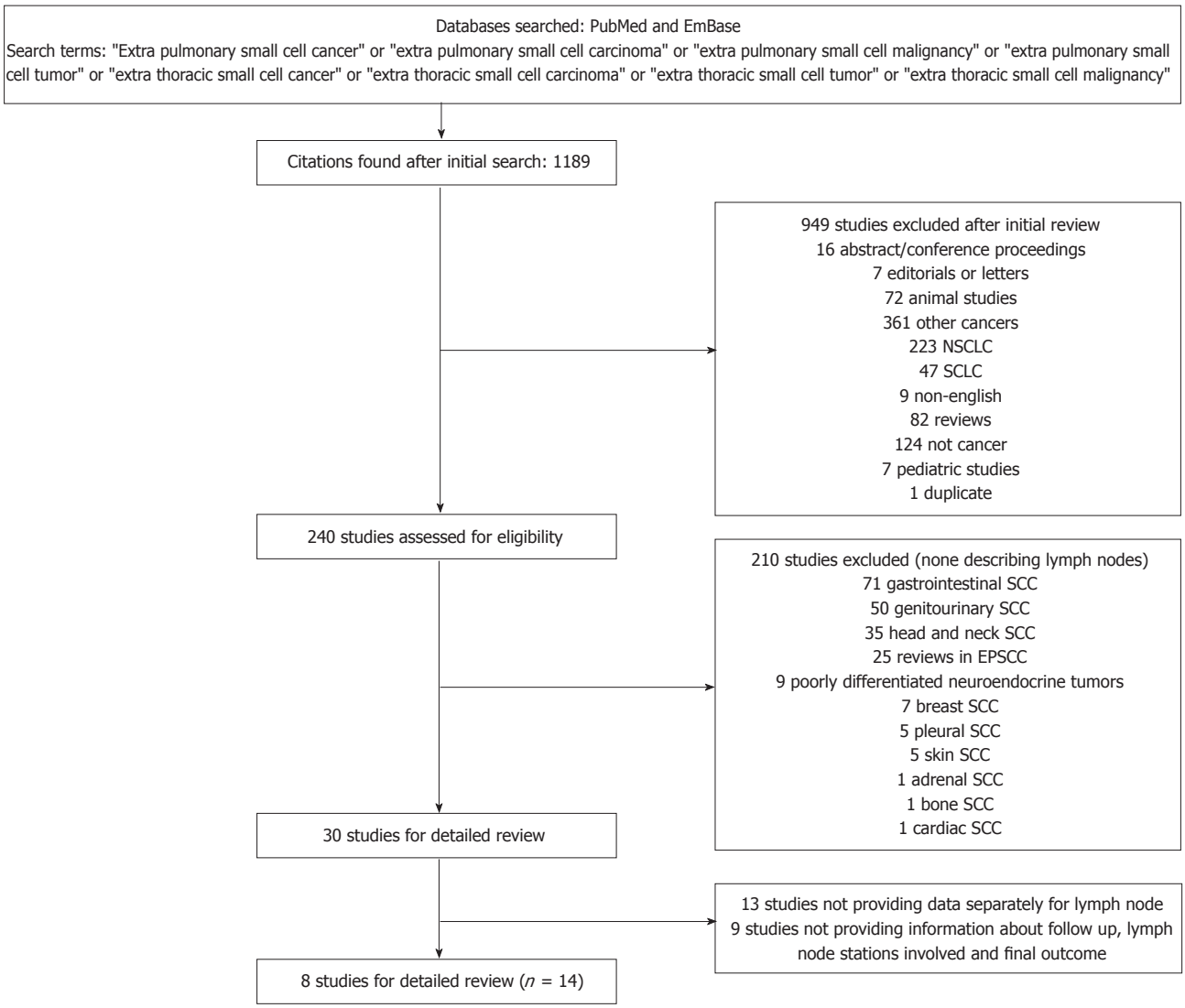


Figure 6 Study selection process for systematic review. NSCLC: Non small cell lung cancer; SCLC: Small cell lung cancer; SCC: Small cell cancer; EPSCC: Extrapulmonary small cell cancer.

chemotherapy with or without radiation.

Small cell carcinoma of the lung (SCLC) accounts for 18%-20% of all lung cancers and is invariably associated with a dismal prognosis[51,52]. Median overall survival is 9-10 mo despite treatment in extensive SCLC and 18-24 mo in case of LD SCLC[53,54]. Role of surgical resection in SCLC is limited with treatment being primarily combined chemoradiation[54]. However, SCC has also been described in various other parts of the body including the gastro-intestinal tract, male and female genital tract, musculoskeletal system and others[5,15,55-57]. Extrapulmonary SCLC, especially in the lymph nodes, is an extremely rare entity. Infact, in our more than two decade of experience with lung cancer, we have only seen three patients. However, this is an important condition as if identified then the treatment can result in good response and clinical outcomes, in contrast to SCLC. This is likely to benefit in the patient care and management.

The exact pathogenesis of LNEPSCC is controversial, although several hypotheses exist[28,58,59]. It is believed to arise from multipotent stem cells in the LN and hence a slow growing nature of the LNEPSCC. It can also be due to a primary elsewhere in the body with secondary metastasis to the LNs and spontaneous regression of the primary tumor. However, the fact that patients with LD LNEPSCC had a prolonged survival (median survival 22 mo) makes this theory unlikely. Further, LNEPSCC is cytogenetically different from SCLC as the loss of chromosome 3p, 10q and deletion of chromosome 13 are not seen in EPSCC[60]. It may also be plausible that EPSCC is derived from neuroendocrine amine precursors uptake and decarboxylation cells as neurosecretory granules are frequently seen in the tumor[60].

Apart from pathogenesis, the schema for the staging of LNEPSCC is also uncertain. In the systematic review we could only identify 14 patients with LNEPSCC suggesting it to be a rare disease. An attempt was made to stage LNEPSCC in the current analysis on the basis of ability to perform curative surgery and the number of LN regions involved, and involvement of other organs. If TMN staging process was to be followed, then the stage

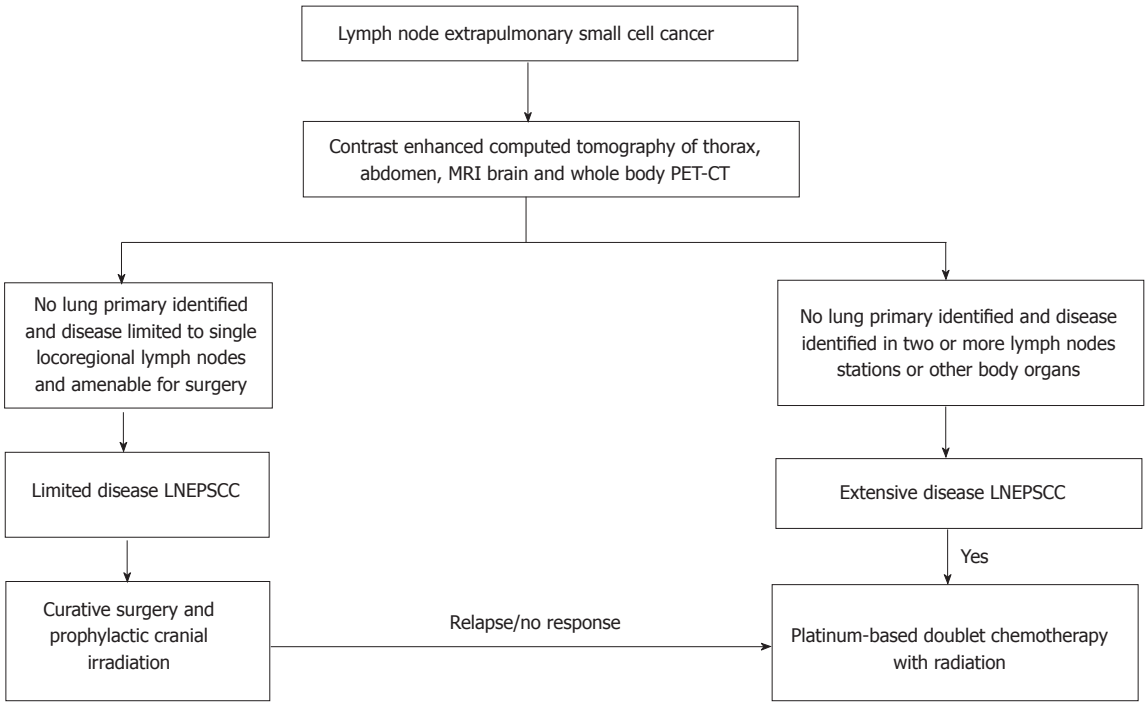


Figure 7 Algorithm for the diagnostic work-up and treatment of lymph node extrapulmonary small cell carcinoma. LNEPSCC: Lymph nodes extrapulmonary small cell carcinoma; PET: Positron emission tomography; CT: Computed tomography; MRI: Magnetic resonance imaging.

of all the LNPSCC would have been stage Ⅳ disease or ED that is associated with a poor prognosis and survival outcome[54]. However, this was in contrast to our index cases and the results of the systematic review, where patients with LD LNEPSCC had a longer survival. The PFS and OS in both the LD LNEPSCC and in ED LNEPSCC was higher than that of the SCLC. Thus the staging schema adopted in the current analysis seems appropriate. This has important clinical implications as contrary to LD SCLC where surgery has no role, surgery has a definite role in patients with LD LNEPSCC[18,20,22].

SCC limited to LNs usually develops slowly as highlighted by a prolonged stable disease despite no treatment[22]. However, at an unpredictable time there may be rapid dissemination of the disease and the tumor enters its aggressive phase with an outcome similar to progressive SCLC[20,22]. This was highlighted in one of the index cases that had a fulminant course after diagnosis. Hence, patients presenting with LD LNEPSCC should be treated early with curative surgery (regional LN dissection)[18]. Patients with ED LNEPSCC should also be treated aggressively and early in contrast to SCLC where despite treatment the results are dismal[54]. Treatment with combination chemotherapy, concurrent chemoradiation resulted disease stabilization in five of the six patients with ED LNEPSCC. However, due to the paucity of data and various chemotherapy regimen used with or without radiotherapy, the best treatment option for EDLNSCC remains uncertain. Thus, patients with ED LNEPSCC may be treated similar to patients with ED SCLC until further good quality evidence is available.

Finally, the role of FDG-PET CT (18F-fluorodeoxyglucose positron emission tomography) in evaluating the cases of LNEPSCC needs to be discussed. The studies included in the current systematic review do not clarify this issue. The authors in the various studies have used a combination of chest radiograph, CECT thorax and abdomen and bone scan along with blind mucosal biopsies to investigate the patients with LNEPSCC. This seems reasonable as most patients who were still alive in the studies did not demonstrate primary in the lung or at any other site. However, the advent of PET CT and its inclusion in the diagnostic algorithm will further clarify and enable a better staging process[61,62]. This was highlighted in one of the index cases where performance of PET CT upstaged the tumor to ED with FDG uptake in liver and the entire skeletal system. Hence, future studies should include PET-CT in the diagnostic evaluation of patients with presumed LNEPSCC.

Our systematic review has several limitations. Most of the studies included were either case reports or retrospective data and included only a small number of patients. Further, most studies did not have a complete follow-up data and hence the interpretation of overall survival may not be correct with most patients still alive with disease at the time of publication of studies. However, in rare diseases it is not possible to conduct randomized trials and generate good quality evidence. Also, most reports did not utilize PET-CT in the diagnostic evaluation of the cases. In current era, the authors believe that PET-CT may enable a better understanding and staging of disease and may also pick up small primaries in the lung and should be incorporated in the diagnostic algorithm (Figure 7). Although, Cisplatin and Etoposide is now the current standard chemotherapy regimen, at our center a

combination of irinotecan and cisplatin is preferred and was used in the three illustrative cases[52]. This is because this combination is cost effective and is better tolerated by our patients as the patients. The use of this combination might have resulted in unfavorable outcomes in the illustrative cases.

In conclusion, LNEPSCC is a rare disease and seems to be distinct from SCLC and other EPSCCs. LNEPSCC that remains confined to single group of LN region should be considered for surgical resection with curative intent combined with chemotherapy. Those patients who present late or with an ED should be offered treatment with a combination of concurrent chemoradiation. Prognosis of LNEPSCC seems to be better than that of SCLC in general emphasizing the need for recognition of this unusual entity.

COMMENTS

Background

Lymph node extrapulmonary small cell carcinoma (LNEPSCC) is a rare disorder that is characterized by histological evidence of small cell carcinoma in lymph nodes without a primary in the lungs.

Research frontiers

Contrary to small cell carcinoma of the lungs, LNEPSCC is associated with better overall survival. However, due to the rarity of this disease much needs to be ascertained regarding the staging schema and the appropriate management of patients affected with this entity.

Innovations and breakthroughs

The current study provides a pooled analysis of all the reported cases in literature and provides a schema for management of subjects with LNEPSCC. The study highlights that subjects who are affected with LNEPSCC have a better overall survival than those with extensive disease (ED) small cell lung carcinoma. For staging purpose, a primary in the lungs should be ruled out with the help of contrast enhanced computed tomography of thorax and a whole body 18F-fluorodeoxyglucose positron emission tomography/computed tomography (FDG-PET/CT) to detect the overall extent of the disease. LNEPSCC should be staged as limited if it involves either a single LN station or if surgical resection with curative intent can be undertaken. The disease is staged extensive if it involves two or more LN regions and/or other body organs. Subjects who have disease limited to only one LN region should be offered surgical management consisting of radical neck dissection or regional LN dissection with a curative intent. Subjects with ED at presentation respond favorably to chemotherapy with or without radiation.

Applications

This review suggests that LNEPSCC is a rare disease and seems to be distinct from small cell lung cancer (SCLC) and other EPSCCs. The diagnostic algorithm for future studies should include whole body PET-CT. LNEPSCC that remains confined to single group of LN region should be considered for surgical resection with curative intent combined with chemotherapy. Those patients who present late or with an ED should be offered treatment with a combination of concurrent chemoradiation.

Terminology

Prognosis of LNEPSCC seems to be better than that of SCLC in general emphasizing the need for recognition of this unusual entity.

Peer-review

This review is well-written.

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Awareness that early cancer lump is painless could decrease breast cancer mortality in developing countries

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Abstract

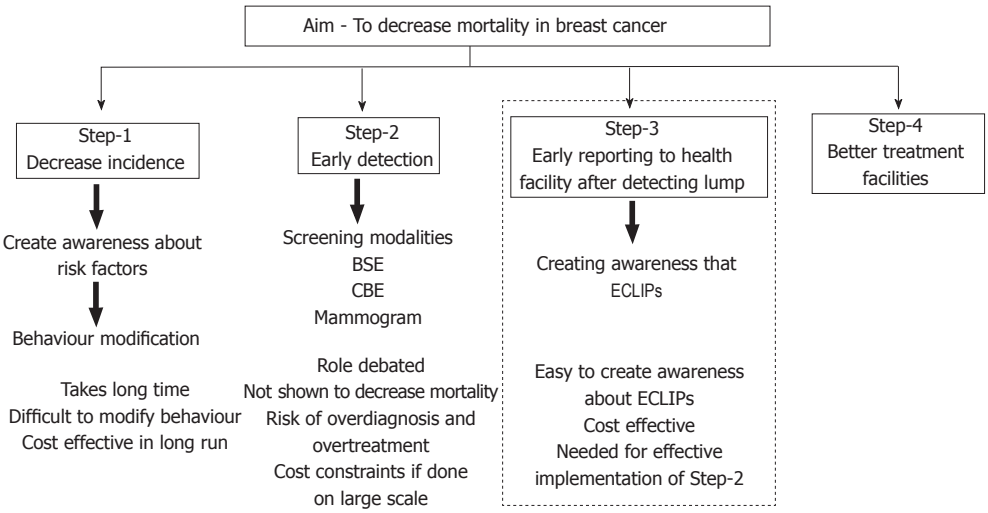
There are several factors which contribute to patients' reporting late to healthcare facility even after detecting the breast lump (patient delay). Amongst these, one of the important factors in low- and middle-income countries is lack of awareness that early cancer lump is painless (ECLIPs). Pain is often taken as a danger sign and absence of pain is often not taken seriously. The studies have shown that up to 98% of women in low-income countries are unaware that a painless lump could be a warning sign of early breast cancer. This fact is significant because this could be one of the prime reasons for the women having discovered a painless lump in the breast, accidentally or by breast self-examination, presume it to be harmless and don't report early to health care facility. Therefore, creating awareness about ECLIPs could be an effective strategy to reduce mortality due to breast cancer in low- and middle-income countries. Moreover, unlike modifying risk factors which requires long term behavior modification, creating awareness about ECLIPs is easy and cost effective.

Key words: Breast; Cancer; Screening; Lump; Pain; Painless

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Core tip: Breast cancer mortality is quite high in low- and middle-income countries (LMICs) despite low incidence levels in these countries. One of the major reasons for this is late presentation of patients to clinicians in LMICs. The late presentation can be due to either inability to detect lump early or late realization that the detected lump can be cancerous. For the latter, lack of awareness that early cancer lump is painless (ECLIPs) is one major reason. Moreover creating awareness about ECLIPs is easy and cost effective. Therefore ECLIPs awareness should be part of breast cancer national programs in LMICs.

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Step-3	More relevant in low-income countries (high levels of illiteracy)
Step-1	More relevant in developed countries (high incidence)
Step-2 and 4	Important in developed and low-income countries

Figure 1 Role of awareness about early cancer lump is painless in overall management of breast cancer. BSE: Breast self-examination; ECLIPs: Early breast cancer lump is painless; CBE: Clinical breast examination.

TO THE EDITOR

I read with great interest the article titled "Lay perceptions of breast cancer in Western Kenya". The article highlights the dismal level of awareness about breast cancer causes and symptoms in low- and middle-income countries (LMICs)[1]. Primary as well as secondary prevention can play an important part to decrease mortality due to breast cancer. In this context, an important aspect of secondary prevention [awareness about the fact that early breast cancer lump is painless (ECLIPs)] assumes quite importance in LMICs where the level of education is quite low.

The incidence of breast cancer in low-income countries is three to four times lower as compared to the developed countries (25.8 *vs* 95 per 100000)[2]. But the pressing issue in low-income countries is high mortality despite low incidence (12.7 *vs* 17.1 per 100000)[2]. The main reasons for this are delay in detecting a lump, delay in availing treatment even after detecting a breast lump (patient delay), and inadequate specialized healthcare facilities (hospital delay and inadequate treatment).

The strategies to tackle this are modifying risk factors (to reduce incidence), regular screening (to detect early), decrease patient delay, and provide better treatment facilities (Figure 1).

Modifying risk factors require behavior modification which is difficult and take much longer time. Regular screening by mammography, and clinical breast examination and provision of better healthcare facilities is hindered by logistical constraints[3]. Breast self-examination (BSE), though cheap, has sensitivity of only 12%-14%, has no positive effect compared to those who are not performing it and it also leads to false positives[4].

Amongst the patient delay factors, one of the important factors is lack of awareness about ECLIPs[5]. In low-income countries, due to high prevalence of illiteracy, pain is usually taken as a danger sign and presence of a painless lump is often not taken seriously and is ignored[5]. In these countries, up to 75% of women perceived breast pain as a symptom of breast cancer[6] and up to 98% of women were unaware that a painless lump could be the first warning sign of a breast cancer[7]. This fact is significant because this could be one of the prime reasons for the women having discovered a painless lump in the breast, accidentally or by any other screening method, presume it to be harmless and don't report early to health care facility. Therefore, creating awareness about ECLIPs could be an effective strategy to reduce mortality due to breast cancer in LMICs. Moreover, creating awareness about ECLIPs is easier. It is a single small fact which can easily reach large populations if highlighted properly at various platforms including media. This step (by decreasing patient delay) would also increase the efficacy of screening. Therefore ECLIPs awareness is a logical, easy, and cost effective strategy to decrease breast cancer mortality in LMICs and should be a part of national cancer management strategies in these countries.

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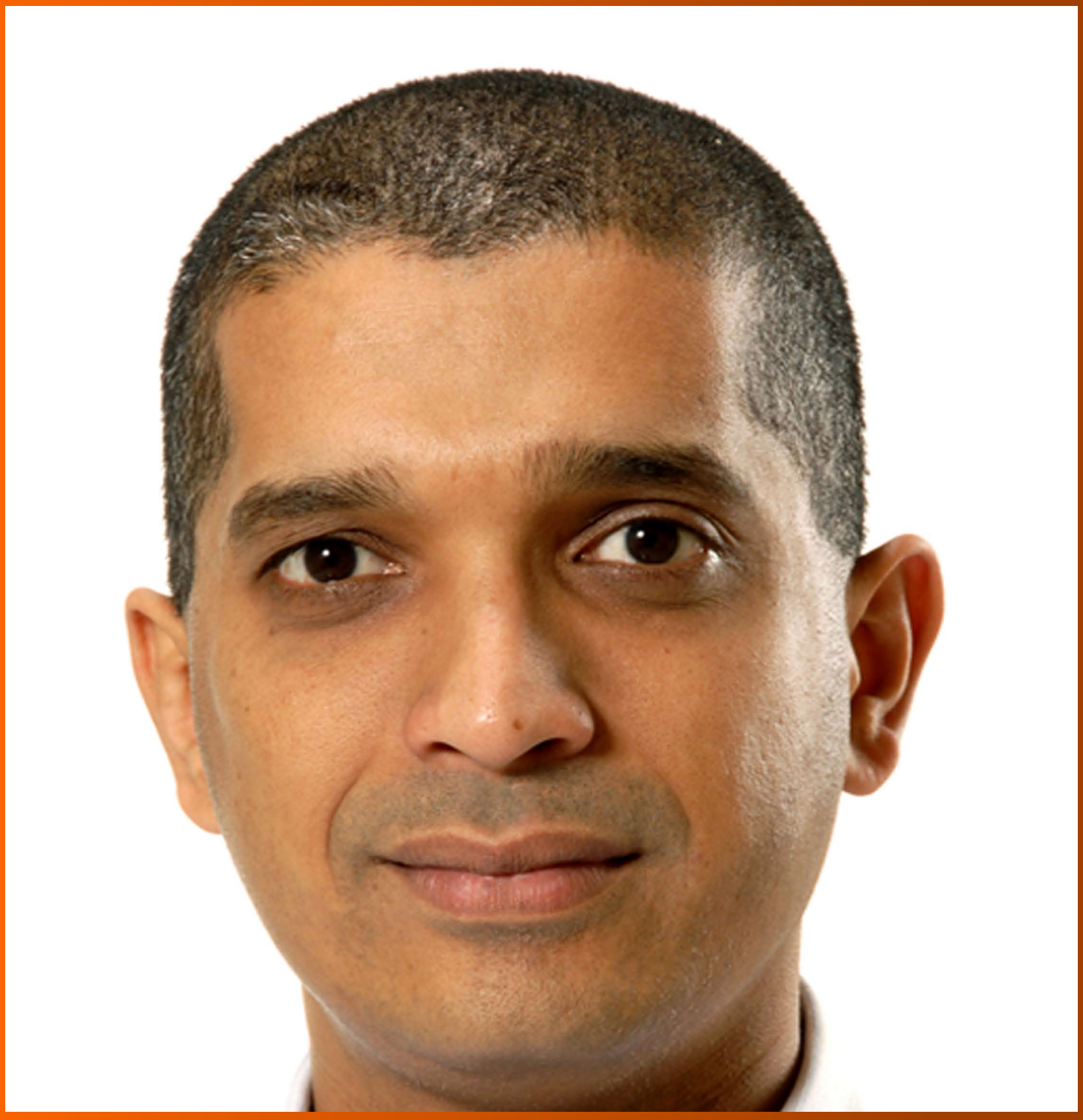


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ORIGINAL ARTICLE

Retrospective Study

Risk of second primary malignancies in a population-based study of adult patients with essential thrombocythemia

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Abstract

AIM: To determine the risk of second primary malignancy (SPM) and survival of patients with essential thrombocythemia (ET).

METHODS: We identified all patients with ET diagnosed during 2001 to 2011 from the Surveillance, Epidemiology and End Results (SEER) 18 database. Actuarial and relative survival methods were used to calculate the survival statistics. We utilized the SEER 13 database to calculate SPM. We used multiple primary standardized incidence ratio (SIR) session of the SEER*Stat software (version 8.1.5) to calculate SIR and excess risk of SPM for ET patients.

RESULTS: Age standardized five-year cause-specific survival was greater for patients < 50 years *vs* those ⩾ 50 years (99.4% *vs* 93.5%, $P < 0.01$). Five-year cause-

specific survival was lower for men *vs* women (70.2% *vs* 79.7%). A total of 201 patients (2.46%) developed SPM at a median age of 75 years. SPMs occurred at an observed/expected (O/E) ratio of 1.26 (95%CI: 1.09-1.45, P = 0.002) with an absolute excess risk (AER) of 37.44 per 10000 population. A significantly higher risk was noted for leukemia (O/E 3.78; 95%CI: 2.20-6.05, P < 0.001; AER 11.28/10000).

CONCLUSION: ET patients have an excellent cause-specific five-year survival but are at an increased risk of SPM, particularly leukemia, which may contribute to excess deaths.

Key words: Essential thrombocythemia; Second primary malignancy; Survival

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Core tip: Second primary malignancy (SPM) contributes to worse survival in essential thrombocythemia (ET). We utilized the Surveillance, Epidemiology and End Results database to analyze the risk of SPM in ET patients diagnosed during 2001-2011. Two hundred and one patients (2.46%) developed SPM at a median age of 75 years. SPMs occurred at an observed/expected (O/E) ratio of 1.26 (95%CI: 1.09-1.45, P = 0.002) with an absolute excess risk of 37.44 per 10000 population. A significantly higher risk was noted for leukemia (O/E 3.78; 95%CI: 2.20-6.05, P < 0.001). An increased risk of SPM, particularly leukemia, may contribute to excess deaths in ET.

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INTRODUCTION

Essential thrombocythemia (ET) is a subtype of myeloproliferative disorder, which lacks BCR-ABL fusion transcript[1,2]. In United States, the estimated annual incidence is approximately 2.5 cases per 100000 population, whereas the prevalence is estimated to be approximately 24 cases per 100000 population[3,4]. Patients with ET generally have an excellent survival. A large study has shown a median survival of 20 years for the entire cohort of patients with ET and a median survival of 33 years for those < 60 years. Nonetheless, the life expectancy of patients with ET is inferior to sex- and age-matched population[5]. Another study has also demonstrated a median survival of approximately 19 years but the survival was worse than the general population, particularly after the first decade[6]. Risk factors associated with inferior survival include advanced age at diagnosis, leukocytosis and thrombosis; the mutational status of janus kinase 2 (*JAK2*) and calreticulin (*CALR*) genes may not definitely influence survival[7-9]. The thrombohemorrhagic events and second primary malignancy (SPM) are among the frequent causes of death in ET[10-12]. ET may progress to other myeloid malignancies such as myelofibrosis, myelodysplastic syndrome (MDS) and acute myeloid leukemia (AML)[2,12]. The probability of transformation to leukemia is 1%-5% during the first decade but increases significantly in the subsequent decades[2,6]. Additionally, patients with ET may also develop lymphoid malignancies such as non-Hodgkin's lymphoma (NHL) and solid organ malignancies[13,14]. The treatment with cytotoxic chemotherapy decreases the incidence of thrombohemorrhagic events but may increase the risk of hematological SPM[2]. The risk of SPM may be high in patients exposed to alkylating agents and radioactive phosphorus, more so when used in high doses[2,12,15,16]. A study from Italy has demonstrated that the use of alkylating agents such as melphalan may be associated with a higher risk of developing second hematologic malignancies but not non-hematological malignancies[17]. Prior studies assessing the risk of SPM in ET utilized data mainly from outside the Unites States. Although the risk of MDS/AML in ET has been studied, the risk of other malignancies is not well determined in United States population-based studies. This United States population-based database analysis aimed to determine the probability of SPM and survival in patients with ET[18]. The influence of age at diagnosis and disease duration on the probability of SPM was also analyzed.

MATERIALS AND METHODS

We utilized Surveillance, Epidemiology and End Results (SEER) database to extract data on all ET patients diagnosed and treated between 1973 and 2011. The SEER is a program of the National Cancer Institute (NCI) that provides cancer data from population-based cancer registries and covers 28% of the total United States population. The database covers data from 25% of all White population, 26% of African Americans, 38% of Hispanics, 50% of Asians, 44% of American Indians and Alaska Natives and 67% of Hawaiian/Pacific Islanders[19]. Patients with ET were identified using International Classification of Diseases for oncology, 3rd edition (ICD)-O-3 code 9962/3 from the SEER 18 registry. Prior studies have used a similar approach in identifying patients with ET from the SEER database[20,21]. Cases with unknown age or survival time and those diagnosed at autopsy and by death certificate only were excluded. SEER started reporting data on chronic myeloproliferative disorders including ET from 2001; as a result our analysis was restricted to cases diagnosed after 2001 only[21]. Actuarial and relative survival methods were used to calculate the survival statistics. We computed

Table 1 Characterstics of patients with essential thrombocythemia

Characteristics	*n* (%)
Age	
< 50 yr	1600 (19.7)
50-70 yr	2958 (36.5)
> 70 yr	3558 (43.8)
Sex	
Male	3195 (39.4)
Female	4921 (60.6)
Race	
White	6332 (78)
African American	971 (12)
Other[1]	576 (7.1)
Unknown	237 (2.9)
Marital status	
Single/unmarried	1152 (14.2)
Married	3815 (47)
Divorced/separated/widowed	2125 (26.2)
Unknown	1024 (12.6)
Year at diagnosis	
2001-2005	3035 (37.3)
2006-2010	4251 (52.4)
2011-present	840 (10.3)

[1]Other race indicates Asian, Hispanic, Native North American and other racial groups.

age-standardized cause-specific survival using the cause-specific death classification available from the SEER registry. Cause-specific survival is a net survival measure that computes survival from cancer related causes of death in the absence of other causes of death. A prior study from SEER registry has shown that cause-specific survival rates may be a reliable alternative to relative survival methods when suitable life tables are not available[22]. Relative survival rate, which measures net cancer survival controlling for differences in mortality for causes other than cancer, was defined as the ratio of observed survival of a group of cancer patients to the expected survival of a comparable cohort of cancer free patients. Expected survival was computed using the Ederer Ⅱ method. All calculations were age standardized to the International Cancer Survival Standard for age 15+ years[23].

For the calculation of SPM, we utilized the SEER 13 registry using patients with ET diagnosed between 2001-2010. Using Warren and Gates criteria as modified by NCI[24], SPM was defined as a metachronous malignancy developing at least six months after the diagnosis of ET. A similar approach for the definition of SPM has been used in prior SEER based studies[25,26]. We used multiple primary standardized incidence ratio (SIR) session of the SEER*Stat software (version 8.1.5) to calculate SIR and absolute excess risk (AER) of SPM for ET patients. SIR is obtained by dividing the observed number of second malignancies by the expected number of cases that would occur in a reference population without the index malignancy. Confidence intervals (at 95%) and *P*-values were calculated using Poisson exact methods for the ratio of observed to expected events. AER was defined as the excess (observed-expected) number of second cancers in patients with index ET, per 1000 person years at risk. For patients who developed more than one malignancies after the primary disease, all of the subsequent malignancies were counted in the numerator for the calculation of SIR. The strata for SPMs were defined as a priori, and we included at least ten observed occurrences in each stratum.

Statistical analysis was done using SEERstat version 8.1.5 (National Cancer Institute, Bethesda, Maryland) and STATA (Stata-Corp, College-Station, Texas). Differences between survival rates of two groups were analyzed using the *Z* test for comparison of population proportions. All *P*-values were two sided and the level of significance was chose at 0.05. Institutional review board waiver was obtained from the University of Nebraska Medical Center Institutional Review Board prior to conducting this study.

RESULTS

A total of 8152 cases were identified from the SEER 18 registry, out of which 36 cases met the exclusion criteria (23 had unknown survival and 13 cases diagnosed prior to 2001). The remaining 8116 cases included 39.4% males (n = 3195) and 60.6% (n = 4921) females, and had a median age at diagnosis of 68 years (range < 1-107) (Table 1). Only 17 patients were less than 18 years old (0.7% of total). Ethnicity included 78% Whites, 12% African Americans, 7% others (American Indian/Alaska Native or Asian/Pacific Islander) and 3% unknown. The median year at diagnosis was 2007 (range 2001-2011).

The overall 1-year and 5-year age-standardized cause-specific survival of the study cohort was 99.1% and 94.9% respectively. The 5-year cause-specific survival was significantly different for patients below 50 years *vs* those 50 years and above (99.4% *vs* 93.5%; P < 0.01; Figure 1A) as well as for men *vs* women (93.1% *vs* 96.0%; P < 0.01; Figure 1B) (Table 2). Similarly, the age-standardized 1-year and 5-year relative survival rates were 96.6% and 88.4% respectively.

At a median follow-up of 3 years (range, 6-129 mo), 201 patients (2.46%) out of 2913 patients with ET developed SPM. The median age at diagnosis was 75 years (range, 39-94 years). None of the patients less than 18 years old developed SPM. SPMs occurred at an SIR of 1.26 (95%CI: 1.09-1.45; P = 0.002) with an AER of 37.44 per 10000 population. The risk for developing leukemia (SIR 3.78; 95%CI: 2.20-6.05, P < 0.001; AER 11.28/10000), particularly AML (SIR 7.74; 95%CI: 3.71-14.24, P < 0.001; AER 7.86/10000), and kidney cancer (SIR 2.40; 95%CI: 1.15-4.42, P = 0.01; AER 5.27/10000) was high (Table 3).

The risk of SPM was higher after 24 mo of diagnosis of ET (Table 4). Before 24 mo, the risk of development of leukemia (O/E 3.88, CI: 1.42-8.44, P < 0.05), particularly AML (O/E 9.02, CI: 2.46-23.09, P < 0.05) was noted to be higher than expected. After a follow-up

Table 2 Age standardized cause-specific survival rates of the study population

Survival at defined time period	Entire cohort	Male	Female	Patients < 50 yr	Patients ⩾ 50 yr
12 mo	99.10%	98.80%	99.30%	99.90%	98.90%
24 mo	98.30%	97.80%	98.70%	99.80%	97.90%
36 mo	97.20%	96.20%	97.90%	99.70%	96.50%
48 mo	96.10%	94.80%	96.90%	99.70%	95.00%
60 mo	94.90%	93.10%	96.00%	99.40%	93.50%

The percentage indicates cause-specific survival of specified population at defined time period.

Table 3 Risk of second primary malignancies among the study population

SPM	Observed	SIR	95%CI	AER	*P*-value
All sites	201	1.26	1.09-1.45	37.44	0.002
Solid	162	1.15	0.98-1.35	19.43	0.074
Colorectal	12	0.66	0.34-1.15	-5.62	0.916
Lung/bronchus	31	1.31	0.89-1.86	6.67	0.133
Breast	24	1.06	0.68-1.58	1.29	0.743
Prostate	24	1.11	0.71-1.65	2.14	0.594
Kidney	10	2.40	1.15-4.42	5.27	0.014
Lymphoma	12	1.59	0.82-2.78	4.02	0.127
Leukemia	17	3.78	2.20-6.05	11.28	< 0.001
AML	10	7.74	3.71-14.24	7.86	< 0.001

AER: Absolute excess risk; SIR: Standardized incidence ratio; SPM: Second primary malignancies.

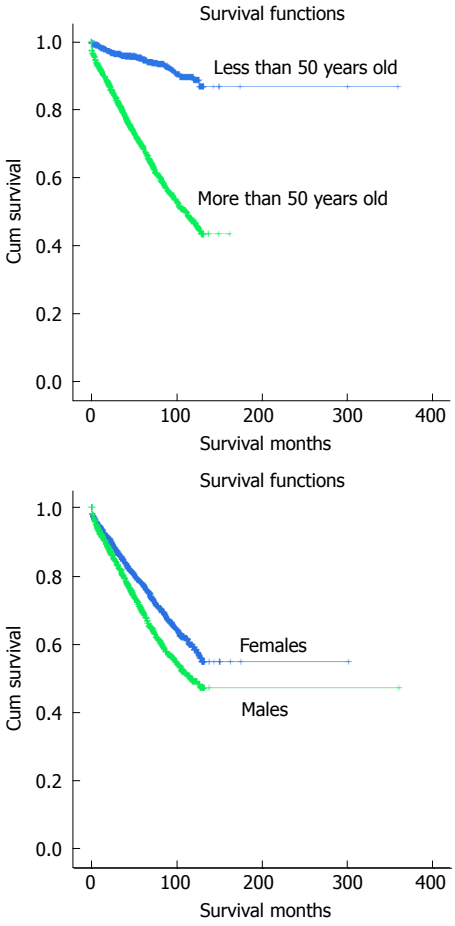


Figure 1 Cumulative survival (Kaplan-Meier estimates) of patients with essential thrombocythemia based on age (A) and gender (B). The log rank test was statistically significant with a *P*-value of < 0.01.

of 24 mo, the risk of SPM in general (O/E 1.31, 95%CI: 1.1-1.55, $P = 0.002$), kidney cancer (O/E 3.27, 95%CI: 1.5-6.21, $P = 0.003$), and leukemia (O/E 3.72, 95%CI: 1.86-6.66, $P < 0.001$), particularly AML (O/E 7.08, 95%CI: 2.6-15.4, $P < 0.001$) was determined to be higher.

The risk of AML was noted to be higher among all age groups (Table 5). The risk of SPM in general (O/E 1.78), solid tumors (O/E 1.75), and AML (O/E 12.7) were noted to be higher among patients 18-60 years old. Among patients ⩾ 60 years old, the risk of kidney cancer (O/E 2.42) and leukemia (O/E 3.78), particularly AML (O/E 7.06) was higher.

DISCUSSION

Our study demonstrates excellent age-standardized cause-specific five-year survival with 99.4% of patients below 50 years of age and 93.5% of patients above 50 years of age alive at 5 years. The fiver-year overall survival was significantly lower in patients over 50 years of age as well as in males as compared to females.

A Mayo clinic study demonstrated significantly higher OS in patients younger than 60 years *vs* those 60 years or older (32 years *vs* 19 years, $P < 0.001$). While OS was similar to general population in the first decade of disease [risk ratio (RR), 0.72; 95%CI: 0.50-0.99], OS was significantly worse after the first decade (RR, 2.21; 95%CI: 1.74-2.76, at 20 years and RR, 3.37; 95%CI: 1.84-5.65, at 30 years). This is consistent with fact that the incidence of SPM such as leukemia and myelofibrosis is higher after the first decade[6,10,27]. A number of factors may determine the OS of these patients. The prognostic factors associated with the poor outcomes are age at diagnosis of ⩾ 60 years, leukocyte count more than 15×10^9/L, a history of thrombosis, diabetes, hypertension and tobacco smoking[6].

In our study, a significantly elevated risk was noted for the development of AML and kidney cancer and especially after 24 mo of diagnosis of ET. Such increased risk has been noted in other studies also. In a retrospective population-based Danish cohort study, patients with ET, compared to those without, had a higher risk for developing hematological and solid malignancies with the SIR of 5 (95%CI: 3.6-6.9) and 1.2 (95%CI:

Table 4 Risk of second primary malignancies based on the follow-up duration

SPM	Overall SIR (95%CI)	SIR at 6-23 mo follow-up (95%CI)	SIR at > 24 mo follow-up (95%CI)
All sites	1.26 (1.09-1.45)[1]	1.16 (0.9-1.49)	1.31 (1.1-1.55)[1]
Solid malignancies	1.15 (0.98-1.35)	1.09 (0.82-1.43)	1.18 (0.97-1.43)
Colorectal	0.66 (0.34-1.15)	0.47 (0.10-1.38)	0.76 (0.35-1.44)
Lung/bronchus	1.31 (0.89-1.86)	1.48 (0.76-2.58)	1.23 (0.74-1.92)
Breast	1.06 (0.68-1.58)	0.91 (0.37-1.88)	1.14 (0.66-1.82)
Prostate	1.11 (0.71-1.65)	1.03 (0.45-2.03)	1.15 (0.66-1.87)
Kidney	2.40 (1.15-4.42)[1]	0.71 (0.02-3.95)	3.27 (1.5-6.21)[1]
Hematologic malignancies	2.36 (1.63-3.29)[1]	2.03 (0.97-3.73)	2.53 (1.62-3.76)[1]
Lymphoma	1.59 (0.82-2.78)	1.56 (0.42-3.99)	1.61 (0.69-3.17)
Leukemia	3.78 (2.2-6.05)[1]	3.88 (1.42-8.44)[1]	3.72 (1.86-6.66)[1]
AML	7.74 (3.71-14.24)[1]	9.02 (2.46-23.09)[1]	7.08 (2.6-15.4)[1]

[1]Indicates a statistically significant value. AML: Acute myeloid leukemia; SIR: Standardized incidence ratio; SPM: Second primary malignancies.

Table 5 Risk of secondary primary malignancies based on age at the time of diagnosis of essential thrombocythemia

SPM	Overall SIR (95%CI)	SIR for age group, 18-60 yr (95%CI)	SIR for age group, > 60 yr (95%CI)
All sites	1.26 (1.09-1.45)[1]	1.78 (1.31-2.36)[1]	1.15 (0.98-1.35)
Solid tumors	1.15 (0.98-1.35)	1.75 (1.27-2.35)[1]	1.03 (0.85-1.23)
Colorectal	0.66 (0.34-1.15)	1.33 (0.27-3.88)	0.56 (0.26-1.07)
Lungs/bronchus	1.31 (0.89-1.86)	2.36 (0.86-5.13)	1.19 (0.77-1.75)
Breast	1.06 (0.68-1.58)	1.08 (0.40-2.35)	1.06 (0.63-1.67)
Prostate	1.11 (0.71-1.65)	1.91 (0.82-3.76)	0.92 (0.52-1.49)
Kidney	2.40 (1.15-4.42)[1]	2.32 (0.28-8.37)	2.42 (1.05-4.78)[1]
Lymphoma	1.59 (0.82-2.78)	1.72 (0.21-6.23)	1.57 (0.75-2.88)
Leukemia	3.78 (2.2-6.05)[1]	3.74 (0.45-13.51)	3.78 (2.12-6.24)[1]
AML	7.74 (3.71-14.24)[1]	12.73 (1.54-45.98)[1]	7.06 (3.05-13.90)[1]

[1]Indicates a statistically significant value. AML: Acute myeloid leukemia; SIR: Standardized incidence ratio; SPM: Second primary malignancies.

1.0-1.4) respectively[13]. Another retrospective study from Italy (n = 331) showed cumulative incidence of SPM of 13% from the time of diagnosis of ET[17]. The study determined a particularly higher incidence of AML[17]. The risk of developing AML in ET depends on multiple factors such as old age, decreased hemoglobin and increased platelet count ≥ 1000 × 10^9/L[28]. Additionally, the risk increases with longer follow-up, with one study demonstrating a relatively low probability of developing leukemia in the first decade (1.4%) but significantly increased risk in the second (8.1%) and third decades (24.0%)[6]. The probability of occurrence of AML may also increase with the use of leukemogenic therapy such as radioactive phosphorus (P^{32}) and alkylators such as chlorambucil[2,7,29,30]. Although the presence of JAK2V617F mutation and cytogenetic abnormalities may not increase the probability of developing leukemia[2], the probability is increased in the presence of 2 or more somatic mutations[31].

In our study, the risk of SPM varied based on the age at diagnosis of ET and the follow-up duration. The risk of developing leukemia especially AML was increased after 6 mo of diagnosis of ET and persisted beyond 24 mo of follow-up, consistent with prior studies[6]. Although patients < 60 years had a higher risk of overall SPM, the increased risk of kidney cancer and leukemia was noted in those above 60 years. Prior studies have shown that the risk of SPM differed based on the age of patients. In Passamonti study, age > 65 years was determined to be a risk factor for developing SPM including leukemia[32]. A Danish study demonstrated that the probability of non-hematologic and hematologic malignancies was higher in younger patients (20-49 years *vs* 50-69 and > 70 years)[13]. Such conflicting results may be accounted by the differences in the characteristics of patients and other risk factors for SPM in different studies.

Potential limitations of this research are its retrospective study design, possibility of coding errors in the SEER database and inability to obtain data on potential impact of other factors such as therapy or other risk factors for SPM such as smoking. Detection bias is another potential shortcoming of the study, since many asymptomatic malignancies may be over diagnosed because of closer follow-up of patients following the diagnosis of ET. The strength of this study includes real world data from a relatively large number of ET patients. Our study demonstrated that patients with ET have an excellent five-year survival. Besides bleeding and thrombotic events, patients diagnosed with ET are also at risk of developing SPM. The risk of leukemia, particularly AML, can affect patients of all age groups and persists over time. Clinical trials on early detection of SPM may be considered in patients with ET. Additionally, patients with ET should be encouraged to undergo age and sex-

appropriate cancer screening tests.

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COMMENTS

Background

Patients with essential thrombocythemia (ET) have an excellent cause-specific five-year survival but are at an increased risk of second primary malignancy (SPM), particularly leukemia, which may contribute to excess deaths.

Research frontiers

A few United States studies have evaluated the risk of myelodysplastic syndrome/acute myeloid leukemia (AML) in ET but not the risk of other hematological or solid-organ malignancies.

Innovations and breakthroughs

This large Surveillance, Epidemiology and End Results database-based study confirmed an increased risk of SPM in patients with ET. A significantly higher risk was noted for leukemia. An increased risk of SPM, particularly leukemia, may contribute to excess deaths in ET. The risk of developing leukemia especially AML was increased after 6 mo of diagnosis of ET and persisted beyond 24 mo of follow-up, consistent with prior studies. Prior studies have shown conflicting results on the impact of age on the risk of SPM. In this study, the risk of overall SPM was higher in patients younger than the age of 60 years, however, the increased risk of kidney cancer and leukemia was noted in those above 60 years. The risk of leukemia, particularly AML, can affect patients of all age groups and persists over time.

Applications

The knowledge of an increased risk of SPM in patients with ET has implications in patient education, planning of preventive cancer screening strategies and may serve as a foundation for future research targeted at understanding the tumorigenesis in these patients.

Terminology

SPM: A metachronous malignancy developing at least six months after the diagnosis of ET; Relative survival rate: Ratio of observed survival of a group of cancer patients to the expected survival of a comparable cohort of cancer free patients. It measures net cancer survival controlling for differences in mortality for causes other than cancer; Cause-specific survival: A net survival measure that computes survival from cancer-related causes of death in the absence of other causes of death; Standardized incidence ratio: Ratio obtained by dividing the observed number of second malignancies by the expected number of cases in a reference population without the index malignancy; Absolute excess risk: Excess (observed-expected) number of second cancers in patients with ET, per 1000 person years at risk.

Peer-review

The paper is about an interesting topic. It is well written and the methods are sound. The conclusions are consistent with the results.

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Sorafenib in breast cancer treatment: A systematic review and overview of clinical trials

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Abstract

AIM: To evaluate the current role of sorafenib, an oral multikinase inhibitor in the treatment of breast cancer.

METHODS: An extensive search of the literature until March 2016 was carried out in Medline and clinicaltrials.gov, by using the search terms "sorafenib" and "breast cancer". Papers found were checked for further relevant publications. Overall, 21 relevant studies were found, 18 in advanced breast cancer (16 in stage Ⅳ and two in stages Ⅲ-Ⅳ) and three in early breast cancer.

RESULTS: Among studies in advanced breast cancer, there were two trials with sorafenib as monotherapy, four trials of sorafenib in combination with taxanes, two in combination with capecitabine, one with gemcitabine and/or capecitabine, one with vinorelbine, one with bevacizumab, one with pemetrexed and one with ixabepilone, three trials of sorafenib in combination with endocrine therapy and two trials in women with brain metastases undergoing whole brain radiotherapy. In addition, there was one trial of sorafenib added to standard chemotherapy in the adjuvant setting, and two trials in the neoadjuvant setting. In general, sorafenib was well tolerated in breast cancer patients, though its dosage had to be adjusted in some trials, and discontinuation rates were high, particularly for the combination of sorafenib with anastrozole. Sorafenib monotherapy and combinations with taxanes, bevacizumab and ixabepilone showed inadequate efficacy, while efficacy results from combinations with gemcitabine and/or capecitabine and possibly tamoxifen were more promising.

CONCLUSION: At present, sorafenib should not be

used for the treatment of breast cancer outside of clinical trials and more clinical data are needed in order to support its standard use in breast cancer therapy.

Key words: Breast cancer; Sorafenib; Kinase inhibitors; BRAF; Mitogen-activated protein kinase

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Core tip: In this systematic review of the literature, the role of sorafenib in the treatment of breast cancer has been evaluated. Regarding toxicity, sorafenib was generally well tolerated in breast cancer patients, while in terms of efficacy the most promising results came from clinical trials evaluating sorafenib in combination with gemcitabine and/or capecitabine and possibly tamoxifen. Efficacy was inadequate with sorafenib monotherapy and combinations with taxanes, bevacizumab and ixabepilone.

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INTRODUCTION

Breast cancer is the most frequently diagnosed malignancy and the leading cause of cancer death among women worldwide. It is estimated that each year breast cancer will be diagnosed in 1.7 million women and cause 520000 deaths around the world. Furthermore, it is estimated that breast cancer alone accounts for 25% of all cancer cases and 15% of all cancer deaths among females[1]. Despite the widespread use of breast cancer screening, leading to detection at early stages[2] and despite ongoing progress in adjuvant treatment strategies[3], still many women will develop metastatic disease and eventually die from breast cancer. Hence, new treatment modalities are needed, especially in order to treat breast cancer metastases.

Sorafenib (Nexavar®, Bayer and Onyx Pharmaceuticals) is a small molecule, which acts as an inhibitor of various tyrosine kinases, including BRAF, C-RAF, vascular endothelial growth factor receptor and platelet-derived growth factor receptor, RET, c-KIT and FMS-like tyrosine kinase-3[4-6]. Sorafenib is administered orally, and it is approved for the treatment of advanced renal cell carcinoma, advanced hepatocellular carcinoma, and advanced thyroid cancer[6-8]. Its possible mechanisms of anti-tumor action include inhibition of tumor growth and tumor progression, inhibition of metastasis and angiogenesis, as well as down-regulation of mechanisms that protect tumors from apoptosis[6-8]. The clinical application of sorafenib, especially in the treatment of renal cancer, is based on its activity on angiogenesis inhibition, and parallels other anti-angiogenetic agents[9]. Since targeting angiogenesis has been also a valid approach in the treatment of advanced breast cancer, the role of sorafenib, was investigated for this indication as well[10]. The aim of the present study was to evaluate the current role of sorafenib in the treatment of breast cancer, especially its efficacy and safety profile, based on available evidence from clinical trials.

MATERIALS AND METHODS

An extensive search of the literature was carried out in the bibliographic database Medline for articles ever published until March 2016, by using the search terms "sorafenib" and "breast cancer" in the field "Title". No other limits or filters were used, including no limits for language and year of publication. A further search was conducted in clinicaltrials.gov, a public database of registered clinical trials, by using the same key-words, *i.e.*, "sorafenib" and "breast cancer".

Inclusion criteria included published and ongoing clinical trials evaluating the efficacy and toxicity of sorafenib in early and advanced breast cancer. Exclusion criteria included the following study characteristics: Case reports, animal and *in vitro* studies, clinical trials with inconclusive information and clinical trials in various cancer types besides breast cancer.

At first, the titles of studies were screened and irrelevant publications were excluded. Evaluation of the abstract of the remaining studies followed. Finally, the content of the main text of identified studies was evaluated. The lists of references of relevant studies, including relevant review articles[11-13], were searched in order to identify possibly additional articles meeting the inclusion criteria. A meta-analysis was not possible due to extensive differences between studies.

RESULTS

Thirty-six papers were found by the Medline search and 30 clinical trials by the clinicaltrials.gov search. After screening the titles, abstracts and/or the full-texts of these studies, 21 relevant trials were identified; most of these studies involved patients with metastatic (16 studies) and/or advanced breast cancer (stages Ⅲ-Ⅳ; two studies), while three studies involved patients with breast cancer stages Ⅰ-Ⅲ. An overview of these clinical trials is presented in Table 1.

Among studies in metastatic and/or advanced breast cancer, there were two trials with sorafenib as monotherapy[13-15], three trials of sorafenib in combination with paclitaxel[16,17], one trial of sorafenib in combination with docetaxel and/or letrozole[18], two trials of sorafenib in combination with capecitabine[19,20] and one with gemcitabine and/or capecitabine[21]. In stage IV disease there was also one trial of sorafenib in combination with vinorelbine[22], one with bevacizumab[23], one with pemetrexed[17] and one with ixabepilone[24].

Regarding endocrine therapy in metastatic breast

Table 1 Overview of clinical trials evaluating sorafenib in breast cancer treatment

Ref.	Regimen	Study design	Number of patients	Disease stage	Toxicity	Clinical efficacy
Moreno-Aspitia *et al*[14]	Sorafenib as monotherapy	Phase Ⅱ; 1 arm	23	Stage Ⅳ	Manageable	Low
Bianchi *et al*[15]	Sorafenib as monotherapy	Phase Ⅱ; 1 arm	54	Stage Ⅳ	Manageable	Low
Gradishar *et al*[16]	Sorafenib + paclitaxel	RCT (*vs* placebo)	237	Stage Ⅳ	Manageable	Better TTP
NCT00622466 (ongoing)[17]	Sorafenib + paclitaxel	RCT (*vs* placebo)	41	Stage Ⅳ	NA	NA
NCT00499525 (ongoing)[17]	Sorafenib + paclitaxel	RCT (*vs* placebo)	180	Stages Ⅲ-Ⅳ	NA	NA
Mariani *et al*[18]	Sorafenib + docetaxel and/or letrozole	RCT (*vs* placebo)	218	Stage Ⅳ	Manageable	Not better
Baselga *et al*[19]	Sorafenib + capecitabine	RCT (*vs* placebo)	229	Stage Ⅳ	High	Better PFS
Baselga *et al*[20] (ongoing)	Sorafenib + capecitabine	RCT (*vs* placebo)	519	Stage Ⅳ	NA	NA
Schwartzberg *et al*[21]	Sorafenib + gemcitabine and/or capecitabine	RCT (*vs* placebo)	160	Stages Ⅲ-Ⅳ	Manageable	Better PFS
Luu *et al*[22]	Sorafenib + vinorelbine	Phase I/Ⅱ; 1 arm	11/35	Stage Ⅳ	Manageable	Low
Mina *et al*[23]	Sorafenib + bevacizumab	Phase Ⅱ; 1 arm	18	Stage Ⅳ	Substantial	Low
NCT02624700 (ongoing)[17]	Sorafenib + pemetrexed	Phase Ⅱ; 1 arm	35	Stage Ⅳ	NA	NA
Yardley *et al*[24]	Sorafenib + ixabepilone	Phase I/Ⅱ; 1 arm	10/76	Stage Ⅳ	High	Low
Isaacs *et al*[25]	Sorafenib + anastrozole	Phase I/Ⅱ; 1 arm	35	Stage Ⅳ	High	Benefit 23%
Massarweh *et al*[26]	Sorafenib + endocrine therapy[1]	Phase Ⅱ; 1 arm	11	Stage Ⅳ	Manageable	Promising[4]
NCT00634634 (ongoing)[17]	Sorafenib + letrozole	Phase I/Ⅱ; 1 arm	54	Stage Ⅳ	NA	NA
NCT01724606 (ongoing)[17]	Sorafenib + WBRT	Phase I	24	Stage Ⅳ	NA	NA
NCT01621906 (ongoing)[17]	Sorafenib + WBRT (18F-FLT-PET *vs* MRI)	Diagnostic	20	Stage Ⅳ	NA	NA
Spigel *et al*[27]	Sorafenib + AC-T[2]	One arm	45	Stages I-Ⅲ	Limited	NA
Loibl *et al*[28]	Sorafenib + EC-T[3]	Phase Ⅱ	36	Stages Ⅱ-Ⅲ	Manageable	pCR 27.7%
Bazzola *et al*[29]	Sorafenib + letrozole + cyclophosphamide[3]	One arm	13	Stages Ⅱ-Ⅲ	Tolerable	Clinical /no pCR

[1]Tamoxifen or anastrozole or letrozole or exemestane or fluvestrant; [2]Adjuvant; [3]Neoadjuvant; [4]With tamoxifen. WBRT: Whole brain radiotherapy; NA: Not available; pCR: Pathological complete response; PET: Positron emission tomography; MRI: Magnetic resonance imaging.

cancer, there were three trials evaluating sorafenib in combination with endocrine therapy: One in combination with anastrozole[25], one in combination with tamoxifen or anastrozole or letrozole or exemestane or fluvestrant[26] and one in combination with letrozole[17]. Finally, two trials were found, involving patients with brain metastases undergoing whole brain radiotherapy[17].

In breast cancer stages Ⅰ-Ⅲ, there was one trial of sorafenib added to standard AC-T chemotherapy in the adjuvant setting[27], and two trials in the neoadjuvant setting in stages Ⅱ-Ⅲ, one in combination with standard EC-T chemotherapy[28] and one in combination with letrozole and cyclophosphamide[29].

DISCUSSION

Sorafenib, a multiple tyrosine kinase inhibitor, is an orally administered small molecule, which has been evaluated in numerous clinical trials in breast cancer patients. The present study aimed to clarify the current role of sorafenib in breast cancer treatment.

Most clinical trials identified in our study involved patients with advanced and/or metastatic breast cancer. Sorafenib as a single agent was administered in two phase Ⅱ clinical trials involving 23[14] and 54 patients[15] with stage Ⅳ breast cancer; in both studies, toxicity was clinically manageable, whereas efficacy was limited, precluding future application of sorafenib monotherapy in breast cancer.

In a multi-national, randomized, placebo-controlled trial, involving 237 patients with metastatic breast cancer, sorafenib was added to paclitaxel[16]; toxicity was manageable with dose reduction; time to progression and overall response were significantly improved; however progression-free survival and overall survival did not differ significantly. Results from two ongoing randomized, placebo-controlled trials (NCT00622466 and NCT00499525[17]) evaluating the same combination are awaited. In another randomized, placebo-controlled trial, published only in abstract form, involving 218 patients with metastatic breast cancer, sorafenib was added to docetaxel and/or letrozole[18]; though toxicity was manageable, no improvement in terms of efficacy was found.

Baselga *et al*[19] reported the results of a phase ⅡB trial, involving 229 patients with HER-2-negative metastatic breast cancer; patients were randomly assigned to first- or second-line capecitabine in combination with sorafenib or placebo; though the addition of sorafenib to capecitabine significantly improved progression free survival, there was no significant improvement for overall survival and overall response, and the dose of sorafenib (400 mg twice daily) resulted in unacceptable toxicity for many patients. These findings led to the initiation of RESILIENCE[20], an ongoing multi-national, randomized, placebo-controlled, phase Ⅲ confirmatory trial in HER-2-negative, stage Ⅳ, breast cancer patients, comparing capecitabine plus a reduced initial sorafenib dose and possible dose-escalation with capecitabine plus placebo; enrollment began in November 2010 with a target of approximately 519 patients. In a similar study, published by Schwartzberg *et al*[21], 160 HER-2-negative patients with locally advanced or metastatic breast cancer whose disease progressed during or after bevacizumab were randomized to chemotherapy plus sorafenib or chemotherapy plus placebo; initially,

chemotherapy was gemcitabine, but later, capecitabine was allowed as an alternative; the addition of sorafenib provided a statistically significant progression-free survival benefit, with manageable toxicities but frequent dose reductions.

Combinations of sorafenib with other agents in metastatic breast cancer have shown less promising results. In particular, the combination of sorafenib with vinorelbine in a phase Ⅰ and Ⅱ study with 11 and 35 patients with metastatic breast cancer, respectively, showed manageable toxicity but low efficacy; the authors concluded that this combination may be of interest if specific biomarkers guiding patient selection can be identified[22]. The combination of sorafenib with bevacizumab in a phase Ⅱ, one arm study, involving 18 patients had substantial toxicity and minimal efficacy[23]. Likewise, in a Phase Ⅰ and Ⅱ study with 10 and 76 patients with metastatic breast cancer, respectively, the combination of ixabepilone and sorafenib was poorly tolerated and the activity of the combination was similar to the activity previously reported with single-agent ixabepilone or taxanes[24]. Results from an ongoing study (NCT02624700)[17] in stage Ⅳ breast cancer evaluating the combination of sorafenib with pemetrexed are awaited.

Regarding sorafenib in combination with endocrine therapy in hormone receptor positive metastatic breast cancer, results from two trials have been reported[25,26]. In a phase Ⅰ/Ⅱ trial, involving 35 patients who had disease recurrence or progression while on aromatase inhibitors, the combination of sorafenib with anastrozole was associated with an encouraging clinical benefit rate of 23%, suggesting that sorafenib may be able to restore sensitivity to hormone therapy; however, this combination was associated with significant toxicity[25]. In a pilot phase Ⅱ study, involving 11 patients, sorafenib was added to endocrine therapy, to tamoxifen in 7 cases, and in one case each to anastrozole, letrozole, exemestane and fulvestrant; toxicity was manageable with promising results in terms of efficacy, since most patients developed stable disease[26]. Results from an ongoing study (NCT00634634) evaluating the combination of sorafenib with letrozole are awaited[17].

A special issue in metastatic breast cancer is the administration of sorafenib to patients with brain metastases, undergoing whole brain radiotherapy (WBRT). In an ongoing phase Ⅰ clinical trial (NCT01724606)[17], the safety and efficacy of this combination is under evaluation. Another ongoing study (NCT01621906)[17] from the same center compares 18F-FLT-PET with MRI as imaging methods in the evaluation of response to treatment with sorafenib and WBRT.

Finally, the addition of sorafenib to chemotherapy in the adjuvant and neoadjuvant setting has been evaluated in three clinical trials[27-29]. In a pilot, one arm study[27], 45 patients with node-positive or high-risk early-stage breast cancer (stages Ⅰ-Ⅲ) received adjuvant doxorubicin and cyclophosphamide followed by paclitaxel and sorafenib; though sorafenib was generally associated with limited severe toxicity, many patients discontinued sorafenib early; the authors concluded that additional studies of sorafenib in breast cancer in the neoadjuvant and triple-negative settings are warranted. In SOFIA[28], a phase Ⅱ, single arm clinical trial, 36 HER-2-negative patients with stage Ⅱ-Ⅲ disease received neoadjuvant EC (epirubicin plus cyclophosphamide) in 3-weekly cycles followed or preceded by 12 wk of paclitaxel; sorafenib was added to EC or paclitaxel or the whole chemotherapy regimen; the authors concluded that the addition of sorafenib to this regimen is feasible if the starting dose is 200 mg, escalated every 3 wk based on patients' individual toxicities. In another single-arm study in the neoadjuvant setting[29], 13 estrogen receptor-positive, postmenopausal, breast cancer patients with stage Ⅱ-Ⅲ disease received for 6 mo the combination of letrozole, metronomic cyclophosphamide and sorafenib; the combination was well tolerated, and although no pathological complete response was found, still clinical response rates were promising.

In conclusion, sorafenib is generally well tolerated in breast cancer patients with either metastatic disease or at earlier stages. However, in some clinical trials sorafenib discontinuation rates were high or sorafenib dosage had to be adjusted, due to unacceptable toxicity. To date, sorafenib as a single agent and combinations of sorafenib with taxanes, bevacizumab and ixabepilone have shown inadequate efficacy. More promising efficacy results came from clinical trials with sorafenib in combination with gemcitabine and/or capecitabine and possibly tamoxifen. At present, sorafenib should not be routinely administered for the treatment of breast cancer outside of clinical trials. More evidence from ongoing and future clinical trials should clarify the possible role of sorafenib in the treatment of breast cancer patients.

COMMENTS

Background

Sorafenib is a small molecule, acting as an inhibitor of various tyrosine kinases; it is approved for the treatment of advanced renal cell carcinoma, advanced hepatocellular carcinoma, and advanced thyroid cancer. Its possible mechanisms of action include inhibition of tumor growth, progression, metastasis and angiogenesis and down-regulation of mechanisms protecting tumors from apoptosis. The clinical application of sorafenib, especially in renal cancer, is based on its anti-angiogenesis activity. Since targeting angiogenesis has been also a valid approach in the treatment of advanced breast cancer, the role of sorafenib, was investigated for this indication as well. The aim of the present study was to evaluate the current role of sorafenib in the treatment of breast cancer, based on available evidence from clinical trials.

Research frontiers

Sorafenib is being evaluated in combination with various chemotherapeutic and/or endocrine agents, especially in the treatment of advanced breast cancer. The current hotspot of research is the clinical application of sorafenib in breast cancer therapy in combination with various agents, in order to improve treatment efficacy with an acceptable toxicity profile.

Innovations and breakthroughs

In this systematic review of the literature, the role of sorafenib in the treatment of breast cancer was evaluated. Overall, 21 relevant studies were identified, 18 in advanced breast cancer (16 in stage IV and two in stages III-IV) and three

in early breast cancer. In advanced breast cancer, there were two trials with sorafenib as monotherapy, four trials of sorafenib in combination with taxanes, two with capecitabine, one with gemcitabine and/or capecitabine, one with vinorelbine, one with bevacizumab, one with pemetrexed, one with ixabepilone, three trials with endocrine therapy and two trials in women with brain metastases undergoing whole brain radiotherapy. In early breast cancer, there was one trial of sorafenib added to standard chemotherapy in the adjuvant setting, and two trials in the neoadjuvant setting. In terms of efficacy, sorafenib monotherapy and combinations with taxanes, bevacizumab and ixabepilone showed inadequate efficacy, while results from combinations with gemcitabine and/or capecitabine and possibly tamoxifen were more promising. In terms of toxicity, sorafenib was well tolerated in breast cancer patients, though its dosage had to be adjusted in some trials, and discontinuation rates were high, particularly for the combination of sorafenib with anastrozole.

Applications

At present, sorafenib should not be used for the treatment of breast cancer outside of clinical trials; more clinical data are needed in order to support its standard use in breast cancer therapy.

Terminology

Sorafenib (Nexavar®, Bayer and Onyx Pharmaceuticals): A small molecule, administered orally. Sorafenib is approved for the treatment of the following types of cancer: Advanced renal cell carcinoma, advanced hepatocellular carcinoma, and advanced thyroid cancer. Sorafenib chemical mechanism of action: Inhibition of various tyrosine kinases, including BRAF, C-RAF, vascular endothelial growth factor receptor and platelet-derived growth factor receptor, RET, c-KIT and FMS-like tyrosine kinase-3. Sorafenib biological mechanism of action: Inhibition of tumor growth, progression, metastasis and angiogenesis and down-regulation of mechanisms protecting tumors from apoptosis. Rationale for clinical application of sorafenib: Mainly its activity on angiogenesis inhibition, especially in advanced renal cancer. Tumor angiogenesis: Formation of new blood vessels by malignant tumors.

Peer-review

This is an excellent and well designed article.

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EDITORIAL

Transanal total mesorectal excision: Myths and reality

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Abstract

Transanal total mesorectal excision (TaTME) is a new and promising approach for the treatment of rectal cancer. Whilst the experience is still limited, there are growing evidences that this approach might overcome the limits of standard low anterior resection. TaTME might help to decrease the conversion rate especially in difficult patients, and to improve the pathological results, while preserving the urogenital function. Evaluation of data from large registries and randomized studies should help to draw firmer conclusions. Beyond these technical considerations, the next challenge seems to be clearly the safe introduction of this approach, motivating the development of dedicated courses.

Key words: Transanal total mesorectal excision; Bottom up; TAMIS; Laparoscopy; Robotic; Outcomes; Rectal cancer

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Core tip: The experience and evidences regarding the use of transanal total mesorectal excision is still scarce but promising. Preliminary data showed excellent results, without sacrificing the pathological and oncological outcomes. Whilst still in its infancy, further investigations should be encouraged. Data from large registries and randomized trials are awaited before to draw definitive conclusions.

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There is no doubt that low anterior resection (LAR) and total mesorectal excision (TME) have revolutionized the management of rectal cancer and improved its oncological outcomes[1]. On the other hand, the introduction of

minimally invasive surgery for oncological rectal resection has not yet completely convinced the most skeptical open surgeons. Whilst potentially better short-term outcomes have been published favoring laparoscopic approach[2,3], the recent ALaCaRT and ACOSOG Z6051 trials failed to show the non-inferiority of laparoscopic LAR in comparison to open surgery[4,5]. Indeed, there is still a degree of uncertainty, notably regarding the risk of incomplete TME specimen, positive margins, and worse long-term oncological outcomes. To fuel the debate further, other large randomized series did not show inferior pathological or oncological outcomes following laparoscopic LAR[6]. Meanwhile, even the amazing introduction of robotics has not significantly improved the outcomes[7].

To overcome the challenges posed by abdominal TME surgery, a transanal approach has been developed over the last decade, with promising early outcomes. There is growing evidence available including our recent review of transanal TME (TaTME) showing excellent results[8]. However, TaTME is still in its infancy and definitively requires more robust data and longer follow-up. Since the first description of TaTME, a number of relatively large series have been published, showing not only the feasibility of the approach, but also its safety[9,10] even in challenging patients. In our own experience, we have recently shown a low conversion rate, low R1 rate, and an excellent completeness of TME[11-13].

Several parameters and factors pose technical challenges and need special consideration when considering planning TME surgery: (1) dealing with "difficult anatomy" (male, obese, narrow pelvis, post radiation); (2) increasing the sphincter-preserving rate; (3) performing a safe distal rectal stapling; (4) avoiding positive margins; (5) reducing the risk of incomplete TME; (6) improving the oncological outcomes; and (7) offering adequate functional outcomes.

TaTME seems to offer a solution for most of these parameters/factors. The narrow pelvis with a bulky irradiated specimen in an obese male patient is no longer a relative contra-indication to laparoscopic surgery. Starting the most difficult part of the dissection (the lowest part of the pelvis) from the distal end offers obvious advantages. First of all, the distal margin can be assessed precisely and secured with a purse-string before performing the rectotomy. This in turns avoids the need for distal cross-stapling, which can be laparoscopically challenging due to the limited angle of the endoscopic stapler and the pelvic morphology. This often results in multiple firing to complete the transection with the associated risk of anastomotic leak after more than 2 reloads[14]. With TaTME, this is no longer a challenge. Different anastomotic techniques have been proposed, guaranteeing a safe and efficient way to rejoin the bowel[15]. Although, this may increase the rate of sphincter-preserving surgery, it is at the cost of a higher rate of coloanal anastomosis.

Beyond these technical considerations, the interest to proceed with a complete TME is important.

The threat of incompleteness of mesorectal excision was recently shown to be significant after LAR and APE (36% and 13% respectively)[16]. The lowest part of the mesorectum is at risk of being left behind, which is unacceptable from an oncological point of view. Again, starting the dissection from below might help to obtain a more complete TME specimen. Moreover, comparative studies have shown better pathological outcomes after TaTME in comparison to laparoscopic TME[17,18]. The awaited results from the large multicenter registry study (LOREC) should hopefully help to draw more definitive conclusions.

The main challenges for the future of TaTME can be summarized in three different categories: (1) the long-term oncological outcomes; (2) the functional outcomes; and (3) the safe introduction of this approach.

Obviously, the technique is still in its infancy and long-term outcomes are not yet available. Early oncological data seem promising[13], but it is too early to draw definitive conclusions. The COLOR Ⅲ study[19], evaluating TaTME *vs* laparoscopic TME, should provide a more comprehensive overview of the added value of the transanal approach. In addition, quality of life and functional outcomes will be assessed. Based on previous reports[20-22], adequate function has been reported. However, still a high rate of coloanal anastomosis is performed and the risk of worse functional outcomes is possible.

As for any new surgical technique, the danger of widespread rapid and unmonitored adoption without proper training exists. The development of a dedicated curriculum should be established in order to avoid unnecessary preventable complications during the early phase of a surgeon-s learning curve. As already mentioned for robotic surgery and other surgical innovations, training is probably the biggest challenge[23]. Dedicated theoretical and practical courses including cadaver workshops as well as live cases proctoring are key to ensuring the safe introduction of a new surgical technique[24].

In conclusion, TaTME is a promising approach, aiming to overcome the limitations of laparoscopic TME. So far, the published data support its use. Excellent pathological and acceptable short-term clinical outcomes have been reported, however long-term oncological and functional data are still awaited. There is no doubt that TaTME will play a significant role in the evolution of rectal surgery as the drive to perfecting TME and improving outcomes continues.

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REVIEW

Oncogenic fingerprint of epidermal growth factor receptor pathway and emerging epidermal growth factor receptor blockade resistance in colorectal cancer

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Abstract

Epidermal growth factor receptor (EGFR) has been an attractive target for treatment of epithelial cancers, including colorectal cancer (CRC). Evidence from clinical trials indicates that cetuximab and panitumumab (anti-EGFR monoclonal antibodies) have clinical activity in patients with metastatic CRC. The discovery of intrinsic EGFR blockade resistance in Kirsten RAS (KRAS)-mutant patients led to the restriction of anti-EGFR antibodies to KRAS wild-type patients by Food and Drug Administration and European Medicine Agency. Studies have since focused on the evaluation of biomarkers to identify appropriate patient populations that may benefit from EGFR blockade. Accumulating evidence suggests that patients with mutations in EGFR downstream signaling pathways including KRAS, BRAF, PIK3CA and PTEN could be intrinsically resistant to EGFR blockade. Recent whole genome studies also suggest that dynamic alterations in signaling pathways downstream of EGFR leads to distinct oncogenic signatures and subclones which might have some impact on emerging resistance in KRAS wild-type patients. While anti-EGFR monoclonal antibodies have a clear potential in the management of a subset of patients with metastatic CRC, further studies are warranted to uncover exact mechanisms related to acquired resistance to EGFR blockade.

Key words: Epidermal growth factor receptor; Oncogenic signature; Kirsten RAS; BRAF; Cetuximab; Panitumumab; Epidermal growth factor receptor blockade resistance

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Core tip: Epidermal growth factor receptor (EGFR) blockade treatment is a well-established targeted therapy in metastatic colorectal cancer (CRC) patients. However, a limited number of patients benefit from EGFR inhibition, with limited time duration of response. This review article

discusses the most recent updates from the current-state-of-the-science related to molecular pathways of EGFR signaling, the mechanism of action and efficacy of EGFR blockade treatment, and possible molecular pathways related to EGFR blockade resistance in CRC. We further discuss potential mechanisms contributing to targeted EGFR inhibition. Lastly, future perspectives are discussed to shed some light on efforts to overcome this potential challenge in the era of targeted treatment.

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INTRODUCTION

Colorectal cancer (CRC) with an incidence of 1.2 million cases per year is now the third most common cancer in males and second in females[1]. Although there is a minor trend "towards" a decrease in the incidence of the disease, it is yet one of the major health care burden in United States[2]. Despite extensive research, a limited number of targeted agents have been shown to be active in CRC.

Cetuximab and Panitumumab are two new generation monoclonal antibodies targeting epidermal growth factor receptor (EGFR) recently approved by the Food and Drug Administration (FDA) for the management of metastatic CRC in the United States. Although their exact mechanism of action is unknown it is hypothesized that the direct interaction of these antibodies with EGFR results in apoptosis. Herein, we discuss the molecular signature of the EGFR pathway, the possible mechanism of action of anti-EGFR monoclonal antibodies, the clinical consequences of these cell-based interactions in treatment of metastatic CRC, along with emerging resistance to these agents during the treatment of CRC.

ONCOGENIC SIGNATURE OF EGFR PATHWAYS IN CRC

EGFR, also known as ErbB, is a member of the receptor tyrosine kinase family; it stimulates multiple intracellular proto-oncogenic signaling mediators including Kirsten RAS (*KRAS*)[3]. As a 170 kDa transmembrane glycoprotein expressed on cell surface, EGFR has physiological roles in many organs including the epithelium of the gastrointestinal system, bronchial tract and cutaneous tissue[4]. In normal homeostasis, EGFR is activated by binding with external ligands such as growth factors (EGF, epiregulin or amphiregulin). This interaction initiates homodimerization and heterodimerization processes *via* a diverse combination of identical and different members of the family such as ErbB2 (HER-2), ErbB3 (HER-3) and ErbB4 (HER-4)[5]. The ensuing phosphorylation of tyrosine kinase domain results in activation of oncogenic pathways including mitogen activated protein kinase (MAPK) and phosphotidylinositol-3-kinase (PI3KCA) pathways (Figure 1). These signaling axes have been shown to function in many critical pro-survival cellular reactions in cancer cells including protein synthesis, cell growth, cell cycle progression, transformation and invasion. KRAS, a critical growth signal response in cancer cells, is an upstream activator of the MAPK pathway[6] (Figure 1). KRAS-driven MAPK translocates into the cell nucleus, initiates a transcription cascade and promotes cell growth[7]. For example, KRAS activation leads to upregulation of c-myc which fuels proliferation of human colon cancer cells and provides a survival advantage[8]. Signal cascades of KRAS also induce cell cycle progression *via* activation of the transcription factor Elk-1, which ultimately increases the expression of cell cycle promoting proteins such Cyclin D1[9]. Moreover, as a part of the complex network of EGFR signaling, the KRAS driven MAPK pathway interacts with JNK signaling to modulate cellular stress responses which enhance cellular plasticity. This response helps malignant cells to adapt to dynamic microenvironmental changes[10]. In transformed cancer cells, *KRAS* mutations abolish regulation *via* the upstream EGFR loop; the MAPK and PI3KCA pathways, and other pro-survival cascades are continuously activated, leading to distinct cellular behavior[11,12].

Phosphatidylinositol 3-kinase (PIK3CA) is another well-studied signal transducer of the EGFR pathway. In normal homeostasis, activation of PIK3CA by EGFR leads to induction of Akt-mTOR pathway which has been shown to be crucial signal for protein synthesis and cell cycle progression[13]. Activation of PIK3CA also abrogates apoptosis and cellular senescence in cancer cells[14]. PIK3CA-driven mTOR activates Bcl-2 and ultimately inhibits apoptosis[15], indicating that PIK3CA signaling may have an important role in the immortality of transformed cells. PIK3CA activation has also been shown to be related to elevated expression of COX-2 which enhances angiogenesis in CRC[16]. Consistent with evidence from preclinical observations, mutant *PIK3CA* is associated with development of various cancers including CRC[17]. Current thinking suggests that the changes in the gene expression profile caused by activating mutations of PIK3CA may culminate in changes in the proteome of cancer cells and that this transformation enhances cellular growth and invasion by creating distinct oncogenic signatures[18].

BRAF, a member of the RAF kinase family, functions as a serine/threonine protein kinase, and gets activated by the upstream Ras oncogene (Figure 1)[19]. Activating mutations of the BRAF oncogene occur in the kinase domain and the V599E mutation accounts for the vast majority of point mutations (approximately 80%)[20]. Mutant *BRAF* propagates Raf-MAPK signaling in the absence of upstream stimulation and ultimately induces cell growth and proliferation in malignant clones[21].

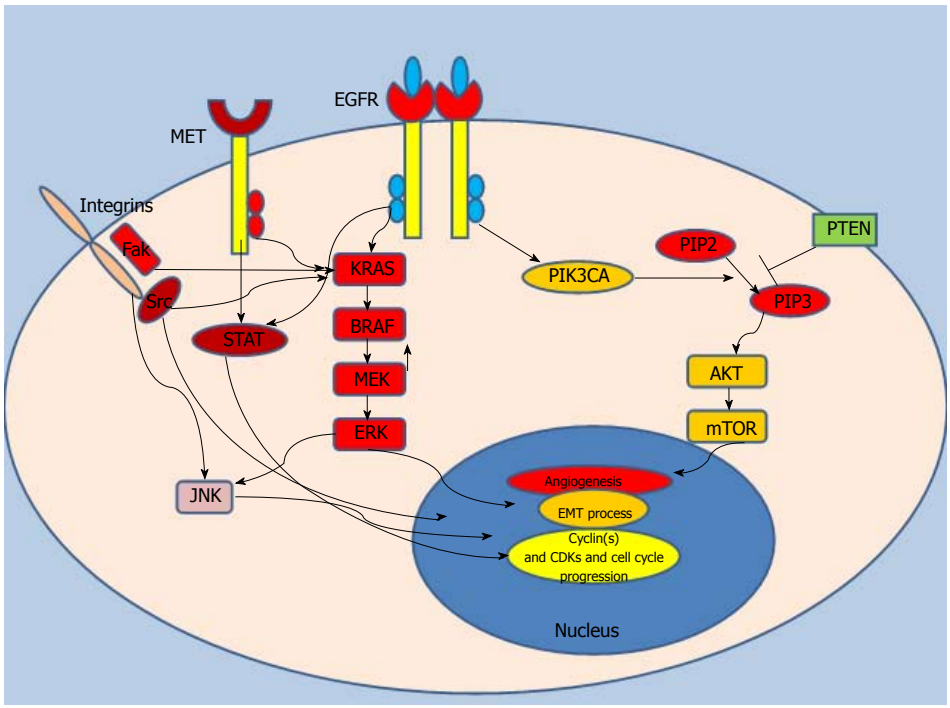


Figure 1 Epidermal growth factor receptor signaling along with co-activated other receptor tyrosine kinases. EGFR: Epidermal growth factor receptor; PIK3CA: Phosphatidylinositol 3-kinase; mTOR: Mechanistic target of rapamycin.

Similar to *PIK3CA* mutations, *BRAF* mutations also transform the protein expression profiles of cancer cells and alter internal metabolism. For example, CRC cells with mutant *BRAF* were found to be more resistant to apoptosis compared to those carrying wild-type BRAF[22]. Moreover, *BRAF* may increase the expression of cell cycle promoting proteins which further enhance the expansion of selected clones[23]. *BRAF* mutations have also been shown to be associated with constitutively activated NF-κB[24], leading to tumor angiogenesis that optimizes the microenviroment for cancer cells[24]. All this evidence suggests that activation of the BRAF oncogene may add further distinct characteristics to the cancer cells' genomic fingerprint.

Src and *STAT*, two other critical oncogenes, have been demonstrated to be involved in the development and progression of epithelial tumors along with cancer angiogenesis, and both mediators operate in the signaling cascade of the EGFR pathway[25]. A study showed that EGFR overexpressing cancer cells bear a 10-fold increase in Src activity compared to low EGFR expressing cancer cells[26], and that increased Src activity has been associated with highly aggressive tumor behavior and metastatic potential in CRC cells[27]. Oncogenic Src, as a non-receptor tyrosine kinase (nRTK), turns on many downstream survival pathways such Ras-MAPK pathway. It further activates receptor tyrosine kinases which create continuous growth signals for cancer cells[28]. Activating mutations of *Src* are related to adverse outcomes in CRC[29,30]. STATs are also activated by EGFR[25] and function as transcriptional factors in downstream pathways of receptor tyrosine kinases and cytokine receptors[31]. Induction of STATs through EGFR signaling[32] may also fuel angiogenesis in the tumor microenvironment[33]. Although activation of STATs has shown to be related to enhanced proliferation in CRC cancer cells[34], the exact role of STATs in development and progression of CRC remains to be elucidated.

Altogether, current evidence indicates intricate EGFR signaling. Variant alterations in the downstream signal transducers of EGFR are likely responsible for the change in expression profiles and molecular behavior of cancer cells.

EMERGENCE OF MONOCLONAL CHIMERIC ANTIBODIES TO EGFR

Considering the diverse oncogenic pathways activated by EGFR, it has become a promising target for therapy in various epithelial tumors[35,36]. The aforementioned studies led to research targeting EGFR signaling *via* different approaches, including small molecule inhibitors of receptor tyrosine kinases such as erlotinib and monoclonal antibodies to neutralize the receptor *via* an internalization and degradation process.

The initial investigations in this field were conducted using murine anti-EGFR antibodies. In these studies, both agonist and antagonist antibodies were tested[37], and antagonist antibodies inhibited the proliferation of the malignant clones with high affinity binding[38]. Since murine antibodies are recognized as foreign antigens in humans, human-mouse chimeric antibodies were generated to further study this effect and they were shown to have superior biological efficacy compared to

murine antibodies in human tumor xenograft models[39]. Cetuximab, a chimeric antibody against EGFR, was demonstrated to have 10 times higher affinity to EGFR compared the murine antibodies (M225)[40], and limited toxicity was observed in phase Ⅰ clinical studies[41].

The mechanism of action of anti-EGFR antibodies was at first attributed to internalization of receptors bound by the anti-EGFR antibodies[42]. Further studies demonstrated that the EGFR blockading agents not only catalyze the removal of the receptors but also inhibit receptor tyrosine kinase activity, and induce apoptosis *via* cell cycle arrest in colonic adenocarcinoma cell lines[43]. Besides its-single agent activity, an enhanced anti-cancer effect of cetuximab was observed in combination with the topoisomerase inhibitor (irinotecan) in human CRC xenograft models[44]. These discoveries led to further studies to elucidate the role of EGFR blockade in modern cancer treatment, and clinical trials were opened for enrollment in different clinical settings.

CLINICAL TRIALS TO ASSESS THE ROLE OF EGFR BLOCKADE IN CRC TREATMENT

To further investigate the promising results observed in preclinical studies, early phase and clinical efficacy trials were designed (Table 1). A phase Ⅰ safety trial was conducted to determine the optimal biological dose, and the doses achieved at maximal systemic clearance were well tolerated in both monotherapy and combined treatment models[45]. In a phase Ⅱ clinical trial of 120 irinotecan-resistant CRC patients with positive EGFR expression, cetuximab reversed the resistance to irinotecan with a 22.5% major objective response rate and 17% radiologic response rate in a combination treatment model[46]. The promising radiological response rate had already resulted in FDA approval for the cetuximab treatment for metastatic CRC patients. In order to solidify the response observed in the original study, cetuximab was investigated as a single agent treatment in patients with advanced stage refractory CRC[47]. In this study, 9% partial response rate along with a median survival of 6.4 mo was reported after cetuximab monotherapy in CRC patients expressing EGFR. To further investigate these findings and to test the superiority of combination treatment to monotherapy, a randomized phase Ⅱ clinical trial was conducted[48]. Patients were randomized to receive cetuximab and irinotecan combination *vs* cetuximab alone. Patients in the combination arm demonstrated a 22.9% partial response compared to 10.8% in the cetuximab monotherapy arm (P = 0.007). While progression free survival (PFS) was significantly improved in patients receiving combination therapy there was no overall survival (OS) difference between the groups. A subsequent study demonstrated limited survival benefit in patients treated with cetuximab compared to best supportive care (6.1 mo *vs* 4.6 mo respectively)[49]. Although EGFR expression was considered as a predictor of EGFR blockade response in early clinical trials, this concept has since changed and EGFR expression is no longer considered a biomarker in CRC patients[50].

The observed improvement in response rate in the treatment of irinotecan-refractory cases led to further studies in treatment-naïve patients. The CRYSTAL study showed significantly improved PFS in metastatic CRC patients who received a cetuximab plus FOLFIRI (bolus 5-fluoruracil/leucovorin chemotherapy plus irinotecan) regimen compared to patients who underwent FOLFIRI treatment alone (Table 1). However, there was no significant difference in OS between the two groups[51]. Subsequent analysis of the study demonstrated improved OS (approximately 3.5 mo) in patients with wild-type KRAS who underwent cetuximab treatment. No significant response was observed in *KRAS*-mutant patients[52]. This study led to the FDA approval of cetuximab as a first line treatment in combination with FOLFIRI in patients with metastatic CRC. In the OPUS study, cetuximab was combined with FOLFOX-4 and an improved PFS [hazard ratio (HR) 0.567, P = 0.0064] was observed in KRAS wild-type metastatic CRC patients who received combination therapy[53]. In order to assess the role of cetuximab in earlier stages of the disease, N0137 trial was conducted: Stage Ⅲ CRC patients were enrolled to receive cetuximab plus FOLFOX-6 combinations *vs* FOLFOX-6 in adjuvant settings after initial resection[54]. After a of median 28 mo follow-up in both wild-type and mutant *KRAS* groups, results of this trial showed no benefit of cetuximab when added to FOLFOX-6 regimen in the setting of locally advance disease. In order to compare the efficacy of cetuximab in combinations with FOLFOX-6 and FOLFIRI regimens as a neoadjuvant treatment in unresectable metastatic CRC patients, a phase Ⅱ clinical trial (CELIM trial) was conducted[55]. Although there was no significant survival difference between the two groups, addition of cetuximab resulted in higher response rates compared to historical controls (FOLFOX and FOLFIRI). A total 36 of 116 patients were able to receive R0 resection. The result of this study was also consistent with previous studies demonstrating improved outcomes limited to patients with wild type KRAS.

Panitumumab, another monoclonal antibody for EGFR blockade, has also been investigated in CRC patients. This agent received FDA approval after prolonged PFS was demonstrated in an open-label phase Ⅲ clinical trial in which patients were enrolled to receive single-agent panitumumab *vs* best supportive care (median PFS, 8 wk *vs* 7.3 wk; HR, 0.54; 95%CI: 0.44-0.66)[56]. Follow-up analysis of this study showed that the survival benefit was again, limited to wild-type KRAS CRC patients[57]. A retrospective study suggested potential resistance in patients with BRAF V600E mutation and requirement of wild-type BRAF for clinical benefit, along with wild-type KRAS[58]. Currently this drug is also approved as a first line agent in combination with FOLFOX4 in metastatic CRC patients with wild-type KRAS due to observed

Table 1 Clinical trials investigating the impact of epidermal growth factor receptor blockades in colorectal cancer patients

Ref.	Year	Sample size	Mutation status	Treatment groups	Special considerations	Summarized findings
Saltz *et al*[46]	2001	120	EGFR +	Cetuximab plus Irinotecan		22.5% major objective response rate 17% radiologic response rate
Saltz *et al*[47]	2004	57	EGFR +	Cetuximab		9% (CI: 3% to 19%) partial response Median survival of 6.4 mo. *P* value ??
Cunningham *et al*[48]	2004	329	EGFR +	Cetuximab plus irinotecan *vs* cetuximab alone		22.9% partial response in combination arm, 10.8% partial response in the cetuximab monotherapy arm No difference in OS
Jonker *et al*[49]	2007	572	EGFR +	Cetuximab compared to best supportive care		6.1 mo OS in treatment arm *vs* 4.6 mo with supportive care. $P = 0.005$ Quality of life was better preserved in the cetuximab group ($P < 0.05$)
Van Cutsem *et al*[56]	2007	463	EGFR+	Panitumumab *vs* best supportive care		Median PFS 8 wk in treatment arm compared to 7.3 wk in patients receiving supportive care (HR 0.54; $P < 0.0001$)
Amado *et al*[57]	2008	427	EGFR+ Wild type KRAS *vs* mutant *KRAS*	Panitumumab *vs* best supportive care	Reanalysis of Van Cutsem 2007	In patients with wild type KRAS median PFS was 12.3 wk for panitumumab *vs* 7.3 wk for supportive care Response rates to panitumumab was 17% for patients with wild type KRAS compared to 0% in patients with mutant *KRAS*
Van Cutsem *et al*[51] CRYSTAL Trial	2009	1198	EGFR +	Cetuximab plus FOLFIRI *vs* FOLFIRI alone		HR for PFS in combination therapy 0.85 ($P = 0.048$) when compared to FOLFIRI alone There was no difference in OS (HR 0.93; $P = 0.31$) Although not significant PFS was improved with cetuximab in patients with wild-type-KRAS (HR 0.68; $P = 0.07$)
Folprecht *et al*[55] CELIM trial	2010	113	Wild type KRAS *vs* mutant *KRAS*	Cetuximab plus FOLFOX *vs* Cetuximab plus FOLFIRI *vs* historical controls	Neoadjuvant setting	No survival difference between the two groups Higher response rates compared to historical controls (FOLFOX and FOLFIRI) Total 36 of 116 patients were able to receive R0 resection Improved outcomes limited to patients with wild type KRAS
Van Cutsem *et al*[52]	2011	1198	EGFR+ Wild type KRAS *vs* mutant *KRAS*	Cetuximab plus FOLFIRI *vs* FOLFIRI alone Wild type KRAS *vs* mutant *KRAS*	Reanalysis of data from CRYSTAL trial	Patients with wild type KRAS had improvements in OS from 20 to 23.5 mo PFS from 8.4 to 9.9 mo and response rates 39.7% to 57.3% with addition of Cetuximab to FOLFIRI
Bokemeyer *et al*[53] OPUS study	2011	315	Wild type KRAS/BRAF *vs* mutant *KRAS/BRAF*	Cetuximab plus FOLFOX		Improved PFS (HR 0.567) and response (OR 2.55) in patients with KRAS wild-type tumors
Alberts *et al*[54] N0137 trial	2012	2686	Wild type KRAS *vs* mutant *KRAS*	Cetuximab plus FOLFOX *vs* FOLFOX alone	Locally advanced disease	No additional benefit of Cetuximab when added to FOLFOX-6 regimen in the setting of locally advance disease

EGFR: Epidermal growth factor receptor; PFS: Progression free survival; OS: Overall survival.

improvement in PFS in a randomized phase Ⅲ clinical trial (PRIME trial; median PFS, 9.6 mo *vs* 8.0 mo; $P = 0.02$)[59]. A small phase Ⅱ trial evaluated Panitumumab in 33 wild-type KRAS metastatic CRC patients whom were deemed ineligible for chemotherapy. The study demonstrated a PFS rate of 36.4%, objective response rate of 9.1% and stable disease in 54.5% of patients. The median PFS was 4.3 mo and OS was 7.1 mo, indicating a role for Panitumumab monotherapy in patients who are not eligible for chemotherapy[60].

EMERGING RESISTANCE TO EGFR BLOCKADE

The promising results observed with monoclonal antibodies either as monotherapy or in combination treatments in metastatic CRC patients were encouraging. However the relatively small magnitude of improvement in PFS was an early sign of emerging resistance to cetuximab therapy. Therefore, further clinical and translational studies were conducted to elucidate possible underlying mechanisms related to EGFR blockade resistance.

EGFR mutations were considered as a possible mechanism for EGFR blockade resistance in CRC patients. However, in a clinical study, only one out of the 293 CRC patients was found to be harboring an EGFR mutation[61]. Considering the rarity of EGFR mutations, they are unlikely to be a common cause of resistance to EGFR blockade in CRC patients.

As mentioned above, a retrospective study first revealed that the *KRAS*-mutant CRC patients did not

benefit from cetuximab therapy[52]. In a study by De Roock *et al*[62], KRAS 13D mutation was reported to be associated with cetuximab response unlike other *KRAS* mutations. However another retrospective study did not confirm this favorable outcome[63]. Whether *KRAS* 13D-mutant CRC tumors have a distinct clinical behavior from other *KRAS* mutations warrants further study. Similar to KRAS, a clinical study found NRAS to be a biomarker of anti-EGFR antibody resistance in CRC patients[64]. This association of NRAS mutations with EGFR blockade resistance needs to be validated in future studies.

Since disruptions downstream of EGFR cause autonomous activation of the signaling pathway and potential intrinsic resistance to EGFR blockade, clinical studies also investigated the utility of *BRAF* mutations as a biomarker in CRC patients (Figure 2). In two clinical trials, patients with CRC harboring *BRAF* mutations were reported to have a poor response to cetuximab; indicating *BRAF* mutations could also limit the efficacy of anti-EGFR treatment[58,65]. A large retrospective multicenter study looking at survival in patients with metastatic CRC and their mutation status; also reported similar findings[64]. Although current evidence strongly suggests that *BRAF* mutations might be a negative biomarker for monoclonal anti-EGFR treatment, guidelines do not currently preclude such patients from this targeted treatment approach.

A study of 23 *PIK3CA*-mutant metastatic CRC patients reported no resistance to anti-EGFR treatment[66]. However, in another cohort of 15 *PIK3CA*-mutant CRC patients, no objective response to panitumumab and cetuximab treatment was observed[67]. A recent study of patients with metastatic CRC harboring wild-type KRAS showed a trend towards improved PFS in patients with wild-type PIK3CA compared to *PI3KCA*-mutant patients who received cetuximab either as a monotherapy or in combination with chemotherapy (P = 0.06)[68]. Furthermore, a meta-analysis of 576 CRC patients reported a significantly lower PFS in patients harboring a *PIK3CA* mutation compared to patients with wild-type PIK3CA when treated with EGFR blockade[69]. Although a majority of the aforementioned studies suggest resistance to EGFR blockade in *PIK3CA*-mutant metastatic CRC patients with wild-type KRAS, the absence of a consensus precludes making a clear conclusion in this specific patient group. A recent study investigated the role of PTEN expression in anti-EGFR blockade therapy and reported potential resistance to cetuximab treatment in metastatic CRC patients with low PTEN expression[70]. Another study also demonstrated a significantly diminished response and worse PFS in patients with loss of PTEN function (32 wk *vs* 14 wk, P < 0.0001)[71]. Loss of PTEN expression was also studied in patients with KRAS wild-type CRC which again suggested a lack of benefit from EGFR blockade[68]. While accumulating evidence supports the hypothesis that PTEN loss may result in potential intrinsic resistance to EGFR blockade in CRC patients, further studies are needed to understand exact role of dysregulation of this gene.

The *MET* proto-oncogene, a receptor tyrosine kinase that belongs to the hepatocyte growth factor receptor, is also related to EGFR blockade resistance in CRC patients[72]. A mechanistic study suggested that, resistance essentially arises in cancer stem cells *via* increased rebound activity of the *MET* oncogene upon inhibition of EGFR signaling by monoclonal antibodies[73]. Another study identified increased Met activity driven by oncogenic Src and this upregulation was found to be related to cetuximab resistance[74]. Increased TGF-α expression mediated by the Met oncogene could be also another oncogenic pathway related to cetuximab resistance[75]. A mechanistic study reported that inhibition of both the Met and EGFR pathways by a bispecific monoclonal antibody may abrogate EGFR blockade resistance[76]. Although these studies suggest that upregulation of this tyrosine kinase pathway may be related to EGFR blockade resistance, the exact mechanism of resistance with Met activity is yet to be elucidated.

POSSIBLE MECHANISMS OF RESISTANCE IN WILD TYPE KRAS PATIENTS

The response to EGFR blockade even in metastatic CRC patients with wild-type KRAS is unfortunately limited: Following an initial honeymoon period, a majority of patients develop disease progression within months of initiation[77]. Researchers investigated pathways possibly related to resistance in wild-type KRAS patients. An animal model study demonstrated that human epidermal growth factor receptor 2 (HER-2) amplification was associated with cetuximab resistance in a subset of metastatic CRC patients with wild-type KRAS, NRAS, BRAF and PIK3CA[78]. The authors also reported prolonged responses with concurrent inhibition of HER-2 and EGFR in their model. Another mechanistic cell line study corroborated the association of aberrant ERBB2 (HER-2) signaling with EGFR blockade resistance[79]. In a retrospective cohort study, HER-3 overexpression was also suggested to be a predictor of EGFR blockade resistance in metastatic CRC patients who underwent cetuximab and irinotecan treatment[80]. Similar to the aforementioned studies, all patients included in this study were also harboring wild-type KRAS. A recent translational study examined the emergence of resistance in CRC cells sensitive to EGFR blockade[81]. The authors examined the ultimate effect of continuous cetuximab treatment on the development of cetuximab-resistant clones. One of the cell lines developed a rescue mechanism by *KRAS* amplification and the other acquired a *KRAS* mutation. In the same study, the authors studied human samples and reported acquisition of *KRAS* mutation in 6 of 10 cases and *KRAS* amplification in another patient who developed resistance to cetuximab after an initial response. In another recent translational study, authors tested their theory with mathematical modeling; they hypothesized that *KRAS*-mutant CRC subclones might exist prior to initiation of anti-EGFR

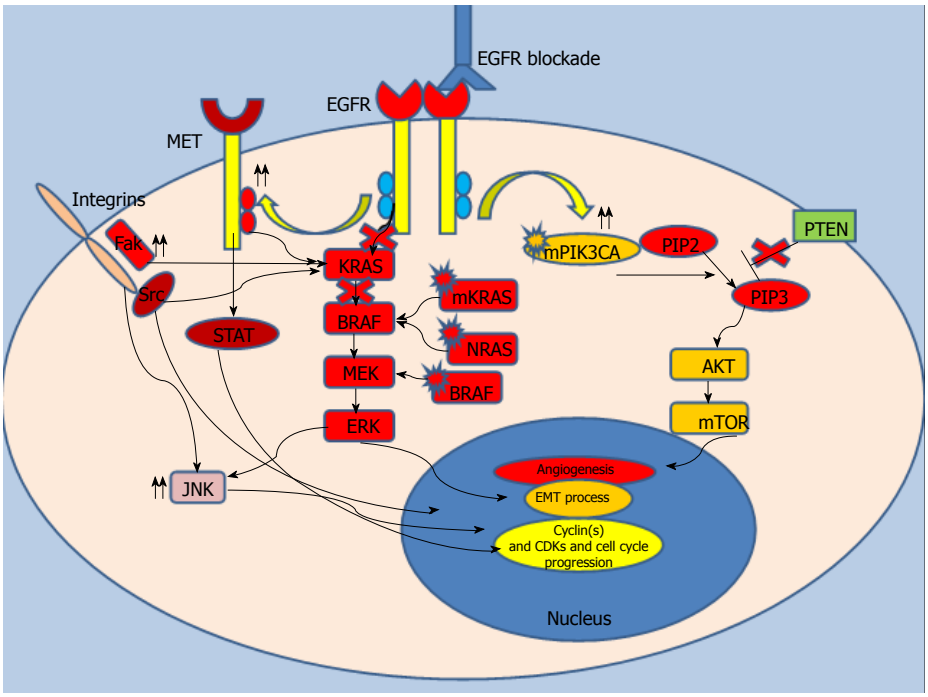


Figure 2 Possible resistance pathways to epidermal growth factor receptor blockade; gain of mutations in KRAS, BRAF, and PIK3CA and upregulation of other receptor tyrosine kinases. EGFR: Epidermal growth factor receptor; mTOR: Mechanistic target of rapamycin; PI3KCA: Phosphotidylinositol-3-kinase.

treatment[82]. According to this model, anti-EGFR monoclonal therapy results in the selective proliferation of resistant subclones. The time required for these subclones to become radiologically detectable corresponds to the time of disease progression and development of EGFR blockade resistance. This hypothesis is also supported by tumor heterogeneity, a consequence of variant clones arising from a single parental clone due to continuous diverse genetic alterations throughout carcinogenesis and metastasis. These diverse genetic signatures have also been demonstrated to cause mixed clinical responses to cytotoxic chemotherapy. Moreover, a recent whole-exome sequencing study of 129 wild-type KRAS CRC tumor samples identified potential resistance mechanisms resulting from mutations in other growth factor pathways such as FGFR, and PDGFRA[83]. This data suggests activation of potential bypass pathways related to other receptor tyrosine kinases abrogating the inhibitory effect of EGFR blockade.

Another possible resistance mechanism to anti-EGFR treatment could be attributed to ineffective structural interaction between the drug and its receptor[84]. Mutations in the extracellular domain of EGFR hindering the binding of cetuximab to EGFR were demonstrated in 2 of 10 study participants with resistance to cetuximab. Moreover, one of those two cases responded to panitumumab. Since the number of the patients in the study was limited, this specific mutation should be further studied in larger populations.

Some recent studies indicate that certain signaling mediators which inhibit apoptosis could potentially rescue malignant cells during initiation of anti-EGFR monoclonal treatments[85,86]. A recent study investigated acquired EGFR blockade resistance by analyzing circulating cancer cells and reported progressive genomic alterations throughout the acquisition of resistance suggesting that genomic instability in cancer cells may execute an important role in EGFR blockade resistance[87]. Activation of other oncogenic tyrosine kinases by the local microenvironment with closed loop feedback might be another key mechanism of resistance in wild type KRAS patients[73]. Further studies are required to elucidate the impact of tumor stroma on EGFR blockade resistance.

FUTURE PERSPECTIVES

Given the limited duration of responses to EGFR blockade and the inevitable development of resistance in both *KRAS* mutant and KRAS wild-type patients, studies were enrolled to optimize treatment outcomes. Based on potential mechanisms of resistance to EGFR blockade identified in preclinical studies, clinical trials have been designed to combat resistance. Considering the role of *PIK3CA* mutations, a phase Ⅱ trial investigated the combination of cetuximab with PX-866, an irreversiblepan-isoform inhibitor of PI3KCA, in 86 patients with KRAS wild type metastatic CRC. The authors reported no improvement in PFS, objective response rate, or OS with the addition PX-866[88]. Combinations of cetuximab with HER-2 and HER-3 targeting agents are currently being studied in clinical trials. The combination of cetuximab with pertuzumab (a monoclonal antibody that disrupts the dimerization of HER-2) showed clinical activity, but produced intolerable side effects and toxicity[89]. Studies

combining cetuximab with HER-3 antagonists have demonstrated promising preliminary data, although final results are yet to be reported[90].

A xenograft study investigated a new agent to overcome *EGFR* mutation-related resistance. Sym004, a novel mixture of two nonoverlapping anti-EGFR monoclonal antibodies, has demonstrated binding and ligand-dependent activation in patients with *EGFR* mutations conferring resistance to conventional anti-EGFR antibodies in functional studies[91]. Phase Ⅰ trials of Sym004 in metastatic CRC patients with wild type *KRAS* mutation and previous EGFR blockade response showed tumor radiologic regression in 17 of 39 patients (44%) and partial response in 5 patients (13%)[92]. Phase Ⅱ clinical trials of Sym004 and early phase studies of other targeting agents of the EGFR pathways are currently being investigated which may enlighten the utility of concurrent/simultaneous inhibition of prosurvival pathways to abrogate EGFR blockade resistance.

CONCLUSION

Current state-of-the-science endorses clinical activity of the EGFR blockade in selected subsets of patients with treatment naïve and refractory metastatic CRC. However, growing evidence has advanced our understanding of the limitations of anti-EGFR treatment.

Although cetuximab and other monoclonal anti-EGFR antibodies effectively inhibit EGFR signaling; their clinical activity is limited to a short period of time. Disease heterogeneity, created by continuous and dynamic genetic alterations, distinct mutational signatures in the EGFR downstream signaling pathways, and rebound activation of other growth signals appears to be the primary driving force rescuing cancer cells from apoptosis. New subclones with new oncogenic fingerprints arising as a consequence of genomic instability, appear to be one of the most challenging factors in the era of targeted treatment. Frequently, this dynamic process and the associated genetic plasticity overcome the inhibitory effect of targeted agents and ultimately, disease progression occurs despite optimal treatment. Moreover, the addiction of cancer cells to a single oncogenic pathway appears to be limited, and the co-activation of rebound survival pathways further limits the impact of a single targeted agent. The lack of durability of the clinical responses to EGFR blockade observed in the aforementioned studies also represents the phenotypic characteristics of dynamic changes in genotype. Whether combinations of targeted agents along with alternate treatment cycles can overcome this acquired resistance requires further clinical studies. Studies are warranted to better understand the underpinnings of dynamic genomic alterations and its role in acquired resistance to targeting agents including EGFR blockades.

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REVIEW

Role of Akt signaling in resistance to DNA-targeted therapy

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Abstract

The Akt signal transduction pathway controls most hallmarks of cancer. Activation of the Akt cascade promotes a malignant phenotype and is also widely implicated in drug resistance. Therefore, the modulation of Akt activity is regarded as an attractive strategy to enhance the efficacy of cancer therapy and irradiation. This pathway consists of phosphatidylinositol 3 kinase (PI3K), mammalian target of rapamycin, and the transforming serine-threonine kinase Akt protein isoforms, also known as protein kinase B. DNA-targeted agents, such as platinum agents, taxanes, and antimetabolites, as well as radiation have had a significant impact on cancer treatment by affecting DNA replication, which is aberrantly activated in malignancies. However, the caveat is that they may also trigger the activation of repairing mechanisms, such as upstream and downstream cascade of Akt survival pathway. Thus, each target can theoretically be inhibited in view of improving the potency of conventional treatment. Akt inhibitors, *e.g.*, MK-2206 and perifosine, or PI3K modulators, *e.g.*, LY294002 and Wortmannin, have shown some promising results in favor of sensitizing the cancer cells to the therapy *in vitro* and *in vivo*, which have provided the rationale for incorporation of these novel agents into multimodality treatment of different malignancies. Nevertheless, despite the acceptable safety profile of some of these agents in the clinical studies, with regard to the efficacy, the results are still too preliminary. Hence, we need to wait for the upcoming data from the ongoing trials before utilizing them into the standard care of cancer patients.

Key words: Phosphatidylinositol 3 kinase/Akt; Platinum; Taxane; Antimetabolite; Radiation

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Core tip: The Akt pathway plays an important role in resistance to several cytotoxic agents, targeted drugs and radiation. Exposure to these drugs will stimulate the Akt survival pathway leading to a decreased response to these drugs. In model systems inhibition of the Akt pathway enhanced the cytotoxicity of drugs like taxanes, antimetabolites, platinum analogs, several targeted drugs

and radiation. Akt inhibitors offer a new opportunity to increase the efficacy of currently used drugs and of radiotherapy.

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AKT PATHWAY SIGNALING OVERVIEW

The Akt signal transduction pathway controls most hallmarks of cancer, including metabolism, cell survival, cell cycle progression, regulation of apoptosis, protein synthesis, motility, and genomic instability by phosphorylation of the substrates[1]. Aberrant loss or gain of Akt activation has been associated with the development of various diseases, *e.g.,* diabetes, autoimmune diseases, and cancer[2-5].

The Akt pathway consists of phosphatidylinositol 3 kinase (PI3K), mammalian target of rapamycin (mTOR), and the transforming serine-threonine kinase Akt protein isoforms (further referred to as Akt), also known as protein kinase B (PKB) and phosphate and tensin homologue (PTEN) as a critical tumor suppressor. PI3K enzymes phosphorylate phosphatidylinositol-4,5-biphosphate (PIP2) to generate phosphatidylinositol-3,4,5-triphosphate (PIP3) at the cell membrane that are required for the recruitment and activation of Akt[6,7] (Figure 1). These phospholipids are constitutively elevated in most cancer cells. Docking of Akt to the cell membrane causes a conformational change, which in turn leads to phosphorylation of the two critical amino acid residues, threonine 308 and serine 473, and finally leads to the activation of Akt[8]. After the activation, Akt is translocated to intracellular compartments where it phosphorylates several substrate proteins. The downstream targets of Akt are numerous due to the multiple interactions with its consensus sequence[1]. In summary, the most important effects of Akt activation are: (1) cell survival through inhibition of BAD, caspase-9, and FOX[9-11]; (2) cell proliferation and gluconeogenesis through inhibition of GSK3, P21, P27, *etc.*[12]; and (3) protein synthesis and cell growth through activation of mTOR[13] (Figure 1).

To date, 3 Akt family members have been identified in mammals, *i.e.,* Akt1 (also known as PKBα), Akt2 (PKBβ) and Akt3 (PKBγ). Having shown highly conserved properties, these homologues may be activated by the same mechanism[14]. However, being encoded by three different regions at 14q32, 19q13, and 1q44, respectively, these three isoforms are distinct substrates with distinct physiological outcomes, and also opposing to each other. Accumulating evidence casts Akt1 and Akt2 function almost in contrary to each other in modulating phenotypes associated with migration and invasion. Akt isoforms contain an N-terminal pleckstrin homology (PH) domain, a central catalytic domain, and a C-terminal regulatory region. The PH domain can bind phosphatidylinositol lipids (*e.g.,* PIP3) with high affinity and targets Akt to the cell membrane[15].

Regulation of Akt, on the other hand, is mainly achieved through PTEN, which antagonizes PI3K. *PTEN* is a tumor suppressor gene that is frequently mutated in different types of cancer, and loss of PTEN leads to elevation of PI3K lipid products and thus activating the Akt pathway[16]. Thus, PTEN negatively regulates the Akt pathway, while loss of PTEN results in overactive Akt, which induces proliferation and promotes survival by inhibiting apoptosis[10,17]. Among the three Akt isoforms, Akt2, is exclusively having carcinogenic properties in PTEN-deficient solid tumors[18].

Despite many breakthroughs in elucidating the cancer behavior and possible mechanisms leading to developing different treatments, resistance is still a problem. The main goal of cytotoxic cancer therapy is to eliminate irregularly dividing cancer cells by targeting DNA synthesis or the mitotic apparatus. Different molecules, genes, proteins and signal transduction pathways are involved in this complicated process[1,19,20]. Resistance is often related to uptake, metabolism or alterations in the target. Besides, many studies demonstrated the modulation of key signaling pathways by the DNA-targeted therapies (reviewed in the following sections). The PI3K/Akt signaling pathway being mutated in a high percentage of malignancies[20] is widely implicated in tumor growth, which may also render tumor cells resistant to chemotherapeutic drugs[5]. Thus, inhibition of this pathway should foil local tumor growth. Many trials are underway to investigate whether adding inhibitors targeting PI3K/Akt pathway may improve the efficacy of the conventional regimen by reducing the apoptotic threshold[21]. Here, we review the literature on the potential value of modulating Akt pathway in view of improving the cytotoxicity of DNA-targeted anticancer drugs and radiotherapy.

METHODS: A SYSTEMATIC BEST EVIDENCE REVIEW

We looked for publications studying the effects of the approved or tested DNA-targeted cytotoxic agents on the Akt signaling using the Medline *via* PubMed database. The inclusion criteria consisted of studies on modulation of Akt signaling by DNA-targeted cytotoxic agents, *i.e.,* platinum agents (cisplatin, carboplatin, oxaliplatin), taxanes (paclitaxel, docetaxel), antimetabolites (gemcitabine, fluorouracil, pemetrexed), and radiation in glioblastoma, mesothelioma and lung, ovary, and pancreas cancers, including their synonyms, with no language restriction as of September 2014. Search terms related to the Akt modulation were "p-Akt" OR "pAkt" OR "phospho-Akt"

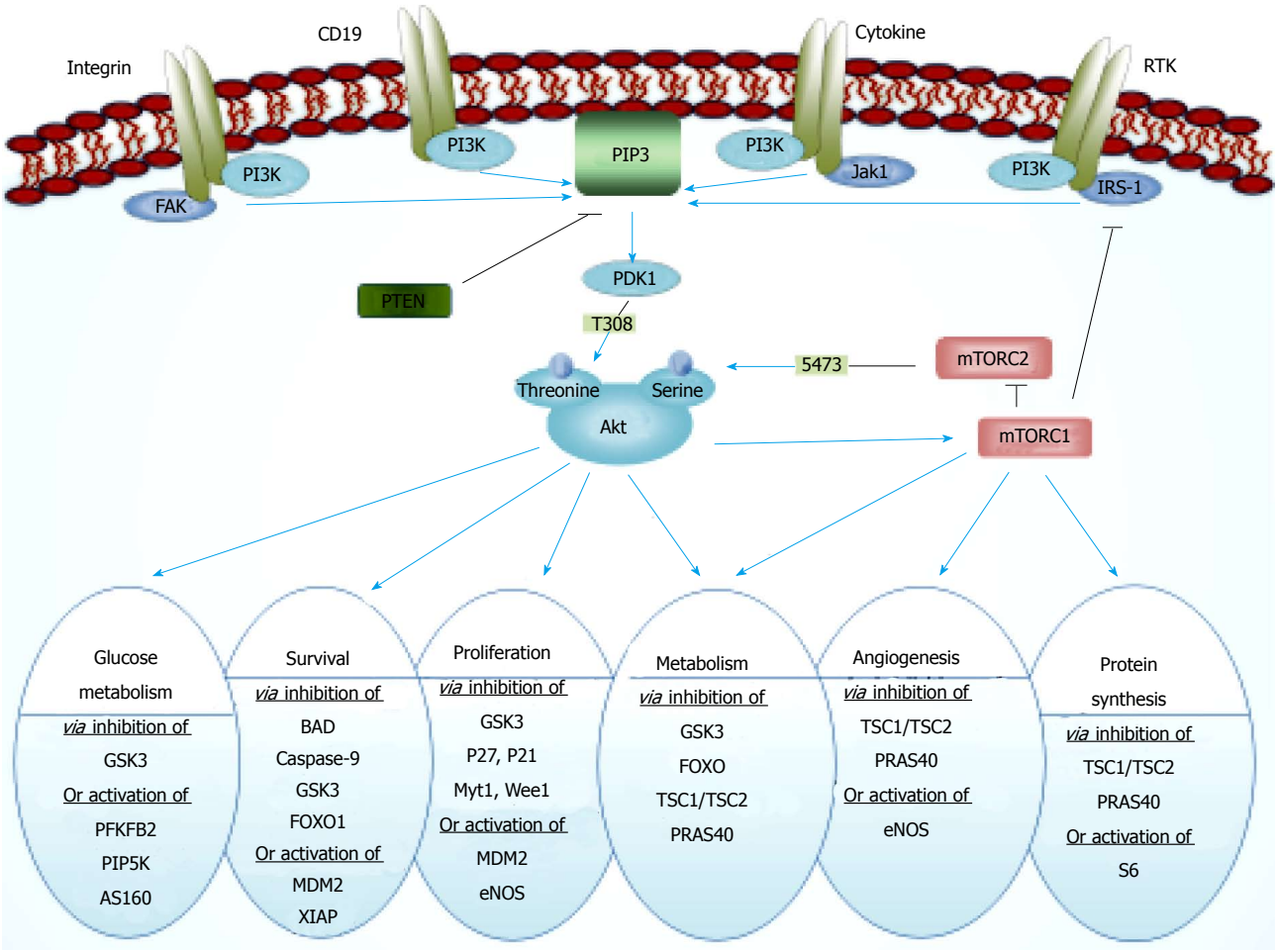


Figure 1 Phosphatidylinositol 3 kinase/Akt pathway. Activated RTKs activate PI3K through direct binding or through tyrosine phosphorylation of scaffolding adaptors, such as IRS1, which then bind and activate PI3K. PI3K phosphorylates PIP2 to generate PIP3, in a reaction that can be reversed by the PIP3 phosphatase PTEN. Activation of PI3K results in membrane recruitment and thus activation of Akt protein. Akt regulates cell growth and many other cellular processes through its effects on mTOR pathways and thus regulates glucose metabolism, protein synthesis, mitochondrial metabolism, lipid metabolism, adipogenesis, lipogenesis, angiogenesis, autophagy, proliferation and cell growth. Other targets of Akt include insulin receptor substrate-1 (IRS-1), glycogen synthase kinase 3 (GSK3), phosphodiesterase-3B (PDE-3), B cell lymphoma-2-associated death promoter (BAD), human caspase-9, Forkhead box (FOX) and nuclear factor kappa-light-chain-enhancer of activated B cells (NFκB) transcription factors, endothelial nitric oxide synthase (eNOS), Rapidly Accelerated Fibrosarcoma (Raf) kinases, P21CIP1/WAF1 (P21; a potent cyclin-dependent kinase inhibitor), P27Kip1, Tuberous Sclerosis Complex 2 (TSC2; also known as Tuberin), X-linked inhibitor of apoptosis protein (XIAP; also known as inhibitor of apoptosis protein 3 and baculoviral IAP repeat-containing protein 4), and Mouse Double Minute 2. Following the activation, Akt phosphorylates and blocks the molecules involved in the apoptotic pathway, including FOX, Caspase-9 and BAD. In addition to the inhibition of proapoptotic factors, Akt can activate the transcription of antiapoptotic genes through the activation of the transcription factor Rel/NFκB. Akt also phosphorylates and activates IκB, which results in IκB degradation by the proteasome. This allows NFκB to translocate from the cytoplasm to the nucleus and activate transcription of a variety of substrates including anti-apoptotic IAP genes, such as the *c-IAP1* and *c-IAP2*. IRS1: Insulin receptor substrate 1; PI3K: Phosphatidylinositol 3-kinase; PIP3: Phosphatidylinositol-3,4,5-trisphosphate; PTEN: Phosphate and tensin homologue; RTK: Receptor tyrosine kinase.

OR "phosphorylat* Akt" OR "Akt phosphorylation" OR "Akt inhibition" OR "Akt modulation" OR "inhibit* Akt" OR "inactivation of Akt" OR "Akt inactivation" OR "activation of Akt" OR "inactivating Akt". Because of a large body of data on this subject, here we narrowed the scope of the current review down to the preclinical and clinical data on the simultaneous administration of cisplatin, paclitaxel, gemcitabine, or pemetrexed with some of the most clinically relevant PI3K/Akt modulators, *i.e.,* PI3K inhibitors, (*e.g.,* LY294002, Wortmannin), PI3K/mTOR inhibitors (*e.g.,* BEZ235), or Akt inhibitors (*e.g.,* perifosine, MK2206), which were tested in combinations with DNA-targeted agents in any of the five types of cancers. We excluded papers not meeting the inclusion criteria as well as retracted publications, duplicates, or non-original papers, *i.e.,* review articles, letters and editorials, comments, and case reports.

For clinical data, we searched for all the registered trials, being planned or performed to study the effect of PI3K/Akt inhibitors, *i.e.,* MK2206, perifosine, LY294002, Wortmannin, BEZ235, in combination with any of the nine DNA-targeted modalities discussed above, *i.e.,* carboplatin, cisplatin, oxaliplatin, paclitaxel, docetaxel, fluorouracil, gemcitabine, pemetrexed, and radiation. All published full text papers or abstracts as well as those with preliminary results in any languages fulfilling selection criteria were

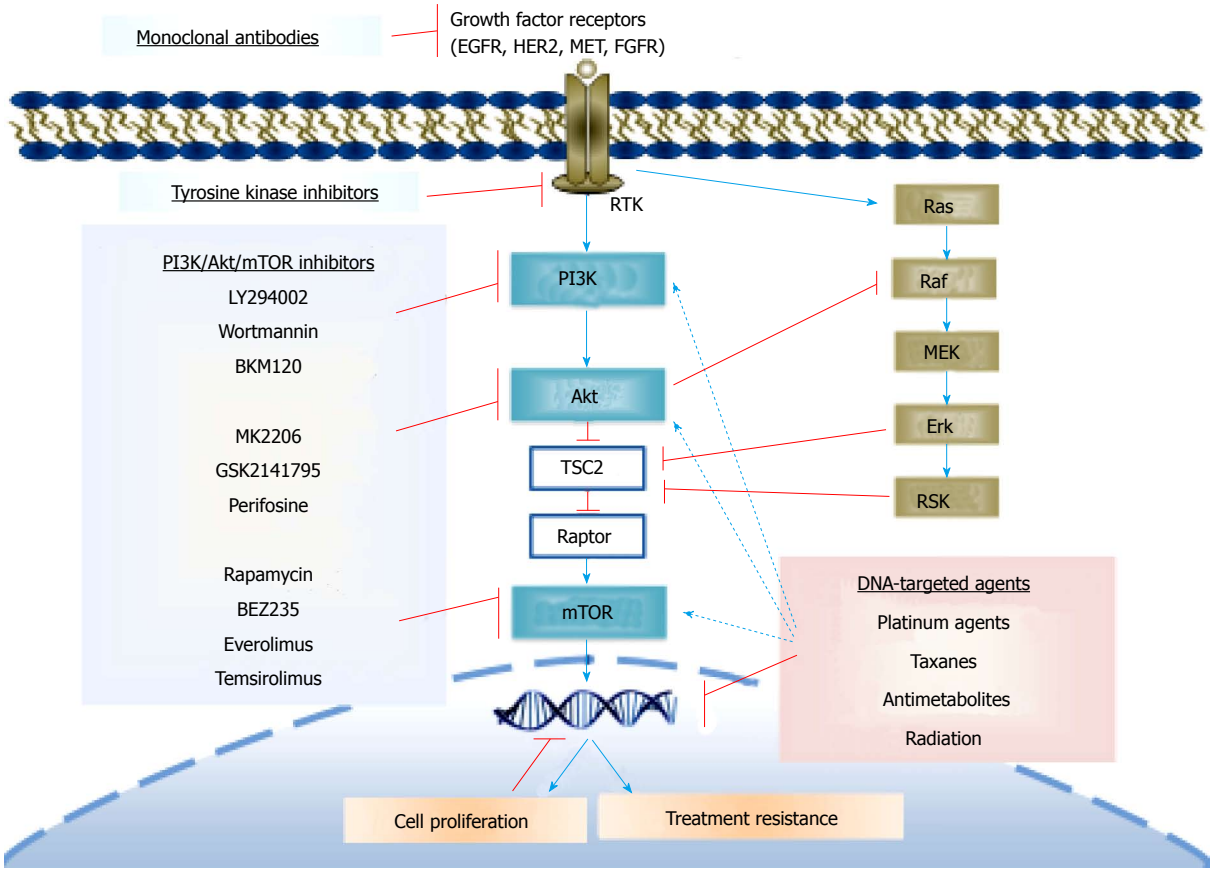


Figure 2 A schematic figure showing the complementary effects of phosphatidylinositol 3-kinase/Akt inhibitors with platinum agents, taxanes, antimetabolites, tumor antibiotics, and radiation resulting in a better cytotoxic profile.

included with no time period restriction. Studies with prior administration of anticancer medications, or combination of drugs targeting other signaling pathways other than Akt, PI3K, and mTOR were excluded. The databases that were used in this phase included the US clinical trials registry (clinicaltrials.gov), NIH clinical research studies (clinicalstudies.info.nih.gov), worldwide clinical trials listings (www.clinicaltrialssearch.org/5-cancer-clinical-trials.html), and the WHO International Clinical Trials Registry Platform (apps.who.int/trialsearch). The latter included 13 registries (Nationalities: Australian, New Zealand, Chinese, American, Canadian, European, Dutch, Brazilian, Indian, Korean, Cuban, German, Iranian, Japanese, Pan African, Sri Lankean, and Thai clinical trials).

AN OVERVIEW ON PI3K/AKT INHIBITORS

Many compounds have been developed to inhibit PI3K, Akt, and mTOR signaling, among which only few were tested in clinical settings (Figure 2). However, they did not yet have significant clinical benefit, except for idelalisib (GS-1101, a p110δ-selective inhibitor), which is the first Food and Drug Administration approved PI3K inhibitor[22], and some mTOR inhibitors, *e.g.,* rapamycin and its analogs. There are six general classes of these agents targeting the Akt network: Pan-class Ⅰ PI3K inhibitors, isoform-selective PI3K inhibitors, rapamycin analogues (rapalogues), active-site mTOR inhibitors, pan-PI3K-mTOR inhibitors and Akt inhibitors[23] (Table 1). Isoformspecific PI3K inhibitors targeting PI3Kβ, inhibitors of ribosomal protein S6 kinase β1 (S6K), PDK1 inhibitors and isoform-selective Akt kinase inhibitors (Akt1 and 2) are also under investigations soon to be tested in the clinic[23].

PI3K is upstream of Akt pathway, and with its inhibition all the subsequent signaling will potentially be down-regulated. Emerging clinical data show limited single-agent activity of inhibitors targeting PI3K. However, the drugs targeting PI3K pathway usually modulate the myriad substrates, including Akt and/or mTOR. LY294002, Wortmannin, and perifosine as PI3K/Akt inhibitors, BEZ235 as dual PI3K/mTOR inhibitor, and MK2206 as a specific Akt inhibitor are some of the commonly used drugs in this category that modulate Akt signaling. LY294002 is a morpholine-containing chemical compound that is a potent reversible inhibitor of PI3K signaling. Wortmannin is a steroid metabolite of the fungi Penicillium funiculosum with an irreversible inhibitory effect on PI3K. Perifosine is an orally active alkylphospholipid analog, which targets cell membrane and modulates different

Table 1 Drugs targeting phosphatidylinositol 3 kinase/Akt/mammalian target of rapamycin pathway

PI3K/Akt/mTOR subgroups	Agents	Clinical Stage
Pan-PI3K inhibitors	XL147	Phase Ⅱ
	BKM120	Phase Ⅲ
	GDC0941	Phase Ⅱ
Rapalogues (mTORC1 inhibitors)	Sirolimus	Phase Ⅲ
	Everolimus	Approved
	Temsirolimus	Approved
	Ridaforolimus	Phase Ⅲ
mTORC1/2 inhibitors	INK128	Phase Ⅱ
	AZD8055	Phase Ⅰ
	OSI027	Phase Ⅰ
PI3K–mTOR inhibitors	BEZ235	Phase Ⅱ
	XL765	Phase Ⅱ
	GSK1059615	Phase Ⅰ
Isoform-specific PI3K inhibitors	CAL-101 (p110δ)	Phase Ⅲ
	INK1117 (p110α)	Phase Ⅰ
	BYL719 (p110α)	Phase Ⅱ
Akt inhibitors	Perifosine	Phase Ⅲ
	MK-2206	Phase Ⅱ
	GDC0068	Phase Ⅱ
	GSK690693	Phase Ⅰ

PI3K: Phosphatidylinositol 3 kinase; mTOR: Mammalian target of rapamycin.

signaling pathways, and Akt in particular[24,25].

INDIRECT ALTERATION OF AKT SIGNALING AND ITS MODULATION

We finally selected 65 papers being suited to this review (Figure 3; Table 2) by which combination of a PI3K/Akt inhibitor with any of cisplatin, paclitaxel, gemcitabine, or pemetrexed was studied. The results are precisely discussed in the following sections.

Effect of platinum analogs on Akt signaling

Cisplatin, carboplatin and oxaliplatin are the 3 most commonly used anticancer platinum analogs. The main antitumor properties of cisplatin are attributed to the formation of platinum-DNA adducts causing DNA bending[26], which interferes with DNA replication, transcription and other nuclear functions leading to the inhibition of cellular proliferation and tumor growth. Intrinsic and acquired resistance limits the efficacy of platinum drugs in cancer treatment. Decreased drug uptake along with increased influx or inactivation by sulfhydryl molecules, such as glutathione, or increased DNA adduct repair can result in platinum resistance[27,28]. Wang and Lippard[29] suggested that induction of signaling pathways might be an alternative mechanism of resistance to platinum analogs.

Some data suggest that cell death induced by cisplatin may occur through regulation of cell cycle[30-32]. Mitsuuchi *et al*[32] demonstrated that PI3K, Akt1, and Akt2 are required for p53 protein expression and the full induction of p21 in ovarian cancer cells treated with cisplatin. Liu *et al*[33] suggested that Akt1 expression regulates cisplatin resistance in lung cancer cells through mTOR pathway, and its inhibition may sensitize cells to cisplatin. Moreover, amplification of a catalytic subunit of PI3K (PIK3CA) was also found to be associated with the risk of resistance to platinum-based chemotherapy in a group of patients with ovarian cancer, but not with the overall survival[34]. Thus vailable data support the probable role of Akt amplification/overexpression in platinum-resistance *in vitro* and *in vivo*[33,35-43].

The PTEN (encoding PIP3 phosphatase) and PIK3CA (encoding the PI3K catalytic isoform p110α) are the two most frequently altered mutated tumor suppressor and oncogenes, respectively[23]. Moreover, a low level of the PTEN expression is associated with amplified PIK3CA expression and finally PI3K/Akt activity[42]. Other data suggest that loss of the FHIT (fragile histidine triad; an inhibitor of Akt signaling) and overexpression of Redd1 (an inhibitor of mTOR signaling) are associated with cisplatin resistance in lung cancer cell lines[44-46]. Furthermore, overexpression of ADAM17 (a disintegrin and metalloproteinase-17) has been found to be associated with hypoxia-induced cisplatin resistance in hepatocellular carcinoma cells through activation of EGFR/PI3K/Akt pathway *in vitro*[47]. ADAM17 is a member of the metalloproteinase superfamily involved in the cleavage of ectodomain of many transmembrane proteins. Besides, prostate apoptosis response-4 (a proapoptotic tumor suppressor protein) downregulation was associated with cisplatin resistance in pancreatic cancer cells through upregulation of PI3K/Akt signaling *in vivo*[31]. Given that cisplatin activates PI3K/Akt signaling, downregulation of this pathway may bypass cisplatin-resistance. Akt pathway overactivation may decrease cisplatin sensitivity and cause treatment resistance even in platinum sensitive cells, whereas downregulation of Akt can boost the drug sensitivity and resistance to platinum compounds like cisplatin[36,39].

Effect of Akt-inhibition on platinum sensitivity

Some studies did not find treatment sensitization by adding LY294002 to cisplatin[48,49]. However, the PI3K/Akt inhibitors, LY294002, Wortmannin, or BEZ235 in combination with cisplatin showed synergistic or additive effects against malignant mesothelioma and lung cancer[44,50-55], pancreatic cancer[30], ovarian cancer[41-43,56-61], as well as glioblastoma[62,63] *in vitro* and *in vivo* (Table 3).

Perifosine increased the antineoplastic activity of cisplatin in ovarian[25,87], endometrial[91], and lung[44,54,55,92] cancer cells by activating apoptotic pathways and thus enhancing the cytotoxicity of cisplatin. Likewise, the specific Akt inhibitor MK-2206 showed synergism when combined with cisplatin in lung cancer[51,55], gastric cancer[93], and nasopharyngeal carcinoma cells[94] *in vitro* and *in vivo*. It can be concluded that activation of the Akt survival pathway plays a pivotal role in platinum resistance, and inhibition of Akt may enhance the effect of this type of anticancer drug.

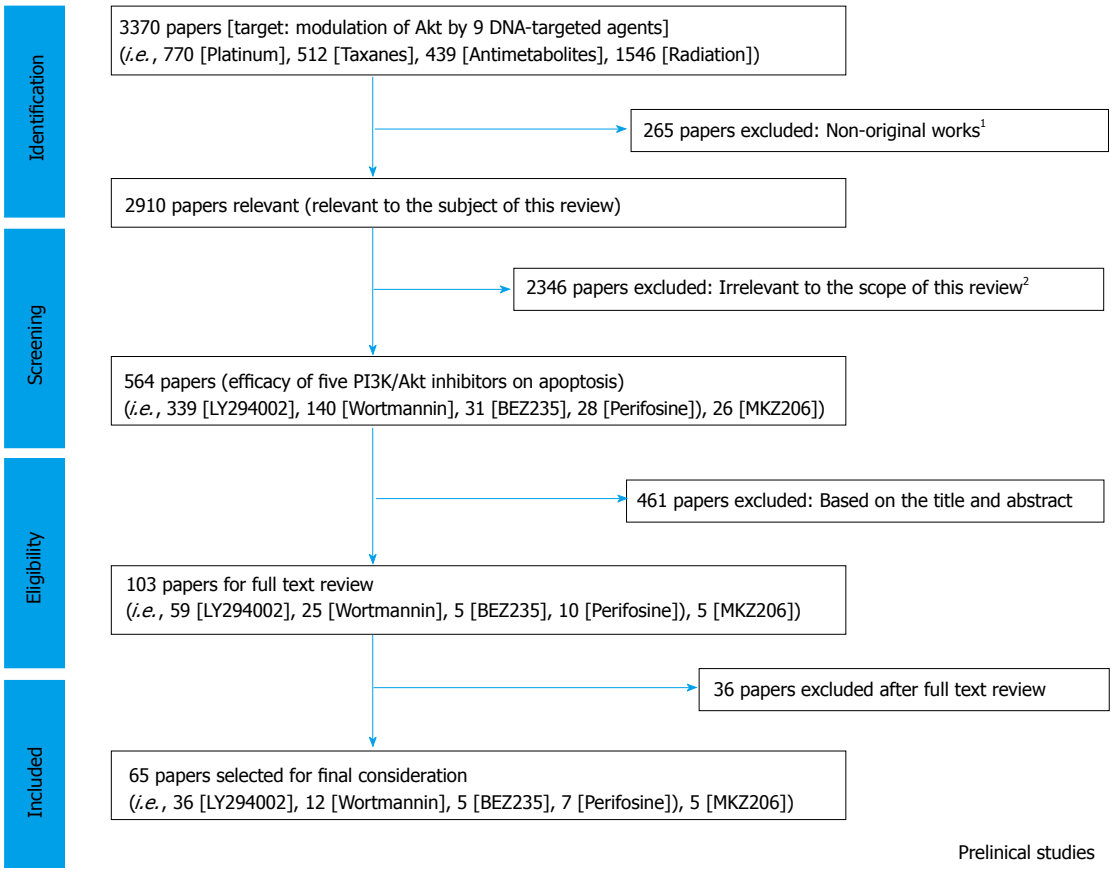


Figure 3 Review flow diagram of the publication selection in preclinical category. [1]Exclusion criteria include the retracted publication, duplicate publication or non-original papers, including review articles, letters and editorials, comments, case reports, *etc.*; [2]According to the scope of the current review: Efficacy of Akt modulation by 9 DNA-targeted agents in 5 types of cancer, including lung cancer, malignant mesothelioma, pancreatic cancer, ovarian cancer, and malignant glioma. PI3K: Phosphatidylinositol 3 kinase; EGFR: Epidermal growth factor receptor; mTOR: Mammalian target of rapamycin.

Table 2 Systematic chart of searching methodology and the results based on PubMed

DNA-targeted therapies	Agents	Akt modulation							
		All papers[1]	Non-original[2]	Relevant papers[3]	PI3K/Akt inhibitors[4]				
					LY294002	Wortmannin	BEZ235	Perifosine	MK2206
Platinum	Carboplatin	68	6	51	3	1	0	0	4
	Cisplatin	631	15	589	85	22	8	9	5
	Oxaliplatin	71	5	59	5	2	0	0	0
Taxane	Docetaxel	149	13	127	9	2	3	1	3
	Paclitaxel	363	18	331	47	15	4	2	5
Antimetabolite	Fluorouracil	191	5	174	18	6	0	2	3
	Gemcitabine	213	14	191	16	8	3	1	1
	Pemetrexed	35	3	32	5	2	1	0	0
Radiation	Irradiation/ radiation	1649	186	1356	151	82	12	13	5
Total articles	(9 agents)	3370	265	2910	339	140	31	28	26

[1]Terms used in the search: Query agent/radiation, and Akt, within whole the article with no language limitation, as of September, 2014; [2]Including the retracted publication, duplicate publication or non-original papers, including Review articles, Letters and Editorials, Comments, Case Reports, *etc.*; [3]Relevant with regard to the modulation of Akt by the query agent. [p-Akt OR pAkt OR "phospho-Akt" OR (phosphorylat* Akt) OR "Akt phosphorylation" OR "Akt inhibition" OR "Akt modulation" OR (inhibit* Akt) OR "inactivation of Akt" OR "Akt inactivation" OR "activation of Akt" OR "inactivating Akt"]; [4]Suppressing Akt cascade by a PI3K/Akt inhibitor to sensitize the query agent. PI3K: Phosphatidylinositol 3 kinase.

Effect of taxanes on Akt signaling

Taxanes, *e.g.*, paclitaxel and docetaxel, are frontline therapy for several cancers. They stabilize microtubules, leading to cell cycle arrest through centrosomal impairment, induction of abnormal spindles and suppression of spindle microtubule dynamics, finally triggering apoptosis[95]. Microtubules are critical for the integrity of the segregated DNA during mitosis. However, inherent or acquired resistance to taxanes

Table 3 Studies evaluating the efficacy of phosphatidylinositol 3 kinase phosphatidylinositol 3 kinase/Akt modulators on the apoptotic profile of cisplatin, paclitaxel, gemcitabine and pemetrexed

PI3K/Akt inhibitor and DNA-targeted agent combination	Akt modulation (phosphorylation)							
	Lung cancer and mesothelioma		Pancreatic cancer		Ovarian cancer		Malignant glioma	
	Synergistic	Antagonistic	Synergistic	Antagonistic	Synergistic	Antagonistic	Synergistic	Antagonistic
LY294002/Cisplatin	1[51]a	1[50]	2[30,31]	-	6[42,56-60]	1[49]	2[62,63]b	1[48]
LY294002/Paclitaxel	2[64,65]	2[50,66]	-	-	7[32,56,67-71]b	-	1[63]	-
LY294002/Gemcitabine	1[72]	1[73]	4[74-77]b	1[78]	1[56]	-	-	-
LY294002/Pemetrexed	1[79]	1[50]	-	-	-	-	-	-
Wortmannin/Cisplatin	1[52]		-	-	3[41-43]	-	-	-
Wortmannin/Paclitaxel	1[80]	-	-	-	1[70]	-	1[80]	-
Wortmannin/Gemcitabine	-	-	5[74,81-84]	-	-	-	-	-
Wortmannin/Pemetrexed	1[79]	-	-	-	-	-	-	-
BEZ235/Cisplatin	1[53]	-	-	-	1[61]	-	-	-
BEZ235/Paclitaxel	-	-	-	-	1[61]	-	-	-
BEZ235/Gemcitabine	-	-	1[85]	-	-	-	-	-
BEZ235/Pemetrexed	1[86]	-	-	-	-	-	-	-
Perifosine/Cisplatin	2[44,54]	-	-	-	2[25,87]	-	-	-
Perifosine/Paclitaxel	-	-	-	-	1[88]	-	-	-
Perifosine/Gemcitabine	1[54]b	-	-	-	1[89]	-	-	-
Perifosine/Pemetrexed	-	-	-	-	-	-	-	-
MK2206/Cisplatin	2[51,55]	-	-	-	-	-	-	-
MK2206/Paclitaxel	-	-	-	-	-	-	-	-
MK2206/Gemcitabine	2[54,90]b	-	-	-	-	-	-	-
MK2206/Pemetrexed	-	1[54]c	-	-	-	-	-	-
Total	17	6	12	1	24	1	4	1

[a]Chowdhry *et al*[51]. Reporting an increased sensitivity, but synergism not evaluated; [b]Shingu *et al*[62,63], Kawaguchi *et al*[69], Pinton *et al*[54] additive enhancement of proliferative inhibition; [c]Holcomb *et al*[76] LY294002 combination with gemcitabine showed additive effects on proliferative inhibition in PANC-1 and synergistic in $PaCa^{2+}$ pancreatic cancer cell lines. However, pAkt levels rebounded at later time points. PI3K: Phosphatidylinositol 3 kinase.

may compromise their therapeutic efficacy[96,97].

Resistance to taxanes includes increased efflux, and modification in tubulins. Akt pathway activation contributed to an increased resistance to paclitaxel or docetaxel in epithelial ovarian cancer, prostate cancer, and breast cancer cells[98-101]. Activation of Akt1 by HER2/PI3K may also lead to taxane resistance in breast adenocarcinoma cells[102]. Moreover, *PIK3CA* gene, encoding a catalytic subunit of the PI3K, is mutated and/or amplified in various neoplasms, such as ovarian cancer. Its amplification strongly decreased the sensitivity and thus response to platinum with/without taxanes in patients with ovarian carcinoma[34].

There are also crosstalks between PI3K/Akt pathway with BAD and ERK[41,68], and inhibition of these cascades sensitized ovarian cancer cells to taxanes. Therefore, in order to sensitize taxane treatment, PI3K/Akt cascade can be considered as a suitable target.

Effect of Akt-inhibition on taxane sensitivity

LY294002, Wortmannin, BEZ235, or perifosine-mediated inhibition of the PI3K/Akt-dependent survival pathway enhanced paclitaxel cytotoxicity in various cancers, *e.g.*, malignant glioma[63,80], lung[50,64-66,80], esophageal[64,80], and ovarian cancer cells[32,56,61,67-70,88] (Table 3). However, there are some data not in favor of the combination. LY294002 did not potentiate cisplatin, pemetrexed, or paclitaxel in A549 lung adenocarcinoma cells harboring K-ras mutation and wild-type EGFR[50]. Likewise, inactivation of PI3K/Akt signaling by LY294002 did not result in significant alteration of sensitivity of human ovarian carcinoma A2780 cells to paclitaxel[48]. Similarly, the combination of paclitaxel with LY294002 was antagonistic *in vitro* when dexamethasone was also administered; although dexamethasone did not alter the Akt activity[66].

Activation of NFκB is linked to Akt-dependent transcription of pro-survival genes[103]. Thus, LY294002-mediated suppression of the PI3K/Akt survival pathway with secondary inhibition of NFκB transcriptional activity is associated with enhancement of paclitaxel cytotoxicity in lung, esophageal and ovarian cancer cells[64,104,105], which indicates that NFκB may be the crucial intermediary step connecting Akt to the intrinsic susceptibility of cancer cells to paclitaxel.

Additionally, the Akt inhibitor MK-2206 augmented the efficacy of paclitaxel and carboplatin combination in gastric cancer[106], breast cancer[107], and melanoma cells[108]. However, addition of MK-2206 to paclitaxel alone had no additive inhibitory effect on growth of nasopharyngeal carcinoma cells *in vitro*[90]. Furthermore, Hirai *et al*[90] found that synergy of MK-2206 with docetaxel was dependent on the treatment sequence, in which a schedule of MK-2206 before docetaxel was not effective in terms of growth inhibition. Dual inhibition of PI3K and mTORC1/2 by BEZ235 may overcome docetaxel resistance in human castration resistant prostate cancer *in vitro* and *in vivo*[109]. Thus, modulation of the PI3K/Akt signaling may increase the efficacy and potency of taxanes according

to *in vitro* and *in vivo* data. However, the effect may have been masked by inclusion of platinum in several studies, indicating that in some studies, the effect might be *via* platinum.

Effect of antimetabolites on Akt signaling

Antimetabolites are a large group of anticancer drugs widely used in combination therapy of various leukemias and solid tumors. They interfere with DNA and RNA synthesis and therefore the growth of tumor[110]. Antimetabolites are categorized as pyrimidine analogs [*e.g.,* 5-fluorouracil (5-FU), gemcitabine], purine analogs (*e.g.,* azathioprine, mercaptopurine), and antifolates (*e.g.,* methotrexate, pemetrexed). In the present review, we mainly focused on two commonly used antimetabolites gemcitabine and 5-FU, as well as the novel anti-folate pemetrexed.

Effect of gemcitabine on Akt signaling and effect of Akt inhibition: Gemcitabine is used in the treatment of various carcinomas, such as lung cancer, bladder cancer, breast cancer, pancreatic cancer, and lymphomas[111]. A substantial number of potential biomarkers for sensitivity or resistance to gemcitabine have been characterized, including ribonucleotide reductase, deoxycytidine kinase, cytidine deaminase and human equilibrative transporter-1[112,113]. Additional mechanisms of resistance may exist, possibly not involving metabolism and direct targets[74,75,112,114].

Gemcitabine resistance in breast cancer cells may also be mediated by activation of the PI3K/Akt signaling pathway through phosphorylated Akt[115], so that inhibitors of PI3K/Akt might reverse the resistance to gemcitabine. Moreover, involvement and overexpression of PI3K and phosphorylated Akt in pancreatic carcinoma tissues has been reported in gemcitabine-resistant cells *in vitro*[73,78,84]. Rad51 overexpression may also mediate gemcitabine resistance through Akt or ERK1/2 activation in non-small cell lung cancer (NSCLC) cells, which could be overcome by downregulation of Rad51 or inhibition of Akt and ERK1/2 proteins[72]. Although Akt phosphorylation status is tailored as a predictive biomarker for gemcitabine resistance in NSCLC patients[116], gemcitabine may also reduce Akt phosphorylation without affecting the Akt overall expression[117]. Wilson *et al*[73] reported a weak correlation between phosphorylated S6K and phosphorylated Akt, suggesting the existence of Akt-independent regulation of mTOR-mediated resistance to apoptosis. Overall, inhibition of PI3K/Akt signaling may enhance the gemcitabine cytotoxic profile.

Wortmannin enhanced the efficacy of gemcitabine by a 5-fold increase of apoptosis in murine pancreatic xenografts[81]. A synergistic effect of Wortmannin, LY29 4002, and BEZ235 with gemcitabine was also reported in ovarian cancer[56] and pancreatic carcinoma[74-77,81-85] *in vitro* and *in vivo* (Table 3). Although gemcitabine induces cell cycle arrest at the G1 and early S phases, PI3K/Akt activation does not seem to influence gemcitabine-induced cell cycle arrest[84]. Likewise, perifosine has shown additivity in combination with gemcitabine by inhibiting Akt1 and Akt3 axis, and interfering Akt upstream, EGFR, and MET phosphorylation[54]. Perifosine also enhanced the gemcitabine-mediated antitumor effect on pancreatic cancer cells through blocking p70S6K1 (S6K1) activation, and thus disrupting S6K1-Gli1 association and subsequent Gli1 activation[89]. Besides, Akt[118], mTOR[119], and MAPK[120] may also activate Gli1. Likewise, the Akt inhibitor MK2206 enhanced the effect of gemcitabine on growth inhibition *in vitro* and *in vivo*[90]. In the contrary, Arlt *et al*[78] found that NFκB, rather than PI3K/Akt, activity conferred resistance to gemcitabine in a panel of five pancreatic carcinoma cell lines, which was strongly diminished by NFκB inhibitors, and not by LY294002. Overall, the PI3K/Akt inhibitors have been efficacious in improving gemcitabine cytotoxicity.

Effect of FU on Akt signaling and effect of Akt inhibition: 5-FU is an antimetabolite that acts by inhibition of thymidylate synthase (TS) and can be incorporated into RNA and DNA altering the cancer cell replication and proliferation[121]. 5-FU-based regimens are often used in adjuvant chemotherapy regimens and treatment of various advanced malignancies, such as colon cancer, head and neck cancer, breast cancer, but depending on the disease and stage of the tumor, intrinsic resistance to 5-FU can be as high as 50%[122]. Resistance to 5-FU has often been associated with an increased TS expression, both transient and permanent[123]. Other factors, such as enzymes involved in pyrimidine metabolism, *i.e.,* increased dihydropyrimidine dehydrogenase, decreased orotate phosphoribosyltransferase, or altered folate metabolism have been associated with 5-FU resistance[121,124,125]. Moreover, 5-FU has major effects on glycosylation pathways as well[126], which may indirectly have effects on signaling pathways. Hence, evidence is accumulating that 5-FU resistance is associated with altered signaling.

Smad4 deficiency may also contribute to 5-FU resistance through upregulation of vascular endothelial growth factor expression, which is associated with increased vascular density[127,128]. Zhang *et al*[129] found that loss of Smad4 in colorectal cancer patients may induce resistance to 5-FU through activation of Akt pathway. Akt can interact with Smad molecules to regulate transforming growth factor beta (TGF-β) signaling that is involved in transmitting chemical signals from the cell surface to the nucleus[130-132]. In summary, suppression of PI3K/Akt signaling may potentiate 5-FU.

The combination of LY294002 with 5-FU was synergistic *via* downregulation of PI3K/Akt signaling in Smad4-deficient colorectal cancer cells[129]. Likewise, sequential combination of 5-FU and LY294002 induced synergistic cytotoxicity and overcame intrinsic and acquired resistance of 5-FU *via* downregulation of Akt and mitochondria-dependent apoptosis in an Epstein-Barr virus positive gastric cancer cell line[133]. Wortmannin also promoted 5-FU antitumor activity in oral squamous cell carcinoma[134] and breast cancer cells[135]. In colorectal cancer cell lines, preclinical

studies indicate that perifosine and BEZ235 may enhance the cytotoxic effects of 5-FU, likely through the NFκB and thus PI3K/Akt pathway[136]. As a result, PI3K/Akt pathway is a rational target for sensitizing the tumor cells to 5-FU.

Effect of pemetrexed on Akt signaling and effect of Akt inhibition: Pemetrexed (Alimta; formerly known as LY231514), a multitargeted antifolate, inhibits thymidylate synthase (TS), dihydrofolate reductase, and the de novo purine nucleotide synthesis[137]. Pemetrexed is currently used as a single agent, but more often in combination with cisplatin for first line treatment of non-squamous NSCLC and malignant pleural mesothelioma[138-140]. Resistance to pemetrexed has been associated with TS upregulation in a colon cancer cell line[123,141], a transport deficiency, decreased activation by folylpolyglutamate synthetase and increased efflux[139]. Members of the ATP-binding cassette (ABC) transporters including P-glycoprotein (Pgp/ABCB1), multidrug resistance proteins (MRPs/ABCC) as well as breast cancer resistance protein (BCRP/ABCG2) as ATP-dependent drug efflux transporters may play roles in pemetrexed resistance[142]. The PI3K/Akt pathway regulates ABCG2-mediated drug efflux, which induces drug resistance[86,143-145].

Pemetrexed can also activate Akt signaling[79,143,146,147], although its molecular mechanisms is not completely understood. Chen *et al*[79] have observed a pemetrexed-induced cell apoptosis and a parallel increase in sustained Akt phosphorylation and nuclear accumulation in the NSCLC A549 cell line, and postulated that the activated Akt may play a proapoptotic role, while Giovannetti *et al*[147] observed that pemetrexed increased EGFR phosphorylation and slightly reduced Akt phosphorylation and enhanced apoptosis in six NSCLC cell lines[143,146] and malignant pleural mesothelioma (MPM) cells, particularly when combined with EGFR inhibitor erlotinib or carboplatin.

Adding a PI3K/Akt inhibitor may further increase pemetrexed antineoplastic effect. LY294002 and Wortmannin decreased the pemetrexed-stimulated Akt and GSK3β phosphorylated activation in the NSCLC A549 cell line[79] (Table 3). Perifosine antagonized the effect of pemetrexed in MPM cells by interfering upstream of Akt, affecting EGFR and MET phosphorylation[54]. Likewise, BEZ235 enhanced the antineoplastic effect of pemetrexed in malignant pleural mesothelioma by decreasing ABCG2-mediated drug efflux at the cell surface[86], which may be of therapeutic value in combination regimens. These data suggest that combining pemetrexed with a PI3K/Akt inhibitor may result in a better antineoplastic effect in various tumors.

Effects of irradiation and chemoradiation on Akt signaling: The combination of radiation with cytotoxic chemotherapy has become a standard treatment option for the majority of inoperable, locally advanced cancers, including brain, head and neck, lung, and gastrointestinal malignancies[148]. However, resistance to irradiation compromises therapeutic efficacy leading to tumor recurrence or metastasis. Tumors that recur after a successful radiation are often associated with radioresistance[149]. Resistance to radiotherapy is predominantly related to efficient repair of the DNA damage induced by X-ray. Both normal and neoplastic cells have several types of repair pathways, usually starting with the recognition and excision of the lesion, and then insertion of a new nucleotide. Regulation of several of these repair enzymes is mediated through methylation of the gene or activation of various protein kinases[150]. Given the complex biology underlying the interactions between the targeted agent and chemoradiation, comprehensive preclinical investigations are critical to design the rational combination[148].

Different combinations of drugs and radiation have been studied to improve efficacy and lessen toxicity. Chemotherapeutic drugs that perturb nucleotide metabolism have the potential to produce substantial sensitization of tumor cells to radiation treatment. Redistribution of cells into S-phase of the cell cycle and depletion of deoxynucleotide pools are probable mechanisms for gemcitabine and 5-FU, which made them potent radiosensitizers[151,152].

Radiation can activate multiple signaling pathways in cells[153], such as EGFR and several downstream proteins, *i.e.,* PI3K/Akt, MAPK JNK, p38, NFκB, *etc.,* stimulating DNA repair and thus causing radioresistance and survival of tumor cells. Loss of PTEN[154], as well as *KRAS* mutations[155,156] and NF-κB activation[157,158] also are associated with radioresistance, making the DNA less susceptible to ionizing radiation. Additionally, the ability of radiation to activate signaling pathways may depend on the expression of growth factor receptors, *RAS* mutation, and autocrine or paracrine ligands such as TGF-α, TGF-β, HB-EGF, neuregulins, and interleukin 6[153].

Effect of Akt-inhibition on radiation sensitivity: Alkylphosphocholines may potentiate the effect of radiation if given before or together with radiotherapy[159]. Targeting the PI3K pathway by LY294002 led to radiosensitization in glioblastoma[154] and human bladder cancer cell xenografts[160] *in vivo.* BEZ235 has also shown a modest antitumor response *in vivo*, while the combination of BEZ235 and ionizing radiation provided a longer survival and led to a smaller tumor volume when compared to radiation alone[161]. Likewise, the PI3K inhibitor BKM120 inhibited the radiation-induced activation of Akt[162]. This induced suppression of DNA-double-strand breaks repair and increased apoptosis, which resulted in increased sensitivity of liver cancer cells to irradiation[162]. Perifosine showed some radiosensitization in squamous cell carcinoma[163-165], malignant glioma[166], lymphoma[167], and prostate cancer[168]. In contrast, one study failed to show any favorable results with perifosine in terms of increasing its anticancer potency, despite a significant reduction in the level of phosphorylated Akt as well as Akt activity *in vitro* and *in vivo*[169]. Overall, given the activation of PI3K/Akt pathway by radiation, addition of a PI3K/Akt inhibitor may potentiate the therapeutic index of the conventional

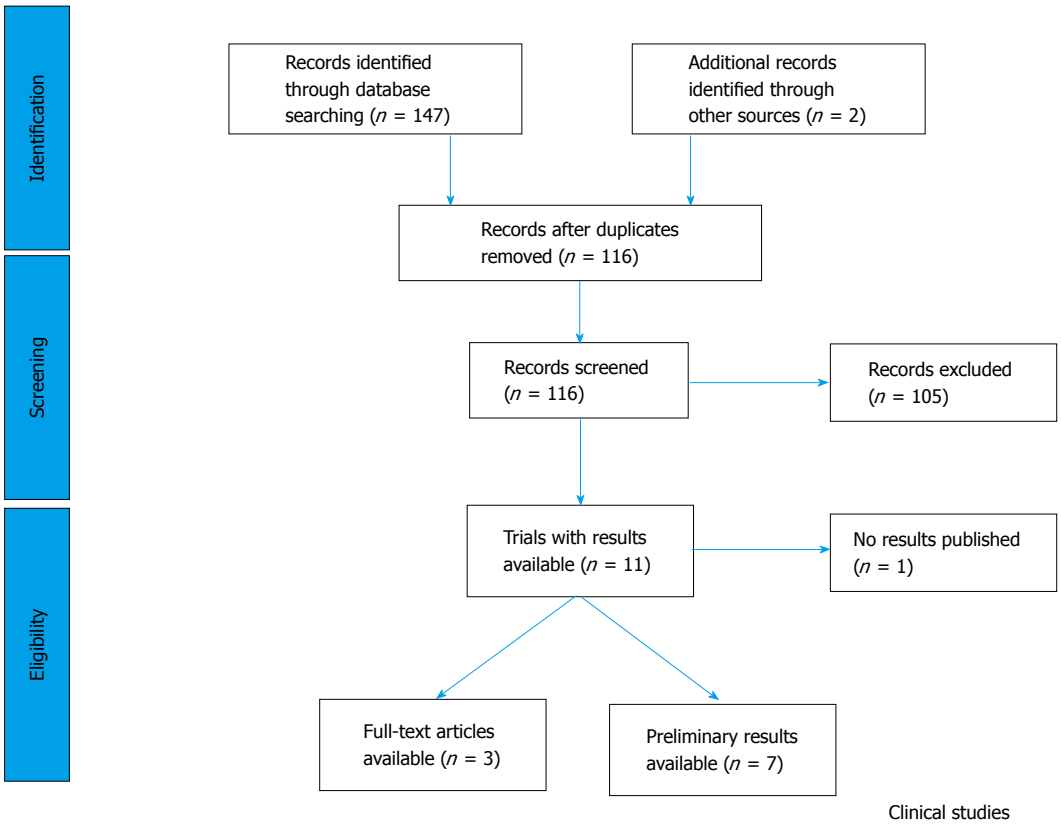


Figure 4 Review flow diagram of the publication selection in clinical category.

chemoradiation therapy.

PI3K/AKT INHIBITORS IN THE CLINIC

In the trial databases, we found 147 studies (Figure 4), which were designed to test the clinical profile of the six PI3K/Akt inhibitors. Only 11 trials - three published - were planned to assess the efficacy and safety of any of the PI3K/Akt inhibitors in combination with DNA-targeted agents (Table 4). We could not find any clinical trials being reported or registered to study the safety and efficacy of LY294002 or Wortmannin.

MK-2206

From 36 trials on MK-2206, registered between April 2008 (the first trial) and May 2013 (the last trial), to evaluate the safety and efficacy of MK-2206; only three suited our selection criteria (Table 4).

In a phase Ⅰ study with 72 patients with advanced solid tumor, Molife *et al*[170] (clinicaltrials.gov; NCT00848718) demonstrated that MK-2206 (45 or 60 mg every other day) plus carboplatin [area under the curve 6.0 mg/mL (AUC 6)] and paclitaxel (200 mg/m^2), docetaxel (75 mg/m^2), or erlotinib (100 or 150 mg daily) was well-tolerated, with early evidence of antitumor activity. The main dose-limiting toxicities included skin rash, febrile neutropenia, tinnitus, and stomatitis. Common drug-related toxicities included fatigue, nausea, and rash.

In a phase Ⅰ trial (NCT01235897) on 17 patients[171], MK2206 in combination with weekly paclitaxel (80 mg/m^2 weekly) with or without trastuzumab (2 mg/kg weekly after a 1-time loading dose of 4 mg/kg) has been tested in patients with human epidermal growth factor receptor 2 (HER2)-overexpressing solid tumor malignancies (11 breast, 3 gastric, 1 esophageal). The highest safe dose of MK-2206 was found to be 135 mg weekly [The Best Disease Response by Response Evaluation Criteria in Solid Tumor (RECIST) scoring was used for evaluation of the treatment toxicity in 15 patients]. Two patients experienced dose-limiting toxicities, while 64% showed tumor response and 29% had no disease progression[171]. Although all patients experienced different adverse events due to the treatment, serious or life threatening adverse events were reported in 5/17 (29.41%) participants. Based on these results, the authors concluded that MK2206 weekly at a dose of 135 mg in combination with weekly paclitaxel and trastuzumab was safe and well tolerated.

A phase Ⅰ trial (NCT01263145) is ongoing to determine the maximum tolerated dose, safety and antitumor activity of MK2206 and paclitaxel combination in patients with locally advanced or metastatic solid tumors or metastatic breast cancer. Thus, based on this scant evidence, we cannot conclude for or against administration of MK-2206 in combination with the available DNA-targeted agents.

Perifosine

From 42 trials on perifosine, registered between June

Table 4 Clinical trials on phosphatidylinositol 3 kinase/Akt inhibitors in combination with DNA-targeted agents

PI3K/Akt inhibitor	Target(s)	Combination arm(s)	Condition	Trial phase/status	Trial/registration
MK-2206	Akt	Carboplatin + paclitaxel, docetaxel, erlotinib	Locally advanced, metastatic solid tumors	Ⅰ/completed (published)	NCT00848718/February 2009[170]
		Paclitaxel, trastuzumab	HER2-overexpressing advanced solid tumors	Ⅰ/completed (abstract is published)	NCT01235897/ November 2010[171]
		Paclitaxel	Adult solid neoplasm, recurrent or metastatic breast cancer	Ⅰ/ongoing (unpublished)	NCT01263145/ December 2010
Perifosine	PI3K/Akt	Docetaxel, prednisone	Neoplasms	Ⅰ/completed (abstract is published)	NCT00399087/ November 2006[172]
		Docetaxel	Recurrent ovarian cancer	Ⅰ/completed (abstract is published)	NCT00431054/February 2007[173]
		Paclitaxel	Neoplasms	Ⅰ/completed (abstract is published)	NCT00399126/ November 2006[174]
		Gemcitabine	Neoplasms	Ⅰ/completed (abstract is published)	NCT00398697/ November 2006[175]
		Radiation	Solid tumors	Ⅰ/published	Vink *et al*[176]
		Radiation	Biochemically recurrent, hormone-sensitive prostate cancer with previous prostatectomy and/or radiation therapy	Ⅱ/published	Chee *et al*[177]
BEZ235	PI3K/ mTOR	BEZ235 + paclitaxel, BKM120 + paclitaxel, BEZ235 + paclitaxel + trastuzumab, BKM120 + paclitaxel + trastuzumab	Metastatic or locally advanced solid tumors	Ⅰ/completed (abstract is published)	NCT01285466/January 2011[178]
		Paclitaxel	Inoperable locally advanced breast cancer, metastatic breast cancer	Ⅰ and Ⅱ/completed (abstract is published)	NCT01495247/ September 2011[179]

HER2: Human epidermal growth factor receptor 2; PI3K: Phosphatidylinositol 3 kinase; mTOR: Mammalian target of rapamycin.

2000 (first trial) and August 2014 (last trial), only four studies as well as two published papers met our selection criteria for the current review.

Three trials evaluated the safety and efficacy of docetaxel or paclitaxel with perifosine (50, 100, and 150 mg/d). However, the results, despite the completion of the studies, have not yet been published (Table 4). In the first open-label study (NCT00399087) on 39 patients[172], daily doses up to 150 mg perifosine in combination with 75 mg/m^2 per 3 wk docetaxel with/without prednisolone was tolerated. Furthermore, the maximum tolerated dose for the weekly perifosine was 1200 mg. In the second trial (NCT00431054)[173] the safety and efficacy of the combination of docetaxel and perifosine were studied on 22 patients with epithelial cancer of the ovary, fallopian tube cancer or gynecologic primary peritoneal cancer, which were platinum resistant or refractory. The third phase Ⅰ trial (NCT00399126)[174] studied the gastrointestinal toxicity and cancer progression on the combination of daily perifosine with weekly or every 3-wk paclitaxel in 12 cancer patients. The preliminary results showed that the combination was well tolerated without increasing the toxicities being expected from using each drug alone.

Perifosine in combination with gemcitabine has also been studied in a non-randomized open-label phase Ⅰ trial (NCT00398697) on 22 patients[175]. The preliminary results showed that 150 mg daily perifosine might have some manageable toxicities without affecting the pharmacokinetic of perifosine.

The feasibility and tolerability of daily perifosine and radiation combination have also been studied in two independent successive trials. Vink *et al*[176] tested this combination in 21 radio-naive patients with solid tumors, of which 17 had NSCLC. Dysphasia and pneumonitis were the main complications of radiotherapy, and nausea, fatigue, vomiting, diarrhea, and anorexia as major drug-related toxicities in the population. One hundred and fifty milligram daily perifosine combined with fractionated radiotherapy was considered a safe modality. Chee *et al*[177] conducted a phase Ⅱ trial in 25 patients with biochemically recurrent, hormone-sensitive prostate cancer with previous prostatectomy and/or radiation therapy. However, only 20% of patients met the primary endpoint of prostate-specific antigen reduction, defined as a decrease by $\geqslant$ 50% from the pretreatment value. Accordingly, we should wait for the results of ongoing and future studies before coming to the conclusion if perifosine may add to the potency of DNA-targeted therapies.

BEZ235

From 23 trials on BEZ235, registered between February 2008 (first trial) and December 2013 (last trial), only two met our selection criteria for the final review, evaluating the safety and efficacy of the combination with any of the named DNA-targeted agents.

A phase Ⅰ multi-center, open-label, 4-arm dose-escalation study (NCT01285466)[178] is ongoing to evaluate the safety and efficacy of oral BEZ235 and

BKM120 in combination with weekly paclitaxel in patients with advanced solid tumors and weekly paclitaxel/trastuzumab in patients with HER2$^+$ metastatic breast cancer. The preliminary results[178] showed that of 35 patients who received BEZ235 at 400-800 mg/d and paclitaxel at 70-80 mg/m^2 per week dose-limiting toxicities occurred in 5 patients, 29% discontinued due to adverse effects and 63% due to progressive disease. Thus, they concluded that the safety over efficacy of this regimen would be questionable.

Additionally, a dose-finding phase Ⅰ followed by an open-label, randomized phase Ⅱ trial (NCT01495247)[179] of oral BEZ235 given twice daily (bid) with paclitaxel in patients with HER2 negative, locally advanced or metastatic breast cancer have recently been completed. The preliminary results with 18 patients showed that the determined maximum tolerated dose of BEZ235 (200 mg bid) in combination with paclitaxel (80 mg/m^2 per week) was not reached, and the trial has been terminated[179]. Thus, based on these two preliminary results, BEZ235 seems not safe in combination with paclitaxel for patients with solid tumors.

CONCLUSION

The Akt pathway is clearly important for the regulation of cell proliferation and survival. Its activation is an additional resistance mechanism for current chemoradiotherapy. Therefore, modulation of Akt activity is an attractive strategy to enhance the efficacy of treatment. However, insight in the mechanism of protection is incomplete and warrants further research. This lack of knowledge hampers to properly evaluate combinations in clinic, while current clinical trials are too preliminary to draw conclusions, despite having several drugs that are relatively safe and efficacious. Thus, the Akt modulation is an attractive target to improve the toxicity and safety profile of classical antitumor compounds and irradiation. Nevertheless, studies were inadequate and therefore inconclusive regarding the additive effect of PI3K/Akt inhibitors to the standard regimens. Accordingly, more in-depth preclinical and clinical studies as well as a critical appraisal are warranted to find congruent rational avenues to designing solid studies on any of the combinations.

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Accelerated partial breast irradiation: Past, present, and future

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Abstract

Accelerated partial breast irradiation (APBI) focuses higher doses of radiation during a shorter interval to the lumpectomy cavity, in the setting of breast conserving therapy for early stage breast cancer. The utilization of APBI has increased in the past decade because of the shorter treatment schedule and a growing body of outcome data showing positive cosmetic outcomes and high local control rates in selected patients undergoing breast conserving therapy. Technological advances in various APBI modalities, including intracavitary and interstitial brachytherapy, intraoperative radiation therapy, and external beam radiation therapy, have made APBI more accessible in the community. Results of early APBI trials served as the basis for the current consensus guidelines, and multiple prospective randomized clinical trials are currently ongoing. The pending long term results of these trials will help us identify optimal candidates that can benefit from ABPI. Here we provide an overview of the clinical and cosmetic outcomes of various APBI techniques and review the current guidelines for selecting suitable breast cancer patients. We also discuss the impact of APBI on the economics of cancer care and patient reported quality of life.

Key words: Breast cancer; Intracavitary brachytherapy; Accelerated partial breast irradiation; Interstitial brachytherapy

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Core tip: Given that accelerated partial breast irradiation (APBI) is becoming increasingly utilized in the management of early breast cancer patients, it is crucial to address the evolution of studies that led to the current guidelines in identifying the suitable group of patients who obtain the most benefit clinically and cosmetically. We, herein, discuss the available clinical and cosmetic outcomes of different APBI techniques in addition to details of ongoing phase Ⅲ randomized clinical trials. We also discuss the effects of APBI on breast cancer patient quality of life.

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INTRODUCTION

Breast conservation surgery (BCS) has been offered to newly diagnosed breast cancer patients as early as the 1950s[1]. BCS with adjuvant whole-breast irradiation (WBI), collectively referred to as breast conservation therapy (BCT), is one of the acceptable standard of cares. Numerous prospective randomized studies, with long term follow-up, have shown the equivalence of BCT to modified radical mastectomy in overall survival (OS) and disease-free survival (DFS)[2-5].

Standard WBI usually consists of 6-7 wk of daily radiation treatments to the whole breast with doses of 45 to 50 Gy. WBI typically includes a 10 to 16 Gy boost to the lumpectomy cavity for many patients to further reduce local recurrence. Local recurrence can also be reduced by tamoxifen or aromatase inhibitors in estrogen receptor positive breast cancer patients[6-9]. Hypofractionated WBI has recently been accepted as a treatment option in BCT, with local control (7.5% 10-year local recurrence rate)[10] and treatment toxicities comparable to conventional fractionation.

RATIONALE FOR ACCELERATED PARTIAL BREAST IRRADIATION

Accelerated partial breast irradiation (APBI) delivers radiation to the tumor bed at a higher dose per fraction. The radiobiologic model of the linear quadratic equation serves as the basis for APBI. A shorter radiation treatment course, given at higher dose per fraction, could achieve the same therapeutic effect as a longer treatment course, given at lower dose per fraction, based on the concept of radiobiologic equivalence. Ipsilateral breast tumor recurrences (IBTR) develop in and around the tumor bed in 44%-86% of cases[11-14], and treatment to the whole breast may be unnecessary. Therefore, by focusing the radiation to the area of potential recurrence, much of the surrounding tissues (including the lung, heart, uninvolved ipsilateral breast, contralateral breast, and skin) could be spared, reducing toxicity and improving cosmetic outcome[15-19].

EARLY APBI TRIALS

In the earliest prospective, randomized study, Christie Hospital (Manchester, United Kingdom) enrolled 708 patients, 355 of which were treated with wide-field (WF) irradiation and 353 treated with limited-field (LF) irradiation, from 1982 to 1987[20]. The study included patients younger than 70 years with tumor size $\leqslant$ 4 cm, and all women underwent tumorectomy "with gross or macroscopic clearance" only. The WF group received 40 Gy in 15 fractions over 21 d to the whole breast through parallel opposed tangent fields with a single matched anterior field covering the axillary, infraclavicular, and supraclavicular regions. The accelerated, partial breast LF group received 40 to 42 Gy in 8 fractions delivered over 10 d to the tumor bed only. At 8-year median follow-up, the survival in the two groups was the same (72%); however, the LF group showed a local recurrence rate of 25% *vs* 13% in the WF group (P = 0.00008)[21]. The authors concluded that APBI was possible, but would need more stringent selection of patients.

The next APBI trial was conducted by Guy's Hospital (London, United Kingdom) beginning in the late 1980s and used low dose rate (LDR) brachytherapy to deliver focal radiation. Twenty-seven non-randomized patients received BCS and axillary clearance immediately followed by placement of brachytherapy needles in a multi-planar arrangement around the surgical cavity. Iridium-192 seeds were loaded into the needles to deliver 55 Gy over 5 d to a 2 cm margin around the tumor bed[22]. Results showed good to excellent cosmesis in 80%-96% of patients at 27 mo of median follow-up; however, 37% of patients suffered local regional failure at 72 mo of median follow-up[23]. The high rate of local regional recurrences was attributed to the inclusion of subjects with recognized risk factors, such as positive margins and node positive disease.

Three additional trials explored dose escalation using interstitial brachytherapy for APBI at the Careggi Hospital (Florence, Italy), Royal Devon and Exeter Hospital (Exeter, England), and, again, Guy's Hospital (London, United Kingdom). Similarly, these studies included patients with unknown or positive margins, resulting in high local recurrence rates[24,25]. Around the same time period, the Milan group reported a much lower IBTR rate of 4.8% with WBI[26]. In summary, these studies demonstrated the feasibility of APBI and provided a basis for the design of subsequent APBI trials with young age, positive margin status, larger tumors, high nuclear grade, extensive ductal carcinoma *in situ*, invasive lobular carcinoma, involved nodes, and lymphovascular invasion (LVSI) established as risk factors for recurrence.

TRIALS WITH MODERN APBI TECHNIQUES

Brachytherapy

Multicatheter interstitial brachytherapy: Investigators at Ochsner Medical Institutions conducted a pilot trial, enrolling 50 patients from January 1992 to October 1993 in a phase Ⅰ/Ⅱ study of multicatheter interstitial brachytherapy (MIB), after segmental mastectomy, for invasive or intraductal tumors $\leqslant$ 4 cm with negative inked margins and $\leqslant$ 3 involved axillary lymph nodes[27]. Patients were treated to the target volume with continuous

LDR brachytherapy of 45 Gy over 4 d or fractionated HDR brachytherapy of 32 Gy in 8 fractions, given twice daily over 4 d. Cosmetic evaluation at median follow-up of 20 mo showed good to excellent cosmetic result in 75% of patients in both arms. At 75-mo median follow-up, there were 4 local-regional failures (8%). In another study, William Beaumont Hospital accrued patients between 1993 and 1999 for an APBI trial with stringent patient selection criteria: Tumor size ≤ 3 cm, age ≥ 40 years, and no extensive DCIS or lobular histology[28]. All patients had lumpectomy and axillary node dissection with ≥ 2 mm clear microscopic margin of the lumpectomy cavity. Patients with 1-3 involved nodes were initially included but were later excluded. The early phase of the trial delivered 50 Gy of continuous LDR brachytherapy over 5 d with iodine-125 sources[29]. The later phase of the trial used HDR brachytherapy with iridium-192 to deliver 32 Gy in 8 twice daily fractions or 34 Gy in 10 twice daily fractions[30]. The planned treatment volume was the lumpectomy cavity with additional 1 to 2 cm margin. With 5.7 years of median follow-up, 90% (total 199 patients) of patients had good to excellent cosmesis with comparable complications to matched WBI treated patients. The 5-year actuarial recurrence rate was 1.2%. These studies, using multicatheter interstitial brachytherapy, were followed by other successful, non-randomized studies listed in Table 1, and ultimately led to multi-institutional trials.

Radiation Therapy Oncology Group (RTOG) 9517 was opened as a multi-institutional phase Ⅰ/Ⅱ MIB-based APBI trial, and enrolled patients with unifocal tumors < 3 cm, negative margins, and axillary lymph-node sampling, with involvement of ≤ 3 involved nodes with no extra-capsular extension[31]. One hundred patients were accrued between 1997 and 2000, and 99 patients were evaluated. Thirty-three patients received 45 Gy in 3.5-5 d with LDR, and 66 patients to 34 Gy in 10 twice-daily fractions with HDR. In both cases, the target volume was the lumpectomy cavity with 2 cm margin peripherally and 1 cm superficially and deep. The 5-year actuarial in-breast failure rates were 6% and 3% for LDR and HDR brachytherapy, respectively[32]. Acute toxicities, including pain, tenderness, erythema, edema, and infection, were followed, and 3 of 33 patients receiving LDR APBI and 2 of 66 patients receiving HDR APBI experienced grade 3 or 4 adverse effects. These rates of toxicity were similar to earlier single institution trials. Reported late toxicities included breast tenderness, skin thickening, and fibrosis, and the LDR group suffered more frequent late toxicities than the HDR group (18% *vs* 4%)[31].

The first phase Ⅲ trial included patients treated with MIB-based APBI[33]. A total of 258 patients, with T1N0-1mi, grade 1-2 non-lobular breast cancer with negative resection margins and no extensive intraductal component, were randomized to partial breast irradiation (PBI) or WBI between 1998 and 2004. PBI included either LF external-beam irradiation of 50 Gy in 25 fractions for patients who were technically unsuitable for HDR MIB or HDR MIB of 5.2 Gy for 7 fractions. One hundred thirty-three patients were accrued in WBI group and 128 in PBI group (88 HDR MIB and 40 LF external-beam PBI). The 10-year actuarial local recurrence rate (5.9% PBI *vs* 5.1% WBI) was similar for the two arms (P = 0.77). The rates of good to excellent cosmetic outcome were 81% in the PBI groups together and 63% in the WBI group (P < 0.01). HDR MIB APBI demonstrated superior cosmesis compared to LF external-beam PBI, with 85% *vs* 72.5% good to excellent cosmesis[34].

A collaborative effort in Europe recently reported a phase Ⅲ, randomized, non-inferiority trial, using solely MIB[35]. A total of 1184 patients between April 2004 and July 2009, with favorable invasive carcinoma and DCIS, were randomized to either WBI (551 patients) or MIB APBI (633 patients). The primary endpoint was local recurrence. Five patients in WBI group and 9 patients in APBI group had local recurrence at 5-year follow-up. The cumulative incidence of local recurrence of APBI was 1.44% *vs* 0.92% with WBI. The 5-year rate of grade 2-3 late toxicities to the skin was 5.7% with WBI *vs* 3.2% with APBI (P = 0.08), and the 5-year rate of grade 2-3 subcutaneous tissue late side-effects was 6.3% *vs* 7.6% (P = 0.53). The incidence of severe grade 3 fibrosis was 0.2% with WBI at 5 years and 0% with APBI (P = 0.46). There were no grade 4 late toxicities. The study concluded that the 5-year LC, DFS, and OS were similar for MIB APBI and WBI after BCS for patients with early breast cancer.

Intracavitary brachytherapy (balloon and hybrid applicators): The success of MIB APBI is highly dependent on center expertise; therefore, it is not easily accessible to the general population. This led to the development of a more user-friendly brachytherapy approach with flexible balloon catheter. The Mammo Site® (Hologic Inc., Marlbourough, MA) intracavitary breast brachytherapy applicator was approved by the FDA in 2002 and simplified APBI administration. The deflated, single-channel balloon catheter is positioned into the lumpectomy cavity after resection at the time of surgery or post-operatively *via* a subsequent procedure. The balloon is then inflated with a mixture of saline and radio-opaque contrast to fill the lumpectomy cavity. CT imaging is used for assessment of catheter positioning and to assure appropriate skin spacing of at least > 5 mm or > 7 mm optimally. A computer-controlled remote after-loader is used to insert iridium-192 source into the balloon catheter to deliver 34 Gy in 10 twice daily fractions (prescribed to 1 cm from the balloon surface). The catheter is removed after the final fraction and deflation of the MammoSite® balloon.

The MammoSite® Breast Brachytherapy Registry Trial enrolled 1449 patients and had a median follow-up of 63.1 mo with 5-year actuarial rate of IBTR of 3.8%. Tumor size and the lack of estrogen receptor expression were found to be associated with IBTR. At 84 mo, 90.6% of patients had good to excellent cosmesis[36].

William Beaumont Hospital enrolled 45 patients in a phase Ⅰ/Ⅱ study using MammoSite balloon brachytherapy with an alternative fractionation schedule[37]. A total

Table 1 Additional selected, non-randomized clinical experience with interstitial brachytherapy with more than 5 years follow-up

Ref.	No. of patients	Follow-up interval (yr)	Modality	Scheme	Total dose (Gy)	5-yr LR (%)	Good/excellent cosmesis
Strnad *et al*[57]	274	5.25	PDR/HDR	PDR = 0.6 Gy/h HDR = 4 Gy × 8	PDR = 50 Gy HDR = 32 Gy	2.9%	90%
Rabinovitch *et al*[32,58]	98	11.3	LDR/HDR	LDR = 3.5-5 d HDR = 3.4 Gy × 10	LDR = 45 Gy HDR = 34 Gy	4%	68%
Shah *et al*[59,60]	199	12.0	LDR/HDR	LDR 0.52 Gy/h × 96 h HDR = 4 Gy × 8 HDR = 3.4 Gy × 10	LDR = 50 Gy HDR = 32 Gy HDR = 34 Gy	5% (12-yr 5%)	99%
King *et al*[27]	51	6.25	LDR/HDR	LDR = 4 d HDR = 4 Gy × 8	LDR = 45 Gy HDR = 32 Gy	3.9%	75%
Ott *et al*[61,62]	274	5.33	PDR/HDR	PDR = 0.6 Gy/h HDR = 4 Gy × 8	PDR = 49.8 Gy HDR = 32 Gy	2.3%	92%
Polgár *et al*[63]	45	11.1	HDR	4.33 Gy × 7 5.2 Gy × 7	30.3 Gy 36.4 Gy	4.4% (12-yr 9.3%)	78%

HDR: High-dose rate; LDR: Low dose rate; LR: Local recurrence; PDR: Pulsed-dose rate.

dose of 28 Gy in 4 fractions were given in 2 d. At ≥ 6 mo, 2% had grade 2 induration, radiation dermatitis, or hyperpigmentation and 2% grade 3 breast pain. There were 4 cases of fat necrosis. Cosmesis was good to excellent in 96% of cases. The investigators concluded that the 2-d dose schedule resulted in acceptable toxicity rates.

Efforts were made to improve the conformity of radiation delivered *via* balloon applicators with a multi-catheter design. The SAVI® (Strut Assisted Volume Implant), which was FDA approved in 2006, is a bundle of flexible, tiny catheters that can be expanded uniformly to conform to the size and shape of tumor cavity. Fisher *et al*[38] compared outcomes for 117 patients; 77 of whom received APBI *via* MammoSite® device and 40 patients *via* the SAVI® APBI device. None of the patients implanted with the SAVI device required explantation due to skin proximity. This compared to 57% of the patients implanted with the Mammo Site® device, whose skin to target distance was < 7 mm, had explantation. The closest target-to-skin distance treated with the SAVI® device was 2 mm. Good to excellent cosmesis was reported in the 12 patients who had limited skin spacing treated with SAVI®. Contura is another commercially available multilumen balloon breast brachytherapy catheter device, and investigators conducted a multi-institutional phase Ⅳ registry trial for this device, enrolling 342 evaluable patients between January 2008 and February 2011. The median follow-up was 36 mo, and the 3-year local recurrence-free survival was 97.8% and good to excellent cosmesis in 88% of the patients. The incidence of infection was 8.5%, and 4.4% of patients suffered symptomatic seroma[39]. Patients treated at high-volume centers had a superior cosmetic outcome, with 95% of those patients with good to excellent overall cosmesis, indicating that cosmetic outcome is variable among centers.

External beam radiation therapy

External beam radiation therapy (EBRT) includes 3D-conformal radiation therapy (3D-CRT) and intensity modulated radiation therapy (IMRT) defined by the inverse planning of radiation fields. EBRT delivers radiation to a clinical target volume, which for APBI is the tumor bed with 10 to 15 mm. An additional 5 to 10 mm margin was added for set-up errors and target motion. Patients receiving APBI can be set up either supine or prone and are typically treated with four or five non-coplanar beams. A potential advantage of EBRT is that it is widely available. RTOG 0319, a phase Ⅰ/Ⅱ trial, sought to evaluate the efficacy and toxicity of 3D-CRT APBI. The trial enrolled 52 evaluable patients with tumors ≤ 3 cm, ≤ 3 positive nodes, and negative margins. Patients received 38.5 Gy in 10 twice daily fractions. With median follow-up of 4.5 years, the 4-year estimates of IBTR, DFS, and OS were 6%, 84%, and 96%, respectively. Only 4% of patients suffered grade 3 toxicities[40]. RTOG 0319 demonstrated the feasibility of 3D-CRT APBI, and the effectiveness of EBRT was further explored in subsequent trials.

The phase Ⅲ study, NSABP B39/RTOG 0413 is the largest ongoing randomized trial of WBI *vs* APBI. The APBI techniques utilized in the trial are multicatheter brachytherapy (34 Gy), MammoSite (34 Gy), and EBRT (38.5 Gy), given twice daily for 10 fractions, with at least 6 h in between.

While the oncology community waits for the results of NSABP B39/RTOG 0413, interim results from other randomized studies of EBRT APBI have been presented. The Ontario Clinical Oncology Group sponsored RAPID, a randomized trial of APBI using 3D-CRT *vs* WBI. The study enrolled 2135 patients between 2006 and 2011, and an interim cosmetic and toxicity report demonstrated increased adverse cosmesis at 3 years for patients receiving APBI as compared with WBI evaluated by trained personnel (29% *vs* 17%, $P < 0.001$), by patients (26% *vs* 18%, $P = 0.0022$), and by review of imaging by physicians (35% *vs* 17%, $P < 0.001$). Grade 3 toxicities were uncommon in the 2 treatment arms (1.4% for APBI *vs* 0% for WBI)[41]. In another study, the University of Florence (Florence, Italy) recently reported the result

of a phase Ⅲ randomized controlled trial comparing IMRT *vs* WBI. A total of 520 patients were randomized with 260 patients in each arm between March 2005 and June 2013[42]. At a median follow-up of 5.0 years, the IBTR rate was 1.5% in the APBI and WBI groups. The 5-year OS was 96.6% for the WBI group and 99.4% for APBI group. Patients treated with APBI demonstrated significantly less acute and late toxicity and better cosmetic outcome.

Other groups are investigating alternative external beam fractionation regimens. The ACCEL Trial (NCT02681107), sponsored by AHS Cancer Control Alberta, is a phase Ⅱ study evaluating patients treated with EBRT APBI to a prescribed dose of 27 Gy over 5 fractions delivered daily. The Mayo Clinic is sponsoring a phase Ⅱ trial evaluating APBI given in 3 fractions of 7.3 Gy using EBRT or 7 Gy using catheter-based brachytherapy (NCT02453737).

ADDITIONAL APBI TECHNIQUES

Intraoperative radiation therapy

Intrabeam: Intraoperative radiation therapy (IORT) refers to radiation treatment of the tumor bed in a single treatment delivered in the operating room after resection and prior to closure. The rationale for IORT is that a single fraction delivered at the time of surgery makes post-operative radiotherapy unnecessary. In the past, the popularity of IORT was limited because of the expense and impracticality of the specialized radiation delivery devices, but more recently advances in technology have made IORT devices more mobile and available[43]. The first widely available IORT device, Intrabeam®, was first used introduced 1998. Since then, at least two mobile IORT-capable linear accelerators, the Mobetron and Novac-7 systems have become available. While Intrabeam® is a kilovoltage photon system, Mobetron and Novac-7 generate megavoltage electrons.

Intrabeam® (Oberkochen, Germany) uses spherical applicators to deliver kilovoltage photons once inserted into the surgical cavity for uniform dose deposition. The estimated time required to deliver APBI using this device is 20 to 35 min in a single application (this is comparable to the treatment times for each of the 10 fractions delivered for EBRT) making this type of treatment more convenient in some setting. In addition, it has been hypothesized that single fraction IORT has a better therapeutic index[44].

The TARGIT-A trial randomized 3451 patients to either EBRT or TARGIT-A (20 Gy IORT with 50 kV photons). Patient eligibility criteria included age ⩾ 45 years, tumor size ⩽ 3.5 cm, N0-1, M0, and unifocal invasive ductal carcinoma[45]. TARGIT-A patients with adverse risk factors identified on final pathology were given an additional 50 Gy equivalent of EBRT. At 29 mo of median follow-up, the 5-year recurrence rates for patients treated with TARGIT-A and WBI were 3.3% and 1.3%, respectively (P = 0.042). Wound complication rates between the 2 groups were similar; however, grade 3 or 4 skin complications were lower with TARGIT-A *vs* EBRT (P = 0.029). Twenty-one percent of prepathology TARGIT-A patients received 5 wk of EBRT. Patients who received only TARGIT-A had 3 times the recurrence rate of those who received TARGIT-A plus EBRT (2.7% *vs* 0.9%). Breast cancer mortality was similar between two groups; however, the number of non-breast cancer deaths was lower in the TARGIT-A group (1.4% *vs* 3.5%, P = 0.0086). The study concluded that longer follow-up is needed, but the results are promising, given the good survival rate and low recurrence rate. Importantly, some of the patients included in the trial might not be suitable for APBI according to current guidelines.

Mobetron: The Mobetron consists of a mobile robotic arm linear accelerator with multiple electron energies. The Mobetron device is inserted into the surgical cavity for the delivery of electron radiation. An acrylic resin-copper disk may be placed between the breast tissue and the underlying muscle to protect the thoracic wall. A phasee Ⅰ/Ⅱ single arm dose-escalation study treated patients with 19 to 21 Gy at the 90% isodose line[46]. Selection criteria for the study included age > 50 years, tumors < 2.5 cm, surgical margins > 1 cm, no extensive intraductal component, no prior chest irradiation, and free surgical margins by intraoperative pathology. The target volume is lumpectomy cavity with 2 cm margin. 6-12 MeV electrons were used for treatment. With only 9 patients and an average follow-up of 11.3 mo, conclusions are limited; however, the largest dose of 21 Gy seemed to be well-tolerated. The authors used Common Terminology Criteria for Adverse Events v3.0 for reporting toxicities and reported grade 1 hematoma in 1 of 3 patients, grade 1 soft tissue infection in 1 of 3 patients, and grade 2 soft tissue necrosis in 2 of 3 patients[46].

Novac7: Novac7 (Hitesys, Latina, Italy) is also a mobile linear accelerator with electrons of multiple energies delivered *via* a cylindrical perspex applicator with a diameter of 4 to 10 cm. The unit is mounted on a robotic arm for positioning. The phase Ⅲ Electron IntraOperative Therapy (ELIOT) trial randomized 1305 patients, who were ⩾ 48 years with tumors ⩽ 2.5 cm, to either a single intraoperative dose of 21 Gy or to EBRT of 50 Gy WBI with a 10 Gy boost all delivered over 6 wk[47]. The trial employed the Novac7, as well as a similar device, the Liac. At 5.8 years of median follow-up, the 5-year recurrence rates for ELIOT and EBRT were 4.4% and 0.4% respectively (P = 0.0001). A low risk ELIOT group had a 5-year recurrence rate of 1.5%. The ELIOT group had significantly less skin toxicity (erythema, dryness, hyperpigmentation, or itching), but a higher incidence of fat necrosis.

Proton therapy

Bush *et al*[48] reported the 5-year results of a phase Ⅱ trial using proton beam radiation to deliver APBI in patients with invasive nonlobular carcinoma with a

Table 2 Accelerated partial breast irradiation patient selection criteria according to American Society for Radiation Oncology consensus statement[52]

Factors	Suitable	Cautionary	Unsuitable
Age (yr)	> 60	50-59	< 50
BRCA1/2 mutation	Not present	NS	Present
Tumor size	< 2 cm	2.1-3.0 cm	> 3 cm
T stage	T1	T0 or T2	T3-4
Margins	Negative (> 2 mm)	Close (< 2 mm)	Positive
Grade	Any	NS	NS
LVSI	No	Limited/focal	Extensive
ER status	Positive	Negative	NS
Multicentricity	Unicentric only	NS	Present
Multifocality	Clinically unifocal with total size < 2 cm	Clinically unifocal with total size 2.1-3.0 cm	Microscopically multifocal > 3 cm in total size or if clinically multifocal
Histology	Invasive ductal or other favorable subtypes	Invasive lobular	NS
Pure DCIS	Not allowed	< 3 cm	> 3 cm
EIC	Not allowed	< 3 cm	> 3 cm
Associated LCIS	Allowed	NS	NS
LN status	pN0 (i-, i+)	NS	pN1, pN2, pN3, or if not evaluated
Neoadjuvant therapy	Not allowed	NS	If used

DCIS: Ductal carcinoma *in situ*; EIC: Extensive intraductal component; ER: Estrogen receptor; LCIS: Lobular carcinoma *in situ*; LVSI: Lymphovascular space invasion; LN: Lymph node; NS: Not specified.

maximal dimension of 3 cm, negative axillary lymph nodes on sampling, and negative surgical margins. Proton therapy was given to the surgical bed with 40 Gy in 10 fractions, once daily over 2 wk, using skin-sparing techniques. The study enrolled 100 patients. At median follow-up of 60 mo, the 5-year actuarial rates of IBTR-free survival, DFS, and OS were 97%, 94% and 95%, respectively. There were no grade 3 or higher acute skin reactions, and patient- and physician-reported cosmesis was good to excellent in 90%[48]. In addition, Chang *et al*[49] reported results of prospective study of 30 patients treated with 30 cobalt gray equivalent in 6 fractions delivered daily over 5 consecutive days. At 59 mo of median follow-up, no patients had local or metastatic recurrence, and all patients were alive at the last follow-up. Qualitative physician cosmetic assessments of good to excellent were 69% at 3 years[49].

CyberKnife stereotactic APBI

With technological advances in stereotactic radiotherapy, CyberKnife has been investigated as a method to deliver APBI. CyberKnife provides for real-time tracking, respiratory motion management, and submillimeter accuracy and allows for treatment intensification while reducing dose to surrounding normal structures[50]. Georgetown University Hospital treated 10 patients, who were $\geqslant$ 48 years with DCIS or invasive non-lobular carcinoma $\leqslant$ 2 cm in maximum diameter and $\geqslant$ 2 mm of negative margin, using CyberKnife[51]. The planning target volume was delineated on CT scans with 5 mm expansion, and 30 Gy was delivered in daily fractions for 5 consecutive days to the planning target volume. All 10 patients experienced good to excellent cosmetic outcomes with no breast events recorded at median follow-up of 1.3 years. The authors concluded that CyberKnife was reliable in delivering APBI that was well-tolerated; however, the study was limited by its small sample size and brief follow-up.

CURRENT PATIENT SELECTION GUIDELINES

The initial APBI trials have demonstrated the importance of patient selection. With more strict criteria, APBI has been shown to have comparable local recurrence rates in addition to better cosmetic outcome. The most recent American Society for Radiation Oncology (ASTRO) consensus guidelines were published in 2009[52]. Patients were classified into three groups: Suitable, cautionary, and unsuitable. The specific criteria are listed in Table 2. In addition, Table 3 compared the guidelines from different task groups.

OTHER CONSIDERATIONS

Patient reported quality of life

Quality of life is a vital consideration when patients are choosing their breast cancer treatments. Bitter *et al*[53] analyzed self-reported cosmetic outcomes for the treated breast and quality of life for patients treated with WBI or APBI *via* single and multilumen HDR brachytherapy. Two hundred and forty-two patients between 2004 and 2014 with early breast cancer treated with APBI were compared to 59 matched patients treated with WBI from 2012 to 2014. They were evaluated with modified Functional Assessment of Chronic Illness Therapy breast quality of life questions which measured pain, lymphedema, energy level, self-consciousness, and breast cosmesis. Compared to APBI eligible patients treated with WBI, the APBI cohort experienced significantly better lymphedema ($P = 0.0002$), self-consciousness ($P = 0.0004$), and energy level ($P = 0.009$) scores during the first year after treatment. The APBI group reported significantly better breast cosmesis

Table 3 Accelerated partial breast irradiation patient selection criteria from selected organizations

Organization	Age	Tumor size	Margin	Histology	LN status
American Brachytherapy Society[64]	> 50	< 3 cm	Negative (at inked margin)	Invasive ductal carcinoma	pN0; by SLN or axillary dissection
American Society of Breast Surgeons[36]	> 45	< 2 cm	Negative (> 2 mm)	Invasive ductal carcinoma or DCIS	pN0; by SLN or axillary dissection
ASTRO[52]	> 60	< 2 cm	Negative (> 2 mm)	Invasive ductal or other favorable subtypes (mucinous, tubular, and colloid)	pN0; by SLN or axillary dissection

ASTRO: American Society for Radiation Oncology; DCIS: Ductal carcinoma *in situ*; SLN: Sentinel lymph node.

Table 4 Phase III prospective randomized trials evaluating the equivalence or non-inferiority of accelerated partial breast irradiation with whole-breast irradiation

Trial	No. of patients	Follow-up interval (yr)	Inclusion criteria	APBI technique	5-yr LR APBI *vs* WBI (%)
TARGIT-A[45]	3451	2.4	Age ⩾ 45 yr; T1, small T2, N0, N1; ductal; non-lobular and no EIC	20 Gy in 1 fraction, IORT low energy X-rays (50 kV)	3.3 *vs* 1.3
ELIOT[47]	1305	5.8	Age ⩾ 48 yr; T ⩽ 2.5 cm, N0; invasive carcinoma; quadrantectomy	21 Gy in 1 fraction, IORT, electrons up to 9 MeV	4.4 *vs* 0.4
RAPID (OCOG)[41]	2135	Pending	Age > 40 yr; T ⩽ 3 cm, N0; DCIS or invasive carcinoma; negative margins	38.5 Gy in 10 fractions (5-8 d) using 3D-CRT	Pending
GEC-ESTRO[35]	1184	5.0	Age ⩾ 40 yr; T ⩽ 3 cm, pN0-Nmi; stage 0, I, II; DCIS, ductal or lobular carcinoma; margin ⩾ 2 mm	32 Gy in 8 fractions or 30.3 Gy in 7 fractions MIB HDR or 50 Gy MIB PDR (1 pulse/h, 24 h/d; 0.6-0.8 Gy/h)	1.4 *vs* 0.9
Florence (NCT02104895)[42]	520	5.0	Age > 40 yr; T < 2.5 cm; clear margins > 5 mm	IMRT 30 Gy in 5 daily fractions	1.5 *vs* 1.5
IMPORT-LOW	2018	Pending	Age ⩾ 50 yr; T ⩽ 3 cm, node negative; invasive adenocarcinoma; margin ≥ 2 mm	IMRT; Arm 1: 40 Gy in 15 fractions to primary tumor region + 36 Gy in 15 fractions to low-risk region (EBRT) Arm 2: 40 Gy in 15 fractions to primary tumor region (EBRT)	Pending
IRMA (NCT01803958)	3302 (Currently Enrolling)	Pending	Age ⩾ 49 yr; T < 3 cm, N0; invasive carcinoma; margins ⩾ 2 mm	38.5 Gy in 10 fractions using 3D-CRT, BID	Pending
SHARE (NCT01247233)	1006	Pending	Age ⩾ 50 yr; invasive carcinoma; T ⩽ 2 cm; margin⩾ 2 mm; pN0 (i+/-)	3D-CRT 40 Gy in 10 fractions, BID	Pending
NSABP B-39/RTOG 0413	4300	Pending	Age ⩾ 18 yr; DCIS or invasive adenocarcinoma; stage 0, I, II (T < 3 cm); lumpectomy; margins free of tumor; ⩽ 3 positive nodes	34 Gy in 10 fractions using MIB or MammoSite® /MammoSite® ML/SAVI® or 38.5 Gy over 10 fractions using 3D-CRT	Pending

3D-CRT: 3D conformal external-beam radiation; BID: Twice daily; DCIS: Ductal carcinoma *in situ*; EBRT: External beam radiation therapy; EIC: Extensive intraductal component; HDR: High-dose rate; MIB: Multicatheter interstitial brachytherapy; ML: Multilumen; IMRT: Intensity modulated radiotherapy; IORT: Intraoperative radiotherapy; PDR: Pulsed-dose rate; WBI: Whole-breast irradiation.

during the second year after treatment. There were no significant differences in the recurrence rates ($P > 0.05$)[53]. Moreover, analyses of late toxicities and cosmesis of patients treated with APBI on RTOG 0319 demonstrated good to excellent cosmesis in 82% and 64% of patients at 1 year and 3 years, respectively. When questioned at 3 years, 31 patients were satisfied with their treatment, 5 were not satisfied but would choose 3D-CRT again, and no patients would elect standard radiation therapy[54].

Economics of treatment

In addition to identifying the group of patients with the appropriate breast cancer biology, it is important to consider other factors, such as socioeconomic issues. Shah *et al*[55] reported results of cost-efficacy of multiple APBI techniques compared with WBI. Their analyses included cost minimization, incremental cost-effectiveness ratio (ICER), and cost per quality adjusted life year (QALY) analyses. For 1000 patients treated, the cost savings would be $6.0 million (APBI 3D-CRT), $2.0 million (APBI IMRT), and $0.7 million (APBI interstitial) with the utilization of APBI compared to WBI 3D-CRT. The cost per QALY was $54698 and $49009 for APBI multilumen and APBI 3D-CRT, respectively, when incorporating the cost of recurrences and non-medical costs[55].

CONCLUSION

APBI has gained acceptance for appropriately selected cases of early stage breast cancer, as outlined by cu-

rrent guidelines. Shaitelman *et al*[56] showed increased utilization of APBI from 3.8% of breast cancer radiation in 2004 to 10.6% in 2011 ($P < 0.0001$), with most of the APBI given *via* brachytherapy. The proliferation of APBI demonstrates its acceptance by patients in the modern era owing in part to its increased convenience and potential for reduced toxicities. As the use of APBI expands, the need for patient selection guidelines and consensus statements becomes even more important. There are many ongoing phase Ⅲ trials that are testing the non-inferiority and equivalence of various forms of APBI compared to WBI (Table 4). Some of these ongoing studies have reported results of interim analyses. As the data matures, we will be able to more appropriately select the specific patients benefiting most from APBI. Furthermore, as patient reported outcome measures, such as quality of life, gain traction in parallel to outcome studies, this data should be incorporated into shared decision making with patients.

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Granulocyte colony-stimulating factor-producing hepatocellular carcinoma with abrupt changes

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Abstract

Granulocyte colony-stimulating factor (G-CSF)-producing tumor is one of the rare types of cancer clinically characterized by an elevated fever and white blood cell (WBC) increment. Although G-CSF producing tumors have been reported in several types of cancer including those of the lungs, cervix and bladder, G-CSF producing hepatocellular carcinoma is extremely rare. Here, we report the case of a rapidly growing and poorly differentiated hepatocellular carcinoma producing G-CSF. The patient showed symptoms of continuous high fever, stomach pain and cough, and high serum WBC counts, C-reactive protein (CRP) and G-CSF levels were found in laboratory tests. After a radical hepatectomy, the patient completely recovered from the above symptoms and inflammatory state. The serum levels of G-CSF were reduced to normal levels after radical surgery. An immunohistochemical analysis revealed the overexpression of G-CSF in the cytoplasm of certain hepatocellular carcinoma (HCC) cell. The patient's serum WBC, CRP and G-CSF levels remained within normal levels in the six months after surgery without recurrence. This is the 9th case report of G-CSF producing hepatocellular carcinoma in English literature. We review the clinical characteristics of the G-CSF producing HCC and discuss a possible treatment strategy.

Key words: Granulocyte colony stimulating factor; Granulocyte colony-stimulating factor producing tumor; Hepatocellular carcinoma; Immunohistochemistry; Sarcomatous changes

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Core tip: Granulocyte colony-stimulating factor (G-CSF)-producing tumor is one of the rare types of cancer clinically characterized by an elevated fever and white blood cell increment. Although G-CSF producing tumors have been reported in several types of cancer including those of the lungs, cervix and bladder, G-CSF producing hepatocellular carcinoma (HCC) is extremely rare. This is the 9th case report of G-CSF producing HCC in English literature. We report our case and review reported literatures with special reference to the clinical characteristics of the G-CSF producing HCC and a possible treatment strategy.

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INTRODUCTION

Granulocyte colony-stimulating factor (G-CSF) is a naturally produced glycoprotein that is synthesized by stromal cells in bone marrow. G-CSF stimulates progenitor cells to differentiate and enhances the functions of neutrophils. The G-CSF producing tumor is characterized by leukocytosis without infection and high serum G-CSF levels. In 1977, the G-CSF producing tumor was first reported in lung cancer[1]. After that, several G-CSF producing tumor cases were reported for cancers of the bladder[2,3], lung[4], thyroid[5], gallbladder[6] and uterine cervix[7]. Among them, the G-CSF producing HCC is extremely rare and is generally reported as having a poor prognosis because of its dramatic tumor progression. Liver cancer including hepatocellular carcinoma (HCC) is the second cause of cancer death worldwide[8]. It is common that HCC develops in the patient with chronic hepatitis caused by viruses, especially hepatitis B virus (HBV). The development of the HCC is driven by the genetic factor, epigenetic factor, environmental factor and viruses. Although, the novel factors such as hematopoietic stem cells and non-coding RNA are reported in the recent researches to be involved in hepatocarcinogenesis[9-11], the mechanisms of the carcinogenesis of G-CSF producing HCC remains unclear.

We report a G-CSF producing HCC that was radically resected and diagnosed by pathological and serological findings. We review previous reports regarding the clinical behaviors of the G-CSF producing HCC, including our case.

CASE REPORT

A 79-year-old man was admitted to our hospital with a continuous fever, cough and high degree of serum C-reactive protein (CRP). A physical examination revealed a hard, fixed mass palpable on the right upper quadrant of the abdomen. Laboratory tests showed an increased degree of serum CRP (17.3 mg/dL) and white blood cell (WBC) counts, and a worsening of anemia compared with the patient's initial examination. In addition, a higher level of serum G-CSF (42 pg/mL) was detected. A preoperative computed tomography (CT) examination revealed an irregular mass in segment Ⅳ of the liver, approximately 60 mm in diameter with peripheral enhancement (Figure 1A). Tumor markers, such as the absence of protein-induced vitamin K or antagonist (PIVKA)-Ⅱ level, α-foetoprotein (AFP) level, carcinoembryonic antigen (CEA) level and carbohydrate antigen 19-9 (CA19-9) levels, were within the normal range. Further evaluations of the liver mass were performed.

Detailed CT examination during arterial portography (CTAP), computed tomography during hepatic arteriography (CTHA), magnetic resonance cholangio pancreatography (MRCP), and gadoxetic acid-enhanced MRI (Gd-EOB-MRI) revealed that the liver mass was a poorly differentiated carcinoma, rather than a liver abscess. The tumor partially occupied segment IV of the liver and protruded toward the abdominal cavity (Figure 1A and B).

Four days after admission, the patient continued to have an intermittent fever (Figure 2A) and the tumor size became drastically enlarged within a short period; therefore, we decided to perform surgery. The surgery was a complete resection with a segment Ⅳ partial hepatectomy. There was ascites around the tumor in the abdominal cavity, but a cytological analysis revealed that was no malignant cells in it.

After the radical hepatectomy, the patient's fever gradually dropped to a normal temperature and the other symptoms, such as cough and abdominal pain, ceased (Figure 2A). The laboratory data, such as WBC count and neutrophil percentage returned to the normal range by postoperative day 5 (from 13020/μL to 6180/μL, 88.3% to 68.5%, respectively). The serum CRP level dropped gradually from 28.7 mg/dL to 1.5 mg/dL by postoperative day 12. The patient had an uneventful postoperative recovery and was discharged on postoperative day 12. Afterward, serum WBC counts, CRP and G-CSF returned to normal levels (Figure 2B and C).

The pathological findings of the resected specimen showed that the tumor size was 12.0 cm × 10.0 cm × 10.0 cm, and the gallbladder and partial greater curvature were also resected with the main tumor (Figure 1C). The cut surface of the tumor was white with an irregular margin and vast necrotic tissue was observed inside the tumor (Figure 1D). Microscopic findings revealed that the tumor was mainly composed of poorly differentiated hepatocellular carcinoma (Figure 3A) and partially sarcomatous spindle-shaped malignant cells (Figure 3B) were detected. Moreover, a drastic neutrophil infiltration within the hepatocellular carcinoma cells was

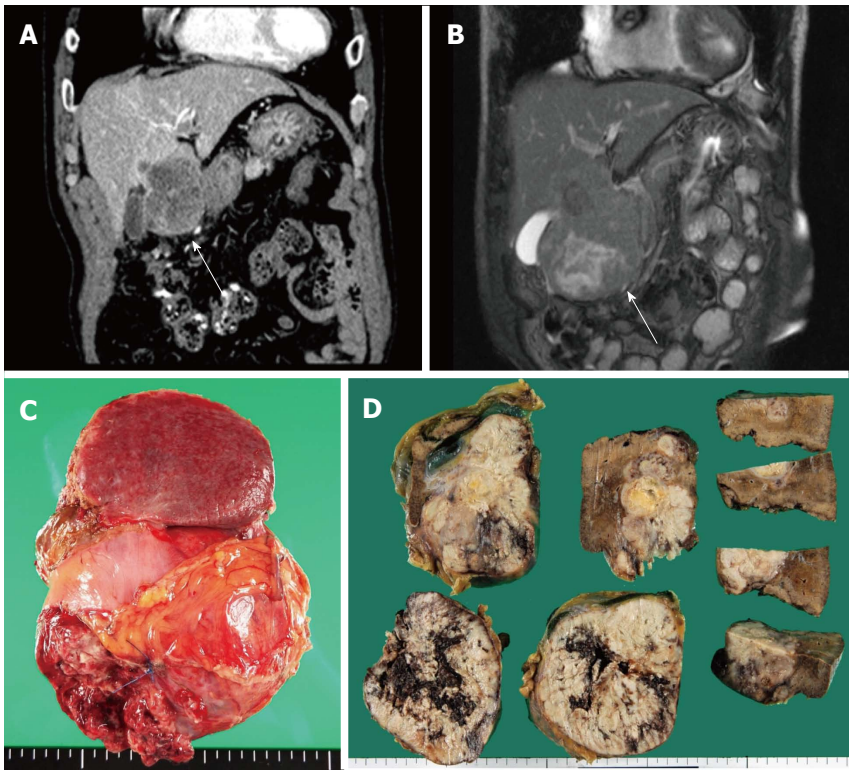
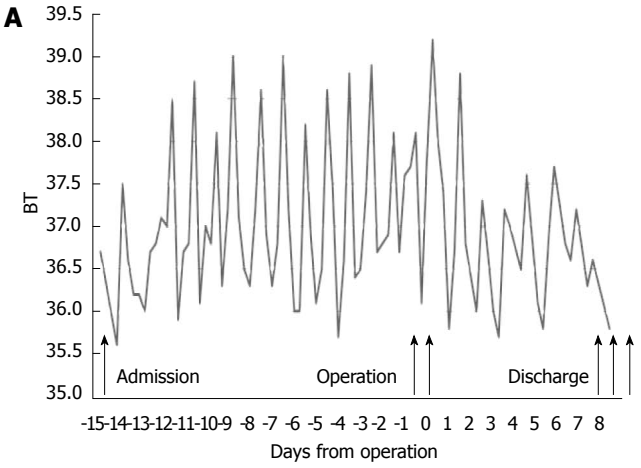
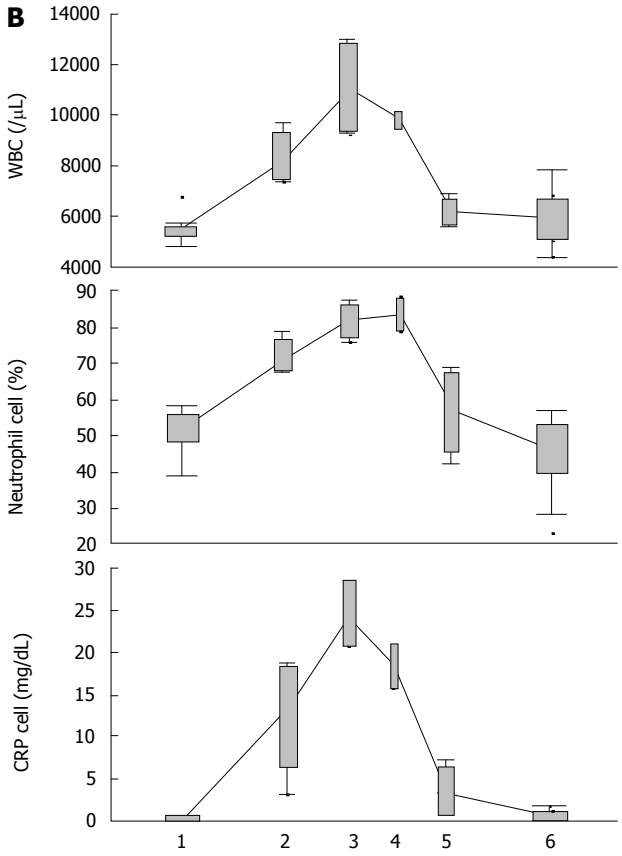


Figure 1 Imaging and macroscopic findings of granulocyte colony-stimulating factor producing hepatocellular carcinoma. A: CT scan one month before operation showed an irregular liver mass located in segment IV, approximately 60 mm in diameter with peripheral enhancement (white arrow head); B: T2-WI MRI one week before operation showed the rapidly growing liver mass with a 100 mm diameter (white arrow head); C: Macroscopic examination showed a large tumor (100 mm × 100 mm) that protruded through segment Ⅳ of the liver to the greater omentum; D: The irregular liver tumor in segment Ⅳ showed a central necrosis.





C

	2 d before operation	2 wk after operation	1 mo after operation	6 mo after operation
G-CSF (pg/mL)	42.0	26.9	25.2	24.0

Figure 2 Physiological and laboratory changes during the treatment. A: Changes in body temperature during the treatment; B: Laboratory changes during the treatment; 1: Steady state; 2: Admission; 3: Pre operation; 4: Post-operation; 5: Within 2 mo after operation; 6: More than 2 mo after operation; C: White blood cell count, neutrophil proportion and C-reactive protein were collected at various treatment points including "steady-state" (more than six months before admission), "before admission" (within six months of admission), "pre-operation" (from admission until operation), "post-operation" (from operation until discharge), "within two months of surgery" and "more than two months after operation".

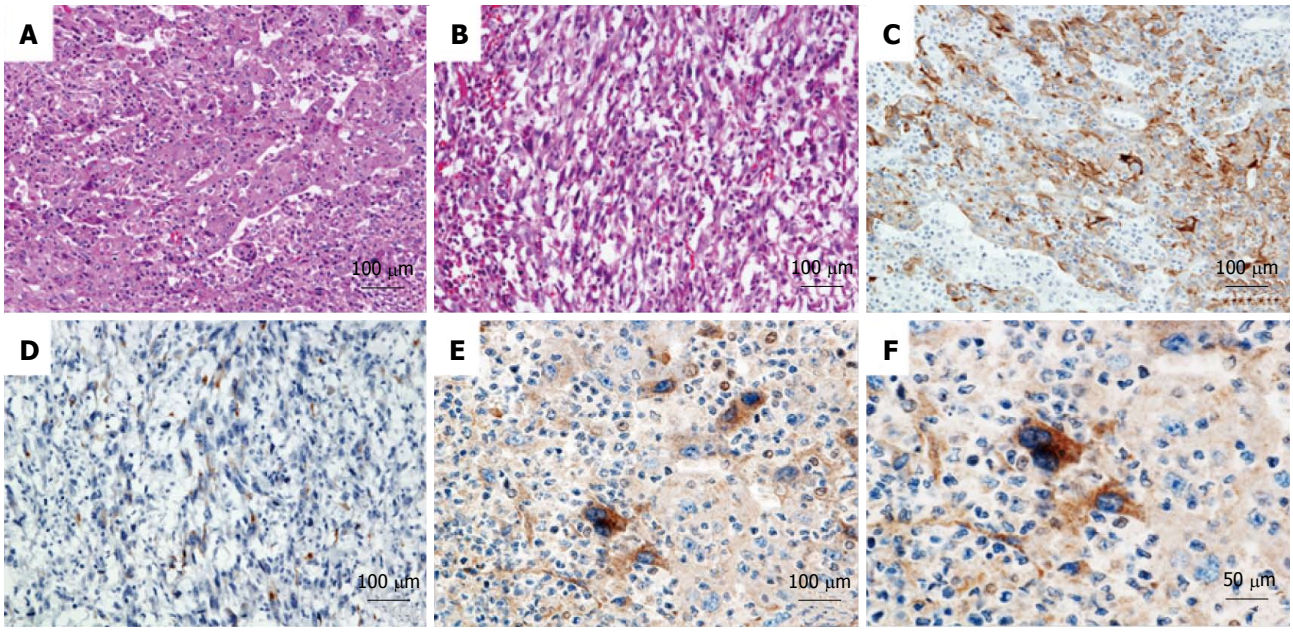


Figure 3 Histopathologic findings. Microscopic findings showed atypical poorly differentiated cells with a sheet structure (A); HCC tumor was also composed of sarcomatous spindle-shaped cells (B); in both samples, a drastic infiltration of the neutrophils was found (H and E, × 20). Immunohistochemical findings showed CAM5.2 positive in the moderately to poorly differentiated HCC lesion (C) and negative in the spindle-shaped cell lesion (D) (CAM5.2, × 20). Immunohistochemical examination showed that G-CSF was positive in the moderately to poorly differentiated HCC lesion (E, F) (G-CSF, × 20 and × 40). G-CSF: Granulocyte colony-stimulating factor; HCC: Hepatocellular carcinoma cell.

noted (Figure 3A and B). Immunohistochemistry showed the ordinary HCC cells to be positive for CAM5.2 (Figure 3C) and the sarcomatous area was positive for vimentin. The HCC cells were positive for G-CSF. These findings were supportive for the diagnosis of G-CSF producing HCC (Figure 3).

G-CSF

G-CSF is a glycoprotein (19.6 kDa) that stimulates cell proliferation and differentiation of precursor cells in the bone marrow. G-CSF is major extracellular regulator of hemopoiesis and the immune system, first named in the 1980s[12,13]. It not only changes mature precursor cells into fully differentiated neutrophils, but also enhances their functional activity[14]. These mechanisms have been exploited to produce a drug to increase neutrophils in patients with chemotherapy-induced neutropenia. Granulocyte colony-stimulating factor receptor (G-CSF R) is also member of the cytokine receptor family and functions in some cell surface adhesion or recognition process. This protein is essential for granulocytic maturation and plays a crucial role in the proliferation, differentiation and survival of cells along the neutrophilic lineage. Furthermore, there are reports on the relationship between G-CSF and cancers[15-17].

CLINICAL BEHAVIOR OF G-CSF PRODUCING TUMOR

Some cancers have been reported to produce certain humoral factors including cytokines, such as G-CSF, granulocyte macrophage colony-stimulating factor (GM-CSF), erythropoietin or parathyroid hormone, which cause paraneoplastic syndrome[18-21]. Paraneoplastic syndrome presents as various clinical disorders, such as anemia, hypercalcemia, erythrocytosis, granulocytosis and thrombocytosis, and is often reported in lung cancer[20,22]. Asano *et al*[1] first reported G-CSF producing lung cancer in 1997. After this report, various cases were reported with G-CSF producing tumors in lung, bladder, sarcoma, cervical and gallbladder cancers[3,6,7,20,22]. The G-CSF producing tumor has been described as having (1) a drastic WBC increase; (2) an elevation of G-CSF activity; (3) WBC decrease after tumor resection; and (4) evidence of G-CSF production in the tumor tissue[1]. In our case, high WBC counts and fever elevation were present without a bacterial infection preoperatively. Also, a contrast enhanced CT image revealed a non-typical and poorly differentiated HCC tumor. After radical hepatectomy, the serum WBC level and G-CSF activity were decreased to normal levels. Finally, immunohistochemical staining showed G-CSF production in the tissue inside the tumor. These findings fit the above definition and strongly suggested that our case was a G-CSF producing HCC[27-31].

PREVIOUS REPORTS OF G-CSF PRODUCING HEPATOCELLULAR CARCINOMA, INCLUDING OUR CASE

G-CSF producing HCC is extremely rare and only eight cases have been documented in the English literature

Table 1 Previous reported cases of Granulocyte colony-stimulating factor producing hepatocellular carcinoma

Case	Ref.	Year	Age	Sex	WBC[1] (/μL)	G-CSF[2] (pg/mL)	HCV	HBV	Pathology	Sarcomatous change	Treatment	IHC	Prognosis[3]	
1	Yamamoto *et al*[19]	1999	67	M	234000	251	+	-	Poorly dif. HCC	-	TAE + Chemotherapy	+	5 mo	Dead
2	Amano *et al*[20]	2005	70	M	26400	308	-	-	Poorly dif. HCC/CCC	+	Palliative surgery	+	1 mo	Dead
3	Aita *et al*[21]	2006	74	M	71700	286	-	-	Poorly carcinosarcoma	+	TAE	+	2 mo	Dead
4	Araki *et al*[22]	2007	66	M	45200	178	-	-	Poorly dif. HCC	+	Radical surgery + TAE	+	4 mo	Dead
5	Joshita *et al*[23]	2010	66	M	25450	62	-	-	Moderately dif. HCC	-	Radial surgery	+	4 yr	Dead
6	Kohno *et al*[24]	2012	46	M	51670	195	-	+	Moderately to poorly dif. HCC	+	Radical surgery + TAE + Chemotherapy	+	7 mo	Dead
7	Snyder *et al*[25]	2012	47	F	40000	58.2	-	-	Poorly dif. HCC	unknown	Radical surgery	unknown	1 mo	Dead
8	Ito *et al*[26]	2012	37	M	51600	342	-	+	Moderately to poorly dif. HCC	-	Radical surgery + Chemotherapy	+	2 yr	Alive
9	Our case	2016	79	M	13020	42	-	-	Poorly dif. HCC	+	Radical surgery	+	6 mo	Alive

[1]White blood cell count (normal value: 4000-8000/μL); [2]granulocyte-colony stimulating factor (normal value: < 39 pg/mL); [3]prognosis after diagnosis. HBV: Hepatitis B virus; HCV: Hepatitis C virus; WBC: White blood cell; G-CSF: Granulocyte-colony stimulating factor; HCC: Hepatocellular carcinoma; CCC: Cholangiocellular carcinoma; TAE: Transcatheter arterial embolization.

(Table 1). G-CSF producing tumors, including HCC, generally grow rapidly and have a poor prognosis. G-CSF is reported to be linked to tumor cell growth and progression[31,32]. Also, there might be a relationship between the secretion of G-CSF and the degree of cell differentiation[23]. Wang *et al*[33] compared the production of G-CSF between well- and poorly differentiated HCC using cell lines and concluded that only poorly differentiated HCC tends to produce G-CSF. As shown in Table 1, of nine reports including our case, six cases 76% (6/9) were pathologically diagnosed as poorly differentiated HCCs and two cases were moderate to poorly differentiated HCCs.

Tachibana *et al*[3] reported that the G-CSF production and G-CSF receptor expression exhibited by cancer cells both play crucial roles in mediating the malignant progression of nonhematopoietic cancer cells. Baba *et al*[34] and Segawa *et al*[35] both reported G-CSF as an autocrine growth factor considered to be necessary for tumor proliferation and metastasis[3,7,34,36]. As these reports demonstrate, the prognosis of patients with G-CSF producing HCCs is indeed very poor[26-28]. Specifically, in more than one-half of the cases, the patients died within approximately within six months after diagnosis. Therefore, some authors have suggested that surgical resection is not an effective strategy considering the poor outcome of this G-CSF producing HCC[26] because most patients were diagnosed in a far-advanced stage. Whereas, our case was diagnosed as a curatively resectable stage, such as a stage-Ⅱ (T2N0M0 UICC 7th), and the preoperative serum WBC counts and G-CSF levels were relatively lower than in previous reports (Table 1). Therefore, in our case, radical tumor resection was effective.

The serum levels of G-CSF are positively correlated with WBC counts[20,37]. Also, in our case, serum WBC counts, CRP and G-CSF levels were shifting in parallel during the treatment course and the trend seemed to be correlated with the growth of the liver tumor (Figure 2B and C). To date, these marker levels are being maintained at normal levels and will continue to be monitored, and the patient has had no recurrence in the six months following surgery.

G-CSF producing HCC as one of the differential diagnosis of fever of unknown origin

Fever of unknown origin (FUO) remains to be of considerable clinical importance. Classical FUO was defined by Petersdorf and Beeson[38] in 1961. In recent study about FUO, Bleeker-Rovers *et al*[39] showed that infection was the cause of FUO in 16% of the patients, cancer in 7% and non-infectious inflammatory diseases in 22%. Their report showed that in over 50% of the cases, the cause of fever was not found. Not only hematological malignancies, but also varieties of solid neoplastic diseases have been reported as occasionally associated with FUO without any associated infection[40]. Therefore, more physicians should include G-CSF producing tumors in the differential diagnosis of FUO.

CONCLUSION

Although G-CSF producing tumors are extremely rare, clinicians should consider this diagnosis for a patient with a continuous high fever of unknown origin and leukocytosis without evidence of infection. Early laboratory and imaging examinations should also be performed for an early diagnosis, effective treatment

and improved prognosis. Radical resection in the early stage of a G-CSF producing HCC might provide a more favorable outcome. Nevertheless, further studies and the accumulation of clinical cases are required to establish appropriate treatment strategies for patients with G-CSF producing HCCs.

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ORIGINAL ARTICLE

Basic Study

Tumor infiltrating lymphocytes in triple negative breast cancer receiving neoadjuvant chemotherapy

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Informed consent statement: Not needed due to the non-clinical study design.

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Data sharing statement: No data were created so no data are available.

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Abstract

AIM

To determine influence of neoadjuvant-chemotherapy (NAC) over tumor-infiltrating-lymphocytes (TIL) in

triple-negative-breast-cancer (TNBC).

METHODS

TILs were evaluated in 98 TNBC cases who came to Instituto Nacional de Enfermedades Neoplasicas from 2005 to 2010. Immunohistochemistry staining for CD3, CD4, CD8 and FOXP3 was performed in tissue microarrays (TMA) sections. Evaluation of H/E in full-face and immunohistochemistry in TMA sections was performed in pre and post-NAC samples. STATA software was used and *P* value < 0.05 was considered statistically significant.

RESULTS

Higher TIL evaluated in full-face sections from pre-NAC tumors was associated to pathologic-complete-response (pCR) (*P* = 0.0251) and outcome (*P* = 0.0334). TIL evaluated in TMA sections showed low level of agreement with full-face sections (ICC = 0.017-0.20) and was not associated to pCR or outcome. TIL in post-NAC samples were not associated to response or outcome. Post-NAC lesions with pCR had similar TIL levels than those without pCR (*P* = 0.6331). NAC produced a TIL decrease in full-face sections (*P* < 0.0001). Percentage of TIL subpopulations was correlated with their absolute counts. Higher counts of CD3, CD4, CD8 and FOXP3 in pre-NAC samples had longer disease-free-survival (DFS). Higher counts of CD3 in pre-NAC samples had longer overall-survival. Higher ratio of CD8/CD4 counts in pre-NAC was associated with pCR. Higher ratio of CD4/FOXP3 counts in pre-NAC was associated with longer DFS. Higher counts of CD4 in post-NAC samples were associated with pCR.

CONCLUSION

TIL in pre-NAC full-face sections in TNBC are correlated to longer survival. TIL in full-face differ from TMA sections, absolute count and percentage analysis of TIL subpopulation closely related.

Key words: Triple-negative breast cancer; Survival; Tumor-infiltrating lymphocytes; Neoadjuvant therapy

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Core tip: We evaluated a series of 98 triple negative breast cancer cases who received neoadjuvant chemotherapy. Pre-neoadjuvant chemotherapy and post neoadjuvant chemotherapy samples were analyzed. We compared tumor-infiltrating lymphocytes evaluated in whole slides *vs* in tissue microarray slides. We also compared subsets of tumor-infiltrating-lymphocytes (TILs) evaluated through an absolute counting *vs* a percentage calculation. Our results confirm the predictive and prognostic role of tumor infiltrating lymphocytes, and evaluated the behavior of tumor infiltrating lymphocytes and lymphocyte subsets during chemotherapy in triple negative breast cancer. Our results increase the understanding of methodological issues for TIL evaluation as well as provide information about variation of the whole TIL population and TIL subpopulation during chemotherapy.

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INTRODUCTION

Triple negative breast cancer (TNBC) represents 10% to 20% of breast cancer (BC) and is a biologically aggressive tumor with high response to Neoadjuvant chemotherapy (NAC) but poor outcome[1].

Prognostic and predictive role of tumor infiltrating lymphocytes (TILs) have been extensively studied in BC and different studies have found they are associated to better outcome in TNBC. Denkert *et al*[2] found that a high TIL percentage evaluated by Hematoxilin and Eosin (H and E) in full-face sections of pre-NAC samples can predict pathologic complete response (pCR) in a series of more than 1000 BC cases. Loi *et al*[3] evaluated the prognostic role of TIL percentage in more than 2000 node-positive BC and found that high TIL percentage are associated to better outcome only in the TNBC (*P* = 0.023). Adams *et al*[4] evaluated the role of TIL in two phase III adjuvant randomized BC trials and also found a better outcome only in the TNBC (*P* = 0.02)[5]. Finally, a recent meta-analysis and a retrospective analysis of 8897 TNBC confirm the prognostic value of TIL in TNBC[5,6]. Recent studies suggest that tumor infiltration by CD8 cytotoxic lymphocytes and absence of FOXP3 immunosuppressive regulatory cells could control tumor growth and carry a better prognosis[7-12].

Despite the international TILs working group defined harmonization criteria to evaluate TIL, there is some areas that still require a better understanding. One of these areas is the value of TILs in small pieces of tumor like tissue microarrays (TMA), as well as the value and the appropriate methodology to evaluate TIL subpopulations in TMA.

Finally, some recent studies also suggest a prognostic role of TIL in post-NAC samples, however, there is need for more information in this area because evaluation of these samples has special challenges to pathologists as neoadjuvant chemotherapy produces a spectrum of histopathologic changes including decrease in cancer cell number and changes in stroma composition that includes fibrosis, elastosis, collagenization, hyalinization, microcalcification, neovascularization, fibrinoid necrosis and mucinous changes[13].

This study aims to evaluate TIL variation during NAC through H and E in full-face and in TMA sections as well as the variation of TIL subpopulations through immunohistochemistry (IHC) staining in TMA sections.

MATERIALS AND METHODS

Patients and sample selection

We retrospectively reviewed the files of all new BC cases which came to the Instituto Nacional de Enfermedades Neoplasicas between 2005 and 2010, and we selected 98 TNBC cases with Clinical Stage Ⅱ-Ⅲ who went to surgery of breast tumor and axilla after receiving NAC. All core biopsy prior to NAC and breast tumor excision specimens were fixed in 10% neutral buffered formalin and embedded in paraffin and stored at Institutes Pathology Department Archive. Institutional review board approved the protocol of this study.

Staining and quantification of H and E, CD3, CD4, CD8 and forkhead box protein 3 IHC staining

Tumor areas were selected and a 0.6 cm punch from the formalin-fixed paraffin-embedded specimens were obtained and organized in 8-10 cylinder cards (TMA). H and E staining was performed in the full-face and in the TMA sections, and TIL was evaluated as the percentage of the stroma area of tumor that contained lymphocytic infiltrate through a 10% increment system under 200 × - 400 × magnification.

Process of IHC preparation included cutting 4 μm sections from the TMA, deparaffinating, rehydrating and processing sections using an automatized stainer (Autostainer Link 48, DAKO, Carpinteria, CA, United States) through standard methods. The following antibodies: CD3 (IS503, Dako), CD4 (IS649, Dako), CD8 (IS623, Dako) and FOXP3 (clone: 236A/E7) were used for staining of TMA section. Lymphocyte subsets were calculated through the percentage between lymphocytes/tumor cells in a 10% increment system, and through the absolute count of the lymphocytes in 5 high power fields under 200 × - 400 × magnification.

Clinical information and pathological response

We obtained clinical information from patient files archived at Instituto Nacional de Enfermedades Neoplasicas. The pathological therapeutic response of the surgically resected tumor was evaluated after NAC. The surgical specimens of breast lesions were cut into 5 mm slices and processed with H and E staining. A pCR was defined as the absence of all invasive cancer cells in breast and axillary lymph nodes, regardless of the presence of non-invasive cancer cells[11].

Statistical analysis

The mean percentage as well as the mean absolute number of immune cells was calculated, and those lesions above this mean were graded as increased. Analysis were also performed considering a high percentage of immune cells when were at least 50%. All statistical analyses were performed using STATA software version 12. Associations among variables were evaluated using Fisher's exact test or the χ^2 test. The Mann–Whitney *U* and Spearman's correlation tests were used to compare groups. The measurement of agreements between TIL evaluated in whole slide and TMA was used intraclass correlation coefficient (ICC). Kaplan-Meier estimation curves disease free and overall survival was applied. All tests were two sided, and a $P \leqslant 0.05$ was considered statistically significant.

RESULTS

Assessment of TILs by H and E

The characteristics of 91 pre-NAC and 80 post-NAC TN BC cases are reported in Table 1. Most cases were ductal infiltrating carcinoma (96.9%), inflammatory (29.6%), clinical stage Ⅲ (86.7%) and HGⅢ (75.5%). Most cases received neoadjuvant doxorubicin and paclitaxel (87.7%) and 29.6% obtained pCR. Sixty-six percent of the patients underwent mastectomy. After a median follow-up of 37.5 mo, there were 42% recurrences and 45% deaths. Pathologic complete response was associated to OS ($P = 0.0071$) but was not associated to DFS ($P = 0.1050$).

Median Pre-NAC TIL percentage in the full-face ($n = 91$) and in the TMA section ($n = 30$) was 40 ± 20 and 20 ± 15, respectively. Median post-NAC TIL percentage in the full-face ($n = 80$) and in the TMA section ($n = 58$) was 20 ± 15 and 10 ± 5, respectively (Table 2).

Pre-NAC TIL evaluated in full-face had low grade of agreement with TMA sections ($n = 30$) (ICC = 0.017). Post-NAC samples were larger and allowed to be divided in homogeneous ($n = 26$) and heterogeneous lesions ($n = 26$). Heterogeneous lesions had low level of agreement between full-face and TMA sections (ICC = 0.20), and homogeneous lesions had high level of agreement between full-face and TMA sections (ICC = 0.73).

Higher median pre-NAC TIL ($n = 91$) evaluated in full-face sections was associated to pCR (40% *vs* 30%, $P = 0.0251$), DFS (40% *vs* 20%, $P = 0.0076$) and OS (40% *vs* 30%, $P = 0.0334$); but not to age ($P = 0.1427$) nor inflammatory features ($P = 0.6401$), in the univariate analysis. Association between median TIL and pCR remained significant even with adjustment for age. Higher median pre-NAC TIL ($n = 30$) evaluated in TMA section was only associated to absence of inflammatory features (10% *vs* 30%, $P = 0.0387$).

Median post-NAC TIL evaluated in full-face ($n = 80$) or in TMA section ($n = 58$) was not associated to any of the previously mentioned features. Post-NAC median H and E TIL percentage evaluated in full-face section was similar in residual fibrous lesions ($n = 17$) (pCR) and in residual tumor lesions ($n = 63$) (no pCR) (20% *vs* 20%, $P = 0.6331$) (Table 3).

Classification of TIL with a cut-off of 50% did not identify a population associated to any of the previously mentioned features ($P = 0.16$ for pCR, $P = 0.14$ for DFS and $P = 0.64$ for OS).

NAC produced a statistically significant decrease in median TIL percentage when evaluated in full-face section ($n = 73$ $P < 0.0002$), but not when evaluated in TMA sections ($n = 16$ $P = 0.4321$) (Table 4).

Table 1 Clinical and pathological general features

Features	*n* = 98 (%)
Median age	49 ± 9
Clinical stage	
Ⅱ	13 (13.3)
Ⅲ	85 (86.73)
Histologic grade	
Ⅱ	23 (23.5)
Ⅲ	74 (75.5)
Subtype histologic	
Ductal	95 (96.9)
Lobular	2 (2.1)
Neoadjuvant chemotherapy	
AC	9 (9.2)
AC-Taxane	86 (87.7)
Taxane alone	3 (3.1)
Surgery	
Tumorectomy	29 (29.6)
Mastectomy	65 (66.3)
Unknown	4 (4.1)
Inflammatory	
No	69 (70.4)
Yes	29 (29.6)
pCR	
No	69 (70.4)
Yes	29 (29.6)
Recurrence	
No	56 (57.1)
Yes	42 (42.9)
Death	
No	53 (54.1)
Yes	45 (45.9)

AC: Adriamycin/cyclophosphamide; AC-Taxane: Adriamycin/cyclophosphamide, followed by treatment with taxane; pCR: Pathologic complete response.

Assessment of TIL subsets by IHC

Analysis of TIL subsets through IHC was calculated through percentage calculation and absolute counting methodology in TMA sections. Percentage calculation was significantly correlated with absolute counting for all markers in pre-NAC (CD3 $n = 27$ $r = 0.7182$, CD8 $n = 27$ $r = 0.6064$, FOXP3 $n = 26$ $r = 0.7192$) and in post-NAC (CD3 $n = 55$ $r = 0.7733$, CD4 $n = 30$ $r = 0.6129$, CD8 $n = 55$ $r = 0.7338$, FOXP3 $n = 47$ $r = 0.5387$) TMA sections that had enough material for both quantification methodologies (Table 5). The lymphocyte subset with highest absolute counts in the pre-NAC and post-NAC samples was CD8 (127 ± 193.5 and 156.5 ± 90.5) (Table 4).

Higher absolute counts of CD3, CD4, CD8 and FOXP3 in pre-NAC samples were associated with longer DFS ($n = 28$ $P = 0.003$, $n = 19$ $P = 0.0062$, $n = 28$ $P = 0.0096$ and $n = 29$ $P = 0.0019$; respectively). Higher absolute counts of CD3 in pre-NAC samples had longer OS ($n = 28$ $P = 0.0241$).

Higher absolute counts of CD4 in post-NAC samples was associated with age ($n = 54$ $P = 0.0393$) and pCR ($n = 54$ $P = 0.0095$).

Higher ratio of absolute counts of CD8/CD4 in pre-NAC and post-NAC samples was associated with pCR ($n = 17$ $P = 0.0343$ and $n = 43$ $P = 0.0086$ respectively).

Table 2 Tumor infiltrating lymphocytes in pre-neoadjuvant-chemotherapy and post-neoadjuvant-chemotherapy samples (median ± interquartile range)

TIL population	(Me ± IQR)
Pre-NAC TIL	
Median percentage TIL H and E full-face ($n = 91$)	40 ± 20
Median percentage TIL H and E TMA ($n = 30$)	20 ± 15
Median counting TIL CD3 ($n = 28$)	244.5 ± 253.8
Median counting TIL CD4 ($n = 19$)	48 ± 107.5
Median counting TIL CD8 ($n = 28$)	102 ± 98.8
Median counting TIL FOXP3 ($n = 29$)	22 ± 35
Post-NAC TIL	
Median percentage TIL H and E ($n = 80$)	20 ± 15
Median percentage TIL H and E TMA ($n = 58$)	10 ± 5
Median counting TIL CD3 ($n = 68$)	156.5 ± 200.8
Median counting TIL CD4 ($n = 54$)	12 ± 27.5
Median counting TIL CD8 ($n = 70$)	75.5 ± 93.5
Median counting TIL FOXP3 ($n = 70$)	7.5 ± 14.5

NAC: Neoadjuvant-chemotherapy; TIL: Tumor-infiltrating-lymphocyte; TMA: Tissue microarrays; Me ± IQR: Median ± interquartile range.

Higher ratio of absolute counts of CD4/FOXP3 in pre-NAC sample was associated with longer DFS ($n = 16$ $P = 0.0389$). Higher ratio of absolute counts of CD4/FOXP3 in post-NAC sample was associated with pCR ($n = 30$ $P = 0.003$).

Higher ratio of absolute counts of CD4/CD3 in post-NAC samples was associated with pCR ($n = 48$ $P = 0.0095$).

DISCUSSION

We have evaluated pre and post-NAC samples in an effort to produce a comprehensive analysis of the role of TIL variation during NAC in TNBC samples. Evaluation of pre-NAC H and E staining of full-face sections found that those tumors with higher TILs are associated to both pCR and better outcome. These results are similar to those found by Denkert *et al*[2] in the neoadjuvant setting and by Loi *et al*[3] and Adams *et al*[4] in the adjuvant setting, and confirm accuracy of our methodology.

We did not find an association between prognosis and TILs in post-NAC samples evaluated in full-face or in TMA sections. García-Martínez *et al*[14] evaluated 121 BC cases and found that high TIL level in pre-NAC samples was associated to pCR. TIL in pre-NAC and post-NAC were not associated to outcome. By other side, Dieci *et al*[15] evaluated 278 TNBC with residual disease after NAC and found that those residual lesions with high level of TIL had better prognosis.

We found that TIL percentage evaluated in full-sections were higher in pre-NAC than post-NAC samples. No association between TIL variation (pre- *vs* post-NAC) and response to NAC was found. Post-NAC samples of those cases who obtained pCR were similar to TIL levels in those cases who did not obtain pCR (residual cancer). By other side, Dieci evaluated 19 selected cases with

Table 3 Comparison between tumor-infiltrating-lymphocytes evaluated in full-face and tissue microarrays sections

Features	H and E in the full-face section			H and E in TMA section		
	n	Me ± IQD	*P*-value[2]	*n*	Me ± IQD	*P*-value[3]
Pre-NAC	91			30		
Age			0.1427			0.6313
≤ 49	50	40 ± 20		19	20 ± 15	
> 49	41	20 ± 15		11	20 ± 25	
pCR			0.0251[1]			0.2227
No	63	30 ± 20		15	30 ± 25	
Yes	28	40 ± 17.5		15	20 ± 10	
Inflammatory			0.6401			0.0387[1]
Yes	27	40 ± 15		8	10 ± 6.5	
No	64	35 ± 20		22	30 ± 15	
DFS			0.0076[1]			0.1601
< 32 mo	46	20 ± 20		20	15 ± 13.8	
≥ 32 mo	45	40 ± 20		10	25 ± 25	
OS			0.0334[1]			0.7214
< 41 mo	51	30 ± 20		22	20 ± 15	
≥ 41 mo	40	40 ± 20		8	20 ± 15	
Post-NAC	80			58		
Age			0.8547			0.5684
≤ 49	43	20 ± 15		31	10 ± 15	
> 49	37	20 ± 15		27	10 ± 5	
Inflammatory			0.4582			0.1299
Yes	26	15 ± 10		16	10 ± 0	
No	54	20 ± 15		42	10 ± 15	
pCR			0.6331			0.1299
No	63	20 ± 15		44	10 ± 7.5	
Yes	17	20 ± 10		14	10 ± 7.5	
DFS[3]			0.2450			0.2573
< 32 mo	35	20 ± 15		22	10 ± 0	
≥ 32 mo	28	20 ± 15		22	10 ± 15	
OS[3]			0.5973			0.6948
< 41 mo	40	20 ± 15		27	10 ± 10	
≥ 41 mo	23	20 ± 15		17	10 ± 5	

[1]$P < 0.05$; [2]U Mann Whitney test; [3]Only cases with incomplete pathological response in post NAC. DFS: Disease free survival; Me ± IQD: Median ± interquartile deviation; OS: Overall survival; pCR: Pathologic complete response; NAC: Neoadjuvant-chemotherapy.

Table 4 Comparison of tumor-infiltrating-lymphocyte evaluated in pre- *vs* post-neoadjuvant-chemotherapy samples

Population of lymphocytes	*n*	Pre-NAC Me ± IQR	Post-NAC Me ± IQR	*P*-value[2]	*P* corrected value[3]
Percentage					
H and E whole slide	73	40 ± 15	20 ± 15	< 0.001	< 0.002[1]
H and E TMA	16	30 ± 17.5	15 ± 15	0.4321	
Absolute counting					
CD3	22	244.5 ± 315.5	255.5 ± 267	0.8583	
CD4	7	14 ± 94	32 ± 43	0.6721	
CD8	21	127 ± 193.5	156 ± 90.5	0.7544	
FOXP3	21	18 ± 31	12 ± 19.5	0.0917	

[1]$P < 0.05$; [2]Wilcoxon signed rank test; [3]Bonferroni correction. NAC: Neoadjuvant-chemotherapy; TMA: Tissue microarrays; Me ± IQR: Median ± interquartile range.

high TIL level in post-NAC samples and found that lower TIL increased during NAC[15], and Demaria *et al*[16] found that those cases with higher response have an increase of TIL during NAC in a series of 25 BC.

Although lymphocytic infiltration has demonstrated to behave as a prognostic and predictive marker in breast cancer, there are some aspects without standardization. We evaluated TIL in the 0.6 cm TMA, and we found that the TIL percentages differ from those found in the full-face sections. TIL percentage in TMA was not associated to pCR nor prognosis. Breast tumors and especially TNBC are heterogeneous lesions and our findings indicate that TILs concentration is also heterogeneous inside the different tumor areas. The evaluation of only one region of the tumor through a TMA cylinder appears not to produce confident information about immune reaction against the whole tumor.

Different articles have evaluated the role of TIL

Table 5 Relationship between percentage and absolute counting methodologies of tumor-infiltrating-lymphocyte subpopulations

Population of lymphocytes	*n*	rho[2]	*P*-value	*P* corrected value[3]
Pre-NAC				
CD3	27	0.7182	< 0.001	< 0.004[1]
CD4	17	0.5071	0.0378	0.1512
CD8	27	0.6064	0.0008	0.0032[1]
FOXP3	26	0.7192	< 0.001	< 0.004[1]
Post-NAC				
CD3	55	0.7733	< 0.001	< 0.004[1]
CD4	30	0.6129	0.0003	0.0012[1]
CD8	55	0.7338	< 0.001	< 0.004[1]
FOXP3	47	0.5387	0.0001	0.0004[1]

[1]$P < 0.05$; [2]Spearman correlation coefficient test; [3]Bonferroni correction. NAC: Neoadjuvant-chemotherapy.

subpopulations (IHC staining), however some of them have measured them by percentage (resembling methodology used for TIL evaluation with H and E)[2,3] and other have measured by an absolute counting[7,8,17-21].

We compared both methodologies in 0.6 cm TMA tumor samples for CD3, CD4, CD8 and FOXP3 lymphocyte subpopulations and we found a significant correlation between both methodologies.

We also evaluated the association between levels of CD3, CD4, CD8 and FOXP3 lymphocyte subpopulations (absolute counting) and clinical features. Although our sample size for evaluating lymphocyte subpopulations is small, we found that higher absolute counts of CD4 lymphocytes in post-NAC samples were associated with pCR. Higher absolute counts of CD3, CD4, CD8 and FOXP3 lymphocytes in pre-NAC samples were associated with DFS, and higher absolute counts of CD3 lymphocytes in pre-NAC samples had longer OS.

Finally, we also found an association of pCR with higher ratio of absolute counts of all CD8/CD4 in pre-NAC, and CD8/CD4, CD4/CD3, CD4/FOXP3 in post-NAC samples. Higher ratio of absolute counts of CD4/FOXP3 in pre-NAC sample was associated with longer DFS.

The role of one TIL subpopulation and the role of the relationship between two TIL subpopulations over tumor behavior have been previously described and some of authors confirm our findings. Rathore *et al*[9] found that higher levels of $CD3^+$, $CD4^+$ and $CD8^+$ TILs was significantly associated with good prognosis in a series of 123 early BC cases. Kim *et al*[22] reported that lower number of $CD8^+$ TILs in breast tumors were significantly associated with lymph node metastasis, higher stage and high proliferative index in a series of 72 early BC cases. Increased number of FOXP3 lymphocytes was associated to lymph node metastasis, high proliferative index and shorter DFS. Ladoire *et al*[10] evaluated 56 BC cases who went to NAC and found that high CD8 and absence of FOXP3 infiltration was associated to pCR. Miyashita *et al*[11] evaluated 131 TNBC patients treated with NAC and found that high CD8 TIL levels and high CD8/FOXP3 ratio in residual tumor had better outcome. García-Martínez *et al*[14] described a decrease of CD4, an increase of CD8 and an absence of changes of FOXP3 during NAC. High levels of CD3 and CD4 in pre-NAC were associated to pCR. A decrease of CD3 and CD4 during chemotherapy was associated to pCR. A decrease of CD3 during NAC was also associated to better DFS and OS. They also found that higher ratio of CD4/CD8 in pre-NAC was associated to a pCR. They also evaluated six public genomic datasets with around 1000 BC patients treated with NAC and found that higher CD4 count in post-NAC samples was associated to pCR. Finally, they found that high levels of CD3 in post NAC was associated to better DFS[14]. Other authors have also evaluated the role of TIL ratios, such as CD8/CD4[23] or FOXP3/CD3[12], as an alternative approach to better integrate the information provided by each TIL subpopulation[14].

Differences among mentioned authors and our findings could be explained by our small population size, analysis of a not representative area in the TMA samples or changes in $CD4^+$ TILs phenotype from effectors to suppressors. Therefore, our results need to be validated in larger series. Remarkably, our work is the first to our knowledge to evaluate TIL in BC tumors from Latinoamerican women. And, it is the first to compare the evaluation of TIL percentage in full-sections and in TMA sections, as well as to compare the evaluation of TIL levels through percentage analysis and through absolute counting.

Identification of biomarkers and evaluation of therapy in the neoadjuvant setting has become a major challenge in BC since they could speed-up the development and approval of new drugs[24]. pCR is a validated surrogate for drug efficacy in the neoadjuvant setting but its specificity needs still to be improved. The finding of a biomarker related to host immunity in the pre or post-NAC samples could have the benefit to predict response not only to chemotherapy but also to immune checkpoint modulators.

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COMMENTS

Background

Triple-negative-breast-cancer (TNBC) is associated to poor outcome and is highly prevalent among Latinoamerican women. Tumor-infiltrating-lymphocytes (TILs) have been associated to higher response to chemotherapy and better outcome in TNBC when evaluated in retrospective and prospective series as well as meta-analysis. An international TILs working group defined harmonization criteria to evaluate them in 2015, however, there is still some areas requiring a better understanding. There is not information describing TILs in small pieces of tumor, and the value and the appropriate methodology to evaluate TIL subpopulations in tissue microarrays (TMA). There is small information describing variation of TIL during chemotherapy.

Research frontiers

TNBC has higher prevalence in Latinoamerican women and is a poor prognostic malignancy without target therapy. Chemotherapy is the only available treatment for TNBC. TIL appears to identify prognosis in TNBC and some recent studies are evaluating if it predicts response to chemotherapy or immunotherapy. Therefore, information about TIL variation during chemotherapy is an important issue as it is the scenario we need to improve treatment efficacy.

Innovations and breakthroughs

As TNBC malignancy and role of TIL as biomarker are important issues, more research about relevance of TIL in TNBC is needed. Therefore, the authors revealed that TIL evaluated in a small area of tumor differs to those evaluated in full-face samples, and loss their prognostic and predictive value. The authors also found that evaluation of lymphocyte subsets can be equally performed through absolute counting or percentage calculating, and can provide prognostic information.

Applications

Based on the present study, the authors can suggest that full-face samples (and not core samples) are used for TIL evaluation in H and E. Absolute counting and percentage calculating could be considered appropriate for evaluation of TIL subsets.

Terminology

TIL is a biomarker with current strong evaluation in different malignancies including TNBC. TIL is accepted as those stromal mononuclear cells inside the tumor but not in contact with cancer cells nor inside tumor niches. TMA is a technique allowing immunohistochemistry staining of many tumor samples at the same time.

Peer-review

The paper is very interesting and well written.

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ORIGINAL ARTICLE

Case Control Study

Cigarette smoking, dietary habits and genetic polymorphisms in *GSTT1*, *GSTM1* and *CYP1A1* metabolic genes: A case-control study in oncohematological diseases

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Abstract

AIM

To analyze the association between oncohematological diseases and *GSTT1*/*GSTM1*/*CYP1A1* polymorphisms, dietary habits and smoking, in an argentine hospital-based case-control study.

METHODS

This hospital-based case-control study involved 125 patients with oncohematological diseases and 310 control subjects. A questionnaire was used to obtain sociodemographic data and information about habits. Blood samples were collected, and DNA was extracted using salting out methods. Deletions in *GSTT1* and *GSTM1*

(null genotypes) were addressed by PCR. *CYP1A1 MspI* polymorphism was detected by PCR-RFLP. Odds ratio (OR) and 95%CI were calculated to estimate the association between each variable studied and oncohematological disease.

RESULTS

Women showed lower risk of disease compared to men (OR 0.52, 95%CI: 0.34-0.82, P = 0.003). Higher levels of education (> 12 years) were significantly associated with an increased risk, compared to complete primary school or less (OR 3.68, 95%CI: 1.82-7.40, P < 0.001 adjusted for age and sex). With respect to tobacco, none of the smoking categories showed association with oncohematological diseases. Regarding dietary habits, consumption of grilled/barbecued meat 3 or more times per month showed significant association with an increased risk of disease (OR 1.72, 95%CI: 1.08-2.75, P = 0.02). Daily consumption of coffee also was associated with an increased risk (OR 1.77, 95%CI: 1.03-3.03, P = 0.03). Results for *GSTT1*, *GSTM1* and *CYP1A1* polymorphisms showed no significant association with oncohematological diseases. When analyzing the interaction between polymorphisms and tobacco smoking or dietary habits, no statistically significant associations that modify disease risk were found.

CONCLUSION

We reported an increased risk of oncohematological diseases associated with meat and coffee intake. We did not find significant associations between genetic polymorphisms and blood cancer.

Key words: Cancer; Oncohematological disease; Case-control study; Lifestyle; Diet; Tobacco; Xenobiotic metabolizing genes; *GSTT1*; *GSTM1*; *CYP1A1*

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Core tip: Cancer is considered as a multi-factorial disease. Except certain genetic abnormalities, viruses, environmental exposures and chemotherapeutic agents, it is not well defined which are the risk factors for these diseases (leukemia, lymphoma, multiple myeloma, among others). Here, we analyzed lifestyle and genetic polymorphisms as risk factors for blood cancer. We reported an increased risk of disease associated with meat and coffee intake. No significant associations were found between metabolic gene polymorphisms and disease. Our study offers relevant insights into diverse aspects of oncohematological diseases etiology, particularly genes and environmental factors, in an Argentinean population.

Cerliani MB, Pavicic W, Gili JA, Klein G, Saba S, Richard S. Cigarette smoking, dietary habits and genetic polymorphisms in *GSTT1*, *GSTM1* and *CYP1A1* metabolic genes: A case-control study in oncohematological diseases. *World J Clin Oncol* 2016; 7(5): 395-405 Available from: URL: http://www.wjgnet.com/2218-4333/full/v7/i5/395.htm DOI: http://dx.doi.org/10.5306/wjco.v7.i5.395

INTRODUCTION

Xenobiotic metabolizing enzymes (XME), coded by a family of xenobiotic metabolizing genes (XMG), transform endo and exogenous compounds in hydrophilic by-products, which are more easily excreted from the tissues[1]. It is well known since decades that genetic differences in the metabolism of drugs and environmental chemicals exist[2]. These differences are due to pharmacogenetic polymorphisms, which are allele variants that occur with a relatively high frequency in the population, and are generally associated with anomalies in gene expression or enzymatic function. Moreover, pharmacogenetic polymorphisms have been associated with an increased risk of some types of cancer, due to: (1) an impaired ability to inactivate endogenous or exogenous mutagenic molecules; or (2) the conversion of metabolites into highly reactive and toxic compounds. Both polymorphisms and the levels of exposure to their substrates, may impact on cancer susceptibility.

The cytochrome P450 family of enzymes is responsible for catalyzing phase Ⅰ metabolism reactions. CYP1A1 is a member of the CYP family and plays an important role in the metabolism of estrogen and polycyclic aromatic hydrocarbons (PAHs), catalyzing the activation of pro-carcinogenic PAHs[3]. Dysfunction of CYPs enzymes can cause damage to DNA, lipids and proteins, leading to carcinogenesis[4,5]. A commonly studied single nucleotide polymorphism (SNP) in *CYP1A1* gene is the T3801C (also named *MspI* polymorphism, *2A or m1), a T to C mutation in the 3′ flanking region of the gene. The C variant becomes more highly inducible than the T variant[6], which may cause enhanced enzymatic activity, thus modifying susceptibility to adduct formation and cancer risk[7]. In fact, T3801C polymorphism was associated with leukemia and cervical, hepatocellular, lung, prostate, and head and neck cancer[8].

Glutathione S-transferases (GSTs) constitute a superfamily of phase II detoxification enzymes which play a key role in cellular protection against environmental carcinogens, drugs, toxins and by-products of oxidative stress. GSTs catalyze the conjugation of reduced glutathione (GSH) to a wide variety of electrophilic compounds to facilitate their cellular excretion. In addition, as non-enzymatic proteins, GSTs can modulate signaling pathways that control cell proliferation, cell differentiation, apoptosis, anti- and pro-inflammatory functions and DNA damage processing, among other processes[9]. Genetic polymorphisms in *GST* genes are common in the human population. GSTM1 and GSTT1 exhibit variations in copy number due to complete gene deletion, resulting in the loss of enzymatic activity. The absence of enzyme has been associated with lung, breast and gastrointestinal cancer, among others[10], and also with adverse side effects and toxicity in chemotherapies[11].

Lifestyle and dietary habits are additional risk factors for cancer. Diet is known to modulate the immune system, and it may also influence cancer susceptibility through changes in the energy balance and in the levels of carcinogens and anticarcinogens[12]. Cigarette smoke contains more than 7000 chemicals and compounds, from which more than 70 are associated with cancer[13]. Benzene, present in tobacco smoke, is a strong carcinogen associated with leukemia and lymphoma development[14], and has long been recognized as hematotoxic[15].

It should not be forgotten that cancer susceptibility results from genetic and environmental factors, individually or in combination. According to this, it is expected that genetic, dietary and lifestyle factors interact with each others.

Several studies have inquired the epidemiologic risk factors associated with leukemia, lymphoma and/or myeloma. Except certain genetic abnormalities, viruses, environmental exposures and chemotherapeutic agents, little is known about risk factors that develop these onco-hematological diseases.

Argentina is within the range of countries with medium to high incidence of cancer, according to the International Agency for Research on Cancer (IARC) data for 2012. They estimated an incidence of 14.2 new cases/year/100000 persons for Hodgkin lymphoma (HL), leukemia, non-Hodgkin lymphoma (NHL) and multiple myeloma (MM) all together[16]. During 2012, nearly 3830 patients have died because of these diseases according to the Statistics and Health Information Office[17]. Between 2007 and 2011, oncohematological diseases account for the 6.5% of all cancer deaths[18].

The aim of this study was to analyze the association between oncohematological diseases and genetic polymorphisms in *GSTT1*, *GSTM1* and *CYP1A1*, dietary habits and cigarette smoking, in an argentine hospital-based case-control study.

MATERIALS AND METHODS

Subjects

A hospital-based case-control study was performed, involving 125 patients with oncohematological diseases and 310 control subjects. Participants were recruited between June 2013 and March 2015 at the Unit of Diagnosis, Treatment and Support for Hematological Diseases of the Acute Care General Hospital "Prof. Dr. Rodolfo Rossi" (La Plata, Buenos Aires, Argentina). The study was approved by the hospital's Ethics Committee. The International Classification of Diseases for Oncology information was not available.

Cases were patients diagnosed with acute lympho-blastic leukemia (ALL, n = 10), acute myeloblastic leukemia (AML, n = 18), chronic lymphoblastic leukemia (CLL, n = 10), chronic myeloblastic leukemia (CML, n = 20), MM (n = 29), HL (n = 18) and NHL (n = 20). Controls included patients frequently visiting the Unit for routine checks of disorders unrelated to cancer or preoperative blood analyses (*i.e*., for ophthalmic surgeries, pre-employment medical examinations, anemia, among others). All participants reside in Argentina. Cases and controls with previous history of cancer or pathologies closely related to oncohematological diseases were excluded from the study.

Patients were invited to participate in the study and signed an informed consent. A questionnaire was used to obtain sociodemographic data and information about habits, such as cigarette smoking (never, former and current smoker), and consumption of grilled/barbecued meat (times per month), canned food (times per week), alcohol (times per week) and coffee (cups per day). No other food or beverage items were asked. The survey also included weight, height, medication, working conditions, family history of cancer, history of disease (only in cases), and functionality data (quality of sleep, fatigue, changes in appetite and mood, *etc*.). The overall case and control response rate was higher than 90% for both groups.

All surveys were addressed by the same person. Blood samples were collected and kept in Vacutainer tubes with K2-EDTA (3.6 mg), and DNA was extracted from whole blood using salting out methods.

GSTT1 and GSTM1 genotype assay

GSTT1 and *GSTM1* gene deficiency resulted from the deletion of the loci (null alleles). The detection was performed by a multiplex PCR, using GSTT1 forward primer 5'-TTC CTT ACT ggT CCT CAC ATC TC-3' and GSTT1 reverse primer 5'-TCA CCg gAT CAT ggC CAg CA-3' (459 bp product), GSTM1 forward primer 5'-CTg CCC TAC TTg ATT gAT ggg-3' and GSTM1 reverse primer 5'-CTg gAT TgT AgC AgA TCA TgC-3' (273 bp product), and a third pair of primers, forward 5'-TCC AgC AgT TTC ATg AgA TgC-3' and reverse 5'-gAg gTC ATT TCA Tag CTg AgC-3' for a 221 bp product of the gene CLOCK, as an internal control of the reaction (Figure 1). PCR conditions were as follows, in a final volume of 15 μL: 1 × buffer, 50 ng DNA, 0.25 μmol/L each primer, 200 μmol/L dNTPs, 2 mmol/L $MgCl_2$, 0.45 U Taq Platinum Polymerase (Invitrogen, Life Technologies) and H_2O up to 15 μL. PCR cycling consisted in an initial denaturation at 94 ℃ for 5 min, followed by 35 cycles of 1 min at 94 ℃, 1 min at 59 ℃ and 1 min at 72 ℃, with a final extension at 72 ℃ for 5 min. Verification of PCR products, and subsequent identification of genotype, were performed using 2% agarose gels stained with GelRed (Biotium Inc., CA, United States). The absence of PCR product defines the null allele.

CYP1A1 genotype assay

MspI polymorphism was detected by PCR-RFLP. A product of 420 bp was amplified by PCR in a final volume of 15 μL, containing buffer 1 ×, 2 mmol/L $MgCl_2$, 250 nmol/L each primer, 200 μmol/L dNTPs, 0.4 U Taq DNA Polymerase Recombinant (Invitrogen), 30 ng DNA and H_2O up to 15 μL. Primers were: forward

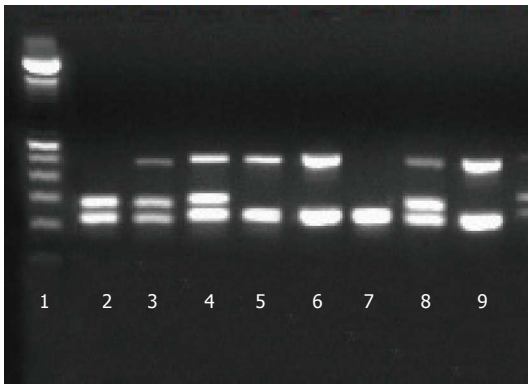


Figure 1 Gel electrophoresis showing PCR products of GSTT1 (459 bp), GSTM1 (273 bp) and CLOCK (221 bp). Numbered lanes represents: 1: 100 bp molecular weight marker; 3, 4, 8: T^+/M^+; DNA from samples with positive GSTT1 and GSTM1 alleles, plus control CLOCK gene band; 7: T^-/M^-; Double null genotype of GSTM1 and GSTT1 (in the presence of CLOCK PCR product); 5, 6, 9: T^+/M^-; GSTT1 positive/GSTM1 null; 2: T^-/M^+; GSTT1 null/GSTM1 positive.

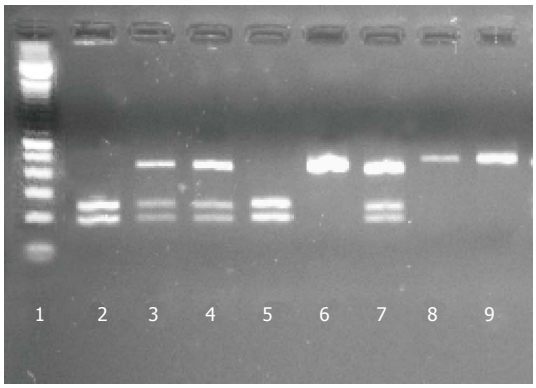


Figure 2 Gel electrophoresis showing PCR-RFLP product of CYP1A1 gene digested with MspI restriction enzyme. *CYP1A1* gene T3801C variant gives the following digestion band patterns: TT, 420 bp; TC, 420 bp and 237 bp + 183 bp; and CC, 237 bp + 183 bp. Numbered lanes represents: 1: 100 bp molecular weight marker; 2, 5: CC homozygous type; 3, 4, 7: TC heterozygous type; 6, 8, 9: TT homozygous type.

5′-ACC CCA TTC TgT gTT ggg TT-3′ and reverse 5′-TAg AgA ggg CgT AAg TCA gCA-3′. Cycling conditions were as follows: An initial denaturation at 94 ℃ for 5 min, followed by 35 cycles of 30 s at 94 ℃, 30 s at 58 ℃ and 40 s at 72 ℃, with a final extension at 72 ℃ for 5 min.

After checking amplification in 2% agarose gel stained with Gel Red, 7 μL of PCR product were digested with 5U of *MspI* enzyme (Thermo Scientific), buffer and H_2O up to 15 μL. Incubation time was 5 h at 37 ℃. Verification of digested products was carried out in 2% agarose gels stained with Gel Red. The C variant has the restriction site, generating 237 and 183 bp products (Figure 2).

Statistical analysis

Odds ratio (OR) and 95%CI were calculated to estimate the association between each variable studied and oncohematological disease. χ^2 test was applied to obtain the statistical significance of the association. Analyses were performed with the softwares STATA 11.1[19] and Epidat 4.0[20]. Genotype and allele frequencies were calculated and tested for Hardy-Weinberg Equilibrium using the software GenAlEx 6.5[21]. The sample size of this survey achieved 80% power to detect an OR = 2. *P*-values ⩽ 0.05 were considered statistically significant. The statistical review of the study was performed by a biomedical statistician (Gili JA, one of the authors).

RESULTS

Study population

In this association study, a total of 125 cases were compared to 310 controls, all of them patients from the Acute Care General Hospital "Prof. Dr. Rodolfo Rossi". Demographic characteristics are listed in Table 1, and were already published by our group in Cerliani *et al*[22]. Missing data for each variable were not included in the analysis, nor are detailed in the tables. The maximum number of missing data in a variable was 18, representing 4.14% of the samples. Of all variables in the questionnaire, only height and weight were excluded due to > 10% missing data. There was no significant difference in the mean age of cases and controls. Women showed lower risk of disease compared to men (OR 0.52, 95%CI: 0.34-0.82, *P* = 0.003). Higher levels of education (> 12 years) were significantly associated with an increased risk, compared to complete primary school or less (OR 3.68, 95%CI: 1.82-7.40, *P* < 0.001 adjusted for age and sex). Marital status did not show association with the disease.

Tobacco, dietary habits and XMG polymorphisms

With respect to tobacco, none of the smoking categories showed association with oncohematological diseases. Regarding dietary habits, consumption of grilled/barbecued meat 3 or more times per month showed significant association with an increased risk of disease (OR 1.72, 95%CI: 1.08-2.75, *P* = 0.02). Daily consumption of coffee also was associated with an increased risk (OR 1.77, 95%CI: 1.03-3.03, *P* = 0.03). Since control patients with gastrointestinal problems could create a spurious OR (given that they may be abstaining from coffee), patients from the Gastroenterology Unit of the hospital were removed from the analysis, as well as control patients under treatment with gastric protectors or medication prescribed for other gastrointestinal issues. Therefore 19 controls were excluded from the analysis; nevertheless, coffee consumption still remains statistically correlated with disease, showing the same OR range (OR 1.01-3.02). No association was observed with consumption of canned food or alcohol. When assessing the risk associated with consumption of coffee and grilled/barbecued meat, within each of the pathologies, results showed no differences between groups for coffee consumption, but CML and MM cases exhibited a significant association with consumption of grilled/barbecued meat 3 or more times per month, adjusted for age, sex and educational

Table 1 Demographic characteristics of the population under study *n* (%)

	Cases *n* = 125	Controls *n* = 310	OR (95%CI)	*P*
Age mean (SD)	48.5 (16.6)	51 (18.5)	-	0.507
Sex				
Male	74 (59.2)	134 (43.2)	Ref.	0.003
Female	51 (40.8)	176 (56.8)	0.52 (0.34-0.82)	
Education				
⩽ 7 yr (complete primary school or less)	174 (56.31)	55 (44)	Ref.	
12 yr (complete secondary school)	115 (37.22)	48 (38.4)	1.2 (0.73-1.95)	< 0.001[1]
> 12 yr (complete college)	20 (6.47)	22 (17.6)	3.68 (1.82-7.4)	
Marital status				
Single	79 (25.48)	27 (21.6)	Ref.	
In couple/married	162 (52.26)	86 (68.8)	1.7 (0.99-2.95)	0.05[1]
Divorced/separated	27 (8.71)	5 (4)	0.64 (0.21-1.9)	0.42[1]
Widowed	42 (13.55)	7 (5.6)	0.75 (0.27-2.13)	0.59[1]
Type of malignancy				
ALL	10 (8)			
AML	18 (14.4)			
CLL	10 (8)			
CML	20 (16)			
MM	29 (23.2)			
HL	18 (14.4)			
NHL	20 (16)			

[1]Adjusted for age and sex. $P < 0.05$ considered statistically significant. ALL: Acute lymphoblastic leukemia; AML: Acute myeloblastic leukemia; CLL: Chronic lymphoblastic leukemia; CML: Chronic myeloblastic leukemia; MM: Multiple mieloma; HL: Hodgkin lymphoma; NHL: Non-Hodgkin lymphoma; Ref: Reference category; SD: Standard deviation.

Table 2 Allele and genotype frequencies for *CYP1A1* *2A polymorphism, and *GSTT1/GSTM1* null polymorphisms

	Cases *n* = 125	Controls *n* = 310
Allele frequencies		
CYP1A1		
T	0.7	0.65
C	0.3	0.35
Genotype frequencies (*n*)		
GSTT1*null	0.18 (22)	0.18 (55)
GSTM1*null	0.5 (61)	0.47 (140)
CYP1A1		
TT	0.53 (64)	0.47 (140)
TC	0.34 (42)	0.37 (113)
CC	0.13 (16)	0.16 (47)

level (data not shown). With regard to *GSTT1*, *GSTM1* and *CYP1A1* polymorphisms, results showed no significant association with oncohematological diseases. *CYP1A1 MspI* polymorphism was not in Hardy-Weinberg equilibrium. Allele and genotype frequencies are detailed in Table 2. Results from the association analysis between tobacco, dietary habits, XMG polymorphisms and oncohematological diseases are described in Table 3. When analyzing the interaction between XMG polymorphisms and tobacco smoking or dietary habits, no statistically significant associations that modify disease risk were found (data not shown).

DISCUSSION

Carcinogenesis is considered as a multi-step and multifactorial process that implied different genetic alterations and several biological pathways. Thus, it is expected that cancer risk factors interact with each others. Genetic polymorphisms may play different roles in cancer susceptibility according to the genetic background, environmental exposures and lifestyles[8]. Despite this, we did not find evidence of interaction between genetic and lifestyle factors, probably because the sample size is not big enough for this type of analysis.

Regarding tobacco use and dietary habits, we reported an increased risk of oncohematological diseases associated with grilled/barbecued meat intake 3 or more times per month, and with daily consumption of coffee, after adjustment for age, sex and educational level. Moreover, for both factors that showed an increased risk when analyzing all malignancies combined, we have also assessed the risk associated with each of the major groups within the case category. While for coffee consumption results showed no differences between groups, grilled/barbecued meat intake (3 times or more per month) has shown a significant correlation with CML and MM cases. However, it has to be considered that the sample size becomes small when divided by each pathology. Therefore, the real effect of each risk factor for a specific type of leukemia, lymphoma or myeloma might be determined in future studies by analyzing a larger set of samples.

Although cigarette smoke has been associated with mutagenic/carcinogenic effects, inflammation and immune suppression in animals and humans[23,24], previous studies on cigarette smoking and its association with blood cancers have generated inconsistent findings. Regarding NHL, studies reported little or no association

Table 3 Association between tobacco, dietary habits, xenobiotic metabolizing genes polymorphisms, and oncohematological diseases *n* (%)

	Cases *n* = 125	Controls *n* = 310	OR (95%CI)	*P*
Tobacco smoking status				
Never	49 (39.8)	142 (46.4)	Ref.	
Former	47 (38.2)	100 (32.7)	1.36 (0.84-2.19)	0.202
Current	27 (22)	64 (20.9)	1.22 (0.70-2.13)	0.478
Consumption of canned food				
0-2 times/wk	121 (96.8)	292 (94.8)	Ref.	0.370
3 or more times/wk	4 (3.2)	16 (5.2)	0.60 (0.14-1.92)	
Consumption of grilled/barbecued meat				
0-2 times/mo	69 (55.2)	217 (70.7)	Ref.	0.021[1]
3 or more times/mo	56 (44.8)	90 (29.3)	1.72 (1.08-2.75)	
Alcohol drinking				
0-3 times/wk	110 (88)	268 (86.5)	Ref.	0.665
4 or more times/wk	15 (12)	42 (13.5)	0.87 (0.43-1.68)	
Consumption of coffee				
< 1 cup/d	90 (72)	259 (83.5)	Ref.	0.037[1]
1 or more cups/d	35 (28)	51 (16.5)	1.77 (1.03-3.03)	
GSTT1				
Presence	100 (82)	245 (81.7)	Ref.	0.942
Null	22 (18)	55 (18.3)	0.98 (0.54-1.74)	
GSTM1				
Presence	61 (50)	160 (53.3)	Ref.	0.534
Null	61 (50)	140 (46.7)	1.14 (0.73-1.78)	
CYP1A1				
TT	64 (52.5)	140 (46.7)	Ref.	0.280
TC + CC	58 (47.5)	160 (53.3)	0.79 (0.51-1.24)	

[1]Adjusted for age, sex, and educational level. $P < 0.05$ considered statistically significant. Ref.: Reference category.

with use of cigarettes/tobacco, or patterns related with duration or intensity of exposure (detailed in[25-27]). In a case-control study from Sweden, the effects of smoking on the risk of AML were weak and no significant[28]. However, a cohort study from the same country showed a 50% increased risk of AML for current smokers[29]; this study also indicated no association between current and former smokers with CML, ALL, CLL or MM. An Indian case-control study reported an increase of 2.1 fold in the risk of leukemia in the cigarette smokers, compared to non-smokers[30]. A US cohort study that evaluated risk factors for AML, showed hazard ratios of 1.79, 2.42 and 2.29 for former smokers of > 1 pack/d, current smokers of ⩽ 1 pack/d, and current smokers of > 1 pack/d, respectively[31]. A meta-analysis done by Fircanis *et al*[32] including over 7500 cases of AML, found an increased risk of disease associated with smoking, regardless of sex, geographical region, study design and quality of studies. They also reported a higher risk with higher intensity and longer duration of smoking.

It is possible that the exposure to agents present in the smoke varies given the different usage patterns, exposure pathways and manufacturing processes.

A recent study done by Rubinstein *et al*[33] showed that 29.7% of the general population of Argentina, Chile and Uruguay (n = 7524) smoke cigarettes. The CARMELA study (Cardiovascular Risk Factor Multiple Evaluation in Latin America), performed between 2003 and 2005, reported that Buenos Aires (Argentina, n = 1482) and Santiago (Chile, n = 1655) have the highest smoking prevalence among the seven Latin American cities studied, with no gender differences (38.6% and 45.4% respectively)[34]. Our study reports that 20.9% of the controls and 22% of the cases are current smokers, with higher percentages for former smokers (32.7% for controls and 38.2% for cases). These results are probably due to that this study is based on hospital population, which strongly encourages quitting. Considering the high proportion of people exposed to tobacco in these countries, it would be interesting to continue assessing its impact on the development of blood cancer, given the limited information available.

Diet, probably among the most modifiable environmental factors, contributes to the development of 30%-35% of cancers[35]. Association studies in the field may be inconsistent due to differences in the frequency of food intake, varieties of available food, and different methods of preparation among populations.

According to a recent publication of the IARC Monograph Working Group, consumption of processed meat was classified as "carcinogenic to humans" (group 1), and consumption of red meat as "probably carcinogenic to humans" (group 2A)[36]. Meat processing can result in formation of N-nitroso compounds and PAH, while cooking it can produce heterocyclic aromatic amines and PAH. High-temperature cooking, such as grilling and barbecuing, produces the highest amounts of these carcinogens. Barbecued red meat is a frequent dish

among the Argentinean population, where there is a high consumption of animal protein and fats obtained mainly from red meat[37,38]. Several epidemiologic studies from Córdoba, an Argentinean province, report significant associations between consumption of red meat, or dietary patterns that contains it, and breast, colorectal, prostate, and urinary tract cancers[37-42]. According to Navarro *et al*[38], all meats were associated with an increased risk for colorectal cancer when barbecued, a similar result to that observed in our study. In two other surveys, the Southern Cone dietary pattern (red meat, starchy vegetables and wine consumptions) was associated with higher risk of urinary tract tumors[39] and colorectal cancer[37]. In a case-control study from Uruguay, red meat, lamb, and boiled meat were associated with the risk of squamous cell carcinoma of the esophagus[43].

In relation to oncohematological diseases, there are no studies in Argentina concerning diet and lifestyle as risk factors. Several studies from other countries evaluated this possible association, with inconsistent results. Most of them reported no association between meat consumption and increased risk of NHL or CML[44-46]. A case-control study from US done by Li *et al*[47] reported a positive association between an increased risk of AML and beef intake among women. Our results are the first to report a significant association between oncohematological diseases and consumption of barbecued/grilled meat in our population. However, some bias could exist since portion size was not assessed, and the questionnaire does not differentiate between red and white meat. Despite this, our results are relevant given the fact of a high and frequent consumption of these foods in Argentina.

It has been suggested that light to moderate alcohol consumption has beneficial effects due to advantageous host cellular and humoral immune responses[48]. On the other hand, ethanol was classified as carcinogenic to humans by the IARC. Regarding hematologic malignancies, a pooled analysis from the International Lymphoma Epidemiology Consortium reported that ever or current drinking were associated with a lower risk of NHL, compared with non-drinkers[49]. However, they did not find a dose-response relation or a stronger trend with longer duration. A meta-analysis performed with 18 studies, including 5694 cases with MM and 7142 with leukemia, did not find any association between alcohol drinking and MM or leukemia risks[50,51]. As with beef consumption, Li *et al*[47] reported a positive association between beer and wine intake and AML, only among women. An Italian case-control study showed no clear association between leukemia or NHL and alcohol consumption[27]. In line with some of these reports, we found no association between alcohol consumption and oncohematological diseases in the Argentinean population under study.

Regarding coffee consumption, there is no consistent evidence suggesting protective or deleterious effects. On the one hand, coffee may decrease the risk of cancer through antioxidant, antihormonal, and anti-inflammatory mechanisms[52]. On the other hand, caffeine and Topo Ⅱ inhibitors may elevate cancer risk. A United States cohort study reported no association between coffee intake and risk of all cancers combined, but they observed a decrease in the risk of endometrial cancer for women drinking 1 or more cups per day[53]. Although some studies on solid tumors have reported a protective effect (*i.e*., on liver, colorectal, breast and endometrial cancer[54-57]), studies on hematopoietic malignancies in adults are rare. In our study, daily consumption of coffee was associated with an increased risk of disease. An Indian case-control study showed a 40% reduction in the risk of leukemia for coffee drinkers[30], while an Italian one reported an increase in the risk of NHL[58]. Other studies found no significant associations between coffee consumption and hematologic cancer[44,59,60]. In 1991, the IARC Working Group classified coffee as possibly carcinogenic to humans (group 2B). Given the large number of studies published on the subject since that IARC publication, the IARC Advisory Group recommends a review of the evidence, giving to this exposure high priority for its inclusion in the monographs to be published between 2015 and 2019[61].

Genetic variations in XMG may be important factors in the etiology of onco-hematological diseases. Although they have low penetrance, they are highly prevalent in most populations, giving the chance to identify potential carcinogens and populations at higher risk of cancer[62]. These polymorphisms also interact with other polymorphisms and/or particular environmental factors, which vary between and within ethnic groups[8]. The increased activity/inducibility of CYP1A1*2A may contribute to the accumulation of genetic changes due to an increased production of mutagenic agents. In a similar way, the decreased activity of GSTs due to gene deletions may lead to a more intense cellular oxidative stress, increasing the level of DNA damage.

Regarding *CYP1A1 MspI* polymorphism, allele frequencies vary between ethnic groups, being 0.058, 0.149 and 0.218 for the C allele (*2A) in Caucasians, Asians and Africans, respectively[63]. Roco *et al*[64] reported a *2A allele frequency of 0.37 for a Chilean population. In this study, *2A allele frequencies were 0.34 for controls and 0.30 for cases, similar to that described for the Chilean population and away from that reported for Caucasians. Our genotypic data about this polymorphism deviate from Hardy-Weinberg equilibrium; this may be because the sample does not represent the entire population variability, or due to genotyping errors that create a bias towards increased homozygosity.

Our association analysis between *MspI* polymorphism and blood cancer showed an OR = 0.79 (95%CI: 0.51-1.24), with no statistical significance. Association studies between CYP1A1 *MspI* polymorphism and cancer have been inconsistent. A meta-analysis from 268 studies performed by He *et al*[8] showed that the variant *2A was associated with an increased risk of leukemia, cervical, hepatocellular, head and neck, lung and prostate cancer, but not with other cancer types. Another meta-analysis done by Han *et al*[65] reported a higher risk of leukemia associated with this variant, which remains significant for Caucasians when stratified by ethnicity.

They also reported an increased risk for ALL and AML, especially in childhood ALL in Caucasians. Among Asians, Lu *et al*[66] showed that the presence of the *MspI* polymorphism increased the risk of AML. On the other hand, a meta-analysis performed by Zhuo *et al*[67] did not find significant associations between CYP1A1 variant and AML risk.

Many studies have analyzed the possible association between hematological cancer and the deletions of *GSTT1* and *GSTM1*, with disparate results. In this study, frequencies for null genotypes were 0.18 in cases and controls for GSTT1, and 0.5 and 0.47 for GSTM1, in cases and controls respectively. Reported frequencies for GSTT1*null in controls were 0.13-0.26 for Caucasians[63], 0.10-0.12 for Chilean and Argentinean populations[64,68-70], and 0.09-0.24 for Native American Argentineans[71]. GSTM1*null has higher frequencies, with values of 0.42-0.60 for Caucasians[63], 0.36-0.46 for Chilean and Argentinean populations[64,68-70], and 0.29-0.49 for Native American Argentineans[71]. Our study showed an OR = 0.98 (95%CI: 0.54-1.74) for the GSTT1 deletion, and an OR = 1.14 (95%CI: 0.73-1.78) for GSTM1 deletion. He *et al*[62] carried out a meta-analysis, showing that GSTM1*null genotype significantly increased the risk of AML in East Asians, while GSTT1*null increased it in Caucasians. Double-null genotypes were associated with AML in both ethnic groups. Several case-control studies reported significant associations between GSTT1*null genotype and AML, CML, CLL and acute leukemia[72-78]. Conversely, other studies did not find such associations with all leukemia, acute leukemia, CML, AML or MM[79-82]. A similar scenario occurs with GSTM1*null genotype: many studies showed a significant increased risk of NHL, CLL, AML, MM, CML, and acute leukemia associated with the null genotype[72,73,76,78,83,84], while other ones reported no differences in cancer risk between controls and CML, AML, MM, acute leukemia and all leukemia cases[74,75,77,79-82]. In general, studies reported moderate OR, with values from 1 to 3; however, values up to 7 were reached in some assays, with OR values even greater when analyzing double null genotypes. It should be noted that these case-control studies are from different countries with different ethnic backgrounds, and with a variable number of participants.

Our study has strengths that are worth mentioning. Face-to-face interviews were performed by the same person, with all cases and controls, thus addressing reliable data about personal information, habits and lifestyle. Furthermore, although sample size is small, it has the power to detect an OR = 2, a value close to that reported in similar case-control studies. Even though cases and controls were not age- and gender-matched, statistical adjustment were used to minimize potential biases. It should be recall that hospital-based studies may have some bias, due to controls that might have benign diseases which are prone to turn malignant. This study design could reduce the generalization of the results to the general population. Additionally, hematological malignancies are heterogeneous illnesses, with potentially different causes. A larger number of samples will allow us to conduct studies of risk factors for each pathology independently.

There is a lack of studies for oncohematological diseases etiology in an argentinean population, particularly for genes and environmental factors. Taking that into account, our goal through the study was to address and offers relevant insight into diverse aspects for these pathologies in our population.

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COMMENTS

Background

Risk factors for oncohematological diseases are not completely defined. As in other cancer pathologies, blood cancer susceptibility is related to both lifestyle and genetic factors. In regards to the Argentinean population, these pathologies are barely studied, even in relation to highly frequent risk factors for this population.

Research frontiers

In the latest years, although specific diet patterns for Argentina have been evaluated as risk factors related to cancer development by many studies, hematological cancer was not included. Moreover, studies about tobacco, alcohol and coffee as risk factors for oncohematological diseases are still not conclusive; furthermore, the results can vary between different populations and study designs. On the other side, genetic variants in coding genes for enzymes associated to carcinogenic compounds metabolization are known genetics risk factors linked to cancer. Allelic frequencies for these enzymatic variants are different between populations; therefore, it becomes difficult to extrapolate an estimated risk from one population to another. For Argentina, genetic frequencies for metabolizing enzymes are already known; however, available data are limited to just a few cities and, mostly, for healthy population.

Innovations and breakthroughs

In that regards, in this work the authors reported an association between oncohematological diseases with coffee and meat intake. With respect to the latter risk factor, the result becomes extremely relevant given the fact of high meat consumption in Argentina. The results are the first one showing a possible association in the authors' population. Moreover, Argentina has a medium-high cancer incidence; every research implying an evaluation of different risk factors frequently found in the population, could help in contributing with new information, in order to dilucidate which specific factors are really involved in the actual incidence of blood related cancer diseases.

Applications

The result could be joined with the whole set of studies looking to evaluate the impact of genetics and lifestyle factors in cancer development. The results reported for coffee and barbecued/grilled meat intake, could be taken into account to go deeply in future studies by evaluating specific kinds of meat, portion size, other cooking methods, *etc*. This could allow researchers to classify, more specifically, which particular characteristics related to nutrition patterns are associated with cancer development in a population. Additionally, each oncohematological pathology could be also analyzed individually from the main group, due to the fact that each specific genetic or lifestyle factor could be involved in different pathways and/or specific disease stages, independently.

Terminology

Odds ratio (OR), in statistics, the OR is a way to quantify how strongly the presence or absence of property A is associated with the presence or absence

of property B in a given population. Oncohematological diseases or hematology-oncology, the diagnosis, treatment and prevention of cancer developed in blood cells, and the research associated to them. Hematology-oncology includes diseases such as leukemias and lymphomas, as well as other blood disorders (*i.e.*, iron deficiency anemia, hemophilia, sickle cell disease, and thalassemias).

Peer-review

The study indicates an increased risk of oncohematological diseases associated with meat and coffee intake. Overall the manuscript was written in a clear and concise manner.

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ORIGINAL ARTICLE

Observational Study

Factors associated with cervical cancer screening in a safety net population

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Abstract

AIM

To identify factors associated with Papanicolaou-smear (Pap-smear) cervical cancer screening rates in a safety net population.

METHODS

From January 2012 to May 2013, the use of Pap-smear was determined for all patients seen at the breast clinic in a safety net hospital. Health literacy assessment was performed using the validated Newest Vital Sign. The records of patients were reviewed to determine if they had undergone Pap-smears for cervical cancer screening. Sociodemographic information was collected included age, education, monthly income, race/ethnicity, employment, insurance status, and primary care provider of the

patient. Logistic regression analysis was then performed to determine factors associated with utilization of Pap-smears. Crude and adjusted odds ratios derived from multivariate logistic regression models were calculated as well as the associated 95%CIs and *P*-values.

RESULTS

Overall, 39% had Pap-smears in the prior 15 mo, 1377 consecutive women were seen during the study period and their records were reviewed. Significantly more patients with adequate health literacy underwent Pap-smears as compared to those with limited health literacy (59% *vs* 34%, $P < 0.0001$). In multivariate analysis, patients with adequate health literacy, younger patients, and those with later age of first live birth were more likely to undergo Pap-smears. Patients whose primary care providers were gynecologists were also significantly more likely to have Pap-smears compared to other specialties ($P < 0.0001$). Patients younger than 21 years or older than 65 years underwent screening less frequently (11% and 11%, respectively) than those 21-64 years (41%, $P < 0.0001$). Race, ethnicity, language, and insurance status were not associated with Pap-smear screening rates.

CONCLUSION

Patient health literacy and primary care physician were associated with Pap-smear utilization. Development of interventions to target low health literacy populations could improve cervical cancer screening.

Key words: Cervical cancer; Health literacy; Prevention; Screening; Pap-smear

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Core tip: Patient health literacy and type of primary care physician were associated with Papanicolaou-smear utilization. Development of interventions to target low health literacy populations could improve cervical cancer screening and therefore improve screening in populations most at risk for cervical cancer.

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INTRODUCTION

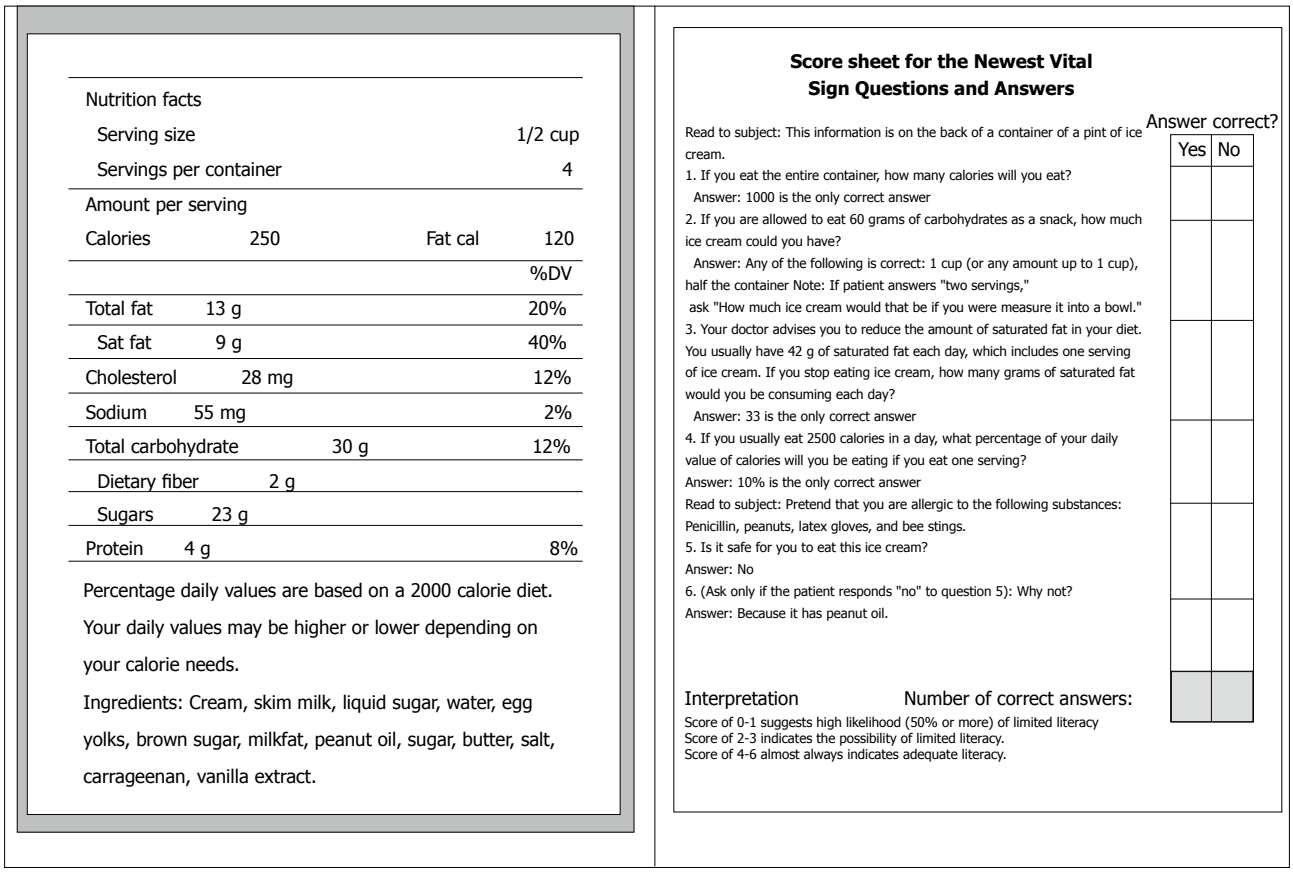
Cervical cancer is one of the most preventable and treatable female cancers. While cervical cancer death rates have decreased, it remains a significant burden in all countries. Cervical cancer screening rates are most suboptimal among recent immigrants to developed countries, in countries without screening programs, among racial and ethnic minorities, women from low socioeconomic backgrounds, and underinsured populations[1-3]. Most studies show higher rates of cervical cancer and lower compliance with cervical cancer screening in these populations[1-3].

Previous studies have found that patients do not understand the concept of screening or did not realize that Pap-smears are a test for cervical cancer. One population studied was less likely to undergo screening because they "didn't have problems/symptoms"[4]. A second study found that patients thought Papanicolaou-smear (Pap-smears) were for infections, gonorrhea, or HIV[5]. Health literacy is the degree to which individuals have the capacity to obtain, process, and understand basic health information needed to make appropriate health decisions[6]. In 2003, the National Assessment of Adult Literacy (NAAL) reported that over 89 million American adults have limited health literacy skills and that individuals with limited health literacy come from all parts of society[7,8]. In fact, the NAAL survey, which rated health literacy skills in four levels ranging from "below-basic" to "proficient", showed that more than 40% of high school graduates and 13% of college graduates have health literacy skills at the lowest two levels and are thus considered to have limited health literacy[8,9]. Similarly, the 2011 Programme for the International Assessment of Adult Competencies which evaluated adults in 23 industrialized countries indicated continuing issues: A significant proportion of adults scored below average for literacy, numeracy, and problem solving in technology-rich environments[10]. The number of patients with low health literacy is increasing and is more common among those with low educational attainment, immigrants, elderly, and racial/ethnic minorities[7,11]. These individuals are less able to navigate the health care system and less likely to participate in preventive health care[12-16]. The current study was performed to identify factors that influence use of Papanicolaou cytology smears for cervical cancer screening in a safety net population.

MATERIALS AND METHODS

This study was conducted at Maricopa Medical Center in Phoenix, Arizona, and reviewed and approved by the medical center's institutional review board. The institutional review board also granted a waiver of informed consent for this study. Maricopa Medical Center is the safety net hospital for Maricopa County, which includes the city of Phoenix and the surrounding metropolitan area. Maricopa County is the state's most populous area with nearly four million of Arizona's 6.5 million inhabitants. Maricopa Medical Center serves a patient population of which 78% of patients are from racial/ethnic minority groups and 79% are underinsured, uninsured, or insured by Medicaid.

Beginning on January 1, 2012 and continuing until May 31, 2013, every patient seen in the Breast Clinic underwent a health literacy assessment as part of their routine history and physical examination. Health literacy



Nutrition facts

Serving size	1/2 cup
Servings per container	4

Amount per serving

Calories	250	Fat cal	120

		%DV
Total fat	13 g	20%
Sat fat	9 g	40%
Cholesterol	28 mg	12%
Sodium	55 mg	2%
Total carbohydrate	30 g	12%
Dietary fiber	2 g	
Sugars	23 g	
Protein	4 g	8%

Percentage daily values are based on a 2000 calorie diet. Your daily values may be higher or lower depending on your calorie needs.

Ingredients: Cream, skim milk, liquid sugar, water, egg yolks, brown sugar, milkfat, peanut oil, sugar, butter, salt, carrageenan, vanilla extract.

Score sheet for the Newest Vital Sign Questions and Answers

Read to subject: This information is on the back of a container of a pint of ice cream.

1. If you eat the entire container, how many calories will you eat?
Answer: 1000 is the only correct answer
2. If you are allowed to eat 60 grams of carbohydrates as a snack, how much ice cream could you have?
Answer: Any of the following is correct: 1 cup (or any amount up to 1 cup), half the container Note: If patient answers "two servings," ask "How much ice cream would that be if you were measure it into a bowl."
3. Your doctor advises you to reduce the amount of saturated fat in your diet. You usually have 42 g of saturated fat each day, which includes one serving of ice cream. If you stop eating ice cream, how many grams of saturated fat would you be consuming each day?
Answer: 33 is the only correct answer
4. If you usually eat 2500 calories in a day, what percentage of your daily value of calories will you be eating if you eat one serving?
Answer: 10% is the only correct answer

Read to subject: Pretend that you are allergic to the following substances: Penicillin, peanuts, latex gloves, and bee stings.

5. Is it safe for you to eat this ice cream?
Answer: No
6. (Ask only if the patient responds "no" to question 5): Why not?
Answer: Because it has peanut oil.

Answer correct?	Yes	No
Number of correct answers:		

Interpretation

Score of 0-1 suggests high likelihood (50% or more) of limited literacy
Score of 2-3 indicates the possibility of limited literacy.
Score of 4-6 almost always indicates adequate literacy.

Figure 1 The newest vital sign instrument for health literacy assessment. Available from: URL: http// www.pfizerhealthliteracy.com/.

assessment involves the use of validated instruments. The Newest Vital Sign was chosen because it can be administered in about 2 min, and therefore is feasible for use in clinical practice rather than strictly a research setting. In addition, it has the advantage of having been validated in both English and Spanish[17-19].

The Newest Vital Sign uses a nutrition label, similar to the nutrition label present on all packaged food in the United States. The nutritional label was tested as one of many scenarios developed by a panel of health literacy experts based on concepts and types of scenarios used in health literacy research and in general literacy assessments. The candidate scenarios tested involved both reading and numeracy skills as this was driven by research indicated that these skills are highly correlated with one another, plus an intuitive understanding that patients must be able to use and understand both text and numbers if they are to successfully deal with today's health care system. Of the candidate scenarios tested, the nutritional label performed the best and has been validated and correlates well with more complicated health literacy instruments[17].

The assessment involves asking the patient five questions (and a sixth qualifier question, if needed) about the nutrition label (Figure 1). One point is awarded for each correct answer. Instructions accompanying the nutrition label specify what the interviewer should ask and they list the only acceptable answer(s). Scoring instructions for the Newest Vital Sign place patients into 3 groups, depending on how many of the 6 questions are answered correctly. A score of 0-1 indicates a high likelihood of limited health literacy. A score of 2-3 indicates the possibility of limited health literacy. Score of 4-6 indicate adequate health literacy. The instrument validation, including its sensitivity and specificity among Spanish speakers has been previously described[17].

The details of incorporating routine health literacy assessment as part of the standard history and physical examination have been described previously[19]. In short, the assessment was performed by the clinician during the history and physical examination. However, if the patient was Spanish speaking, the assessment was performed by a hospital certified Spanish translator or a clinician who was a native-Spanish speaker. No special personnel or other resources were used for performance of the health literacy assessment. Patients were excluded from health literacy assessment if they did not speak English or Spanish, had visual impairment that prevented them from viewing the nutritional label, or had cognitive limitations due to developmental delay, dementia, psychiatric illness, or head injury.

The records of patients receiving care during the study period were reviewed to determine if they had undergone Papanicolaou cytology smears (Pap-smears) for cervical cancer screening. If there was no record in the medical system of a Pap-smear, the patient was

Table 1 Patient sociodemographics

	All patients (n = 1318)
Mean age, years (SD)	45.0 (13.1)
Race/ethnicity	
Non-Hispanic White	230 (17%)
African American	131 (10%)
Hispanic	888 (67%)
Other	69 (5%)
Language, English	591 (45%)
Mean age of menarche (SD)	12.8 (1.88)
Mean pregnancies (SD)	3.3 (2.32)
Mean live births (SD)	2.7 (1.97)
Mean age of first live birth (SD)	21.0 (4.43)
Education, years	
6 or less	286 (22%)
7-11	340 (26%)
High school/equivalent	324 (25%)
Some college	368 (28%)
Adequate health literacy	229 (17%)
Body mass index in kg/m^2 (SD)	29.3 (7.57)
Marital status - married	555 (42%)
Employment, employed	456 (35%)
Insurance status	
Commercial	60 (5%)
Medicare	45 (3%)
Medicaid	312 (24%)
None	900 (68%)
Monthly income $US (SD)	1099 (878.46)
Screening mammography (age ≥ 40 yr)	253/899 (28%)
Pap smear in last 15 mo	514 (39%)

SD: Standard deviation; $US: United States dollars.

asked about their last screening and documentation was obtained *via* contact with prior health facilities or providers to confirm the date and results.

Patients were excluded and no attempt was made to obtain Pap-smear results on patients if they had undergone hysterectomy for reasons other than cervical cancer or had not yet initiated sexual activity. All other patients were considered to have undergone screening if they had done so in the year based on the more guidelines and recommendations for annual Pap-smears as of January 2012. Many insurance payors, however, will not pay for repeat screenings in durations even one day less than one calendar year. Therefore patients will often be scheduled for subsequent screenings in 13 or 14 mo. To account for this issue, a 15 mo time period was chosen to give some room for error on the "annual screening".

For all patients, sociodemographic information was collected and included age, education, self-reported monthly income, race/ethnicity, employment status, and insurance status. Patients were also queried about their reproductive status, height and weight, current smoking status, and use of alcohol. Finally, data were collected on whether or not the patient had a primary care provider and the type of provider.

Statistical analysis

Age, education, body mass index (height/weight^2), number of live births, and estimated monthly income were analyzed as continuous variables. Race/ethnicity (non-Hispanic white *vs* other, Hispanic *vs* non-Hispanic), employment status, insurance status (uninsured *vs* insured), and primary care provider were analyzed as categorical variables. Adequacy of health literacy was analyzed as a categorical variable. Patients with Newest Vital Sign 4-6 were categorized as having "adequate health literacy". Patients with Newest Vital Sign scores of 0-1 and 2-3 had similar use of Pap-smears and were therefore combined into a single group labeled "low health literacy". Analysis of the data did not demonstrate differences when the patients were categorized in three groups or in the two groups as presented. A two-sample *t*-test was used to determine if there were significant differences in continuous variables between women who did and did not undergo Pap-smears. A Fisher's exact test was used to assess differences in the categorical variables. All statistical tests were two sided and significance levels were set at 0.05.

Logistic regression analysis was then performed with the dependent variable being whether or not a patient had undergone Pap-smears. Independent variables included health literacy (adequate *vs* low literacy) and the sociodemographic variables listed in Table 1. Factors shown to have $P < 0.1$ in univariate analysis were included in the multivariate model. Crude and adjusted odds ratios (ORs) derived from multivariate logistic regression models were calculated as well as the associated 95%CIs and *P* values.

The statistical methods of this study were performed and reviewed by a biomedical statistician (CHH).

RESULTS

A total of 1377 consecutive patients were seen from January 1, 2012 and continuing until May 31, 2013. Fifty-nine patients were excluded because they were not yet sexually active or had hysterectomy for an indication other than cervical cancer. The remaining 1318 patients made up the study population (Table 2). The average age of the women was 45 years and the minority was non-Hispanic White (17%), while 10% were African American and 67% were Hispanic. Only 45% spoke English as their primary language. The vast majority (92%) were underinsured (24% Medicaid and 68% uninsured). The mean monthly income was $1099. Patients completed an average of 10 years of education.

Overall, 39% underwent a Pap-smear in past year. Table 1 shows the rates of Pap-smear use according to various sociodemographic variables, health literacy, and other factors. Several factors were significantly associated with Pap-smear uptake, including age, education, employment status, number of pregnancies, age at first live birth, and menopausal status. When evaluated by health literacy, significantly more patients with adequate health literacy underwent Pap-smears as compared to those with low health literacy (59% *vs*

Table 2 Patient factors associated with use of Pap-smear for cervical cancer screening

Variables	Unadjusted			Adjusted[2]	
	Rate	OR	*P*-value[1]	OR	*P*-value
Age (per year)	45.17 ± 12.64	0.97 (0.96, 0.98)	< 0.0001	0.97 (0.96, 0.99)	< 0.0001
Non-Hispanic White race		0.92 (0.68, 1.23)	0.6029	-	
No	429/1088				
Yes	86/230				
Hispanic ethnicity		1.16 (0.91, 1.47)	0.2294	-	
No	158/430				
Yes	357/888				
Pregnancies (per pregnancy)	3.31 ± 2.28	0.95 (0.90, 0.99)	0.0277	1.07 (0.95, 1.20)	0.2537
Live births (per birth)	2.70 ± 1.95	0.91 (0.85, 0.96)	0.0011	0.90 (0.77, 1.04)	0.1556
Age of 1st live birth (per year)	20.96 ± 4.42	1.05 (1.02, 1.07)	0.0013	1.04 (1.01, 1.08)	0.0121
Body mass index, kg/m^2 (per unit)	29.29 ± 7.57	0.988 (0.972, 1.003)	0.1224	-	
Language		1.09 (0.87, 1.36)	0.4622	-	
English	235/619				
Other	280/699				
Education (per year)	10.40 ± 3.80	1.05 (1.02, 1.08)	0.0012	1.00 (0.96, 1.04)	0.9472
Low HL	368/1056	2.73 (2.04, 3.66)	< 0.0001	2.05 (1.38, 3.03)	0.0003
Adequate HL	136/229				
Married		1.19 (0.95, 1.48)	0.1375		
No	285/763			-	
Yes	230/555				
Menopausal		0.42 (0.32, 0.54)	< 0.0001	0.88 (0.58, 1.31)	0.5192
No	410/909				
Post	105/409				
Employed		1.39 (1.11, 1.76)	0.0053	1.04 (0.78, 1.37)	0.8023
No	313/862				
Yes	202/456				
Income (per $1000US/mo)	1.10 ± 8.78	1.44 (1.26, 1.64)	< 0.0001	1.29 (1.09, 1.51)	0.0024
Insured	141/418	1.40 (1.10, 1.78)	0.0076	1.22 (0.90, 1.65)	0.1977
Uninsured	374/900				
Current smoker		0.80 (0.60, 1.06)	0.1211		
No	423/1054			-	
Yes	92/264				
Screening mammography		1.46 (1.11, 1.92)	0.0081	1.69 (1.22, 2.35)	0.0018
No	397/1064				
Yes	118/254				
Primary care provider	427/1182	3.24 (2.24, 4.70)	< 0.0001	2.81 (1.84, 4.29)	< 0.0001
Others gynecologist	88/136				

[1]Derived from a Fisher's exact test for categorical variables and a logistic regression model for continuous variables; [2]Adjusted for age, pregnancies, live births, age of 1st live birth, years of education, HL, menopause, employment, income, insurance, use of screening mammography, and type of primary care provider. HL: Health literacy as measured by Newest Vital Sign.

34%, $P < 0.0001$). Although patients without a primary care provider had Pap-smear rates similar to those with such a provider, the type of provider made a difference. Women who had a gynecologist for their primary care provider were significantly more likely to have Pap-smears (65%) as compared to those who had an internist (23%) or family practitioner (42%, $P < 0.0001$). Patients less than 21 years of age and those older than 65 years underwent screening less frequently (11% and 11%, respectively) than those age 21-54 years (41%, $P < 0.0001$).

In multivariate analysis, older age was significantly associated with lower likelihood of undergoing Pap-smear use ($P < 0.001$), whereas older age of first live birth ($P < 0.01$) and higher income were associated with higher use ($P < 0.0096$). Patients who underwent screening mammography were more likely to undergo Pap-smears (OR = 1.69; 95%CI: 1.22-2.35, $P = 0.0018$). The type of primary care provider also had a significant effect as patients whose primary care provider was a gynecologist were significantly more likely (OR = 2.81; 95%CI: 1.84-4.29, $P < 0.0001$) to undergo screening than those with other types of providers. Level of health literacy also affected use of Pap-smears as those with adequate health literacy were twice as likely to participate as those with low health literacy (OR = 2.05; 95%CI: 1.38-3.03, $P = 0.0003$).

DISCUSSION

Results of this study show that the use Pap-smears in this underinsured population was suboptimal, at 39%. We identified several factors that were independently associated with Pap-smear utilization. Younger patients, patients with later first live birth, and those who participated in screening mammography were more

likely to utilize Pap-smears. Women with a gynecologist as their primary care provider and those with adequate health literacy were most likely to undergo Pap-smears.

The finding that Pap-smear screening is underutilized in an underinsured population is not new[20]. However, the factors associated with low screening rates within these populations can provide insight into factors that may improve compliance or to provide targeted screening. In particular, women with adequate health literacy underwent Pap-smear screening twice as much as those with low health literacy. On the other hand, many sociodemographic factors which are thought to influence use of preventative services, such as race/ethnicity, education, employment, and insurance status were not found to be associated with Pap-smear use in the current study[21-23]. We previously reported that limited health literacy was the strongest predictor of non-use of breast cancer screening[14]. In that study, 57% of patients did not realize they should undergo screening mammography or did not understand the concept of screening[14]. In the current study, we found that participation in screening mammography was associated with higher rates of Pap-smear screening, suggesting that understanding the concept of preventive care and the ability to navigate the health care system - both key components of health literacy - are critical to Pap-smear screening. Since it is unclear whether an individual's level of health literacy can be modified, development of strategies to increase awareness of the importance of prevention in populations with health disparities and limited literacy are critical to improve compliance[24].

In our population comprised of a significant proportion Hispanic women, ethnicity was not a predictor of use of Pap-smears. It is possible that level of acculturation, which we did not assess, may influence screening uptake in these women. However, health literacy may be an adequate proxy for acculturation, as it has been associated with factors related to acculturation (*i.e.,* language, education, employment)[19]. In the current population, patients with adequate health literacy, had reproductive behavior consistent with higher levels of acculturation (fewer pregnancies 2.7 *vs* 3.6; fewer live births 1.8 *vs* 3.1; and later age of first live birth 22 years *vs* 20 years; $P < 0.01$ for all three factors). Patients with characteristics associated with higher levels of acculturation, were more likely to utilize Pap-smears suggesting that acculturation might play a role[25].

Although having a primary care provider did not influence Pap-smear use, the type of provider was significant. Patients whose primary provider was a gynecologist were three times as likely to undergo screening with Pap-smears compared to those with other providers. It also appears that Pap-smears were not over-utilized as patients under the age of 21 years or over the age of 65 years were less likely (both 11%) to undergo Pap-smears compared to the rest of the population (41%). The time frame of this study was 3 years after the 2009 American College of Obstetrics and Gynecology (ACOG) Practice Bulletin, which was when these age recommendations were changed[26]. This study indicates reasonable assimilation of the 2009 recommendations.

There are limitations to the current study. First, because we asked patients about their use of Pap-smears prior to verifying use, it is possible that some patients were unable to recall this information, and this could underestimate the use of Pap-smears. Not being able to recall that the test was completed or the results, however, somewhat defeats the purpose of screening, since not knowing the results would make patients unaware of necessary follow-up. A second limitation is that in November 2012, there was a change in recommendations for screening to every 3 years[20]. It is possible that clinicians and practitioners immediately instituted the recommendations and could have contributed to the low use. Implementation of new guidelines into clinical practice, however, is frequently inefficient and often requires several years to be implemented[27,28]. Further, there were no differences in the use of Pap-smears before (38%) or after (41%) the change in recommendations, indicating this did not affect the results. A third limitation is that Human papilloma virus testing was not included as part of the study and results may have been different had we included this evaluation. Human papilloma virus testing alone, however, is not recommended for cervical cancer screening in any age group. Pap-smear cytology alone remains the recommended (age 21-29 years) or acceptable (age 30-65 years) method for screening[20].

Most clinicians feel that patients with limited health literacy do not exist in their practice. Surveys in the United States and internationally, however, demonstrate that significant proportions of adults in all countries have limited health literacy skills[7-11]. Patients with limited health literacy are found in all clinical practices and the number of patients with limited health literacy is increasing, particularly with the aging population, increasing number of immigrants, and patients with low educational attainment[7,8,10,11]. No strategies have yet been proven to improve or increase health literacy. Therefore increased awareness of patients with limited health literacy is important in all clinical settings.

The current study found that specialty of the primary care physician, health literacy, along with the patient's age and older age at first childbirth were associated with Pap-smear utilization. Patients with low health literacy exist in all countries and clinical practices and their numbers are increasing. Development of interventions to target low health literacy populations could improve cervical cancer screening.

COMMENTS

Background

Cervical cancer is one of the most preventable and treatable female cancers. Cervical cancer screening rates are most suboptimal among recent immigrants to developed countries, in countries without screening programs, among racial

and ethnic minorities, women from low socioeconomic backgrounds, and underinsured populations. Most studies show higher rates of cervical cancer and lower compliance with cervical cancer screening in these populations.

Innovations and breakthroughs

Previous studies have found that patients do not understand the concept of screening or did not realize that Papanicolaou-smears (Pap-smears) are a test for cervical cancer. The number of patients with low health literacy is increasing worldwide and these individuals are less able to navigate the health care system and less likely to participate in preventive health care. The current study is the largest series of consecutive patients at a single institution to evaluate the association of Pap-smear utilization with health literacy and other sociodemographic factors.

Applications

This study found that specialty of the primary care physician, health literacy, along with the patient's age and older age at first childbirth were associated with Pap-smear utilization. Patients with low health literacy exist in all countries and clinical practices and their numbers are increasing. Development of interventions to target low health literacy populations could improve cervical cancer screening.

Terminology

Health literacy is the degree to which individuals have the capacity to obtain, process, and understand basic health information needed to make appropriate health decisions. In 2003, the National Assessment of Adult Literacy reported that over 89 million American adults have limited health literacy skills and that individuals with limited health literacy come from all parts of society. Similarly, the 2011 Programme for the International Assessment of Adult Competencies which evaluated adults in 23 industrialized countries indicated similar issues: A significant proportion of adults scored below average for literacy, numeracy, and problem solving in technology-rich environments.

Peer-review

Few papers have examined the association of health literacy and use of Pap-smears for cervical cancer screening. The authors of the current study evaluated sociodemographic factors which affected use of Pap-smears. This study found that patients with adequate health literacy and specialty of their primary care provider were more likely to use Pap-smears than other patients.

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ORIGINAL ARTICLE

Observational Study

Bone and soft tissue tumors presenting as sciatic notch dumbbell masses: A critical differential diagnosis of sciatica

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Abstract

AIM

To study the clinical findings and characteristic features in sciatic notch dumbbell tumors (SNDTs).

METHODS

We retrospectively reviewed the clinical outcomes and characteristic features of consecutive cases of SNDTs (n = 8).

RESULTS

Buttock masses occurred in three patients with SNDT (37.5%). Severe buttock tenderness and pain at rest were observed in seven patients with SNDTs (87.5%). Remarkably, none of the patients with SNDTs experienced back pain. Mean tumor size was 8.4 ± 2.0 cm (range, 3.9 to 10.6 cm) and part of the tumor mass was detected in 2 patients in the sagittal view of lumbar magnetic resonance imaging (MRI).

CONCLUSION

The clinical information regarding to SNDTs is scarce. The authors consider that above mentioned characteristic findings may facilitate the suspicion of pelvic pathology and a search for SNDT by MRI or computed tomography should be considered in patients presenting with sciatica

without evidence of spinal diseases.

Key words: Sciatic notch; Dumbbell masses; Sciatica; Differential diagnosis; Bone and soft tissue tumor

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Core tip: The author retrospectively studied the clinical outcomes and characteristic findings of consecutive cases of sciatic notch dumbbell tumors (SNDTs) and found that buttock mass, severe buttock pain at rest and lack of back pain may facilitate the suspicion of pelvic pathology and a search for SNDT by magnetic resonance imaging or computed tomography should be considered in patients presenting with sciatica without evidence of spinal diseases.

Matsumoto Y, Matsunobu T, Harimaya K, Kawaguchi K, Hayashida M, Okada S, Doi T, Iwamoto Y. Bone and soft tissue tumors presenting as sciatic notch dumbbell masses: A critical differential diagnosis of sciatica. *World J Clin Oncol* 2016; 7(5): 414-419 Available from: URL: http://www.wjgnet.com/2218-4333/full/v7/i5/414.htm DOI: http://dx.doi.org/10.5306/wjco.v7.i5.414

INTRODUCTION

Sciatica is a very common disorder. Accurate diagnosis of sciatica is problematic, since it may be caused by various pathologies, including lumbar disc herniation (LDH), spinal degeneration, inflammatory diseases, trauma, metabolic or circulatory events, and tumors[1].

In particular, intra- and extra-pelvic sciatic notch dumbbell-shaped tumors [sciatic notch dumbbell tumors (SNDTs)] may cause classic sciatica by invading the sacrum, sacral plexus, and sciatic nerve. The lifetime incidence of LDH was considered to be 2%-3% and that of spinal tumor was 1 per 100000. Meanwhile, the occurrence of SNDTs is considered to be rare and there were no reports of case series with large number and the accurate incidence rate of SNDT remains unknown[2-4], and most spine and orthopedic surgeons therefore have little or no experience with sciatica caused by undiagnosed SNDTs.

Retroperitoneal bone and soft tissue sarcomas may present as SNDTs. These have a poor prognosis because of high rates of local recurrence, mortality, and surgical morbidity[5]. Early recognition of malignant SNDTs should increase the chance of survival, and thus it is essential to promptly and accurately diagnose sciatica caused by such tumors. However, very little clinical information regarding SNDTs has been published thus far[6].

In this study, we studied retrospectively the clinical outcome of 8 patients suffered from sciatica due to SNDTs. We also evaluated the characteristic features of these tumors on physical examination and imaging analysis, with the aim of improving the differential diagnosis of sciatica.

MATERIALS AND METHODS

Clinical findings

This study was approved by institutional review board in our hospital. The clinical findings and surgical records of consecutive eight cases of SNDTs were retrospectively reviewed. The following information was retrieved from medical records: Demographic details, disease history, imaging manifestations, tumor pathology, details of surgical treatment, and postoperative survival and tumor recurrence. SNDT specimens were used for histopathological analysis and final diagnosis. To establish the tissue diagnosis, either computed tomography (CT)-guided needle biopsy or open biopsy were carried out. All patients underwent a clinical examination in which the chief complaint and mode of illness onset were noted in a standardized manner. Physical examination was performed at the first medical visit. Patient delay was defined as the duration between the onset of each patient's initial symptoms and their first physician consultation, while physician delay was defined as the period from the patient's first medical visit for their symptoms until the date of accurate diagnosis.

Review of radiographic images

X-ray, magnetic resonance imaging (MRI), and CT images of the pelvis were taken in all cases. Lumbar MRI was performed in 7 patients. Two orthopedic surgeons with more than 5 years' experience independently investigated all imaging results: For X-rays, bone destruction and matrix mineralization; for MRIs, tumor size, tumor boundaries, and relation between the sciatic nerve and mass on lumbar MRI sagittal imaging; and for CT, osteolytic bone destruction, tumor calcification, and enlargement of the sciatic foramen.

RESULTS

Clinical features of SNDTs

There were 8 cases of SNDTs (5 males and 3 females). Patients' ages at their first medical visits ranged from 12 to 81 years, with a mean age of 35.6 ± 21.2 years (mean ± SD). With regard to the McCormick scale[7], one case was grade Ⅰ, grade Ⅱ in 3, grade Ⅲ in 2, and grade Ⅳ in 2. Histologically, 2 SNDTs were undifferentiated pleomorphic sarcomas, and there was one case each of osteosarcoma, Ewing sarcoma, malignant peripheral nerve sheath tumor, neurinoma, carcinosarcoma, and solitary fibrous tumor. The patient delay averaged 11.8 mo (2 to 26 mo), while the physician delay averaged 2.6 mo (1 to 7 mo). Table 1 presents a summary of the patients' clinical demographics.

Chief complaints and physical examination findings at the first medical visit

All 8 cases presented with sciatica. A palpable mass in one buttock was detected in 3 of the 8 cases. Of the 8

Table 1 Clinical features of 8 patients with sciatic notch dumbbell tumors

Case	Age/sex	McCormick scale	Histology	Patient delay (mo)	Physician delay (mo)
1	41/F	Ⅱ	Solitary fibrous tumor	26	7
2	12/F	Ⅰ	UPS	12	4
3	27/F	Ⅲ	Neurinoma	20	3
4	36/F	Ⅱ	Carcinosarcoma	2	1
5	81/M	Ⅳ	Osteosarcoma	5	2
6	15/F	Ⅳ	UPS	2	1
7	35/F	Ⅱ	Ewing's sarcoma	15	2
8	38/M	Ⅲ	MPNST	12	1

UPS: Undifferentiated pleomorphic sarcoma; MPNST: Malignant peripheral nerve sheath tumor; M: Male; F: Female.

Table 2 Summary of chief complaints and physical examination findings in patients with sciatic notch dumbbell tumors

	SNDT (n = 8)
Sciatica	8
Pain at rest	7
Back pain	0
Palpable mass in the buttock	3
Buttock tenderness	7
Motor weakness	4
Sensory loss testing	6
Positive SLRT	6

SNDT: Sciatic notch dumbbell tumor; SLRT: Straight leg rising test.

cases, 7 reported severe buttock tenderness, 4 reported muscle weakness, 7 reported pain at rest, and 6 reported a positive straight leg raise test (SLRT) (Table 2). Six cases experienced unilateral sensory disturbances of the lower limb. The dermatomal distribution of symptoms was as follows: L5 in 2 cases, L5 to S1 in 2 cases, S1 in one case, and S2 in one case. A positive SLRT was noted in 6 of the 8 cases. Remarkably, no patients experienced back pain. Taken together, the clinical features suggestive of SNDTs were as follows: A chief complaint of pain at rest, lack of back pain, a unilateral buttock mass, and severe buttock tenderness.

Imaging features of SNDTs

On initial plain radiographs, osteolytic bone destruction was detectable in 2 of the 8 cases, while matrix mineralization was observed in one case. In each of the 8 cases, MRI of the pelvis showed a large intrapelvic and extrapelvic tumor adjacent to the sciatic notch. Maximal diameters of the mass ranged from 3.9 to 10.6 cm (mean 8.4 ± 2.0 cm). Tumor borders were clearly defined in 3 cases and poorly defined from the adjacent organ in 5 patients. The sciatic nerves were clearly connected to the tumors in 3 cases. Lumbar MRI was performed in 7 patients; importantly, in 2 of these, part of the tumor mass was detected in the sagittal view but not in axial views. On CT, destructive invasion to the bone by the tumor progression was found in 5 cases. Ectopic calcification was observed in one case and enlargement of the sciatic foramen was found in 4 cases. Imaging features of SNDTs are summarized in Table 3.

Table 3 Radiological features of 8 patients with sciatic notch dumbbell tumors

Modalities	Descriptions	Value
X-ray	Bone destruction	2
	Matrix mineralization	1
MRI	Tumor size (cm)	8.4 ± 2.0 (range, 3.9 to 10.6)
	Indistinguishable tumor boundary	5
	Connection of the sciatic nerve	3
	Mass on lumbar MRI sagittal image	2
CT	Osteolytic bone destruction	5
	Tumor calcification	1
	Enlargement of sciatic foramen	4

MRI: Magnetic resonance imaging; CT: Computed tomography.

Treatment and clinical outcomes of SNDTs

Two of the 8 cases were managed with surgery, one using a wide surgical margin and the other *via* intralesional resection. Chemotherapy was administered to the 5 patients who did not undergo surgical tumor resection. Combination of adriamycin, ifosfamide, cisplatin, and etoposide were used for chemotherapy. Chemotherapy resulted in a complete response in one case, a partial response in 2 cases, stable disease in one case, and progressive disease in one case. Five patients received radiotherapy, 3 patients underwent conventional radiotherapy, and 2 patients were treated with carbon-ion curative radiotherapy. Local recurrence occurred in 2 cases and distant metastases to the lung were observed in one case. At final follow-up, one of the 8 patients had died of disease, one was continuously disease-free, one showed no evidence of disease, and 5 were alive with disease.

Case presentations

Case 1: An SNDT in a 41-year-old woman. She suffered from neurogenic claudication and sciatica for 9 mo. Examination showed moderate sensory loss in the right S1 dermatome and a nerve stretch test was positive.

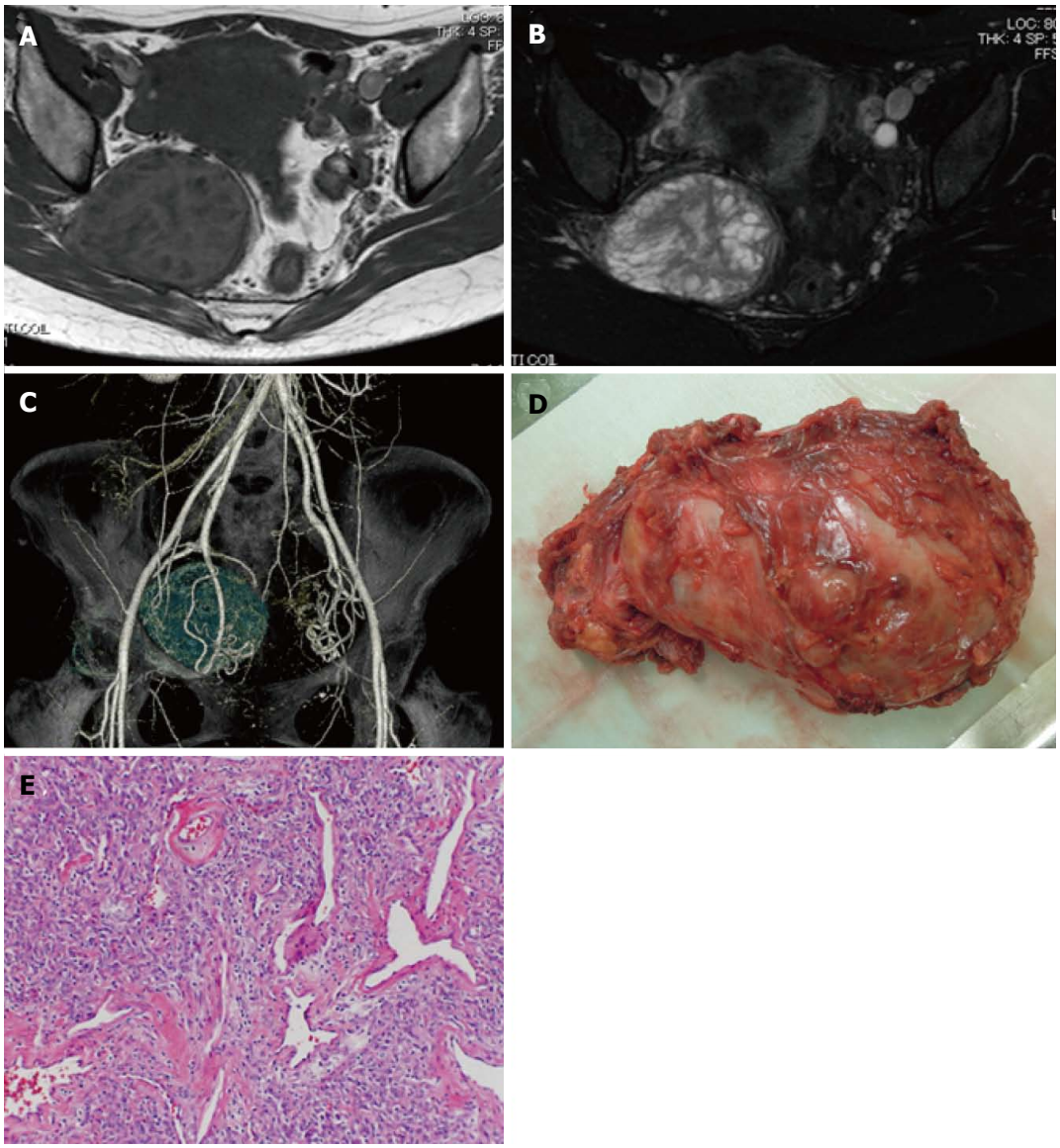


Figure 1 Case 1: An sciatic notch dumbbell tumor in a 41-year-old female. A and B: Axial MRI revealed an SNDT with a 7.7-cm diameter. The tumor showed a mixed intensity signal on T1- (A) and T2-weighted (B) images; C: 3D-CT angiography clearly demonstrated the relationship between the tumor and major vessels; D: Macroscopic appearance of the resected tumor showing a gray-white, dumbbell-shaped mass with surrounding soft tissue; E: Postoperative pathology confirmed the diagnosis of solitary fibrous tumor. The specimen showed cellular proliferation of mildly atypical spindle or oval cells arranged in short fascicles that were associated with dilated sclerotic blood vessels displaying a hemangiopericytoma-like appearance. Hematoxylin and eosin, original magnification 100 ×. MRI: Magnetic resonance imaging; SNDT: Sciatic notch dumbbell tumor; CT: Computed tomography.

Lumbar MRI revealed no pathological findings; however, an intrapelvic dumbbell-shaped mass compressed lumbosacral plexus (Figure 1A and B). The maximal diameter of the mass was 7.7 cm, and the lesion showed mixed signal intensity both on T1- and T2-weighted images. 3D-CT angiography clearly demonstrated the relationship between the tumor and major vessels (Figure 1C). A diagnosis of solitary fibrous tumor was made by CT-guided biopsy. The tumor was resected by a one-stage combined transabdominal and transgluteal (extrapelvic) approach (Figure 1D). A postoperative surgical specimen showed cellular proliferation of mildly atypical spindle or oval cells arranged in short fascicles that were associated with dilated sclerotic blood vessels displaying a hemangiopericytoma-like appearance (Figure 1E). The surgical margin was negative and the postoperative course was uneventful. The patient noted a significant improvement in pain. There was no local recurrence or distant metastasis at 25 mo after surgery.

DISCUSSION

Common symptoms of unilateral sciatica include pain radiating down the lower extremity, pain with motion of the hip joint, and pain in the buttock, groin, and low back. LDH is by far the most common and well-known cause of sciatica[8]. Other frequent causes include hip diseases[9], degenerative lumbar spinal disease, spinal infection, spinal and spine tumors[10], and vascular diseases[11]. Local compression of sciatic nerve by tumors

and/or trauma may cause sciatica. Importantly, several reports have demonstrated that benign, malignant, and metastatic bone and soft tissue tumors in the pelvis also cause symptoms that are typical and suggestive of sciatica[12]. In particular, pelvic tumors arising adjacent to the sciatic notch have the ability to form huge dumbbell-shaped tumors[5], and compress the lumbosacral plexus, thus causing sciatica.

However, SNDTs are quite rare and there are few previous studies on these tumors. Thomas *et al*[13] presented 35 cases of neurogenic tumors around the sciatic nerve, 11 of which occurred at the sciatic notch. There have been few reports of SNDTs producing sciatica; these include lipoma[14] and a range of cancers[15]. Due to this rarity, SNDTs are rarely considered in the potential diagnosis of sciatica.

Physical examinations may be insufficient to distinguish SNDTs from other cause of sciatica. From the finding in this study, the authors suggest that the diagnosis of SNDT should be considered in sciatica with the following characteristics: (1) a palpable mass in one or both buttocks; (2) severe buttock tenderness; and (3) chronic pain at rest. Importantly, lack of back pain is another characteristic feature of sciatica caused by SNDTs and this should alert the clinician to consider for alternative diagnosis for sciatica. Overall, the combination of the aforementioned features may warrant the consideration of sciatic nerve compression by an SNDT, a diagnosis that may be clarified preoperatively by MRI or CT of the pelvis.

This case series demonstrated the detailed imaging features of SNDTs, and we found that in certain cases SNDTs could be identified by careful inspection of pelvic X-ray or lumbar MR images. Specifically, 2 patients showed abnormal X-ray findings and 2 demonstrated tumor masses on sagittal sections of lumbar MR images. These findings may be helpful for physicians who suspect the existence of SNDTs in patients with sciatica. SNDTs often form extraordinarily large, asymptomatic soft-tissue masses before the lesions become evident on clinical examination, even in cases of benign neurogenic tumors.

The presence of inadequate operative margins has been proven to be an independent and adverse prognostic factor in local recurrence of sarcoma of the pelvis[16]. Tumor location is a critical factor for tumor resectability. In cases of SNDTs, *en bloc* resection of SNDTs is not feasible because of the complex anatomic features of the surrounding organs, including the pelvic bone, lumbosacral nerve plexus, and large blood vessels. A recent report described a safe resection of certain cases of SNDTs by combination of one-stage transabdominal and transgluteal approach[5]. Accordingly, we applied this method in one case and achieved complete tumor resection with no impaired neural function. Thus, we believe that SNDTs can be resected safely and completely if the tumor displaces rather than directly involves the lumbosacral plexus (including the sciatic nerve).

In conclusion, early recognition and treatment is important in bone and soft tissue tumors. Thus in patients presenting with sciatica without evidence of spinal diseases such as LDH, prompt and accurate diagnostic strategies should include the suspicion of pelvic pathology and a search for SNDT by MRI or CT.

COMMENTS

Background

Sciatica, defined as pain radiating from the back into the buttocks and lower extremities, is a very common disorder. The differential diagnosis of sciatica is difficult, since it may be caused by various pathologies including sciatic notch dumbbell tumors (SNDTs).

Research frontiers

There is little clinical information relating to SNDTs.

Innovations and breakthroughs

This study clearly describes clinical outcomes and characteristic features of SNDTs and this may improve the differential diagnosis of sciatica.

Applications

Practical clinical tips for differential diagnosis of sciatica.

Terminology

SNDTs: Sciatic notch dumbbell tumors.

Peer-review

This is a very good article.

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CASE REPORT

Male papillary breast cancer treated by wide resection and latissimus dorsi flap reconstruction: A case report and review of the literature

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Abstract

Breast cancer (BC) in men represents between 0.5% and 1% of all BC diagnosed each year. We report a case of advanced BC in a 62-year-old male treated at our interdisciplinary Breast Cancer Center. The patient presented with a newly diagnosed large, symptomatic mass in his left breast. Clinical examination showed a not movable mass of 16 cm diameter, deforming the whole breast; the overlying skin was livid and hypervascularized. Enlarged lymph nodes were palpable in the axillary pit. He had no concomitant diseases at time of presentation. He denied any first- or second degree family medical history of cancer of any type and he never received radiotherapy. Ultrasound guided minimal-invasive 14-gauge core biopsy revealed a moderately differentiated encapsulated papillary carcinoma with high expression of estrogen and progesterone receptors (both > 80%, IRS 12) and HER2-negative. Because of the tumor size a mastectomy with axillary dissection and chest wall reconstruction using a latissimus dorsi flap was performed. Histological analysis showed invasive growth besides typical (non-invasive) papillary carcinoma and was classified as invasive solid papillary carcinoma; pT3 (10 cm), pN0 (0/15), M0,

R0; OncotypeDX Recurrence Score indicated low risk (RS: 2). After discussion in the interdisciplinary tumor board meeting, radiation therapy and tamoxifen were recommended. The patient had an uneventful recovery and is disease-free after two years of follow-up. Male BC is typically diagnosed at an advanced stage, most likely due to a lack of awareness that men can develop BC. Therefore, in case of a large tumor, a flap-based thoracic reconstruction may be required.

Key words: Male breast cancer; Papillary carcinoma; Reconstruction; Latissimus dorsi flap; Rare tumors

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Core tip: Male breast cancer (BC) is typically diagnosed at an advanced stage, most likely due to a lack of awareness that men can develop BC. Therefore, in case of a large tumor, a flap-based thoracic reconstruction may be required.

Banys-Paluchowski M, Burandt E, Banys J, Geist S, Sauter G, Krawczyk N, Paluchowski P. Male papillary breast cancer treated by wide resection and latissimus dorsi flap reconstruction: A case report and review of the literature. *World J Clin Oncol* 2016; 7(5): 420-424 Available from: URL: http://www.wjgnet.com/2218-4333/full/v7/i5/420.htm DOI: http://dx.doi.org/10.5306/wjco.v7.i5.420

INTRODUCTION

Breast cancer (BC) in men represents between 0.5% and 1% of all breast cancers diagnosed each year[1]. Few epidemiological or clinical trial data on male BC are available. The disease appears to share similar risk factors and characteristics with postmenopausal BC in women[2]. Favorable parameters, such as low nuclear grade and positive hormone receptor status, are more common for men and postmenopausal women than for premenopausal women. As in women, the most common subtype is invasive ductal carcinoma, while lobular tumor types are rarely diagnosed in men. In contrast to female BC, men are significantly more likely to present with a more advanced stage at diagnosis and with lymph node involvement[3]. We report an interesting case of a large papillary invasive breast cancer in a 62-year-old man.

CASE REPORT

A 62-year-old Caucasian male presented at the certified Breast Cancer Center, Klinikum Pinneberg, Germany, with a newly diagnosed large, symptomatic mass in the left breast. Clinical examination showed a not movable mass of 16 cm diameter, deforming the whole breast (Figure 1); the overlying skin was livid and hypervascularized.



Figure 1 Photodocumentation at time of presentation.

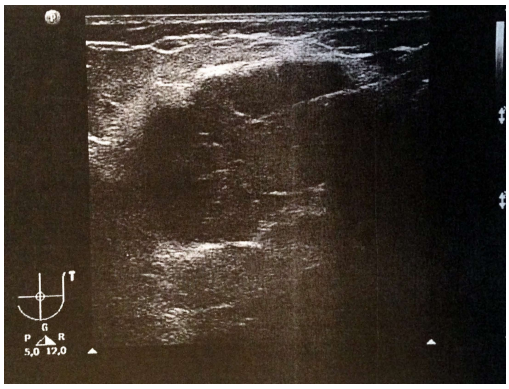


Figure 2 Breast ultrasound shows a large irregular structure of low echogenicity with spiculae measuring > 15 cm × 15 cm (BI-RADS 5).

Enlarged lymph nodes were palpable in the axillary pit. He had no concomitant diseases at time of presentation; his previous surgeries included circumcision as a child and he was a nonsmoker. He denied any first- or second degree family medical history of cancer of any type and he never received radiotherapy. His serum estradiol levels were normal. The patient reported that he had noticed the tumor one year prior to presentation; the reason for eventually seeing a doctor was an increasing pain, most likely due to the pressure on the skin by the growing tumor. There was no nipple discharge. At ultrasound, the lesion was scored BI-RADS 5 (Figure 2). Axillary lymph nodes were suspicious on sonography. Mammography was not possible due to tenderness on palpation.

Ultrasound guided minimal-invasive 14-gauge core biopsy revealed a moderately differentiated non-invasive encapsulated papillary carcinoma with high expression of estrogen and progesterone receptors (both > 80%, IRS 12) and HER2-negative (Figure 3). Because of the tumor size a mastectomy with axillary dissection and chest wall reconstruction using a latissimus dorsi (LADO) flap was performed. Histological analysis showed invasive growth besides typical non-invasive papillary carcinoma and was classified as invasive solid papillary carcinoma (Figures 4 and 5). The TNM stage was pT3 (10 cm), pN0 (0/15), M0 and the resection margins were at least 5 mm in all directions. After discussion in our interdisciplinary

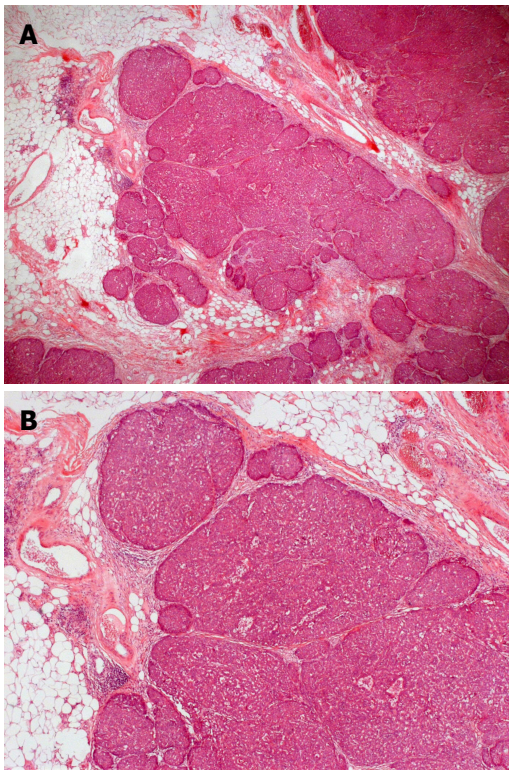


Figure 3 Solid papillary carcinoma (*in situ*), composed of expansile rounded nodular epithelial masses. A: Low magnification 12.5 ×; B: Low magnification 25 ×.

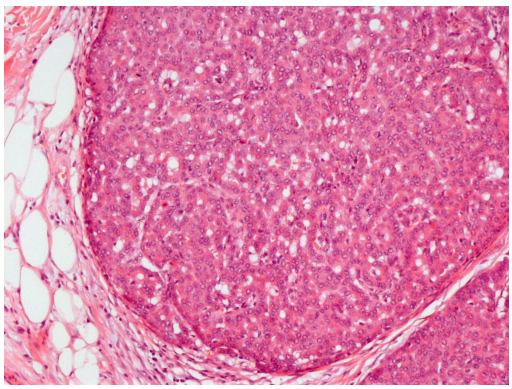


Figure 4 Relatively bland tumor cells with ovoid nuclei and indistinct nucleoli. Fine fibrovascular septa are seen within the epithelial islands (medium magnification, 100 ×).

tumor board we conducted the OncotypeDX test which indicated low risk (Recurrence Score: 2). Radiation therapy and tamoxifen were recommended. The patient was referred for genetic counseling but decided against genetic testing. He had an uneventful recovery and is disease-free after two years of follow-up (Figure 6).

DISCUSSION

To our knowledge, this is the first case report on a giant invasive papillary carcinoma in a man treated by LADO flap-based reconstruction of the thoracic wall. Papillary

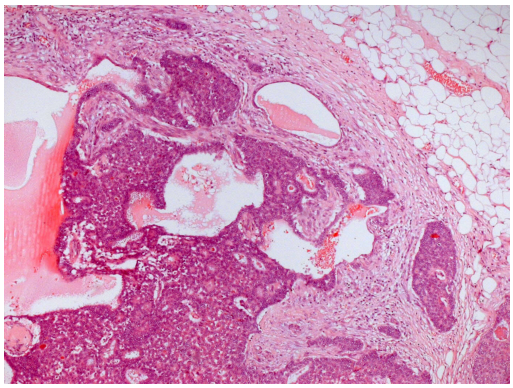


Figure 5 Solid papillary carcinoma (invasive) - tumor cell islands with irregular jagged contours within a desmoplastic stroma (medium magnification, 50 ×).



Figure 6 Postoperative clinical presentation.

breast cancer is a very rare type of BC with an estimated incidence of approximately 1% of all breast cancer cases[4]. In terms of histopathology, papillary carcinoma refers to a morphologically heterogeneous group of lesions, all of which are characterized by arborescent fibrovascular stalks lined by epithelial cells. In most cases of invasive papillary carcinoma, ductal carcinoma *in situ* is also present. Available epidemiological data suggest an improved survival of patients with papillary carcinoma in comparison to the more common invasive ductal cancer[4]. In men, papillary tumor type is more common than in women: In a large series of 778 men with invasive BC, 34 (4.4%) were diagnosed with this tumor subtype[5]. In case of our patient, the minimally invasive core biopsy showed in situ papillary cancer, while the examination of the whole surgical specimen revealed invasive growth as well.

According to the epidemiological data from the Surveillance, Epidemiology, and End Results Program of the American National Cancer Institute, men tend to be older than women at the time of diagnosis, with a median age of 67 years compared with 62 years for women[3]. Further, men are more likely to be diagnosed with advanced disease: At time of diagnosis, 20% of women had tumors smaller than 1 cm compared with only 9.8% of men, 38% of men had regional lymph node involvement compared with 29% of women, and more

Table 1 The comparison of male and female breast cancer with respect to diagnostics and therapy

	Female breast cancer	Male breast cancer
Epidemiology	Very common (125 new cases per 100000 women per year)	Very rare (1.2 new cases per 100000 men per year)
Average age at diagnosis	62 yr	67 yr
Diagnostics	Mammography, sonography; in selected cases MRI	Sonography; mammography if possible; in selected cases MRI
Association with BRCA mutation	5%-10% of all cases are BRCA-positive	10%-20% of all cases are BRCA-positive
Surgery	Breast-conserving surgery (70%-80% patients) or mastectomy; sentinel node biopsy in cN0	Mastectomy; sentinel node biopsy in cN0
Reconstruction techniques	Implant or flap-based reconstruction after mastectomy	Flap-based reconstruction of thoracic wall in case of a large tumor
Chemotherapy	Recommendation depends on tumor biology and tumor load; adjuvant or neoadjuvant use; usually anthracycline- and taxane-based	
Endocrine therapy	Recommended in hormone receptor positive tumors; tamoxifen or aromatase inhibitor	Recommended in hormone receptor positive tumors; tamoxifen
HER2-targeted treatment	Trastuzumab recommended in HER2-positive tumors	
Radiation therapy	Recommended after breast-conserving surgery; thoracic wall or lymph node radiation in case of higher tumor load	Thoracic wall or lymph node radiation in case of higher tumor load

MRI: Magnetic resonance imaging; HER2: Human epidermal growth factor receptor 2.

men had distant metastasis at time of diagnosis than women. On the other hand, tumor biology appeared to be more favourable in men than in women: Men have a significantly higher proportion of hormone receptor positive tumors than women (91% of men and 76% of women present with ER-positive disease)[3]. With regard to molecular gene expression assays, there is limited evidence available related specifically to men, although tumors in men display very similar gene signatures to those in women[6] and OncotypeDX-based clinical trials such as the Ontario trial include male BC patients as well[7]. With respect to risk factors, genetic factors including BRCA mutations, family history, age, androgen/estrogen imbalance, radiation therapy and environmental exposures seem to predispose to male breast cancer[8]. In context of genetic counseling, BRCA2 germline mutation leads to a 100-fold increase in breast cancer risk in male carriers while this association is less established for the BRCA1 mutation. The cumulative risk of BC for male BRCA1 mutation carriers at age 70 years is 1.2% compared to 6.8% for BRCA2 mutation carriers[9]. Another risk factor, the excessive estrogen stimulation, may be due to exogenous hormonal exposure (*i.e.*, hormonal treatment or estrogen-containing compounds/testosterone), overweight, chronic liver diseases and thyroid disease[10]. Men with Klinefelter syndrome are more at risk for developing breast cancer as well[11].

The approach to men with a suspicious breast mass is similar to that of women and includes mammography, ultrasound and biopsy (Table 1). As in women, male breast cancer is classified according to the TNM staging system and tumor biology (estrogen receptor, progesterone receptor and HER2 status) is crucial for choosing adequate systemic therapy. Most men with early stage disease undergo a simple mastectomy. Flap-based reconstruction of the thoracic wall may be necessary in case of a very large tumor[12]. Both the LADO flap and the abdominal tissue transfer, such as transverse rectus abdominis muscle flap or deep inferior epigastric perforator flap, are reliable techniques. The use of LADO flap in a variety of reconstructive settings has been described since early 20th century[13]. In this surgical technique, the pedicled musculocutaneous flap is dissected along with the fat and skin overlying the vascularized muscle and tunneled subcutaneously under the axilla to be transposed into the wound in the thoracic wall. Beyond breast or thoracic wall reconstruction, the LADO flap may be used in men for phalloplasty after penile trauma or in case of congenital anomalies of the penis; in this setting, the flap is not pedicled but is transferred as a "free flap" to another body site and the circulation is re-established *via* microsurgical anastomosis between the flap and the femoral vessels[14].

Most experts agree that sentinel node biopsy should be performed in early male breast cancer in absence of clinically or sonographically suspicious nodes; this approach is in accordance with the ASCO clinical guidelines[15]. Although large clinical trials on sentinel node biopsy in men have not been carried out, smaller studies confirm this technique to be as feasible as in women[16,17].

As in women, adjuvant therapy of male breast cancer may include radiation therapy, endocrine therapy, chemotherapy, and HER2-targeted treatment. In the absence of large clinical trials focusing on male BC, therapy recommendations radiation therapy, chemo- and HER2-therapy mirror those for women. In the context of endocrine therapy, the use of tamoxifen rather than an aromatase inhibitor is recommended[18].

COMMENTS

Case characteristics

A 62-year-old male with a newly diagnosed large, symptomatic mass in the left

breast.

Clinical diagnosis
A not movable mass of 16 cm diameter, deforming the whole breast; the overlying skin livid and hypervascularized; enlarged lymph nodes in the axillary pit.

Differential diagnosis
Benign breast tumor.

Imaging diagnosis
Ultrasound: Large irregular structure of low echogenicity with spiculae (BI-RADS 5), suspicious axillary lymph nodes.

Pathological diagnosis
Core biopsy: Moderately differentiated non-invasive encapsulated papillary carcinoma with high expression of estrogen and progesterone receptors (both > 80%, IRS 12) and HER2-negative. Surgical specimen: Invasive solid papillary carcinoma, pT3, pN0 (0/15), M0, OncotypeDX low risk (Recurrence Score: 2).

Treatment
Mastectomy with axillary dissection and chest wall reconstruction using a latissimus dorsi flap.

Experiences and lessons
This case report describes a rare case of male papillary breast cancer and emphasizes the importance of flap-based reconstruction in case of a large tumor.

Peer-review
This is a case report of rare men breast cancer and then the author gave a short literature review. The review provides some useful information.

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Basic Study

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

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Abstract

AIM

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

METHODS

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

RESULTS

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

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

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

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

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

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

INTRODUCTION

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

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

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

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

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

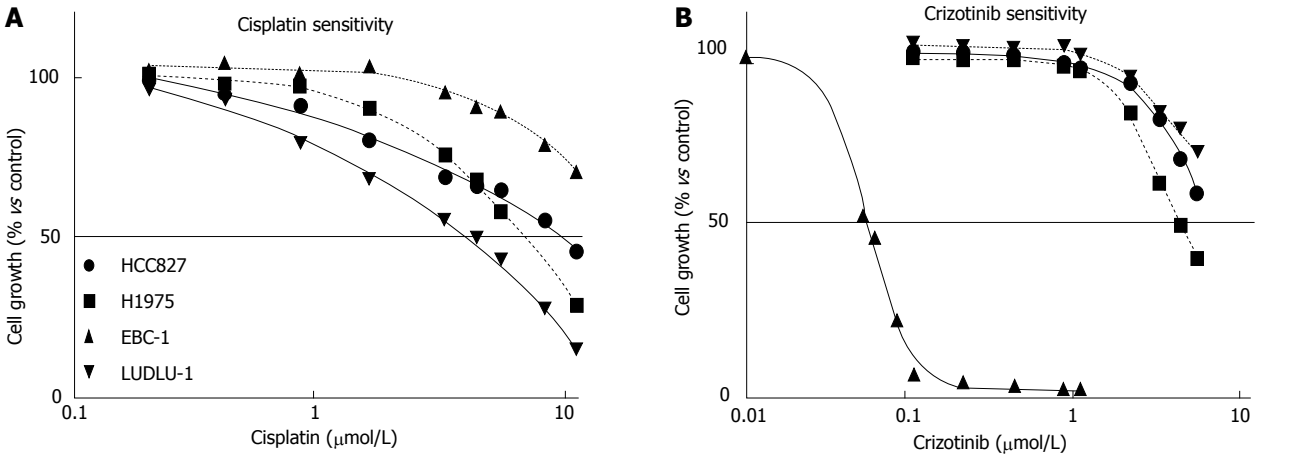


Figure 1 Sensitivity of several non-small cell lung cancer cell lines to cisplatin (A) and crizotinib (B) monotherapy. Cells were exposed to the drugs for 72 h. Cisplatin and crizotinib concentrations are depicted in μmol/L. Values are means of at least 3 separate experiments. The maximal SEM was ± 9%.

Table 1 Cell line properties and drug sensitivity

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

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

MATERIALS AND METHODS

Cell lines and reagents

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

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

Cell proliferation assay: Sulforhodamine B assay

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

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

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

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

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

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

Statistical analysis

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

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

RESULTS

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

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

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

DISCUSSION

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

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

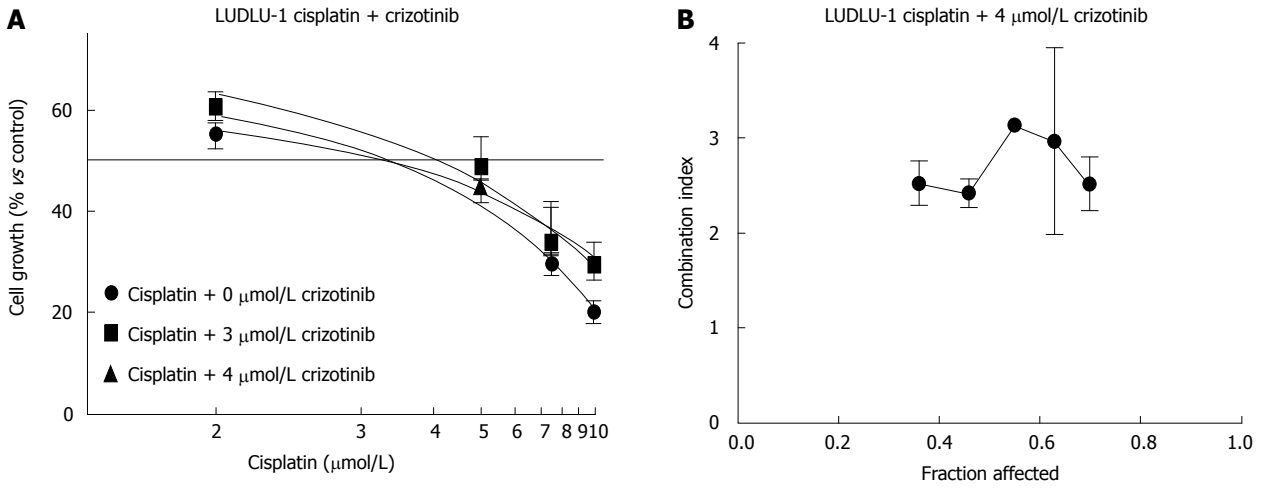


Figure 2 Evaluation of the combination of cisplatin and crizotinib in non-small cell lung cancer cell lines using the fraction affected combination index method. A combination index > 1.2 is antagonistic. A: Growth curves of LUDLU-1 for cisplatin and crizotinib simultaneous treatment during 72 h; B: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

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

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

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

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

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

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

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

COMMENTS

Background

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

Research frontiers

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

Innovations and breakthroughs

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

Applications

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

Terminology

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

Peer-review

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

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ORIGINAL ARTICLE

Retrospective Study

Circulating cytokeratin-positive cells and tumor budding in colorectal cancer

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

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Abstract

AIM

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

METHODS

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

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

RESULTS

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

CONCLUSION

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

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

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

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INTRODUCTION

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

MATERIALS AND METHODS

Patients

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



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

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

Blood sample collection

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

Blood sample preparation and immunocytochemistry

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

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

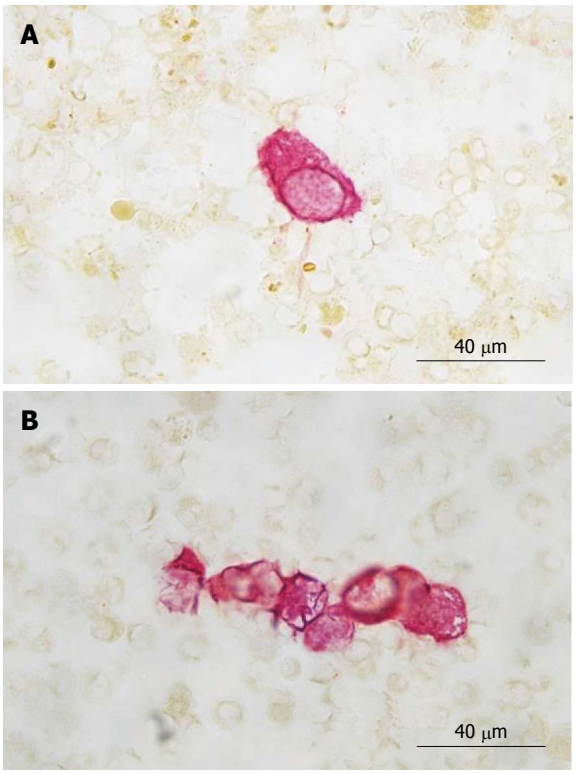


Figure 2 Cytokeratin 18 (Clone cytokeratin 2) cytochemistry. A: A single CK^+ cell is shown in this image; B: A CK^+ cell cluster is shown in this image. CK^+: Cytokeratin-positive.

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

Histopathological evaluation, immunohistochemistry and tumor budding

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

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

Statistical analysis

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

Table 1 Clinicopathological data

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

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

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

RESULTS

Patients

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

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

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

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

Survival analysis

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

DISCUSSION

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

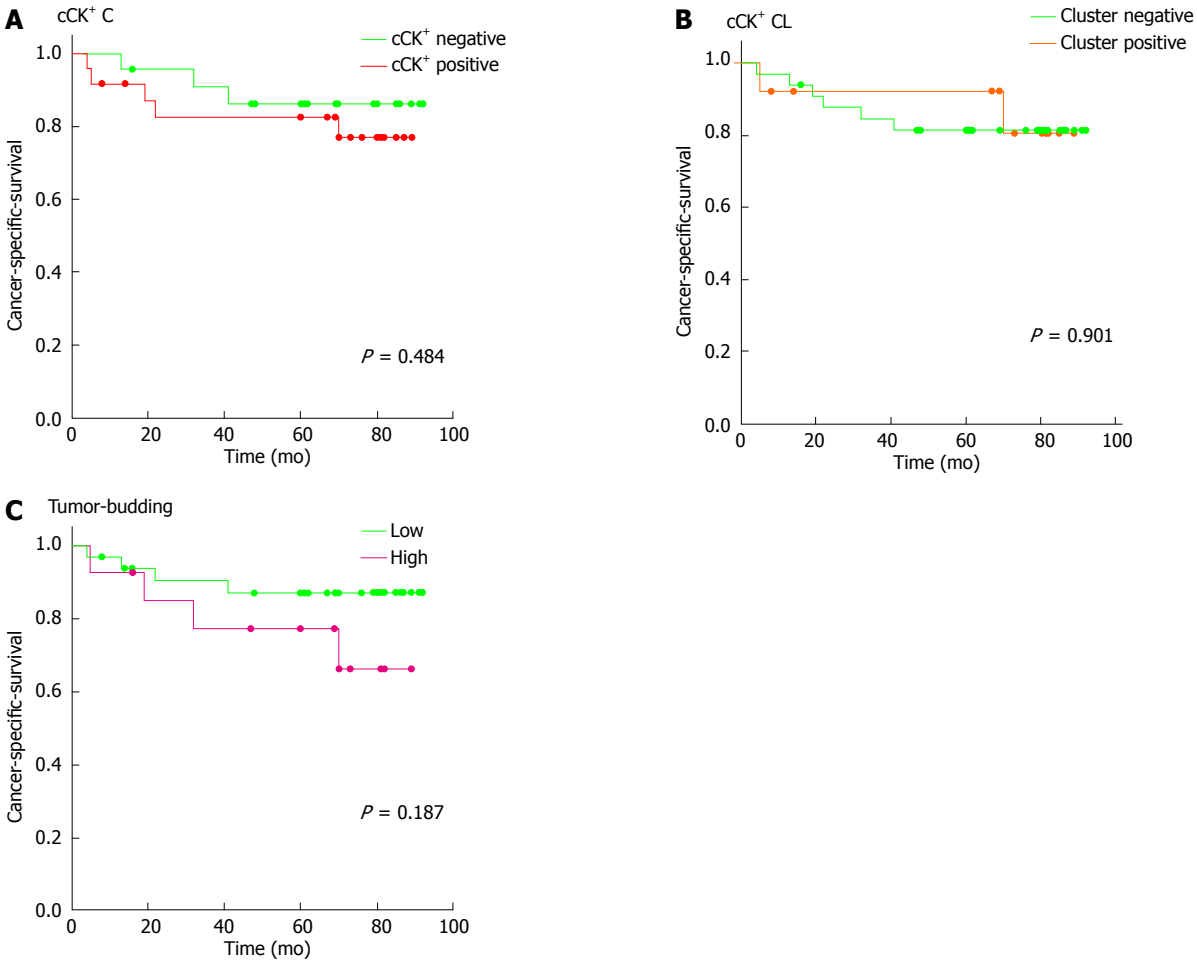


Figure 3 Cancer specific survival. A: Circulating CK$^+$ cell negative *vs* positive; B: Circulating CK$^+$ cell cluster negative *vs* positive; C: Tumor budding negative/low grade *vs* high grade. CK$^+$: Cytokeratin-positive.

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

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

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

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

Table 2 Literature: Circulating tumor cells in the mesenteric blood

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

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

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

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

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

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

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

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COMMENTS

Background

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

Research frontiers

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

Innovations and breakthroughs

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

Applications

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

Terminology

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

Peer-review

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

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