

# World Journal of *Gastrointestinal Oncology*

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2016-2019

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### Abstract

Hepatocellular carcinoma (HCC) is one of the leading causes of cancer related mortality worldwide. HCC incidences have increased worldwide though more prevalent in Asia and Africa. Hepatitis B virus and hepatitis C virus infections are mostly responsible of increased number of HCC cases. Biomarkers can help early detection and improve treatment regimen in patients as advanced stage is chemo-refractive with limited treatment options. Potential of proteomics in finding new biomarkers for early detection has been explored more recently. Future developments in this area rely on how efficiently we manage vast amount of data generated by these techniques and speed up the clinical trials to improve patient care.

**Key words:** Proteomics; Cancer; Biomarker; Hepatocellular carcinoma

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**Core tip:** Despite ongoing development in treatment for hepatocellular carcinoma (HCC), effective biomarkers for diagnosis and treatment for HCC are not available. Profiling of proteins puts proteomics on the forefront to understand promising new biomarkers and drug targets for HCC. HCC proteome database would be an important step towards identifying tumor associated proteins as potential therapeutic targets in the treatment of HCC.

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Hepatocellular carcinoma (HCC) is most common in



underdeveloped and developing countries<sup>[1,2]</sup>. Etiological agents of HCC vary in different part of world. HCC is more common in Asia due to chronic hepatitis B virus (HBV) infections whereas hepatitis C virus (HCV) infection is the major cause of HCC in western countries and Japan. Besides infection, alcoholic fatty liver disease, nonalcoholic fatty liver disease as well as nonalcoholic steatohepatitis also accounts for HCC in developing and developed countries. Aflatoxins produced by *Aspergillus flavus* and *Aspergillus parasiticus* are the risk factors for HCC in China and sub-Saharan Africa. Inherited metabolic diseases, against a background of cirrhosis or without cirrhosis are associated with HCC. Smoking, estrogens, androgens, and thorium oxide (thorotrast) are also associated with HCC. Alcohol is responsible for HCC in both developing and developed countries<sup>[3]</sup>. Less common and emerging risk factors also include diabetes and obesity.

Curative surgery is not possible in HCC patients due to late diagnosis and/or advanced underlying liver cirrhosis<sup>[4]</sup>. Only limited treatment options such as resection and transplantation, radiofrequency ablation and transarterial chemoembolization with marginal clinical benefits are available for majority of patients. The cytotoxic systemic therapy options usually fail in patients with HCC due to the chemoresistance<sup>[5]</sup>. Though most commonly used drug is Doxorubicin<sup>[6]</sup>, a combination of Sorafenib and Doxorubicin therapy is more beneficial in patients with advanced HCC<sup>[7]</sup>. Recent reviews summarized a list of agents for the treatment of HCC<sup>[8,9]</sup>. Despite ongoing development in treatment for HCC, effective biomarkers for diagnosis and treatment for HCC are not available.

Alpha-fetoprotein (AFP) is a biomarker, currently used for screening patients at-risk of HCC. AFP is not a good marker as many other liver diseases can also increase blood level of AFP. Furthermore, AFP is not always elevated in early stages of cancer development, when therapy is mostly effective. Other serum markers for HCC have now been identified in addition to AFP. These are, for example, an abnormal prothrombin molecule, des- $\gamma$ -carboxyprothrombin, cell-surface proteoglycan, glypican-3, glycoprotein, osteopontin, golgi protein 73, microRNA-21,  $\alpha$ -1-fucosidase, human telomerase reverse transcriptase, squamous cell carcinoma antigen, and transforming growth factor- $\beta$ 1. Data suggest that a combination of biomarker may be more effective<sup>[10]</sup>. More importantly, nine Food and Drug Administration-approved blood based cancer markers are used to monitor the treatment<sup>[11]</sup>. Profiling of proteins puts proteomics on the forefront to understand promising new biomarkers and drug targets for HCC.

HCC proteome database would be an important step towards identifying tumor associated proteins as potential therapeutic targets in the treatment of HCC<sup>[12]</sup>. Basic proteomic approaches including 2-dimensional electrophoresis (2DE), reversed-phase high performance liquid chromatography, size-exclusion chromatography, free-flow electrophoresis, capillary electrophoresis, ion-

exchange chromatography along with tools such as MS-based imaging of tissue biopsies, plasmon resonance technique coupled to MS, matrix assisted laser desorption/ionization-time of flight mass spectrometry, surface-enhanced laser desorption/ionization time of flight mass spectrometry (SELDI-TOF MS), 2DE-LC-MS/MS and laser capture microdissection enable the study of cancer proteomics<sup>[13]</sup>. Most significant advancement in 2DE is two-dimensional difference gel electrophoresis (2D-DIGE), with greater sensitivity and dynamic range due to use of fluorescent cyanine dyes (Cye2, Cye3, and Cye5)<sup>[14]</sup>. Analysis of HBV related HCC tumor and non-tumor tissues using 2D-DIGE revealed increased expression of heat-shock proteins (hsp70, hsp90) and heterogeneous nuclear ribonucleoproteins (C1 and C2) as tumor biomarkers<sup>[14]</sup>.

Bioinformatics is essential for proteomic analyses. The Human Proteome Organization (HUPO) has developed the standards for experimental strategies and data exchange<sup>[15]</sup>. An open basic XML (extensible markup language) representation of MS data, named mzXML, helped accelerate data management, interpretation and dissemination using different instrumentation platforms<sup>[16]</sup>. Commercial tools available to the proteomics community to analyze two-dimensional electrophoresis protein patterns include Delta2D (Decodon), BioNumerics 2D (Applied maths), Melanie (GeneBio), Imagemaster 2D (GE healthcare), Progenesis Samespots (NonLinear Dynamics), PDQuest (BioRad Laboratories), REDFIN (Ludesi), ProteinMineTM (Scimagix), and the Z3:2D-Gel image Analysis System (Compugen Limited).

Global analysis of proteins faces several challenges, for example, tertiary structure of proteins, detection of low abundance proteins and reversible modifications such as glycosylation and phosphorylation when compared to studies of genes and transcripts. Furthermore, RNA splicing can produce splice variants that are homologous but differ in function. The revolution in the field of proteomics can hopefully overcome some of these hurdles. In last forty years, only few new tests have been added in clinics. A four-way collaboration is required between the research laboratory (for developing the fundamental concept), the diagnostic lab or industry (converting the concept into a hands-on reliable tool), the clinical laboratory (assessing the tool in real life practices), and the clinicians (providing clinical specimens) for bringing a biomarker from the research lab successfully into the clinical practice<sup>[17]</sup>.

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## New therapeutic approaches to metastatic gastroenteropancreatic neuroendocrine tumors: A glimpse into the future

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### Abstract

Neuroendocrine (NE) gastroenteropancreatic tumors are a heterogeneous group of neoplasias arising from neuroendocrine cells of the embryological gut. Their

incidence have increased significantly over the past 3 decades probably due to the improvements in imaging and diagnosis. The recent advances in molecular biology have translated into an expansion of therapeutic approaches to these patients. Somatostatin analogs, which initially were approved for control of hormonal syndromes, have recently been proven to inhibit tumor growth. Several new drugs such as antiangiogenics and others targeting mammalian target of rapamycin pathways have been approved to treat progressive pancreatic neuroendocrine tumors (NETs) although their role in non-pancreatic is still controversial. The treatment of NETs requires a coordinated multidisciplinary approach. The management of localized NETs primarily involves surgical resection followed by surveillance. However, the treatment of unresectable and/or metastatic disease may involve a combination of surgical resection, systemic therapy, and liver-directed therapies with the goal of alleviating symptoms of peptide release and controlling tumor growth. This article will review the current therapeutic strategies for metastatic gastroenteropancreatic NETs and will take a glimpse into the future approaches.

**Key words:** Gastroenteropancreatic neuroendocrine tumors; Peptide receptor radionuclide therapy; Somatostatin analogs; Octreotide; Transarterial chemoembolization; Carcinoid syndrome; Setotonin; Chromogranin

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**Core tip:** The management of localized NETs is straight forward, however, the treatment of advanced tumors involves several disciplines and requires a coordinated multidisciplinary approach. Recent advances in molecular biology have expanded the therapeutic arsenal. Somatostatin analogs, initially approved for control of hormonal syndromes, have recently proven to inhibit tumor growth. Several new drugs, antiangiogenics,

mTOR inhibitors have been tested with promising results and some of them have already been approved. Several trials are still under way but the future should focus on patient selection, predictive markers, and tolerability improvement as critical aspects to continue advancing.

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## INTRODUCTION

Neuroendocrine (NE) gastroenteropancreatic tumors are a heterogeneous group of neoplasias arising from neuroendocrine cells of the embryological gut<sup>[1]</sup>. Their incidence have increased significantly over the past 3 decades with a crude incidence of 5.25/100000 per year. This is probably due to the improvements in imaging and diagnosis<sup>[1-4]</sup>.

Usually, the primary lesion is located in the gastric mucosa, the small and large intestine, rectum and pancreas<sup>[2,3]</sup>. These tumors can appear at all ages, but the highest incidence is after the fifth decade. The carcinoid of the appendix is an exception as its highest incidence is at around 40 years of age<sup>[1]</sup>. Those patients with multiple endocrine neoplasia type 1 or von Hippel-Lindau's disease, may have a clinical onset 15-20 years earlier than patients with sporadic neuroendocrine tumors (NETs)<sup>[5]</sup>.

The recent advances in molecular biology have translated into an expansion of therapeutic approaches to these patients. Somatostatin analogs, which initially were approved for control of hormonal syndromes, have recently been proven to inhibit tumor growth<sup>[6]</sup>.

Several new drugs such as antiangiogenics and others targeting mammalian target of rapamycin (mTOR) pathways have been approved to treat progressive pancreatic NETs although their role in non-pancreatic is still controversial<sup>[7]</sup>.

The treatment of NETs requires a coordinated multi-disciplinary approach. The management of localized NETs primarily involves surgical resection followed by surveillance. However, the treatment of unresectable and/or metastatic disease may involve a combination of surgical resection, systemic therapy, and liver-directed therapies with the goal of alleviating symptoms of peptide release and controlling tumor growth<sup>[7]</sup>.

Several completed and ongoing studies are evaluating somatostatin analogs (SSAs), vascular endothelial growth factor (VEGF) pathway inhibitors, mTOR inhibitors, cytotoxic chemotherapy, and peptide receptor radionuclide therapy (PRRT)<sup>[7]</sup>.

This article will review the current therapeutic strategies for metastatic gastroenteropancreatic NETs

and will take a glimpse into the future approaches.

## MANAGEMENT OF ADVANCED NETS

NETs present in up to 40% of cases with metastases at diagnosis (mainly in the liver). If metastatic disease is localized or if > 70% of tumor burden can be resected, cytoreductive surgery should be considered. This approach has shown to reduce local symptoms and also systemic endocrine symptoms<sup>[8]</sup>.

NETs can arise in different organs and from different cell types, and so present a clinical challenge due to their diversity and the variety of symptoms they can cause. Functioning NETs are characterized by the hormones they produce and/or the symptoms they cause; these tumors usually produce clinical symptoms following dissemination to the liver<sup>[8,9]</sup>.

### Carcinoid syndrome

Many functioning NETs release vasoactive peptides and amines (such as serotonin and tachykinins), into the systemic circulation. These can cause a group of symptoms known as "carcinoid syndrome", which appear in 10% of cases of metastatic NETs. This syndrome is characterized by flushing, diarrhea, abdominal pain, telangiectasia and bronchoconstriction<sup>[8,9]</sup>. Carcinoid crisis are believed to be caused by a massive release of bioactive products from the tumor and can occur spontaneously or more frequently after stress, chemotherapy, surgery or anesthesia. These episodes are life-threatening<sup>[10]</sup>. The clinical picture represents an exacerbation of the usual symptoms of carcinoid syndrome, including severe flushing with/without bronchospasm, tachycardia and hypo/hypertension<sup>[10]</sup>.

This needs prompt and effective management to prevent any carcinoid heart disease, though 10%-20% of patients suffer from this issue at diagnosis<sup>[11]</sup>. This is characterized by fibrous thickening of the endocardium (classically on the right heart)<sup>[12]</sup>, tricuspid and pulmonary valves<sup>[12]</sup>.

### Other syndromes

Pancreatic NETs can cause several other syndromes, such as Zollinger-Ellison syndrome, which is characterized by peptic ulcers, diarrhea and abdominal pain and caused by gastrinomas. Glucagonomas which produce hyperglycemia, leading to diabetes mellitus and also a chronic necrolytic migratory erythema. Insulinomas cause hypoglycemia and VIPomas a Verner-Morrison syndrome with severe watery diarrhea (10-15 L/d) and flushing<sup>[13]</sup>.

### Nonfunctioning NETs

These are not associated with hormonal syndromes, thus they become more difficult to diagnose and patients present with advanced disease. Anyway, these tumors may secrete bioactive hormones or amines at subclinical levels<sup>[13]</sup>.

**Table 1** Systemic treatment

Ref.	Type of tumor	Treatment	RR %	PFS (mo)	OS (mo)
Moertel <i>et al</i> <sup>[41]</sup>	Pancreatic	STZ	42		16.5
		STZ + 5FU	42		26
Moertel <i>et al</i> <sup>[39]</sup>	Poorly differentiated	CIS + ETO	18		19
Chan <i>et al</i> <sup>[58]</sup>	Carcinoid	TMZ + beva	0		18.8
	Pancreatic		33		41.7
Yao <i>et al</i> <sup>[72]</sup>	Pancreatic	Everolimus		9.7	
		Everolimus + octreotide		16.7	
Yao <i>et al</i> <sup>[74]</sup>	Midgut carcinoid	Everolimus + octreotide		16.4	
		Octreotide		11.3	
Yao <i>et al</i> <sup>[78]</sup>	Pancreatic	Everolimus	34		
		Placebo	9		
Yao <i>et al</i> <sup>[80]</sup>	Lung/GI NETs	Everolimus		11	
		Placebo		3.9	
Ahn <i>et al</i> <sup>[84]</sup>	Carcinoid	Pazopanib	0		
	Pancreatic		21.9		
Kulke <i>et al</i> <sup>[81]</sup>	Pancreatic	Sunitinib		11.4	
		Placebo		5.5	

PFS: Progression-free survival; STZ: Streptozocin; CIS: Cisplatin; ETO: Etoposide; 5FU: 5-fluorouracil; TMZ: Temozolomide; beva: Bevacizumab.

## SOMATOSTATINE ANALOGS: PAST, PRESENT AND FUTURE

Most NETs express G-protein-coupled transmembrane somatostatin receptors (SSTRs)<sup>[14]</sup>. There are five subtypes of SSTRs, and different NETs have different proportions of receptors expression<sup>[7]</sup> (Table 1).

Somatostatin analogs bind to G-protein-linked receptors on the cell surface and inhibits the release of NE hormones. However, somatostatin has a short half-life *in vivo* (< 3 min)<sup>[7]</sup> and therefore, synthetic somatostatin analogs have been developed for NET symptom control. These analogues form the first-line medical step for well-differentiated NETs<sup>[3,15,16]</sup>.

They bind with high affinity to the five SSRT (sstr<sub>1-5</sub>) on secretory NE cells<sup>[3,16,17]</sup>, which have different inhibitory effects in the body. Subtypes sstr<sub>2</sub> and sstr<sub>5</sub> are the most important in inhibiting hormonal secretions in functioning NETs, thus dual inhibition of both may have a higher inhibitory benefit<sup>[3,16,17]</sup>. These two subtypes may also mediate antiproliferative effects<sup>[7]</sup>. Octreotide and lanreotide bind to the SSTR and decreased hormonal secretion, growth and proliferation, increased apoptosis, inhibit protein synthesis and have a direct antiproliferative activity<sup>[17,18]</sup>.

There is evidence that octreotide controls severe diarrhea and flushing in carcinoid syndrome<sup>[14,19]</sup>.

It has long been suggested that somatostatin analogs may exert antitumor effects for NETs<sup>[20,21]</sup>. Moreover, there may inhibit the release of growth factor and trophic hormones, angiogenesis and modulation of the immune system.

Octreotide is the first somatostatin analogue available commercially, and it is a sstr<sub>2</sub>-preferring agonist, although it has also moderate affinity for sstr<sub>3</sub> and sstr<sub>5</sub><sup>[22,23]</sup>. It has a much longer half-life than somatostatin (2 h).

Lanreotide was the second analogue available and

has a similar binding profile to octreotide.

Octreotide was introduced in clinical practice in the 1987 as it confirmed ability to palliate carcinoid syndrome, as well as other hormonal syndromes caused by metastatic gastroenteropancreatic NETs. Several clinical trials of SSAs tested their ability to inhibit the release of NE hormones such as serotonin, glucagon, insulin, gastrin and vasoactive intestinal peptide (VIP)<sup>[14]</sup>.

Survival rate at 5 years of 67% have been reported in patients receiving somatostatin analogues compared with 18% for historical controls<sup>[3]</sup>.

Several years after the approval of octreotide, evidence of its antineoplastic activity emerged. Although objective radiographic responses (ORR) were rare, many cases of prolonged stable disease (SD) were documented, leading to the hypothesis that SSAs exert an inhibitory effect on tumor growth<sup>[24-27]</sup>.

Recently, this has been tested in a phase III trial. Initial evidence demonstrating that octreotide can reduce symptoms of carcinoid syndrome and decrease 5-HIAA levels was shown with the subcutaneous formulation<sup>[28]</sup>.

The first controlled study of octreotide LAR for treating carcinoid syndrome was conducted in 93 patients with NETs over at least 20 wk<sup>[29]</sup>.

There was a significant decrease in the number of daily stools and incidence of flushing. Treatment success was obtained in 66% of patients receiving octreotide LAR 10-30 mg/mo. It also decreased 5-HIAA levels by 50%<sup>[29]</sup>.

This study demonstrated that monthly octreotide LAR was at least as effective as subcutaneous octreotide for symptom control. Its efficacy for the symptomatic and biochemical control in NETs have subsequently been demonstrated in other studies<sup>[21,22]</sup>.

The mechanism by which somatostatin analogues normalize bowel function is not clear, however, it is hypothesised that involves inhibition of gut hormone

secretion, lengthening of intestinal transit time, increased water and electrolyte absorption and reduced splanchnic blood flow<sup>[23-26]</sup>. Treatment with octreotide improves survival in patients with carcinoid crisis<sup>[27]</sup>. Therefore, its prophylactic use is mandatory to prevent the development of a crisis. It is generally well tolerated, being the most common side effects, abdominal discomfort and bloating, generally mild and resolve spontaneously within the first week<sup>[27]</sup>.

Gallstones can develop, although only a small proportion of patients develop clinical symptoms. Local pain at the injection site has also been reported<sup>[27]</sup>.

A second somatostatin analog, lanreotide, was licensed in Europe in 1998 for the treatment of symptoms associated with NETs (particularly carcinoid).

Lanreotide is less widely studied than octreotide for symptomatic and biochemical control and no directly comparative trials have been conducted. The effects of lanreotide on symptom relief are comparable with those of octreotide<sup>[28]</sup>.

Ruszniewski *et al*<sup>[29]</sup> carried out a study with 71 patients who received lanreotide for 6 mo and reported that 65% of the patients documented a 50% or greater reduction in flushing episodes, and 18% had a 50% or greater reduction in diarrhea episodes. The biochemical response rate is similar to octreotide, with higher responses in patients naive to somatostatin analogue therapy<sup>[30]</sup>.

Somatostatin analogs have got minimal adverse effects and have demonstrated antiproliferative activity *in vitro*<sup>[23]</sup>.

These have been used for patients with metastatic disease when surgical cure is not possible and have been also indicated for the relief of symptoms in patients with functionally active NETs<sup>[31]</sup>.

It has been controversial if somatostatin analogs control the growth of well-differentiated metastatic NETs. Uncontrolled studies have shown tumor shrinkage in response to somatostatin analogs<sup>[32]</sup> and their combination with interferon alfa<sup>[18]</sup>.

Later trials were only able to confirm tumor stabilization in up to 50% of patients, but these studies were not placebo controlled<sup>[30-35]</sup>.

In 2009, Panzuto *et al*<sup>[36]</sup> carried out a prospective, phase IIIB, double-blind, placebo-controlled trial to check the effect of octreotide LAR in the control of tumor growth in patients with well-differentiated metastatic midgut NETs. Treatment-naïve patients were randomly assigned to either placebo or octreotide LAR 30 mg intramuscularly in monthly intervals until tumor progression or death.

The primary end point was time to tumor progression. Most patients (75%) had evidence of somatostatin receptor expression as evidenced by radiotracer uptake on Octreoscan. Thirty-eight percent had carcinoid syndrome (flushing and/or diarrhea associated with elevation in urine 5-HIAA). Only patients with mild carcinoid syndrome who tolerated flushing without intervention or responded to treatment with loperamide

and/or cholestyramine in cases of diarrhea were included. The trial showed a median time to tumor progression of 14.3 mo in the octreotide LAR compared to 6 mo in the placebo arm (HR = 0.34; 95%CI: 0.20-0.59,  $P = 0.00072$ ). Functionally active and inactive tumors responded similarly. Chromogranin A or age did not make any impact on the result either. At 6 mo, tumor progression was seen in 24% of patients on the octreotide LAR arm vs 66% of patients receiving placebo ( $P = 0.0079$ )<sup>[36]</sup>.

Serious adverse events were balanced (11 patients in the octreotide LAR 30 mg arm and 10 patients in the placebo arm).

The most favorable effect was observed in patients with low hepatic tumor load (10%) and resected primary tumor, however both of these subgroups contained the majority of study patients. Even patients with higher hepatic tumor burden (> 10%) experienced a near doubling in time to progression on the octreotide LAR arm<sup>[36]</sup>.

The small number of deaths in both treatment arms (seven in the octreotide LAR 30 mg arm; nine in the placebo arm) precluded any analysis of differences in survival.

Authors concluded that Octreotide LAR significantly increased the time to tumor progression in patients with functionally active and inactive metastatic midgut NETs.

This PROMID trial unfortunately does not clarify the appropriate timing for the treatment, either at initial diagnosis or at the moment of tumor progression or if these data can be extrapolated to patients with G2 NETs<sup>[36]</sup>.

No major differences in classical efficacy have been seen between octreotide and lanreotide<sup>[36,37]</sup>.

One study has evaluated the antiproliferative efficacy of lanreotide in 25 patients. They found partial tumor remission in one patient and stable disease in seven patients, whereas tumor progression occurred in 14.

The CLARINET study is a randomized, double-blind, placebo-controlled study of lanreotide in advanced, well or moderately differentiated, non-functioning, SSTR-positive NETs (Ki-67 < 10%)<sup>[38]</sup>. Tumors could be in the pancreas, midgut, or hindgut or unknown origin. Patients were randomized to receive an lanreotide 120 mg or placebo every 28 d for 24 mo. The primary end point was progression-free survival (PFS). Secondary end-points were overall survival (OS), quality of life and safety. Thirty-three percent had liver tumor > 25%. Lanreotide significantly prolonged PFS (median not reached vs median of 18.0 mo,  $P < 0.001$ ). The estimated rates of PFS at 24 mo were 65.1% and 33.0% for the lanreotide and the placebo group respectively.

There were no significant differences in quality of life or OS between-groups. The most common adverse effect was diarrhea (26% vs 9% for lanreotide and placebo respectively)<sup>[36]</sup>.

The somatostatin analogue should be the first



approach for Grade 1 and 2 gastrointestinal NETs<sup>[39]</sup>.

Predictive factors for no-response to somatostatin analog are Ki67 > 5%<sup>[39]</sup> and distant extra-hepatic metastasis. In these situations chemotherapy should be considered alternatively<sup>[39]</sup>.

SOM230 (Pasireotide) is a novel multireceptor ligand analogue that has high affinity for four of the five somatostatin receptor SSTR (sstr<sub>1</sub>, <sub>2</sub>, <sub>3</sub> and sstr<sub>5</sub>); it has 40-fold higher affinity and 158-fold higher functional activity for sstr<sub>5</sub> than octreotide<sup>[37,38]</sup>.

## CHEMOTHERAPY: PAST, PRESENT AND FUTURE

Responses to chemotherapeutics are extremely heterogeneous in gastroentero-pancreatic NETs. These responses are influenced by tumor differentiation/grade and primary site. Poorly differentiated gastroenteropancreatic NETs respond typically to platinum-based regimens, and the reported RR > 50%<sup>[40]</sup>.

Though recent data point to the relevance of proliferative rate (Ki-67) as higher proliferative levels (> 55%) are significantly linked to higher response to platinum/etoposide compared with high-grade tumors with lower rates of proliferative activity<sup>[40]</sup>.

Pancreatic NETs are sensitive to alkylating agents, including streptozocin, dacarbazine, and temozolomide, as well as fluoropyrimidines. Streptozocin showed response rates of 63% in combination with fluorouracil vs 36% in monotherapy<sup>[41]</sup>.

When combined with doxorubicin vs streptozocin + 5-FU, the response rates and time to progression benefited the first combination (69% and 20 mo vs 45% and 6.9 mo respectively)<sup>[42]</sup>. However, radiographic assessment was not accurate and this fact makes difficult to draw final conclusions about the efficacy of streptozocin.

Unfortunately the use of streptozocin is limited due to its toxicity such as myelosuppression, nausea, and renal insufficiency.

But the role of chemotherapy in NETs has evolved in recent years. It represents a useful option mainly for symptomatic patients, progressive disease, G2 differentiation, and a more aggressive behavior. It also should be considered when the primary objective is tumor load reduction for bulky lesions.

Single agents such as fluorouracil, dacarbazine, doxorubicin and streptozotocin were initially assessed in midgut carcinoid tumors with little benefit<sup>[43]</sup>. Therefore these monotherapies could be reserved to pretreated patients or for patients with a poor performance status. In fact midgut NETs are particularly chemoresistant, possibly due to their low proliferative activity as well as their high expression of methyl guanine methyl transferase (MGMT), which is a DNA repair enzyme<sup>[44]</sup>. For many years there was no evidence that combination regimens were any more effective. None of the regimens demonstrated a response rate (RR) greater than

15%<sup>[45]</sup>. Though recently this has changed. Combination of chemotherapy and IFN- $\alpha$  therapy does not appear to improve on the results of monotherapy<sup>[46,47]</sup>.

Pancreatic NETs RR of approximately 40% have been reported for streptozotocin in combination with other agents such as 5-fluorouracil, cisplatin or doxorubicin<sup>[22,45]</sup>. Temozolomide has also demonstrated promising anti-tumor effects in pancreatic NETs<sup>[48]</sup>. Kulke *et al*<sup>[49]</sup> carried out a phase II trial of temozolomide and thalidomide in patients with metastatic NETs. This combination was associated with a biochemical (chromogranin A) response of 40%, and radiologic response of 25% (45% among pancreatic NETs, 33% among pheochromocytomas, and 7% among carcinoid tumors). Median duration of response was 13.5 mo, 1-year survival was 79%, and 2-year survival was 61%. The response rate seems to be related to the expression of O6-MGMT. Low expression gives a higher response rate (40%) vs high expression (0%).

Orally administered temozolomide and thalidomide seems to be an active regimen for the treatment of NETs. This regimen appeared more active in pancreatic NETs than in carcinoid tumors<sup>[49]</sup>.

Saif *et al*<sup>[50]</sup> carried out a retrospective study of capecitabine and temozolomide (CAPTEM) in patients with metastatic pancreatic NETs who have failed prior therapies (long-acting release octreotide, chemotherapy and hepatic chemoembolization). Seven patients were treated, and authors reported a total response rate of 43%, and clinical benefit (responders and stable disease) 71%. The median duration of response was 8 mo and the most common toxicities were grade 1-2 neutropenia, fatigue and hand-foot syndrome.

Authors concluded that CAPTEM was well tolerated and further prospective studies are warranted to evaluate this regimen with targeted therapies in pNETs<sup>[50]</sup>.

Recently Ramirez *et al*<sup>[51]</sup> reported the results of another study reviewing the CAPTEM regimen again but in a wide variety of metastatic NETs. Twenty nine patients were included, small bowel (31%), pancreas (52%), lung (10%), and rectum (7%)<sup>[51]</sup>.

Partial response was documented in 17% and stable disease in 48%. According to Ki-67 values, partial response (PR)/stable disease (SD) were noted in 13/63% if Ki-67 < 2%<sup>[51]</sup>. Values 2%-20%, PR/SD 19%/50%. If Ki-67 > 20% PR/SD were 20% each. Authors reported a median PFS of 12 mo. They concluded that this regimen may prolong survival although prospective data are needed. Although adverse reactions were experienced, most patients tolerated this regimen, thus CAPTEM should be considered as a reasonable option for metastatic NET patients<sup>[50]</sup>.

A phase II study carried out by Claringbold *et al*<sup>[52]</sup> assessed the role of the radiopeptide <sup>177</sup>Lu-octreotate and capecitabine as a treatment for progressive disseminated NETs. Thirty-three patients were included to receive four cycles of 7.8 GBq (<sup>177</sup>Lu-octreotate 8-weekly, with 14 d of capecitabine.

Twenty-four percent showed PR, 70% SD. Median PFS and median OS had not been reached at a median follow-up of 16 mo with the survival at 1 and 2 years 91% and 88% respectively. Minimal transient myelosuppression with one grade 3 thrombocytopenia but no neutropenia were seen and nephrotoxicity was absent.

The addition of capecitabine radiosensitizing chemotherapy did not increase the minimal toxicity of <sup>177</sup>Lu-octreotate and led to significant clinical benefit in terms of response and SD in patients with progressive metastatic NETs<sup>[52]</sup>.

A phase I-II study to assess the safety and efficacy of combining lutetium-177 octreotate with capecitabine/temozolomide in advanced low-grade NETs was published in 2012. Thirty-five patients received fixed activities of 7.8 GBq lutetium-177 octreotate each 8 wk, with capecitabine for 4 cycles<sup>[53]</sup>.

In phase I, successive cohorts of patients received escalating doses of temozolomide in the last 5 d of each capecitabine cycle<sup>[54]</sup>.

In phase II, patients were treated with 200 mg/m<sup>2</sup> temozolomide. Adverse events were mild to moderate. Complete response was achieved in 15%, PR 38%, SD 38%. Median PFS was 31 mo and median OS was not reached with 90% surviving at 24 mo. Response rates were higher in patients with gastropancreatic NETs than in those with bowel primaries. This study showed that lutetium-177 octreotate in combination with capecitabine and temozolomide was well tolerated in patients with advanced low-grade NETs with significant tumor control rates<sup>[55]</sup>.

Temozolomide, an oral analog of dacarbazine, has activity against NETs when administered alone or in combination with other agents.

A systematic review of temozolomide in advanced NETs has been published by Abdel-Rahman *et al*<sup>[54]</sup> in 2015. These authors assessed 16 trials including 348 patients. Median PFS reported ranged from 6 to 31 mo. Disease control rate 65%-100%. They found that most frequent toxicities were leukopenia, lymphopenia and elevated transaminases.

The data suggested that temozolomide-based combinations with some antineoplastic agents (especially capecitabine) could be an effective treatment for advanced low-intermediate grade NETs<sup>[54,55]</sup>.

## NEW AGENTS: PRESENT BUT LOOKING MORE INTO THE FUTURE

NETs are highly vascularised tumours that express high levels of the VEGF ligand together with its receptor VEGFR. These tumors may show 30%-40% RR to combination chemotherapies but the response to single-agents is only 10%<sup>[55]</sup>.

### Bevacizumab

Tyrosine kinase inhibitors targeting the VEGF receptor

and bevacizumab, a monoclonal antibody targeting VEGF, have demonstrated activity in NETs.

Bevacizumab has been shown to induce objective tumour responses and improvement in median time to progression in advanced carcinoid tumours<sup>[56,57]</sup>.

Several studies have found that temozolomide had significant effect on NETs.

A previous report examining a variety of NETs suggested that the combination of bevacizumab and temozolomide can be safely administered and showed promising activity in patients who had progressed after prior treatments<sup>[57]</sup>.

A phase II study evaluating the same combination in advanced/metastatic NETs was carried out including 34 patients with carcinoid and pancreatic NETs. All patients received prophylaxis against *Pneumocystis carinii* and varicella zoster. The combination of temozolomide and bevacizumab was associated with grade 3-4 toxicities, including lymphopenia (53%) and thrombocytopenia (18%)<sup>[58]</sup>.

Although overall radiographic response rate was 15%, response rates were different between pancreatic NETs (33%) and carcinoids (0%). The median PFS was 11 mo (14.3 mo for pancreatic NETs vs 7.3 mo for carcinoid tumors). Median OS was 33.3 mo (41.7 mo for pancreatic NETs vs 18.8 mo for carcinoid tumors). Authors concluded that this combination could be safely administered and seemed to be promising in pancreatic NETs<sup>[59]</sup>.

Koumarianou *et al*<sup>[60]</sup> had carried out a similar study where temozolomide was delivered continuously at 100 mg daily, a so-called metronomic schedule, together with bevacizumab 7.5 mg/kg once every 3 wk and somatostatin long-acting release 30 mg once every 4 wk. The number of patients with carcinoids was small but authors found occasional durable responses. In their comment published in JCO 2013, these authors suggested the the necessity of further studies with larger numbers of patients to be able to identify who those patients are.

This combination seems to be an important approach as it uses treatments with possibly direct antiangiogenic action on the endothelial cells together with an antibody that blocks the action of VEGF produced by the tumor cells. And therefore, this dual antiangiogenic activity may prove to be an efficacious therapy in NETs which are highly vascularized tumors<sup>[11]</sup>.

Several combinations with bevacizumab have been studied with different results<sup>[61-66]</sup>.

These approaches are mainly effective in G1 and G2 tumors, with a Ki-67 < 20%. However, it is relevant to identify the patients who will most probably benefit from this approach. Koumarianou *et al*<sup>[60,67]</sup> proposed that this combination should be restricted to advanced NET G1/2 tumors, possibly with a Ki-67 < 20%.

### mTOR inhibitors

mTOR is a key regulator of protein synthesis in cancer,

cell growth, proliferation, angiogenesis and cell metabolism. Abnormal PI3K-Akt/PKB-mTOR pathway signaling has been implicated in the pathogenesis of pancreatic NETs.

Everolimus, or RAD001 is an oral, once-daily mTOR inhibitor that blocks the mTOR pathway by binding to its intracellular receptor, FKBP-12. It has shown synergistic anti-tumor activity when combined with other anticancer therapies<sup>[68,69]</sup>.

In a phase III study, patients with low- and intermediate-grade advanced pancreatic NETs were randomized to receive everolimus 10 mg/d or placebo. Median PFS was significantly prolonged in the everolimus arm, 11 mo vs 4.6 mo<sup>[70]</sup>.

Everolimus may have a similar effect when used in combination with a somatostatin analogue. In the study by Grozinsky-Glasberg *et al*<sup>[71]</sup>, octreotide and everolimus showed significant anti-proliferative effects and they suggested that everolimus could interact with the same pathway at a site or sites similar to octreotide.

A study to assess the antiproliferative effect of combining everolimus with octreotide in patients with metastatic low to intermediate grade NETs was carried out. It enrolled 60 patients. Authors found promising activity in those receiving everolimus 10 mg daily<sup>[72,73]</sup>.

A Phase II trial of everolimus with or without octreotide LAR in patients with advanced pancreatic NETs following chemotherapy failure (RADIANT-1) found that in those receiving everolimus monotherapy, median PFS was 9.7 mo. PR 9.6%, 67.8% SD and 13.9% showed progressive disease. In the combination arm, median PFS was 16.7 mo, 4.4% PR, 80% SD, and no patients with progressive disease<sup>[74]</sup>. Authors found that an early CgA or NSE response was associated with a longer PFS compared with those without an early response<sup>[74]</sup>. Most adverse events were mild to moderate.

A Phase III trial, RADIANT-2, was carried out in advanced (unresectable locally advanced or distant metastatic and disease progression within the past 12 mo) midgut carcinoid tumors with low-grade or intermediate-grade NETs (carcinoid). It compared everolimus 10 mg/d plus octreotide LAR 30 mg every 28 d with placebo and octreotide LAR every 28 d. Four hundred and twenty-nine patients were randomly assigned to study groups. The combination arm showed a median PFS of 16.4 mo vs 11.3 mo for the control arm ( $P = 0.026$ ). This did not meet a prespecified significance level by central review ( $P = 0.024$ )<sup>[75]</sup>.

However, by an investigator review the median PFS was 12.0 mo for the combination arm and 8.6 mo for the control arm ( $P = 0.018$ ).

Authors concluded that everolimus plus octreotide LAR, compared with placebo plus octreotide LAR, improved PFS in advanced NETs associated with carcinoid syndrome<sup>[76]</sup>.

Both everolimus and temozolomide are associated with single-agent activity in patients with pancreatic NETs.

A phase I-II study was performed to evaluate the safety and efficacy of temozolomide in combination with everolimus in advanced pancreatic NET. Patients received temozolomide 150 mg/m<sup>2</sup> per day on days 1 through 7 and days 15 through 21 in combination with everolimus daily in each 28-d cycle.

In cohort 1, everolimus as administered at 5 mg daily. In cohort 2 it was increased to 10 mg daily. Temozolomide was administered for 6 mo<sup>[77]</sup>. Forty-three patients were enrolled. No synergistic toxicities were reported. Forty percent had PR. The median PFS was 15.4 mo. Median OS was not reached. Authors concluded that this regimen could be safely given to advanced pancreatic NETs with significant antitumor activity<sup>[77]</sup>.

RADIANT-3 trial is another phase III prospective, double-blind, randomized, placebo-controlled study carried out in patients with advanced, low or intermediate grade pancreatic NETs. Patients were randomised to receive everolimus 10 mg daily or placebo. Four hundred and ten patients were included. The median PFS was 11.0 mo with everolimus and 4.6 mo with placebo ( $P < 0.001$ ). Estimates of the proportion of patients who were alive and progression-free at 18 mo were 34% with everolimus as compared with 9% with placebo<sup>[78]</sup>.

Adverse events were mostly grade 1 or 2, mainly stomatitis rash, diarrhea, fatigue and infections primarily upper respiratory. Grade 3 or 4 included anemia and hyperglycemia.

Everolimus significantly prolonged PFS among patients with progressive advanced pancreatic NETs and was associated with a low rate of severe adverse events.

Mature data showed a median OS of 44.02 mo in the everolimus arm compared with 37.68 mo in the placebo; However, a high crossover of patients from placebo to everolimus (85%) may have contributed to the long median OS in the placebo arm and may have confounded the ability to detect a difference in the overall survival results<sup>[79]</sup>.

RADIANT-4 is another phase III study assessing the efficacy and safety of everolimus compared with placebo in patients with advanced, progressive, well-differentiated, non-functional NETs of the lung or gastrointestinal tract<sup>[80]</sup>. Patients were randomised to receive everolimus 10 mg per day or placebo. Three hundred and two patients were enrolled. Median PFS was 11 mo in the everolimus group and 3.9 mo in the placebo arm. Everolimus was associated with a 52% reduction in the estimated risk of progression. In the first pre-planned interim OS analysis, the results of everolimus showed a reduction in the risk of death, although not statistically significant. The safety findings were consistent with the known side-effect profile of everolimus.

Authors concluded that everolimus is the first targeted agent to show robust anti-tumour activity with acceptable tolerability across a broad range of NETs (pancreas, lung, and gastrointestinal tract)<sup>[80]</sup>.

### Sunitinib

NETs express VEGF and its receptor VEGFR. Sunitinib malate, an oral multi-targeted tyrosine kinase inhibitor targets VEGFR-1, -2, and -3; platelet-derived growth factor receptor; and c-KIT. Sunitinib is currently approved for treatment of pancreatic NETs. Its toxicity profile includes diarrhea, fatigue, cytopenias, nausea, hypertension, and palmar-plantar erythrodysesthesia. The efficacy of sunitinib was assessed in a two-cohort, phase II study of advanced carcinoid and pancreatic NETs. Patients were treated with repeated 6-wk cycles of oral sunitinib (50 mg/d for 4 wk, followed by 2 wk off treatment). The trial showed an overall response rate of 2.4% and 16.7% in patients with carcinoid tumors and pancreatic NETs, respectively. Median time to tumor progression was 7.7 mo in pancreatic NETs and 10.2 mo in carcinoid. The authors concluded that sunitinib has antitumor activity in pancreatic NETs whereas its activity against carcinoid tumors could not be definitively determined<sup>[81]</sup>.

A phase III randomized, double-blind trial in low and intermediate grade pancreatic NETs with sunitinib 37.5 mg/d orally or placebo showed. PFS of 11.4 mo vs 5.5 mo in the sunitinib and placebo arms respectively was statistically significant<sup>[82]</sup>.

A phase II study testing the efficacy and safety of everolimus and octreotide LAR or everolimus, bevacizumab, and octreotide LAR in advanced pancreatic NET with evidence of progression was presented at ASCO annual conference 2015. PFS was 16.7 mo on everolimus + bevacizumab + octreotide LAR vs 14 mo for everolimus + octreotide LAR. Response rate 31% in triplet arm vs 12% in doublet. Toxicity was significantly higher on the triplet with 81% grade 3-4 adverse events vs 49% on the doublet. Investigators commented on promising results but future trials to learn about patients selection are warranted<sup>[82]</sup>.

### Pazopanib

Pazopanib is an orally bioavailable, multitargeted kinase inhibitor that inhibits VEGF receptors 1, 2, and 3. It has been evaluated in a nonrandomized phase II study of 37 patients with gastroenteropancreatic NETs. The overall response rate was 24% with a median PFS of 9.1 mo<sup>[83]</sup>.

The PAZONET study is another phase II which showed clinical activity of pazopanib in patients with advanced NETs regardless of previous treatments. Authors suggested that circulating tumor cell counts and soluble *VEGFR2* and *VEGFR3* gene polymorphisms could be potential biomarkers for selecting patients for pazopanib<sup>[84]</sup>.

Another phase II study in metastatic or locally advanced grade 1-2 carcinoid tumours or pancreatic NETs, using pazopanib 800 mg orally once per day and octreotide showed a response of 21.9% in pancreatic NETs whereas no responses in carcinoid tumours<sup>[85]</sup>.

Based on all these results, a randomized phase III trial

of pazopanib vs placebo for advanced carcinoid tumors is ongoing and other phase III trials are warranted<sup>[86]</sup>.

There are few effective therapies for pancreatic NETs. Recent placebo-controlled phase III trials of everolimus and sunitinib have reported improved PFS. Preclinical studies have suggested enhanced antitumor effects with combined mTOR and VEGF pathway-targeted therapy. A phase II trial was carried out with a combination of temsirolimus 25 mg intravenously (iv) once per week and bevacizumab 10 mg/kg iv once every 2 wk in well or moderately differentiated pancreatic NETs and progressive disease<sup>[87]</sup>. Fifty-eight patients were enrolled, response rate was 41%, PFS at 6 mo was 79% with median PFS 13.2 mo. Median OS 34 mo. The investigators concluded that this combination had shown significant activity with acceptable toxicity in pancreatic NETs with progressive disease<sup>[87]</sup>.

### Interferon

Interferon therapy is generally recommended as a second-line in patients with functioning NETs and low proliferation<sup>[28-30]</sup>. The benefits of interferons on symptom control is similar to that of somatostatin analogues but they may have higher antiproliferative activity<sup>[30]</sup>. Unfortunately their safety profile is not as favourable, with fever, fatigue, anorexia and weight loss among others<sup>[29,30]</sup>.

IFN- $\alpha$  has shown in a pooled analysis of trials in patients with NETs a 40% of biochemical responses (similar to octreotide and lanreotide) with 10% of objective tumor responses<sup>[9,29,30,87,88]</sup>.

Bondanelli *et al*<sup>[89]</sup> have suggested that a combination of IFN- $\alpha$  with somatostatin analogues might have a synergistic effect. A phase II prospective trial randomized 44 patients to receive bevacizumab or pegylated IFN- $\alpha$  for 18 wk, followed by both agents in combination. At the end of the single-agent administration period, the rate of PFS was 95% in the bevacizumab arm vs 68% in the IFN- $\alpha$  arm. This study demonstrated activity with bevacizumab in patients with carcinoid tumor<sup>[56]</sup>.

A phase III trial comparing bevacizumab vs IFN- $\alpha$  showed that a combination with bevacizumab obtained longer time to failure compared to IFN- $\alpha$  arm. Responses were also higher with bevacizumab. However, it did not meet its primary endpoint of improvement in PFS.

Participants had advanced NETs with poor prognosis, as defined by one or more of the following criteria: Progressive disease, G2 with 6+ lesions, colorectal or gastric primaries.

Toxicity was higher on the interferon arm with 26% grade 3-4 fatigue. Based on these results, the investigators concluded that neither bevacizumab nor IFN  $\alpha$ -2b arm should be used as standard treatment<sup>[90]</sup>.

## LIVER-DIRECTED THERAPIES

NETs present in up to 40% of cases with metastases at



diagnosis (liver mainly). Although radical surgery would be the treatment of choice, however, it is generally not possible. Liver resection is generally advocated in those cases with limited hepatic disease in which more than 90% of tumors can be successfully resected or ablated<sup>[91]</sup>.

Patients with liver metastases may experience symptoms such as pain, anorexia, and weight loss related to tumor burden. Additional symptoms include flushing and diarrhea caused by secretion of hormones directly into the systemic circulation. Medical treatments and locoregional therapies are palliative approaches in symptomatic patients or in cases of progressive disease. As the liver metastases from NET are hypervascular, endovascular treatments are interesting<sup>[92,93]</sup> too as a cytoreductive technique.

Overall hepatic-directed therapies include liver resection or ablation, hepatic artery embolization (transarterial embolization, transarterial chemoembolization and radioembolization) and liver transplantation. These therapies are generally reserved for patients whose tumors are predominantly confined to the liver.

### Ablation

Ablation techniques are generally reserved for unresectable metastases smaller than 5 to 7 cm in diameter. Several ablation techniques have been described. Those include cryoablation, alcohol ablation and radiofrequency ablation (RFA).

There are no randomized studies comparing surgical to nonsurgical treatments and though long survivals have been observed in surgically treated patients, these could be due to the fact those patients have got favourable prognosis as low tumour burden.

RFA has been used with good results and minimal morbidity for the treatment of patients with NET hepatic metastases<sup>[94]</sup>. One disadvantage with this therapy has been the relatively small volume of tissue that can be coagulated and clinical trials with RFA have shown that complete responses are more likely to occur with tumours  $\leq 4$  cm<sup>[95]</sup>.

With the use of simultaneous multiple fiber laser induced thermotherapy or next generation bipolar RFA, some authors have reported ablation of tumours as large as 7 cm in diameter<sup>[95,96]</sup>.

Moreover, up to 7 lesions at one time may be ablated using specialized techniques to increase lesion size<sup>[96,97]</sup>.

The morbidity associated is 5%-10% and mortality rate is about 0.5%<sup>[98,99]</sup>.

Berber *et al*<sup>[98]</sup> reported a total and significant symptom relief in 95% and 80% respectively in 34 patients with NET liver metastases. The median duration of the benefit was 10 mo. These benefits were seen even in patients with extrahepatic disease.

These techniques are suitable for repeated treatments in patients with local recurrence or new metastases.

### Microwave ablation

Microwave ablation (MWA) is more appropriate than RFA to treat tumours next to major hepatic vasculature. In those areas the adjacent blood flow theoretically predisposes RFA to a heat sink effect<sup>[100]</sup>.

Although clinical experience with MWA has mostly involved hepatocellular carcinoma, though NETs have also been included in some series.

Martin *et al*<sup>[101]</sup> reported NET patients undergoing MWA with a 90% success rate for complete ablation with no recurrences at the ablation sites. Most of these patients had MWA performed under ultrasound guidance during open surgery (concomitant hepatectomy and/or extrahepatic metastasectomy). Median overall survival reported was 41 mo. But these results need further studies to be confirmed as these authors only included 11 patients.

There is a lack of data comparing MWA (especially percutaneous) to RFA but geographic patterns of preference have been described. Whereas RFA is widely adopted in the United States, MWA is in Europe and Asia<sup>[101]</sup>.

### Hepatic artery embolization

This technique is performed in patients with diffuse, unresectable liver metastases. The rationale for embolization is related to liver blood supply. Liver metastases get the majority of their blood supply from the hepatic artery, while the normal liver parenchyma gets blood supply primarily from the portal vein. In patients with bilobar hepatic metastases, staged lobar embolizations are typically performed at 4- to 6-wk intervals<sup>[102-104]</sup>. Several techniques have been included such as TAE, TACE and drug eluting beads (DEB)-TACE.

### TACE and DEB-TACE

TACE has been used several decades. It combines the benefits of embolization and locoregional chemotherapy and provides with a high rate of tumour and symptomatic response<sup>[103]</sup>.

TACE follows the same principles as TAE, but the intra-arterial administration of a chemotherapeutic agent is added at the time of embolization. With this technique intratumoral concentrations of the drug are over 20 times higher than those obtained by systemic administration of the same drug. Moreover, with this technique, it exists the further potential clinical benefit of tumour ischaemia as a result of embolization.

This technique is indicated in nonsurgical candidates with progressive or refractory disease despite medical treatment (SSAs) and no contraindication to TACE. The best results are obtained if liver involvement is  $< 60\%$  and good ECOG (0-1).

Conventional TACE uses a mixture of doxorubicin, lipiodol and embolic agent. The symptoms response has been as high as 73% to 100%, objective response 55% to 80% and time to progression from 8 to 42 mo<sup>[103,104]</sup>.

Toxicity profile shows grade 2 alopecia, 2-3 nausea and vomiting, postembolization syndrome, acute meta-

bolic syndrome or infection<sup>[91,104]</sup>. Some of these toxicities blamed the unfavorable pharmacokinetic profile of doxorubicin binding to lipiodol<sup>[105]</sup>.

DEB-TACE more recently has improved the pharmacokinetics of the delivered drug. However, a higher incidence of bile duct injury has made its indication controversial in NET metastasis in some institutions.

DEB is a new product which has been shown to achieve higher intratumoral drug concentration and less concentration in the bloodstream TACE in animal studies<sup>[106]</sup>.

De Baere *et al*<sup>[107]</sup> carried out a study of 20 patients and showed 80% objective tumor response and disease control, with time to progression of 15 mo. Drug toxicity was very low with grade 2 alopecia around 1% and only a few cases of mild nausea and vomiting. They reported high rate of liver infarction and bile duct injuries although most cases were asymptomatic<sup>[107]</sup>.

In normal liver parenchyma, intrahepatic bile ducts do not have a dual blood supply and are fed only from the hepatic arterial branches that form a vascular plexus (peribiliary capillary plexus) around the bile ducts. Therefore, ischemia of the intrahepatic bile ducts can easily occur after TACE<sup>[108]</sup>.

Some authors have suggested that the incidence of DEB-TACE-related bile duct injury is the result of inadvertent retention at the capillary peribiliary network of DEB loaded with doxorubicin. This may occur as a consequence of over-embolization related to a very aggressive TACE resulting in a high DEB dose and/or complete vessel occlusion.

Experienced operators are aware that the technique is different from conventional TACE notably the embolization. In 35 consecutive patients with liver NET treated in a single institution with DEB-TACE, two different embolization endpoints were compared (complete vs limited embolization). The results showed lower rate of adverse events (14% vs 57%,  $P < 0.05$ ) using the latter. No statistically significant difference in response comparing the two endpoints<sup>[109]</sup>.

It seems not to be definitively clear which technique should be used, although DEB-TACE has an excellent pharmacokinetic profile which results only in minimal drug toxicity, but it has shown to increase the risk of biliary tree injury, albeit asymptomatic in most cases.

Both techniques, conventional TACE and DEB-TACE offer a high objective response rate and disease control with satisfactory duration of the response. Some authors have suggested that there seems not to be rational in performing conventional TACE or DEB-TACE, because of doxorubicin has no proven effect in NETs. Moreover, the highest benefit from these techniques seems to be due to the embolization rather than the drug effect<sup>[110]</sup>.

The ablation techniques include cryoablation, alcohol ablation and radiofrequency ablation. These methods are reserved for unresectable oligometastases smaller than 5-7 cm. There are no randomized trials

comparing surgical vs nonsurgical approaches in the management of gastroenteropancreatic NETs with liver metastases<sup>[96,111]</sup>.

## TAE

In targeted embolization of the hepatic artery (TAE) several occlusive materials have been used such lipiodol, gel foam particles, polyvinyl alcohol foam or bland microspheres. It produces tumoral ischemic necrosis while the surrounding liver is perfused by the portal vein. If bilobar metastases, staged lobar embolizations may be needed.

Contraindications to TAE include > 75% replacement of liver parenchyma by tumour, predominant extrahepatic tumour burden, indolent tumours, and hepatic dysfunction.

In cases of revascularization, TAE or TACE can often be repeated. Postembolization syndrome can occur after this technique. It consists of self-limiting pain, fever and nausea/vomiting. This syndrome occurred in most patients, with an 11% major complication rate. There are no completed randomized trials comparing TAE with TACE and the superiority of one technique to another has never been shown<sup>[111]</sup>.

## Selective internal radiotherapy

A novel approach to liver metastases from gastroenteropancreatic NETs involves embolization of <sup>90</sup>Y embedded either in a resin microsphere (SIR-Sphere) or a glass microsphere (TheraSphere)<sup>[112]</sup>.

This technique also known as selective internal radiotherapy (SIRT) will produce tumor necrosis through direct delivery of radiation. Response rates in metastatic GEP-NETs have been encouraging. A retrospective multicenter study of 148 patients treated with SIR-Spheres showed overall response rate of 63%<sup>[113]</sup>.

SIRT has never been compared prospectively to other embolic therapies and long-term toxicities such as radiation fibrosis represent potential risks. Its cost is substantially higher than more traditional embolization therapies, therefore its widespread adoption should await prospective randomized trials.

## HIGH-INTENSITY FOCUSED ULTRASOUND

High-intensity focused ultrasound (HIFU) has been recently introduced for the treatment of pancreatic cancer<sup>[114]</sup>. HIFU is a non-invasive technique for the treatment of several primary tumors and metastases. Wu *et al*<sup>[115]</sup> have reported large areas of coagulation necrosis with this technique in hepatocellular carcinoma. Zhang *et al*<sup>[116]</sup> have documented complete tumour necrosis even in lesions adjacent to major hepatic blood vessels.

HIFU achieves ablation by focused ultrasound energy

from an external source that is targeted within the body and induces thermally necrosis. The acoustic intensity is high only within the focal region and therefore it minimizes the risk of injury to the surrounding tissues.

This technique can reach tumours in unfavourable locations for a needle placement and it has proved to offer better disease control and quality of life.

HIFU appears to be an alternative for pancreatic NETs when no indication for a different minimally invasive approach exists. It may be easily repeated and provides good local tumour control<sup>[116]</sup>.

Moreover, it could be used as a cytoreductive therapy aiming at improving the palliation of patients with locally advanced pancreatic malignancies. However, more studies are needed to evaluate its real impact on survival or quality of life.

Currently and until solid data become available in NETs, HIFU should be reserve for patients whose symptoms cannot be controlled by medical therapy and they are not candidates for surgery or a different minimally invasive therapy<sup>[116]</sup>.

## THE FUTURE

Mutations in the PI3-kinase (PI3K) pathway occur in 16% of patients with pancreatic NETs. Therefore, these tumors are a potential setting for PI3K/AKT/mTOR pharmacological interventions<sup>[117]</sup>.

Everolimus, a mTOR inhibitor, is used to treat patients with advanced pancreatic NETs. However, resistance to mTOR targeted therapy is emerging partially due to the loss of mTOR-dependent feedback inhibition of AKT. In contrast, the response to PI3K inhibitors in pancreatic NETs is unknown<sup>[118]</sup>.

Soler *et al*<sup>[118]</sup> carried out a study to assess the frequency of PI3K pathway activation in human pancreatic NETs and in RIP1-Tag2 mice, which is a preclinical tumor model of pancreatic NETs. They investigated the therapeutic efficacy of inhibiting PI3K in RIP1-Tag2 mice using a combination of pan (GDC-0941) and p110 $\alpha$  selective (GDC-0326) inhibitors and isoform specific PI3K kinase-dead mutant mice. They found that treatment of these mice with GDC-0941 reduced tumor growth without impact on vascular area and the selective inactivation of the p110 $\alpha$  PI3K isoform reduced tumor growth as well as vascular area.

The authors concluded that p110 $\alpha$  could have a role in pancreatic NETs and unravel a new function of this kinase in cancer biology through its role in promoting metastasis<sup>[118]</sup>.

Andersson *et al*<sup>[119]</sup> carried out a study to define the transcriptome of small intestinal NETs to identify clinically relevant subgroups of tumors, prognostic markers and novel targets for treatment.

Genome-wide expression profiling was conducted on biopsies from 33 patients with well-differentiated metastatic NETs of the distal ileum. They identified three groups: The largest, characterized by longer patient survival and higher expression of NE markers,

including SSTR2. Then, tumors with higher grade (G2/3) or gain of chromosome 14 which were associated with shorter survival and increased expression of cell cycle-promoting genes<sup>[118]</sup>.

The prostaglandin E receptor 2 is the most significantly activated regulator in tumors of higher grade, whereas Forkhead box M1 was the most significantly activated regulator in tumors with gain of chromosome 14<sup>[118]</sup>.

Evaluation of candidate drug targets on NET cells (GOT1) showed significant inhibition of tumor cell growth after treatment with tyrosine kinase inhibitors or inhibitors of HDAC, HSP90 and AKT<sup>[118]</sup>.

Authors found specific gene expression patterns associated with tumor grade and chromosomal alterations<sup>[118]</sup>. The results of several practice-changing phase III clinical trials have been presented at The North American Neuroendocrine Tumor Society symposium 2015: The TELESTAR randomized phase III trial of telotristat vs placebo in patients with carcinoid syndrome<sup>[119,120]</sup>.

Telotristat etiprate is an oral inhibitor of tryptophan hydroxylase (This enzyme triggers the excess serotonin production within metastatic NET cells that leads to carcinoid syndrome). It decreased significantly the mean of daily bowel movements by 35% among patient who received 500 mg of the drug three times a day and 29% among those who received 250 mg three times a day, compared with 17% for those who received placebo<sup>[119,120]</sup>.

Urinary 5-HIAA levels were significantly reduced as well, for patients receiving the active drug, suggesting effective inhibition of serotonin production.

Telotristat etiprate has received Fast Track and Orphan Drug designation from the United States Food and Drug Administration. Whereas current treatments for carcinoid syndrome reduce the release of serotonin outside tumor cells, telotristat etiprate works to reduce serotonin production within the tumor cells<sup>[119,120]</sup>.

Currently, there are limited therapeutic options for patients with advanced midgut neuroendocrine tumors progressing on first-line somatostatin analog therapy.

NETTER-1 randomized phase III trial of radiolabelled somatostatin analog 177-Lutetium-dotatate vs high dose octreotide (LAR) 60 mg in patients with progressive midgut NETs. Results showed that the median PFS, the trial's primary endpoint, improved by nearly 80%. The median PFS with high-dose octreotide was 8.4 mo and was not yet reached in the <sup>177</sup>Lu-Dotatate arm at a median follow-up of 18 mo but update data indicate that it will probably be in excess of three years. Although the OS data were not mature enough for a definitive analysis, the number of deaths was 13 in the Lutathera group and 22 in the Octreotide LAR 60 mg group at interim analysis which suggests an improvement in OS. The overall response rate was 18% vs 3%. Safety data confirmed favorable results of the preceding phase I/II studies<sup>[121,122]</sup>.

Serious adverse events related to treatment were 9%

for Lu-Dotatate and 1% for octreotide. Withdrawals due to adverse events were 5% for Lu-Dotatate and did not occur in patients treated with octreotide. Lu-Dotatate is the most advanced candidate in development of PRRTs, which target tumors with radiolabelled somatostatin analog peptides. In April 2015, the FDA granted a fast track designation to Lu-Dotatate for the treatment of inoperable progressive midgut NETs<sup>[122,123]</sup>.

Radiolabeled SSA therapy (also called peptide receptor radiotherapy or PRRT) has shown to be an effective treatment for gastroenteropancreatic NETs, as it allows targeted delivery of radionuclides to SSTR-expressing tumor cells. Selection criteria for PRRT include evidence of strong radiotracer uptake on somatostatin-receptor scintigraphy, ideally higher than in normal liver tissue.

The agents <sup>90</sup>Y-DOTATOC and <sup>177</sup>Lu-Dotatate are the latest generation of PRRT. <sup>90</sup>Y is a high-energy  $\beta$ -particle emitter. Strosberg *et al*<sup>[123]</sup>, Kwekkeboom *et al*<sup>[124]</sup> and Valkema *et al*<sup>[125]</sup> reported ORR > 25%. A later large multicenter trial of 90 patients with metastatic carcinoids showed a RR of 4%, and 70% of SD<sup>[126]</sup>.

<sup>177</sup>Lu emits both  $\beta$  and  $\gamma$  rays. A large nonrandomized trial including 310 patients, has reported a 30% RR with gastroenteropancreatic NETs receiving <sup>177</sup>Lu-octreotate.

Responses were particularly high in patients with pancreatic NETs<sup>[127]</sup>. PRRT toxicities include myelosuppression and renal insufficiency, with the latter generally ameliorated by concurrent amino acid infusion.

## CONCLUSION

NE gastroenteropancreatic tumors are a heterogeneous group of neoplasias arising from NE cells of the embryological gut<sup>[1]</sup> whose incidence have increased due probably to the improvements in diagnosis<sup>[1-4]</sup>. Many recent advances in molecular biology have expanded the therapeutic arsenal and we have shifted from somatostatin analogs<sup>[6]</sup> only, to a new scenario where antiangiogenics and mTOR inhibitors among others have started to take over<sup>[7]</sup>. The treatment of NETs continues being a challenge and requires a coordinated multidisciplinary approach. Although the management of localized NETs involves surgical resection followed by surveillance, the treatment of unresectable and/or metastatic disease may involve several disciplines (surgical resection, systemic therapy, liver-directed therapies)<sup>[7]</sup>.

Several completed and ongoing studies are evaluating somatostatin analogs, VEGF pathway and mTOR inhibitors, cytotoxic chemotherapy, PRRT<sup>[7]</sup>, new liver-directed therapies, *etc.* but future trials should focus on patient selection, predictive markers, and tolerability improvement as these aspects are critical to continue advancing.

The circulating tumour cells (CTCs), which are detectable in the blood of 50% of patients with functioning midgut NETs, are usually related to poor prognosis. The CALM-NET, a phase IV, multicentre, open label, single

group exploratory study to assess the clinical value of enumeration of CTCs to predict clinical symptomatic response and PFS in patients receiving lanreotide to treat the symptoms of functioning midgut NETs is under way. The results of this trial could be valuable as if positive, CTCs could be used as predictive markers to help make therapeutic decisions.

Pancreatic NETs are heterogenous neoplasms still with limited therapeutic options but everolimus has recently been approved for the treatment of progressive, well-differentiated, non-functional, unresectable, locally advanced or metastatic NETs of gastrointestinal or lung origin. This is the first approved treatment for these rare cancers whose prognosis is poor and their options limited.

Alkylating cytotoxic agents, such as streptozocin and temozolomide, play an important role in the treatment of pancreatic NETs, although RR varies widely. Future studies of cytotoxics in gastroenteropancreatic NETs should stratify patients based on primary site and tumor grade. Over the next years, randomized clinical trials are expected to provide more data about role of radiolabeled somatostatin analogs. Predictive biomarkers that would allow for individualized selection of treatments are needed.

New findings have shed light on the biological processes of pancreatic NETs and have identified a tumorigenic cell population that suggest these cells can hide from immune surveillance.

These discoveries will hopefully open the door to new potential therapeutic targets<sup>[128]</sup> which can lead to personalised treatments and optimize the results in this heterogeneous group of tumors.

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## Novel therapeutic approaches targeting L-type amino acid transporters for cancer treatment

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### Abstract

L-type amino acid transporters (LATs) mainly assist the

uptake of neutral amino acids into cells. Four LATs (LAT1, LAT2, LAT3 and LAT4) have so far been identified. LAT1 (SLC7A5) has been attracting much attention in the field of cancer research since it is commonly up-regulated in various cancers. Basic research has made it increasingly clear that LAT1 plays a predominant role in malignancy. The functional significance of LAT1 in cancer and the potential therapeutic application of the features of LAT1 to cancer management are described in this review.

**Key words:** LAT1; Amino acid transporter; Molecular target drug; Amino acid starvation response; Signal transduction

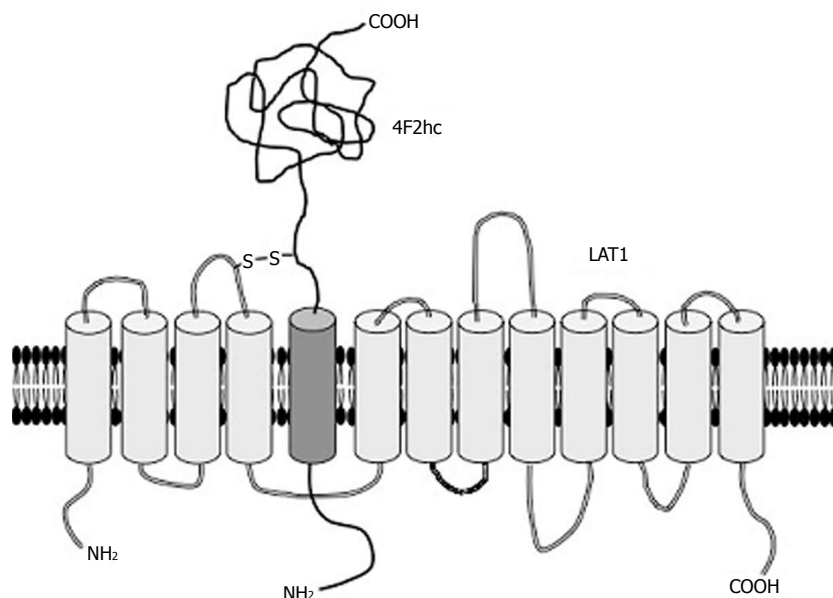
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**Core tip:** The discovery of molecules preferentially expressed in cancer cells is extremely valuable for the development of molecular target drugs in cancer therapy. Amino acid transporters have been receiving a great amount of attention as a candidate of such molecular targets. This review summarizes new initiatives for clinical applications of the basic research relative to L-type amino acid transporters, which are commonly expressed in cancers.

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### INTRODUCTION

Cancers consume a huge amount of materials for biochemical reactions, and a continuous supply of sufficient nutrients is essential for their survival. Hydrophilic nutrients are delivered into cells by transporters.



**Figure 1 Structure of LAT1.** LAT1 is composed of 12 transmembrane helices that are predicted to form a cylindrical conformation penetrating the cellular membrane. LAT1 associates with 4F2hc for stable localization at the cellular membrane. LAT2 is similar in structure to LAT1, whereas LAT3 and LAT4 function independently of 4F2hc.

Recent studies have revealed several transporters preferentially expressed in cancers. Inhibition of cancer specific-nutrient transporters would be a good strategy for cancer management with minimal side effects. Indeed, a therapeutic approach using transporter inhibitors for cancer prevention has been proven to be efficacious in cell lines and animal experiments and is now under evaluation in a clinical trial.

## L-TYPE AMINO ACID TRANSPORTERS

Many cells take advantage of transporters to incorporate what is necessary at the time of need. Transporters fall into two broad categories based on ATP dependency for their transport form<sup>[1]</sup>. ATP-dependent transporters, known as ATP-binding cassette, hydrolyze ATP to obtain the energy for translocation of their substrates across the membrane (active transport). Transporters with no ATPase, called solute carriers (SLCs), facilitate diffusive transport. Each SLC transporter is named in combination with the family numeral based on the sequence similarity and individual number with letter A between them (e.g., SLC3A2), with a few exceptions. Most of the amino acid transporters were formerly categorized into several groups ("System") on the basis of their substrates and sodium dependency (e.g., System L, which incorporates neutral amino acids without sodium), but they are currently classified into SLCs according to their protein homology.

L-type amino acid transporters (LATs) are categorized as system L transporters. LATs mainly deliver neutral amino acids into cells in a sodium-independent manner. So far, four LATs have been identified.

LAT1 (SLC7A5) was identified as the first LAT by

two groups in 1998<sup>[2,3]</sup>. The major substrate of LAT1 is large neutral amino acids as typified by leucine. The expression of LAT1 in normal adults is detected in proliferative zones of gastrointestinal mucosa, testicular sertoli cells, ovarian follicular cells, pancreatic islet cells, and some endothelial cells that serve as a barrier between tissues (blood-brain, blood-retinal and blood-follicle barrier)<sup>[4]</sup>. Recent studies revealed a crucial role of LAT1 in activated T cells<sup>[5,6]</sup>. As described below, LAT1 expression is commonly up-regulated in various cancers.

LAT2 (SLC7A8) was subsequently isolated on the basis of sequence similarity to LAT1<sup>[7-9]</sup>. LAT2 has broader specificity of its substrates including polar uncharged and small neutral amino acids than that of LAT1<sup>[8]</sup>. LAT2 is ubiquitously expressed in normal body<sup>[4]</sup>, though LAT2 knockout mice show a mild phenotype and almost no visible symptoms except aminoaciduria<sup>[10]</sup>. Both LAT1 and LAT2 are composed of 12 transmembrane domains that form the pathway of their substrates<sup>[11]</sup> (Figure 1). They associate with the heavy glycoprotein subunit 4F2hc (SLC3A2) by sulfur bond<sup>[11]</sup>. Although 4F2hc does not seem to have a function to directly transfer the substrates, it makes the localization of its partner LATs more stable at the plasma membrane<sup>[12]</sup>.

LAT3 (SLC43A1) was isolated by expression cloning from hepatocarcinoma cells<sup>[13]</sup>. Sequence analysis revealed that LAT3 was identical to POV1, which was originally identified as a cancer-up-regulated gene<sup>[14,15]</sup>. The substrate selectivity of LAT3 was similar to that of LAT1. LAT3 mRNA is expressed in the liver, skeletal muscle, and pancreas<sup>[16]</sup>. The physiological role of LAT3 in normal individuals of mammals remains unknown,

but it was shown that LAT3 functions for podocyte development in zebrafish<sup>[17]</sup>.

LAT3 appears to behave as a critical transporter in several cancers. LAT3 is up-regulated in response to androgen and knockdown of LAT3 expression by RNA interference (RNAi) significantly inhibits the leucine uptake and cell proliferation in human prostate cancer cell lines *in vitro*<sup>[18]</sup>. Furthermore, high expression of LAT3 is detected in prostate cancer patients, and stably knockdown of LAT3 by RNAi in human prostate cancer cell lines results in decrease of their growth and metastatic potential with alteration of cell cycle gene expression after xenografts into mice<sup>[19]</sup>.

LAT4 (SLC43A2) was identified by searching for sequence homology to LAT3<sup>[20]</sup>. LAT4 is expressed in the basolateral membrane of the small intestine, kidney proximal tubule and thick ascending limb epithelial cells. LAT4 knockout mice are smaller than their controls and die within 9 d, presumably because of defective amino acid absorption<sup>[21]</sup>. Unlike LAT1 and LAT2, LAT3 as well as LAT4 functions independently of heavy chain.

## LAT1

LAT1 is the most extensively studied transporter among LATs. The interest in LAT1 is because of its extremely high expression in diverse human cancers. LAT1 was originally cloned from mRNA of C6 glioma cells<sup>[2]</sup>. Subsequent studies have shown that LAT1 is highly expressed in many cancer cell lines. Histological analysis with qualitatively enhanced antibodies confirmed potent expression of LAT1 in human cancers in a broad range of tissues. The number of cancer types that were reported to express a high level of LAT1 is well above twenty (Table 1). LAT1 is thus a commonly up-regulated amino acid transporter in multiple human cancers. Furthermore, LAT1 expression level appears to be associated with prognosis of cancer patients. For example, elevated expression of LAT1 correlates with an adverse prognosis in prostate<sup>[22]</sup>, gastric<sup>[23]</sup>, and pancreatic cancers<sup>[24]</sup>, suggesting that higher-grade tumors are more dependent on LAT1. Not only the expression of LAT1 but also the functional significance of LAT1 in cancers has been verified by use of its inhibitors, by knockdown with RNAi and by gene disruption. 2-Aminobicyclo (2,2,1) heptane-2-carboxylic acid (BCH) is an inhibitor of system L transporters. BCH inhibits leucine uptake and strongly suppresses the proliferation of many cancer cells (Table 1). Genetic manipulation confirmed the functional significance of LAT1 in cancer cells. Knockdown of LAT1 with RNAi<sup>[25-29]</sup> as well as genetic disruption of *LAT1* by zinc fingers nucleases-mediated gene knockout<sup>[12]</sup> in cancer cells reduces leucine uptake and cell proliferation, indicating that LAT1 is a predominant transporter that is essential for growth of cancers. The reason that so many cancers use LAT1 despite the presence of many other amino acid transporters might be that LAT1 has a prominent capability for substrate transport. Indeed,

the affinity of LAT1 for leucine is much higher than that of LAT2<sup>[30]</sup>, although LAT2 is ubiquitously expressed in the normal body<sup>[4]</sup>. Cancers may therefore be more dependent on LAT1 for rapid uptake of sufficient amino acids, whereas normal cells need less amino acid delivery that can be supported by LAT2.

The definite effect of LAT1 on the growth of various cancer cell lines prompted researchers to apply the LAT1 inhibitor in a clinical setting. However, the concentration of BCH required for suppression of cancer growth is extremely high (usually around 10 mmol/L). Moreover, the unselective effect of BCH that inhibits all LATs is another problem, since LATs other than LAT1 are considered to have functions in the normal body. It has been necessary to develop drugs that act on just LAT1 but not other transporters at a low concentration. In 2010, Endo and colleagues designed a new compound named JPH203 ((S)-2-amino-3-(4-((5-amino-2-phenylbenzo[d]oxazol-7-yl) methoxy)-3,5-dichlorophenyl) propanoic acid)<sup>[31]</sup>. JPH203 has structural analogy to tyrosine, but it inhibits only LAT1 without affecting any other LATs. JPH203 displayed potent suppressive effects on the growth of cancers *in vitro*<sup>[12,32,33]</sup>. Moreover, this compound has the ability to powerfully inhibit the proliferation of tumor cell lines of the colon and leukemia injected into nude mice<sup>[31,33]</sup>. Following improvements in its specificity and pharmacological effect, JPH203 is under evaluation in a phase I clinical trial of cancer patients.

## CLINICAL APPLICATION OF LAT1

### Positron emission tomography

By exploiting the characteristics of LAT1 expression, an approach for the diagnosis of cancers through radiolabeled substrates of LAT1 has been attempted. [<sup>18</sup>F] or [<sup>11</sup>C]-labeled compound administered into the body can be visualized by positron emission tomography (PET)<sup>[34]</sup>. Cancers incorporating an isotopically labeled probe can be located by tracing the body with PET. In the past, 2-<sup>18</sup>F-fluoro-2-deoxy-d-glucose ([<sup>18</sup>F]FDG) was one of the most commonly used probe for diagnosis of cancer with PET. This strategy exploits the characteristic of cancers consuming a huge amount of glucose compared to that consumed by normal cells. Although [<sup>18</sup>F]FDG has been of assistance in the clinical diagnosis of many cancers, it sometimes showed false positive results, especially in brain, because even normal brain cells take up a relatively large amount of glucose. To overcome this problem, amino acids have attracted attention as alternative probes to glucose. Representative amino acids or their analogs developed as probes of PET are L-3-[<sup>18</sup>F]-fluoro- $\alpha$ -methyl tyrosine ([<sup>18</sup>F]FAMT), 6-<sup>18</sup>F-fluoro-L-3,4-dihydroxy-phenylalanine (<sup>18</sup>F-DOPA), L-[<sup>11</sup>C-methyl] methionine ([<sup>11</sup>C]MET) and O-(2-[<sup>18</sup>F]fluoroethyl)-L-tyrosine ([<sup>18</sup>F]FET). If the compounds are delivered into cells specifically through LAT1, those cells are likely to be cancers. Indeed,

**Table 1** Summary of studies for expression and functions of LAT1 in cancers

Cancer	Expression (method of detection)	Inhibition of amino acid uptake by	Growth inhibition by	Ref.
Biliary tract	Immunohistochemistry	BCH	BCH	[71]
Bladder	Northern blot (cell line)	BCH		[72]
Bone	Immunohistochemistry			[73]
Brain	Immunohistochemistry, RT-PCR (cell line), Western blot (tissue, cell line)	BCH	BCH	[74,75]
Breast	Immunohistochemistry, RT-PCR (cell line)	BCH	RNAi, BCH	[29,76-78]
Colon	Western blot (cell line)	Knockout (cell line)	Knockout (cell line) JPH203	[12]
Esophagus	Immunohistochemistry			[79,80]
Hepatocyte	Immunohistochemistry			[81]
Gastrointestine	Immunohistochemistry, Western blot (cell line)		RNAi	[23,45]
Laryngeal	Immunohistochemistry			[82]
Leukemia	RT-PCR (cell line)		BCH, JPH203	[33]
Lung	Immunohistochemistry			[41,83-85]
Melanoma	Immunohistochemistry, Microarray (tissue), Western blot (cell line)	BCH		[86,87]
Myeloma	RT-PCR (cell line)	RNAi		[88]
Neuroendocrine	Immunohistochemistry, RT-PCR (tissue), Western blot (tissue)			[89]
Ovarian	Immunohistochemistry, RT-PCR (cell line), Western blot (tissue, cell line)	BCH	BCH	[47,65,90]
Oral	RT-PCR (cell line)	RNAi	RNAi	[25]
Pancreas	Immunohistochemistry Western blot (cell line)	RNAi	RNAi	[24,27,91]
Pleura	Immunohistochemistry			[92]
Prostate	Immunohistochemistry, Western blot (cell line)	RNAi, BCH	RNAi, BCH	[18,19,22,28]
Tongue	Immunohistochemistry			[93]
Thymus	Immunohistochemistry, Western blot (cell line)	JPH203	JPH203	[94,95]
Urinary tract	Immunohistochemistry			[96]

RT-PCR: Reverse transcription polymerase chain reaction; BCH: 2-aminobicyclo (2,2,1) heptane-2-carboxylic acid.

[<sup>18</sup>F]FAMT images accord well with LAT1 distribution<sup>[35]</sup>. Moreover, FAMT is incorporated by LAT1 but not by other amino acid transporters<sup>[35]</sup>. Although there is still room for improvement in its specificity, this method is powerful tool for diagnosis of cancers including microcarcinoma.

### Boron neutron capture therapy

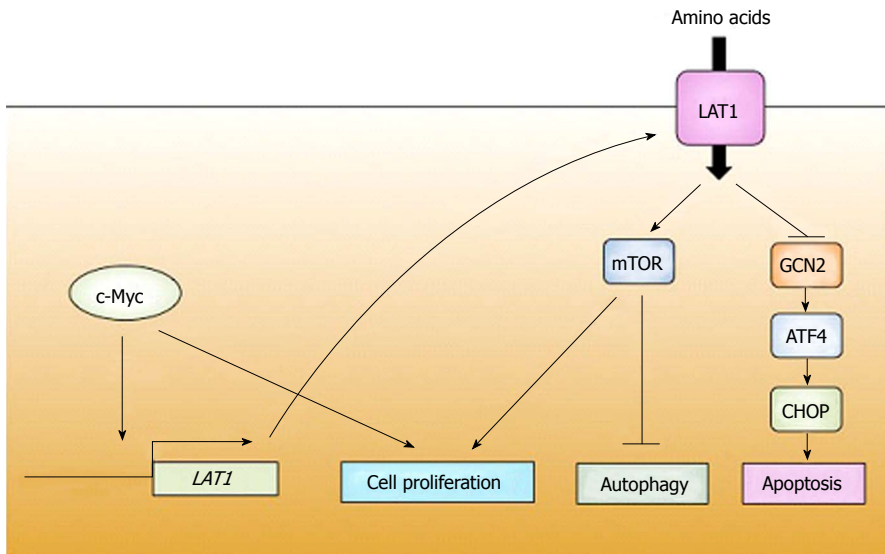
LAT1 is an attractive molecular target for boron neutron capture therapy (BNCT). BNCT is an anticancer therapy that utilizes high linear energy transfer alpha particles. Particle radiation is produced by fission reaction when irradiated thermal neutrons collide with boron incorporated by a malignant tumor. The traveling distance of particle radiation is limited (5-9 μm), and it therefore disrupts only cancer cells incorporating boron without damage to other cells around target cells<sup>[36,37]</sup>. A key component of BNCT success is accumulation of boron specifically in cancer cells. This difficult task could be achieved by the synthesis of a boron compound that is selectively delivered by LAT1. Indeed, p-boronophenylalanine (BPA), a boron compound commonly used in BNCT, is incorporated by LAT1<sup>[38-40]</sup>, suggesting that LAT1 is an optimal mediator for delivery of boron in BNCT. However, since we cannot still completely rule out the possibility of BPA uptake by other transporters, it is necessary to develop compounds that exhibit strict selectivity to LAT1. BNCT has accomplished certain clinical outcomes so far, but the problem in the

past was that it required a large-scale nuclear reactor to generate neutrons. However, a compact accelerator has been developed as an alternative to a nuclear reactor and it can be installed in a hospital, making BNCT easier to perform. Such technology will expand the applications of BNCT in the future.

### LAT1 AND METASTASIS

It has been suggested that LAT1 is involved in cancer metastasis. A number of studies have shown a correlation of increase in LAT1 expression with metastasis of multiple cancers. Lymph node metastasis-positive squamous cell carcinomas express LAT1 whereas there is no positive signal of LAT1 in metastasis-negative cells<sup>[41]</sup>. LAT1 mRNA level was significantly higher in renal cell carcinoma with metastasis<sup>[42]</sup>. A group of cells with high LAT1 expression showed a larger size of the metastatic lesion of gastric carcinoma<sup>[43]</sup>. LAT1 expression in neuroendocrine tumors was significantly associated with lymph node metastasis<sup>[44]</sup>. The potency of the functional significance of LAT1 in metastasis has been shown. Knockdown of LAT1 by RNAi inhibited the migration and invasion of gastric cancer<sup>[45]</sup> and a cholangiocarcinoma cell line<sup>[46]</sup>. BCH inhibited the proliferation and migration of a human epithelial ovarian cancer cell line<sup>[47]</sup>. On the basis of these findings, inhibition of LAT1 will be good strategy to prevent metastasis of cancer. However, it remains to





**Figure 2 Schematic model of acquisition and monitoring of amino acids in cancer.** c-Myc promotes expression of LAT1, which supplies amino acids necessary for growth of cancers. The availability of amino acids is constantly monitored by factors such as mTOR and GCN2. Once amino acid deficiency is detected, cancers suppress their proliferation and, as occasion demands, induce apoptosis. mTOR: Mechanistic target of rapamycin; GCN2: General control non-derepressible 2; ATF4: Activating transcription factor 4; CHOP: C/EBP homologous protein.

be determined whether the metastasis defect is derived from amino acid starvation or from other factors such as an aberrance of adhesion molecules. It would thus be valuable to investigate the relevance of LAT1 and integrin in metastasis, since they form a complex<sup>[48]</sup>.

## MECHANISM OF LAT1 EXPRESSION

Although it remains unknown how LAT1 expression is facilitated in cancers, some possible molecular mechanisms have been proposed. c-Myc, a proto-oncogenic transcription factor, has been demonstrated to be an upstream of LAT1. The expression of c-Myc in normal adults is generally low<sup>[49]</sup>, but overexpression of c-Myc triggered by some cues such as gene amplification, gene translocation or other gene mutations<sup>[50]</sup> is responsible for malignant transformation. Numerous human cancer tissues strongly express c-Myc. Target genes of c-Myc include many factors involved in progression of the cell cycle<sup>[51]</sup>. On the other hand, the consensus binding sequence of c-Myc is also located at the *LAT1* promoter<sup>[27]</sup>. Moreover, knockdown of c-Myc leads to reduction of LAT1 expression in cancer cell lines<sup>[27]</sup>. These results suggest that up-regulation of LAT1 is mediated, at least in part, by c-Myc (Figure 2). Of note is that c-Myc also enhances the metabolic reprogram in cancers by promoting the expression of enzymes of glycolysis and glucose transporter<sup>[52,53]</sup>. This is an ingenious strategy of cancers since they can coordinate multiple events required for cell growth by just one factor.

Some other factors appear to regulate LAT1 expression. Hypoxia-inducible factor (Hif) is a critical regulator in response to hypoxia. Hif2 $\alpha$ , an isoform of the Hif family, binds to the *LAT1* promoter and enhances LAT1 expression in renal carcinoma cell

lines<sup>[54]</sup>. Artificial manipulation to elevate Hif2 $\alpha$  activity induces LAT1 expression in lung and liver tissues, in which LAT1 expression is usually low<sup>[54]</sup>. Aryl hydrocarbon receptor (AHR) is a transcription factor that is activated by interaction with its ligands such as dioxin, and it promotes tumorigenesis<sup>[55]</sup>. AHR binds to its consensus binding sequence in *LAT1* and drives LAT1 expression in breast cancer cell lines<sup>[56]</sup>, suggesting that LAT1 contributes to tumorigenesis induced by an environmental carcinogen. As described previously, T cell activation induces LAT1 expression<sup>[5,6]</sup>. Nuclear factor kappa B, AP-1 and nuclear factor of activated T-cells are critical transcription factors that are activated by T cell stimulation and enhance immunological reactions. The expression of LAT1 is prevented by inhibitors of these transcription factors<sup>[5,6]</sup>. This means that LAT1 expression is induced by the common regulators that also boost immunological reaction in T cells.

## DOWNSTREAM OF LAT1

Ensuring a sufficient supply of nutrients is an issue of vital importance for cancers. The majority of cancers are thought to constantly monitor the availability of amino acids. Starvation of amino acids rapidly induces a stress response that puts a brake on cellular biochemical reactions to avoid wasting energy and materials. The most extensively studied system for monitoring the amino acid availability is mechanistic target of rapamycin (mTOR)<sup>[57]</sup>, a serine-threonine kinase. Plenty of amino acids maintains mTOR kinase activity, resulting in progression of the cell cycle, protein synthesis, or inhibition of autophagy induction (Figure 2). Some mTOR regulators such as SLC38A9<sup>[58-60]</sup>, Cellular arginine sensor for mTORC1 (CASTOR1)<sup>[61]</sup> and Sestrin2<sup>[62]</sup> have

been demonstrated to associate with amino acids to dictate mTOR activity. Dissociation of those interactions caused by amino acid deficiency inactivates mTOR and inverses the reaction of its downstream, resulting in a halt of cancer growth. Growing evidence suggests that LAT1 disruption leads to the inhibition of mTOR. LAT1 inhibition decreases mTOR activity in many cancer cell lines<sup>[28,33,63-65]</sup>. These findings suggest that the arrest of cell growth of cancers by a defect of LAT1 is derived from inactivation of mTOR (Figure 2). mTOR inhibitors are being used in practical trials for therapeutic management of several cancers<sup>[66]</sup>. Application of JPH203 together with an mTOR inhibitor probably creates a synergistic effect and might be useful for maximizing the benefit of treatment with a low-dose drug, which would help to minimize adverse effects.

General control non-derepressible 2 (GCN2) is another factor for detection of amino acid starvation<sup>[67]</sup>. GCN2 is a serine-threonine kinase that is activated by amino acid deficiency. Uncharged tRNAs caused by a decline of amino acid concentration activates GCN2, which eventually induces activity of activating transcription factor 4 (ATF4). ATF4 regulates the expression of genes responsible for coping with amino acid deficiency<sup>[68]</sup>. Several studies have shown that dysfunction of LAT1 initiates the GCN2 signal. JPH203 promotes the expression of C/EBP homologous protein [CHOP, also known as DNA damage inducible transcript 3 (DDIT3)], which is up-regulated by ATF4<sup>[68]</sup> and probably takes part in apoptosis in leukemia<sup>[33]</sup>. Gene disruption of LAT1 in cancer cell lines activates the GCN2-ATF4 cascade<sup>[12]</sup>. Activation of ATF4 by LAT1 defect was also shown in cells other than cancer. JPH203 triggers the expression of CHOP<sup>[5,69]</sup> and homeobox B9<sup>[70]</sup>, a novel target of ATF4, in human T cells to repress cytokine production. These findings suggest that GCN2-ATF4 is another critical system for detecting amino acid deficiency evoked by LAT1 inhibition (Figure 2).

## CONCLUSION

After the importance of LAT1 in cancer cells had been established, basic studies on LAT1 have progressed with remarkable speed. Better still, research achievements are potentially capable of technical developments for the use of LAT1 as a molecular target in clinical practice. However, although JPH203 is more effective and specific than BCH, it still requires a high concentration for sufficient suppression of the growth of cancers, and wariness of adverse effect persists. Nevertheless, such concerns might be overcome, at least for the time being, by virtue of the proper combinational use of multiple drugs with different action points in cellular metabolism (e.g., mTOR inhibitor). However, further improvements in selectivity of the inhibitor, boron donor of BNCT and PET probe to LAT1 will raise the quality of cancer treatment. Besides, although not to the extent to LAT1, there are several cancers that rely on LAT3

for their growth and development of a LAT3-specific inhibitors is also encouraged. Advances in technologies are expected to resolve such issues.

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## Pancreatic resection in very elderly patients: A critical analysis of existing evidence

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### Abstract

The aging of the population results in a rise of number of elderly patients (aged 80 years and older)

with pancreatic or periampullary cancer, and more pancreatectomies could eventually be performed in such complex patients. However, early and long-term results after pancreatic resection in octogenarians are still controversial, and may trouble the surgeon when approaching this type of population. Evaluation of reported experiences shows that for almost all Authors, pancreatectomy can be performed safely in elderly population, although overall morbidity and mortality rates were 34.9% and 13.2% respectively, with a mean length of hospital stay of 18 d. These features appear higher in older patients compared to the younger counterpart. Less than 50% of patients underwent adjuvant therapy after operation. Long-term survival is reported not significantly different in aged 80 years and older patients, with a median overall survival time of 17.6 mo. The quality of life after pancreatic resection is only sporadically evaluated but, when considered, it highlights the need of health facility service after operation for these "frail" patients. Prospective studies on the quality of life of pancreatectomized octogenarians are welcome. Proper selection of patients, geriatric assessment with multidisciplinary approach, centralization of pancreatic surgery in high-volume centres and rehabilitation programs after surgery appear to be crucial points in order to improve surgical treatments of pancreatic tumors in very elderly patients.

**Key words:** Elderly; Octogenarian; Pancreatectomy; Pancreatic neoplasms; Survival

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**Core tip:** Although not statistically significant, pancreatic resection in very older patients carried a greater risk of complications, mortality and nursing facility after discharge than in younger patients. Thus, pancreatectomy in 80 years and older patients, should be performed after careful consideration of potential benefit, surgical risk, and patient's preferences.

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## INTRODUCTION

The number of elderly in Western countries is rapidly increasing and it constitutes the fastest-growing age group of the population<sup>[1]</sup>. In the United States, the proportion of people 65 years of age or older will reach 18.2% by 2025<sup>[2]</sup>, and the oldest elderly (individuals 85 years old or older) will account for 5% of the overall population<sup>[3]</sup>. The number of octogenarian patients referred to surgeons is going to gradually increase as well. This is particularly true for gastrointestinal cancers, which are characterized by the greatest incidence in the elderly, pancreatic cancer included. In past years, the high mortality and morbidity rates associated with pancreatic resections made this kind of surgery a rare indication for elderly people, considering also the limited survival time associated with pancreatic cancer. Recent data have clearly shown that pancreatic surgery is safe and feasible in high-volume centres, with reported mortality rates less than 2% and acceptable morbidity rates<sup>[4,5]</sup>. As postoperative outcome after pancreatic resections improved, many authors began to report pancreatectomies also in elderly patients. However, there are limited data on outcomes in octogenarians patients after pancreatic surgery. So, some crucial points may arise when treating very elderly patients with pancreatic tumors: (1) Is pancreatic resection safe and feasible in octogenarians? (2) Is surgical risk justified by long-term outcome after resection of malignancy? (3) Is quality of life preserved after major pancreatic resection?

The aim of this study was to analyse the existing literature and the available data on early postoperative outcomes and long-term results after pancreatic resection in patients 80 years and older.

## EVIDENCE ACQUISITION

The published Literature was systematically searched using PubMed and free text search engines up to December 2015. The following search terms were used: Pancreaticoduodenectomy, pancreatectomy, duodenal neoplasm/surgery, pancreatic neoplasm/surgery, pancreatic neoplasm/surgery, 80 years of age and over, elderly and octogenarian. The "related articles" function was used to broaden the search and all abstracts, studies, and citations retrieved were reviewed. The preliminary literature search showed 113 studies matching the initial criteria. After screening, 16 studies evaluating octogenarians patients and their outcome

**Table 1 Type of periampullary neoplasms**

Ref.	n	Age (mean)	Benign disease	Malignant disease	Pancreatic adenocarcinoma
Chen <i>et al</i> <sup>[6]</sup>	16	82.3	1	15	5
Makary <i>et al</i> <sup>[7]</sup>	207	82	30	177	96
Finlayson <i>et al</i> <sup>[8]</sup>	2915	NR	0	2915	NR
Riall <i>et al</i> <sup>[9]</sup>	214	NR	50	164	NR
Hardacre <i>et al</i> <sup>[3]</sup>	32	82	2	30	25
Tani <i>et al</i> <sup>[2]</sup>	25	82.3	3	22	10
Lee <i>et al</i> <sup>[10]</sup>	74	82.6	16	58	45
Khan <i>et al</i> <sup>[11]</sup>	53	NR	0	53	53
Stauffer <i>et al</i> <sup>[12]</sup>	32	82.1	11	21	18
Hatzaras <i>et al</i> <sup>[13]</sup>	27	83.4	0	27	24
Melis <i>et al</i> <sup>[14]</sup>	25	83	0	25	25
Oguro <i>et al</i> <sup>[15]</sup>	22	81.5	0	22	8
Turrini <i>et al</i> <sup>[16]</sup>	64	83	0	64	64
Belyaev <i>et al</i> <sup>[17]</sup>	38	82	NR	NR	NR
Beltrame <i>et al</i> <sup>[18]</sup>	23	82.6	1	22	20
Kinoshita <i>et al</i> <sup>[19]</sup>	26	82	0	26	26
Total	3793	82.2	114	3641	419

NR: Not reported.

after pancreatic resections were selected<sup>[2,3,6-19]</sup>. Information about 3793 aged 80-years or older patients who underwent pancreatic resections, were collected (Table 1). There were 13 single institution's series, 2 nation or regional inpatient samples, and 1 multicentric report. In the population selected, there were 1710 male patients (45.1%) and the mean age was 82.2 years. Information about preoperative comorbidities were available for 489 patients. The most frequent reported comorbidities were cardiovascular disease (53.8%,  $n = 263$  patients), in particular hypertension was reported for 168 patients, and coronary disease for 95 patients. Other frequent major comorbidities were diabetes mellitus ( $n = 94$ , 19.2%), pulmonary disease ( $n = 30$ , 6.1%) and chronic renal failure ( $n = 10$ , 2.0%). Elderly patients are often reported to have two or more concomitant major comorbidities. Finlayson *et al*<sup>[8]</sup> and Khan *et al*<sup>[11]</sup> reported a percentage of respectively 67.6% and 51% patients with 2 or more concomitant diseases. Six studies<sup>[3,11,14-16,18]</sup> reported data on the American Society of Anesthesiologists (ASA) score, with ASA grades 3 or 4 more frequently observed (60.3% of patients) (Table 2).

## SURGERY

Thirteen studies<sup>[2,3,6,7,10-16,18,19]</sup> reported the type of pancreatic neoplasm treated ( $n = 626$  patients). In particular, four reports evaluated only outcome after resections for pancreatic adenocarcinoma, whereas other two authors considered also patients with other primary malignancies. Finally, the remaining 7 studies addressed also resections for benign pancreatic conditions. Malignant indications for surgery accounted for 89.8% of cases ( $n = 562$ ), with pancreatic adenocarcinoma being the most frequent primary



**Table 2** Type of surgical resections and American Society of Anesthesiologists score

Ref.	Type of surgical procedure			Vascular resections % (n)	ASA SCORE	
	PD	DP	TP		1-2	3-4
Chen <i>et al</i> <sup>[6]</sup>	16	0	0	NR	NR	NR
Makary <i>et al</i> <sup>[7]</sup>	197	0	10	2.4 (5)	NR	NR
Finlayson <i>et al</i> <sup>[8]</sup>	NR	NR	NR	NR	NR	NR
Riall <i>et al</i> <sup>[9]</sup>	155	48	NR	NR	NR	NR
Hardacre <i>et al</i> <sup>[3]</sup>	26	5	1	12.5 (4)	8	24
Tani <i>et al</i> <sup>[2]</sup>	25	0	0	4 (1)	NR	NR
Lee <i>et al</i> <sup>[10]</sup>	74	0	0	14.9 (11)	NR	NR
Khan <i>et al</i> <sup>[11]</sup>	18	10	4	6.25 (2)	7	46
Stauffer <i>et al</i> <sup>[12]</sup>	20	5	0	NR	NR	NR
Hatzaras <i>et al</i> <sup>[13]</sup>	53	0	0	NR	NR	NR
Melis <i>et al</i> <sup>[14]</sup>	25	0	0	4 (1)	7	17
Oguro <i>et al</i> <sup>[15]</sup>	22	0	0	23 (5)	21	1
Turrini <i>et al</i> <sup>[16]</sup>	56	8	0	11 (7)	37	23
Belyaev <i>et al</i> <sup>[17]</sup>	27	3	8	NR	NR	NR
Beltrame <i>et al</i> <sup>[18]</sup>	21	2	0	8.7 (2)	5	18
Kinoshita <i>et al</i> <sup>[19]</sup>	16	9	1	39 (10)	NR	NR
Total	751	90	24		85	129

PD: Pancreatoduodenectomy; DP: Distal pancreatectomy; TP: Total pancreatectomy; NR: Not reported; ASA: American Society of Anesthesiologists.

tumor (74.6% of malignant cases,  $n = 419$ ), followed by periampullary carcinoma (11.6%,  $n = 65$ ) and cholangiocarcinoma (6.6%,  $n = 37$ ). Other malignant tumor types reported were neuroendocrine tumors, intraductal papillary mucinous neoplasms (IPMNs) with invasive carcinoma, and pancreatic secondary tumors. Among benign neoplasms ( $n = 64$ , 10.2%), the most frequent indications were benign or borderline IPMNs ( $n = 25$ , 39.1%), cystic neoplasms (20.3%,  $n = 13$ ) and neuroendocrine tumors (9.4%,  $n = 6$ ). A total of 3793 resections were performed, with data on 751 pancreatoduodenectomy, 90 distal pancreatectomy and 24 total pancreatectomy (TP). A vascular resection was reported in 48 cases (Table 2).

## EARLY OUTCOME

Overall morbidity and mortality rates were 34.9% and 13.2% respectively, with a mean length of hospital stay of 18 d (Table 3). Detailed information on specific type of postsurgical complications were available for 569 patients. Most frequent complications were pancreatic fistula (12.1%,  $n = 69$ ), delayed gastric emptying (10.9%,  $n = 62$ ) and cardiopulmonary complications (9.3%,  $n = 53$ ). Reoperations rate was 7.5% (Table 4). Four studies<sup>[2,8,9,11]</sup> focused on hospital discharge and the need for skilled nurse facilities after surgical resection. Finlayson *et al*<sup>[8]</sup>, Riall *et al*<sup>[9]</sup> and Khan *et al*<sup>[11]</sup> observed a percentage of respectively 63.3%, 61.8% and 79% of resected patients who were discharged directly home, with or without home health care support. The other patients were discharged to health care facilities (nursing home, skilled care or other intermediate care facilities)

**Table 3** Perioperative outcomes after pancreatic resection

Ref.	Mortality % (n)	Morbidity % (n)	Mean length of stay (d)
Chen <i>et al</i> <sup>[6]</sup>	13.0 (2)	51 (8)	25.0
Makary <i>et al</i> <sup>[7]</sup>	4.0 (8)	53 (109)	11.0
Finlayson <i>et al</i> <sup>[8]</sup>	15.5 (452)	NR	20.4
Riall <i>et al</i> <sup>[9]</sup>	11.4 (24)	NR	15.0
Hardacre <i>et al</i> <sup>[3]</sup>	0	66 (21)	11.0
Tani <i>et al</i> <sup>[2]</sup>	NR	44 (11)	25.0
Lee <i>et al</i> <sup>[10]</sup>	5.4 (4)	47 (35)	10.5
Khan <i>et al</i> <sup>[11]</sup>	2.0 (1)	51 (27)	13.5
Stauffer <i>et al</i> <sup>[12]</sup>	0	50 (16)	13.3
Hatzaras <i>et al</i> <sup>[13]</sup>	3.7 (1)	52 (14)	12.0
Melis <i>et al</i> <sup>[14]</sup>	4.0 (1)	68 (17)	20.0
Oguro <i>et al</i> <sup>[15]</sup>	4.5 (1)	27 (6)	31.5
Turrini <i>et al</i> <sup>[16]</sup>	4.7 (3)	56 (36)	24.9
Belyaev <i>et al</i> <sup>[17]</sup>	11.4 (4)	NR	15.0
Beltrame <i>et al</i> <sup>[18]</sup>	0 (0)	43 (10)	13.5
Kinoshita <i>et al</i> <sup>[19]</sup>	0 (0)	8 (2) <sup>1</sup>	25.8
Total	13.2 (501)	34.9 (306) <sup>2</sup>	18.0

NR: Not reported. <sup>1</sup>Clavien-Dindo classification  $\geq$  III; <sup>2</sup>Riall *et al*<sup>[9]</sup> excluded, because only severe complications were reported.

or required other inpatient acute care hospitals.

## ADJUVANT THERAPY

Only few studies reported data on adjuvant therapy after pancreatic resections for cancer (Table 5). Kinoshita *et al*<sup>[19]</sup> reported 13 out of 26 resected patients (50%) who received adjuvant treatment. Six out of 13 patients completed the planned adjuvant chemotherapy, which was discontinued in the other patients because of poor general conditions, chemotherapy-related adverse-events or postoperative recurrence. Beltrame *et al*<sup>[18]</sup> reported 30% of resected patients who received adjuvant treatment, while in the study by Turrini *et al*<sup>[16]</sup> the patients' rate receiving postsurgical treatment was as low as 23.4%. Finally, Hardacre *et al*<sup>[3]</sup> reported 10/25 patients resected for adenocarcinoma who were administered adjuvant chemotherapy. Specific survival outcome for patients receiving adjuvant treatment were not reported.

## SURVIVAL

Median overall survival was 17.6 mo. One-year and 5-year survival rates were not always reported and varies among different series; at that point it is important to keep in mind that different reports consider different surgical indications. One-year survival rates range from 50% to 75.7%, while 5-year survival rates range from 0% to 46% (Table 5). When considering only those studies focusing on pancreatic adenocarcinoma, median overall survival is 15.4 mo. Melis *et al*<sup>[14]</sup> reported a 1-year and 5-year survival rate of 68.2% and 4.5% respectively. Turrini *et al*<sup>[16]</sup> reported a 1-year survival rate of 75.7% while 5-year survival rate was 0%.

**Table 4 Postoperative complications and reoperation rates**

Ref.	Pancreatic Fistula	Delayed gastric emptying	Postpan- createctomy haemorrhage	Reoperation rate % (n)
Chen <i>et al</i> <sup>[6]</sup>	2	3	3	NR
Makary <i>et al</i> <sup>[7]</sup>	21	32	0	5.6 (11)
Finlayson <i>et al</i> <sup>[8]</sup>	NR	NR	NR	NR
Riall <i>et al</i> <sup>[9]</sup>	NR	NR	NR	NR
Hardacre <i>et al</i> <sup>[3]</sup>	3	4	5	22.0 (7)
Tani <i>et al</i> <sup>[2]</sup>	1	6	0	NR
Lee <i>et al</i> <sup>[10]</sup>	3	NR	NR	5.4 (4)
Khan <i>et al</i> <sup>[11]</sup>	6	9	5	1.9 (1)
Stauffer <i>et al</i> <sup>[12]</sup>	NR	NR	NR	6.2 (2)
Hatzaras <i>et al</i> <sup>[13]</sup>	3	0	NR	4.0 (1)
Melis <i>et al</i> <sup>[14]</sup>	NR	NR	NR	NR
Oguro <i>et al</i> <sup>[15]</sup>	11	5	4	4.5 (1)
Turrini <i>et al</i> <sup>[16]</sup>	10	NR	10	10.9 (7)
Belyaev <i>et al</i> <sup>[17]</sup>	NR	NR	NR	13.1 (5)
Beltrame <i>et al</i> <sup>[18]</sup>	4	0	1	13.0 (3)
Kinoshita <i>et al</i> <sup>[19]</sup>	5	3	1	NR
Total	69	62	29	7.5 (43)

NR: Not reported.

## PROGNOSTIC FACTORS

Six authors<sup>[9,10,13,15,16,19]</sup> examined clinical variables and risk factors that could be associated with poorer survival in octogenarian patients. Hatzaras *et al*<sup>[13]</sup> reported lymphovascular invasion as the only predictor of survival. Oguro *et al*<sup>[15]</sup> found that pancreatic cancer was an independent poor prognostic factor in the multivariate analysis with a hazard ratio of 4.398. Turrini *et al*<sup>[16]</sup> identified 4 independent prognostic indicators of overall survival: Venous invasion, arterial invasion, positive lymph nodes and adjuvant treatment. In their multivariate analysis, Kinoshita *et al*<sup>[19]</sup> indicated that distant metastasis and the conclusion of the planned adjuvant therapy were independent prognostic factors of surgical resection. Lee *et al*<sup>[10]</sup> reported female gender, non-caucasian race and positive lymph nodes as factors associated with shorter survival time in the multivariate model. In none of the aforementioned studies, age 80 or more resulted to be a significant predictor of long-term survival. On the contrary, Riall *et al*<sup>[9]</sup> in a population-based study used logistic regression models to assess the independent effect of age group on mortality. When compared with patients < 69 years of age, age group was an independent predictor of mortality after pancreatic resection.

## QUALITY OF LIFE AFTER RESECTION

Although quality of life (QoL) is an important aspect of surgical result, this point is not evaluated in most of the studies. Gerstenhaber *et al*<sup>[20]</sup> firstly assessed QoL after pancreaticoduodenectomy in 70 elderly patients (aged 70 years and older). Fatigue was the most common symptom for the first 6 mo after surgery, but QoL quickly improved to normal scores. However, it has been reported that patients 80 years or older required

**Table 5 Long-term results**

Ref.	Adjuvant therapy % (NR)	Median overall survival (mo)	1-yr survival rate (%)	5-yr survival rate (%)
Chen <i>et al</i> <sup>[6]</sup>	NR	17.6	NR	NR
Makary <i>et al</i> <sup>[7]</sup>	NR	19	59.1	24.4
Finlayson <i>et al</i> <sup>[8]</sup>	NR	NR	NR	11.3
Riall <i>et al</i> <sup>[9]</sup>	NR	NR	NR	NR
Hardacre <i>et al</i> <sup>[3]</sup>	31.2 (10)	14.4	57.0	24.0
Tani <i>et al</i> <sup>[2]</sup>	NR	NR	NR	NR
Lee <i>et al</i> <sup>[10]</sup>	NR	11.6	NR	NR
Khan <i>et al</i> <sup>[11]</sup>	22	13.5	NR	NR
Stauffer <i>et al</i> <sup>[12]</sup>	NR	NR	67.0	42.0
Hatzaras <i>et al</i> <sup>[13]</sup>	NR	33.3	NR	33.1
Melis <i>et al</i> <sup>[14]</sup>	NR	17.3	68.2	4.5
Oguro <i>et al</i> <sup>[15]</sup>	0 (0)	13.0	NR	46.0
Turrini <i>et al</i> <sup>[16]</sup>	23.4 (15)	30.0	75.7	0
Belyaev <i>et al</i> <sup>[17]</sup>	NR	NR	NR	NR
Beltrame <i>et al</i> <sup>[18]</sup>	30.0 (7)	19.0	NR	NR
Kinoshita <i>et al</i> <sup>[19]</sup>	50.0 (13)	12.4	50.0	NR

NR: Not reported.

discharge to a nursing facility more frequently when compared to younger patients<sup>[21]</sup>. This is obviously due to the need of rehabilitation program both in the physical activity and digestive function.

## DISCUSSION

The higher incidence of morbidity, risk of mortality and of a prolonged recovery in an extended care facility following hospital discharge, made in past years pancreatic surgery a rare indication for older patients. The improved outcomes after pancreatic resections performed in high-volume centres have allowed to broaden the selection criteria for surgery and to include more elderly patients. The first study considering octogenarian patients and pancreatic surgery was published by Sohn *et al*<sup>[22]</sup>. Authors compared postoperative outcome of octogenarian patients undergoing pancreaticoduodenectomy with patients younger than 80 years, and reported similar morbidity and mortality rates in the two different age groups. This observation was then confirmed in other subsequent studies, showing similar results in postoperative outcome in elderly patients<sup>[2,7,11]</sup>. On the contrary, two large population-based studies<sup>[8,9]</sup> showed high mortality rates after pancreatic surgery in octogenarians with a high rate of discharge to health facilities. Sukhramwala *et al*<sup>[23]</sup> performed a systematic review and meta-analysis comparing the results of four studies<sup>[7,11,13,22]</sup> and showed that patients 80-years or older had a higher incidence of postoperative mortality, morbidity and pneumonia in comparison to younger patients. A recent meta-analysis by Casadei *et al*<sup>[24]</sup>, showed a higher postoperative mortality rate in patients 80 years or older when compared to younger patients. These conflicting results may have different possible explanations. First of all, it

has to be considered that the presence of an increased prevalence of preoperative comorbidities, represents a potential selection bias in those studies comparing outcome of elderly to younger patients. Therefore, preoperative studies play a major role in recognizing high-risk patients and in selecting the most appropriate treatment. The identification of modifiable preoperative risk factors for morbidity and mortality would improve the surgical outcome of patients<sup>[25]</sup>. Several scoring systems are available in the clinical practice to assess the surgical risk of old patients: Adult Comorbidity Evaluation-27 (ACE-27)<sup>[26]</sup>, Charlson Comorbidity index<sup>[27]</sup> and G8 geriatric screening tool<sup>[28]</sup>. These tools allow a risk stratification in order to evaluate the impact of age in the surgical management of elderly patients. Old patients require a multidisciplinary evaluation (geriatric assessment) in order to identify those individuals who are at high-risk of complication<sup>[29]</sup>. Another reason of difference in postoperative outcome may be the hospital load for pancreatic resections. In fact, the importance of hospital volume for improving outcome after pancreatic surgery has already been demonstrated<sup>[30]</sup> and better prognosis after centralization of pancreatic cancer resection is reported<sup>[31]</sup>. Riall *et al*<sup>[9]</sup> reported a mortality rate following surgery in octogenarians nearly doubled at low-volume facilities compared to high-volume centres. Management of elderly patients requires a multidisciplinary evaluation prior to surgery in order to have a precise risk stratification and a selection of patients undergoing surgery. Moreover, postoperative care requires a specialized staff (surgeons, anaesthesiologists, interventional radiologists, endoscopists, etc.) and specialized facilities commonly available in high-volume centres. Finally, only few reports<sup>[3,16,18,19]</sup> in the literature reported the patients' rate undergoing adjuvant treatment after surgery and their specific outcome. It is increasingly recognized that elderly patients are underrepresented in cancer trials and that elderly patients are less likely to receive adjuvant chemotherapy<sup>[32,33]</sup>. Reluctance to administer postoperative treatment is often based on the presence of comorbidities in elderly patients and by the perception that there is an increased risk of non-cancer-related cause of death, limiting the overall benefit of adjuvant treatment. Nagrial *et al*<sup>[33]</sup> showed that this is not the case, being cancer the predominant cause of death in older patients. Given the role of adjuvant therapies in prolonging overall survival and delaying time to recurrence in resectable pancreatic cancer<sup>[34,35]</sup>, the advancing age alone should not preclude the use of adjuvant treatment. Although these limitations, most Authors reported that overall survival after resection for pancreatic cancer in octogenarians is not statistically different from younger patients<sup>[18,21,36,37]</sup>.

## CONCLUSION

Although several authors say that major pancreatic

surgery is safe and feasible in very old patients, the risk of postoperative complications and troubled outcome objectively exist, and may explain the reluctance to perform such complex operation in older patients<sup>[38]</sup>. To overcome any prejudice, a careful patient selection is fundamental to avoid or reduce postoperative mortality and morbidity. It seems reasonable to consider elderly patients with 2 or 3 ASA score, with a low rate of comorbidity and good performance status, as good candidate for surgical resection. Even if caution is recommended when treating elderly patients, the morbidity and mortality rates of octogenarians appear within the acceptable range for pancreatic surgery when performed at experienced centres. Geriatric assessment and centralization of pancreatic cancer are key to treatment decisions for patients 80 years and older. Survival after pancreatic resection appears similar in old and young patients, but we are lacking sufficient information about the quality of life of elderly pancreatectomized patients. Additionally, prospective studies are needed in order to determine the quality of life and long-term outcome in this subset of patients, because these features have to be considered in planning of the surgical treatment of octogenarians.

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## Epithelial-mesenchymal transition as a therapeutic target for overcoming chemoresistance in pancreatic cancer

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### Abstract

Pancreatic cancer has one of the worst prognoses among all cancers due to the late manifestation of identifiable symptoms and high resistance to chemo- and radiation therapies. In recent years, a cancer development phase termed epithelial-mesenchymal transition (EMT) has gained increasing research focus. The process is implicated in tumour metastasis, and emerging evidence suggests EMT also contributes or induces chemoresistance in several cancers. Nevertheless, the applicability of therapeutic targeting of EMT faces many challenges. In this mini-review, we summarise the evidence supporting the role of EMT in pancreatic cancer progression, focusing particularly on its association with chemoresistance.

**Key words:** Epithelial-mesenchymal transition; Drug resistance; Pancreatic cancer; Chemotherapy

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**Core tip:** This mini-review examines the role of epithelial-mesenchymal transition in the development of drug resistant pancreatic cancer and a possible use of this process as a drug target.

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## INTRODUCTION

Epithelial-mesenchymal transition (EMT) is a stage of phenotypic alteration in cancer cells instigated by several paracrine and autocrine stimuli, leading to a morphological transformation of epithelial-like cancer cells to an elongated mesenchymal phenotype. The phenotypic change is thought to derive from a shift in the balance between epithelial (*e.g.*, E-cadherin and Claudin-1) and mesenchymal (*e.g.*, N-cadherin, Snail, Zeb-1 and Twist-1) factors. Once described as a key step for successful metastasis in some types of cancers<sup>[1]</sup>, the role of EMT in chemotherapy resistance has attracted much interest recently. Indeed, EMT has been shown to contribute to drug resistance in pancreatic cancer. For instance, in a recent study, the patterns of sensitivity and resistance to three conventional chemotherapeutic agents with divergent mechanisms of action were investigated in several pancreatic cancer cell lines<sup>[2]</sup>. Interestingly, gene expression profiling revealed that the sensitive and resistant cells formed two distinct groups with resistant cells showing several features consistent with EMT. Additionally, an inverse correlation between E-cadherin and its transcriptional suppressor, Zeb-1, was observed in the gene expression. Moreover, silencing of Zeb-1 restored drug sensitivity in pancreatic cancer cells. The implication of this study is that effectors of EMT, in particular Zeb-1, are potential molecules to target to overcome drug resistance. Recently, a study casts doubt on the role of EMT in metastasis, while a separate study strongly corroborates the key role of EMT in drug resistance. Indeed, both Fischer *et al.*<sup>[3]</sup> and Zheng *et al.*<sup>[4]</sup> demonstrated that not only was EMT able to aid chemotherapy resistance in pancreatic and lung cancer cells but that EMT was not needed for metastasis, as inhibition of EMT did not prevent metastasis of pancreatic and lung cancer cells.

## EMT SIGNALLING PATHWAYS IN PANCREATIC CANCER

Several signalling pathways can regulate EMT during tumorigenesis<sup>[5-7]</sup>. Major pathways which can induce EMT include those activated by growth factors such as transforming growth factor- $\beta$  (TGF $\beta$ ), epidermal growth factor and hepatocyte growth factor or the Wnt/beta-catenin and Notch pathway. Notch-2 activation was identified in gemcitabine-resistant (GR) pancreatic cancer cells that have acquired EMT. By using pancreatic cancer cells that have developed gemcitabine resistance through exposure to increasing concentrations of gemcitabine, Wang *et al.*<sup>[8]</sup> were the first to delineate the EMT profile of GR cells. GR but not gemcitabine sensitive (GS) cells had an elongated and irregular shape, increased expression of Zeb-1, vimentin, fibronectin and alpha-SMA, as well as translocation of E-cadherin from the plasma membrane to the nucleus. In concordance with previous reports

regarding the role of Notch signalling in EMT, Wang *et al.*<sup>[8]</sup> further characterised the Notch activation status in GR cells. Notch-2 and its ligands, Jagged-1 and Notch-4, were upregulated in GR cells. The Notch signalling also upregulates NF- $\kappa$ B, a critical mediator of the EMT process. Indeed, NF- $\kappa$ B was upregulated in GR cells as well as its downstream target, matrix metalloproteinase 9 (MMP-9). The activity of MMP-9 is governed by urinary plasminogen activator (uPA), also upregulated in GR cells, and both proteins are known for their role in cancer invasion and metastasis. Using Notch-2 and Jagged-1 siRNA, the interrelation between Notch, EMT and NF- $\kappa$ B were further delineated. Notch-2 and Jagged-1 knock-down in GR cells led to mesenchymal-epithelial transition (MET) morphological changes which include upregulation of E-cadherin, and reduction of vimentin, slug, snail, Zeb-1, and NF- $\kappa$ B (p65 subunit). As expected, Notch-2 and Jagged-1 downregulation by siRNA also led to a reduction in GR cells invasion and migration<sup>[8]</sup>. The study characterised for the first time the Notch-driven EMT pathway in GR pancreatic cancer cells, however, the authors did not address whether silencing or inhibition of Notch could reverse GR.

## EMT AND GEMCITABINE RESISTANCE

The development of gemcitabine resistance and its association with EMT phenotype has drawn attention to a possible role of gemcitabine in inducing EMT. Güngör *et al.*<sup>[9]</sup> examined the role of Midkine (MK) in orchestrating the interplay between Notch, EMT and gemcitabine resistance. MK is a heparin-binding growth factor overexpressed in some types of cancers<sup>[10,11]</sup>. High MK mRNA expression was detected in pancreatic cancer cell lines that developed gemcitabine resistance following repeated exposure to gemcitabine, and in PDAC tumour samples isolated from patients who underwent total pancreaticoduodenectomy. Gemcitabine treatment in GR cells led to a dose-dependent increase in MK mRNA expression and protein secretion, an effect not observed in GS cells. Knockdown of MK with siRNA restored gemcitabine sensitivity in GR cells, while the addition of recombinant human MK (rh-MK) to MK-knockdown or GS cells, restored or induced resistance, respectively. GR cells overexpressing MK displayed EMT characteristics while MK-depleted GR cells displayed MET characteristics as evidenced by downregulation of vimentin and NF- $\kappa$ B, and upregulation of E-cadherin. As expected, there was a reduction in migration and invasion in MK-depleted cells compared to control. As Notch-2 and EMT are associated with gemcitabine resistance, Güngör *et al.*<sup>[9]</sup> further examined the impact of MK on Notch-2 activation. Treatment of GR cells with rh-MK resulted in enhanced cleavage of Notch<sup>ICD</sup> and expression of Hes-1 (Notch-2 target). Expectedly, silencing of Notch-2 improved gemcitabine efficacy in GR cells. Güngör *et al.*<sup>[9]</sup> were the first to pinpoint the role of MK in gemcitabine resistance and its impact on

Notch-2 activation and EMT phenotype.

The role of Notch-2 activation in EMT, metastasis and chemotherapy resistance has attracted attention to target Notch-2 in pancreatic cancer. Palagani *et al.*<sup>[12]</sup> showed the effect of the  $\gamma$ -secretase inhibitor (GSI IX) in preventing Notch-2 activation, EMT, and cancer cell proliferation and migration *in vitro* and pancreatic tumour-initiating CD44<sup>+</sup>/EpCAM<sup>+</sup> xenograft growth and metastasis *in vivo*. Future studies are needed to examine the effect of GSI IX in reducing gemcitabine resistance in pancreatic cancer.

A few microRNAs are implicated in EMT and chemoresistance. Li *et al.*<sup>[13]</sup> were the first to introduce a novel way of reducing gemcitabine resistance in pancreatic cancer (PaCa) through modulation of microRNAs. GR PaCa cells showed downregulation of miR-200 in addition to the typical EMT signature discussed earlier. Upregulation of miR-200 either through the reintroduction of miR-200 or the treatment with the natural compound, isoflavone, resulted in MET as demonstrated by decreased levels of mesenchymal markers (Zeb1, Vimentin and Slug) and induction of epithelial-associated morphological changes, and thus reducing gemcitabine resistance<sup>[13]</sup>. Using a similar methodology, Ma *et al.*<sup>[14]</sup> recently showed a positive influence of miR-233 in EMT, invasion, migration and gemcitabine resistance in PaCa cells.

The conversion in cancer cells from epithelial to mesenchymal phenotype and its requirement for cancer invasion and migration were clearly demonstrated through *in vitro* studies. However, the consequence of EMT on cancer metastasis had not been investigated *in vivo* until recently. Using the well-established mouse model that mirrors human pancreatic cancer (KPC mice), Zheng *et al.*<sup>[4]</sup>, produced KPC mice absent in Snail or Twist protein. Although accumulating evidence suggests the requirement of EMT process for cancer migration, the authors showed the ability of pancreatic cancer to metastasize despite deleted EMT-inducing factors, Snail and Twist. Deletion of either one of these proteins did not affect local invasion, metastasis or overall survival compared to control KPC mice. It also resulted in a reduction in expression of other mesenchymal markers (e.g., Slug, Zeb1 and alpha-SMA) while enhancing the expression of the epithelial factor, E-cadherin. Although the apoptosis of cancer cells was not affected by deletion of Snail or Twist, the proliferation rate of cancer cells significantly increased, while blood dissemination remained unchanged compared to control KPC mice. Examination of EMT profile of the metastatic pancreatic cancer cells at secondary sites (liver, lung and spleen) showed positive for E-cadherin, and negative for Snail or Twist. Moreover, the ability of cancer cells, isolated from either control KPC mice or KPC with deleted Snail or Twist, to form tumour spheres were comparable. In the study, EMT program did not appear essential for primary cancer growth, local invasion, blood dissemination and metastasis. Interestingly, gemcitabine sensitivity was improved in KPC mice with deleted Snail or Twist

compared to KPC control mice which could be explained by a significant upregulation of equilibrative nucleoside transporter 1 and concentrating nucleoside transporter 3 (receptors that mediate uptake of nucleosides) in cancer cells lacking Snail or Twist.

## GENERAL CONSIDERATIONS ON PHARMACOLOGICAL APPROACHES TO TARGET EMT

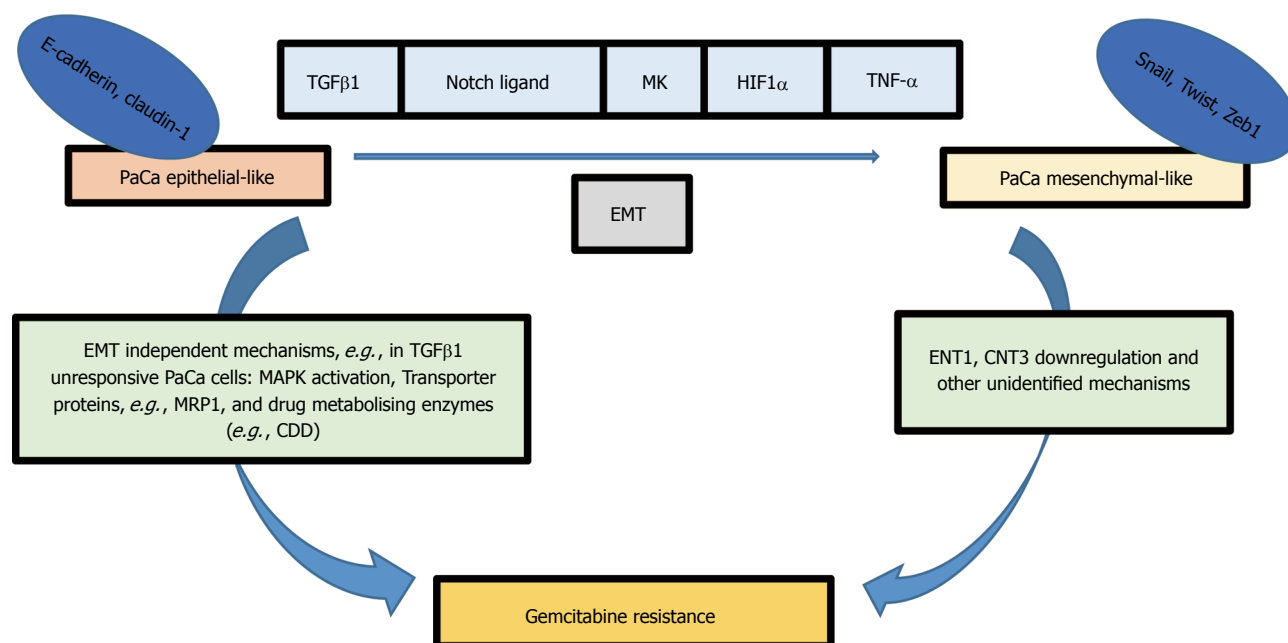
Several strategies have been proposed for the design of EMT-based therapies as recently and extensively described and reviewed by Davis *et al.*<sup>[1]</sup>. While major challenges and questions remain regarding the possibility of targeting EMT to counteract metastasis specifically, stronger evidence is accumulating on the use of anti-EMT agents in cancer chemoresistance settings. However, targeting a single receptor, enzyme or transporter that is associated with EMT faces many limitations since several redundant pathways are involved in this process. Strategies focused on targeting microRNAs regulating EMT such as miR-200, or transcription factors might represent a more effective approach since they influence the process more broadly. In addition, key components of the tumour microenvironment are also attractive targets for therapeutic intervention. Indeed, recent evidence has revealed that local tumour microenvironment represents a main driving force for EMT, chemotherapy resistance and cancer progression. Inflammatory cells such as neutrophils and macrophages are contained in the tumour microenvironment, which offers multidirectional interactions leading in some cases to increased chemotherapy resistance and metastasis<sup>[15-18]</sup>. Neutrophils have been shown to induce EMT in pancreatic cancer while macrophages induce gemcitabine resistance *via* promoting cytidine deaminase mediated drug inactivation<sup>[15,19,20]</sup>. Similarly, platelets were recently shown to be capable of inducing EMT in cancer<sup>[21,22]</sup>. Pancreatic cancer is associated with a high risk of venous thromboembolism (VTE) caused by tumour-derived or tumour-elicited tissue factor which can indirectly induce platelets aggregation<sup>[23,24]</sup>. Whether targeting platelets can offer an indirect way to reduce EMT and chemoresistance as well as the risk of VTE in pancreatic cancer is yet to be demonstrated.

Therefore, a systematic testing of different methods for targeting EMT in combination with existing chemotherapeutic agents is required for each model of therapy relapse. The excitement elicited by the new reinforcement of the link between EMT and chemoresistance will surely result in a surge of studies in this field, and consequently, further in-depth investigations are warranted, especially in pancreatic cancer.

## CONCLUSION

In summary, resistance to treatments such as Gemcitabine in pancreatic cancer can be mediated by several





**Figure 1** Several signalling pathways can induce epithelial-mesenchymal transition in pancreatic cancer cells for example Notch, transforming growth factor- $\beta$ , Midkine, hypoxia-inducible factor-1 $\alpha$  and tumor necrosis factor- $\alpha$ . EMT phenotype is associated with gemcitabine resistance; however, the signalling pathway relating EMT factors (e.g., Snail, Twist and Zeb1) to gemcitabine resistance is not clearly identified, although ENT1 and CNT3 upregulation was observed in KPC mice models with deleted Snail or Twist. EMT independent pathways can also lead to gemcitabine resistance for example MAPK activation, transporter proteins, and gemcitabine metabolising enzymes (e.g., CDD). MRP1: Multidrug resistance protein1; CDD: Cytidine deaminase; TGF $\beta$ : Transforming growth factor- $\beta$ ; MK: Midkine; HIF1 $\alpha$ : Hypoxia-inducible factor-1 $\alpha$ ; TNF- $\alpha$ : Tumor necrosis factor- $\alpha$ ; EMT: Epithelial-mesenchymal transition; ENT1: Equilibrative nucleoside transporter 1; CNT3: Concentrative nucleotide transporter.

EMT-dependent or independent pathways (Figure 1)<sup>[15,25-29]</sup>, making the EMT process an attractive target for reducing chemotherapy resistance in pancreatic cancer. EMT can be regulated by blocking extracellular signalling molecules such as TGF $\beta$ 1 (a cytokine mediator of EMT in many types of cancer) and EMT signal transduction pathways<sup>[1]</sup>. Loss of E-cadherin-mediated cell adhesion is a hallmark of EMT and subsequent invasion and metastasis. Since Snail and Zeb family of transcriptional factors mediate E-cadherin translocation, loss of function or downregulation, they can potentially be targeted to avert EMT at its initial steps. Several studies have reported disruption in TGF $\beta$  signalling in pancreatic cancer<sup>[30-32]</sup>. Therefore, the mesenchymal transcriptional factors may be better druggable targets compared to TGF- $\beta$  receptors to reduce EMT-derived chemoresistance in pancreatic cancer. Despite a significant number of emerging studies examining the role of EMT in cancer, the interplay between different signalling pathways that drive EMT is more complex than we initially thought. The fact that the phenotypic alteration is transient and triggered by several dynamics encountered by tumour cells during their development or metastasis emphasises the challenge of utilising EMT as a lone druggable target. The diversity of EMT-inducing transcriptional factors may enable cancer cells to adapt and survive a single targeted molecular therapy. The association between EMT and chemotherapy resistance is well established in the literature, but it is not well understood how EMT can affect cancer cell survival pathways, drug transporters and drug metabolising enzymes. Delineation of these

interactions may uncover novel approaches to inhibit chemoresistance in pancreatic cancer. Nevertheless, at present, there may be potential benefits in tempering cancer EMT in a combined immunotherapy and molecular-targeted drug strategies to treat pancreatic cancer.

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## Retrospective Study

# Pre-treatment platelet counts as a prognostic and predictive factor in stage II and III rectal adenocarcinoma

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## Abstract

### AIM

To investigate if pre-treatment platelet counts could provide prognostic information in patients with rectal adenocarcinoma that received neo-adjuvant treatment.

### METHODS

Platelet number on diagnosis of stage II and III rectal cancer was evaluated in 51 patients receiving neo-adjuvant treatment and for whom there were complete follow-up data on progression and survival, as well as pathologic outcome at the time of surgery. Pathologic responses on the surgical specimen of patients with lower platelet counts ( $150-300 \times 10^9/L$ ) were compared with these of patients with higher platelet counts ( $> 300 \times 10^9/L$ ) by the  $\chi^2$  test. Overall and progression free survival Kaplan-Meier curves of the two groups were constructed and compared with the Log-Rank test.

### RESULTS

A significant difference was present between the two groups in regards to pathologic response with patients with lower platelet counts being more likely to exhibit a

good or complete response to neo-adjuvant treatment than patients with higher platelet counts ( $P = 0.015$ ). Among other factors evaluated, there was also a significant difference between the carcinoembryonic antigen (CEA) at presentation of patients that exhibited a good or complete response and those that had no response or a minimal to moderate response. Patients with a good or complete response were more likely to present with a CEA of less than 5  $\mu\text{g/L}$  ( $P = 0.00066$ ). There was no significant difference in overall and progression free survival between the two platelet count groups (Log-Rank tests  $P = 0.42$  and  $P = 0.35$ , respectively).

### CONCLUSION

In this retrospective analysis of stage II and III rectal cancer patients, platelet counts at the time of diagnosis had prognostic value for neo-adjuvant treatment pathologic response. Pre-treatment CEA also held prognostic value in regards to treatment effect.

**Key words:** Rectal cancer; Platelets; Prognosis; Treatment response; Neo-adjuvant; Chemoradiation

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**Core tip:** Platelet counts may provide prognostic and treatment efficacy predictive information in various cancers. In this study, platelet number on diagnosis of stage II and III rectal cancer was evaluated in 51 patients before start of neo-adjuvant treatment. A significant difference was present between the two groups, of higher and lower platelets, regarding pathologic response to neo-adjuvant treatment. There was no significant difference in overall and progression free survival between the two platelet count groups. Pre-treatment carcinoembryonic antigen also held prognostic value in regards to treatment effect.

Steele M, Voutsadakis IA. Pre-treatment platelet counts as a prognostic and predictive factor in stage II and III rectal adenocarcinoma. *World J Gastrointest Oncol* 2017; 9(1): 42-49 Available from: URL: <http://www.wjgnet.com/1948-5204/full/v9/i1/42.htm> DOI: <http://dx.doi.org/10.4251/wjgo.v9.i1.42>

### INTRODUCTION

Platelets play a crucial role in maintaining hemostasis and vascular integrity. They are produced from bone marrow precursor cells, megakaryocytes, as fragments breaking off of the megakaryocytes cytoplasm<sup>[1]</sup>. Abnormalities in platelet number, whether an increase or decrease in their circulating number, are associated with many pathologic conditions<sup>[2]</sup>. Cancer is a pathology that is often associated with thrombocytosis as the cytokines that stimulate thrombopoiesis are often elevated in

cancer<sup>[3]</sup>. In addition, thrombocytosis has been found to be an adverse prognostic factor in many common cancers<sup>[4]</sup>.

Colorectal cancer is a common malignancy and one of the leading causes of cancer deaths in both men and women<sup>[5]</sup>. Localized stage rectal cancer is typically treated with neoadjuvant chemoradiotherapy in addition to adjuvant chemotherapy<sup>[6]</sup>. Pathologic stage is the main prognostic factor and treatment modality determinant. Other prognostic factors include positive surgical margins, pre-treatment elevation of carcinoembryonic antigen (CEA), and high tumour grade<sup>[7]</sup>. Prognostic markers of positive pathologic response to neo-adjuvant therapy are also important because such response may be associated with survival outcomes in rectal cancer<sup>[8]</sup>. Moreover, being able to predict which patients would benefit from neo-adjuvant chemoradiation could be important for modification of the treatment plan and sparing of patients predicted not to respond to this therapy and its adverse effects. Thus, additional biomarkers are needed to further promote prognostication of rectal adenocarcinomas. In a previous study in patients with colorectal adenocarcinomas of various stages, pre-treatment thrombocytosis was an independent prognostic factor for overall survival (OS) and progression-free survival (PFS)<sup>[9]</sup>. Nevertheless, another study in colorectal cancer patients did not observe a difference in survival between patients with thrombocytosis and normal platelet counts<sup>[10]</sup>. In the current study, we investigated if pre-treatment thrombocytosis provides prognostic information specifically in patients with stage II and III rectal adenocarcinomas that received neo-adjuvant treatment. We also investigated the effect of thrombocytosis on pathologic outcome at the time of surgery.

### MATERIALS AND METHODS

The case records of 130 patients treated for localized rectal cancer at the Algoma District Cancer Clinic between January 2008 and January 2015 were retrospectively reviewed. Patients were included if they had stage II or III disease, had received neo-adjuvant treatment, and had complete follow-up. Follow-up was considered complete if a patient was followed until his or her death, or was seen within the last six months from data collection. Fifty one patients fulfilled the inclusion criteria and were included in the study. Demographic data, as well as histologic characteristics of tumors, stage, tumor marker CEA, and pathologic response were extracted from the medical records. Platelet number at diagnosis of the 51 patients was also evaluated.

Pathologic response at the time of surgery was categorized in a five tier scale ranging from no response (no evidence of treatment effect on tumor), minimal response (some morphologic effect of treatment evident but no significant regression of tumor areas), moderate



**Table 1** Baseline characteristics of all patients in the series and comparison of the groups with lower ( $\leq 300 \times 10^9/L$ ) and higher ( $> 300 \times 10^9/L$ ) platelet counts *n* (%)

	Total (%) ( <i>n</i> = 51)	$\leq 300$ ( <i>n</i> = 26)	$> 300$ ( <i>n</i> = 25)	$\chi^2$
Age (yr)				
> 60	21 (41.2)	10 (38.5)	11 (44.0)	$P = 0.69$
$\leq 60$	30 (58.8)	16 (61.5)	14 (56.0)	
Clinical stage				
II	25 (49.0)	15 (57.7)	10 (40.0)	$P = 0.21$
III	26 (51.0)	11 (42.3)	15 (60.0)	
CEA ( <i>n</i> = 50)				
> 5	25 (50.0)	11 (44.0)	14 (56.0)	$P = 0.4$
< 5	25 (50.0)	14 (56.0)	11 (44.0)	
Symptoms				
Obstruction/pain	13 (25.5)	6 (23.1)	7 (28.0)	$P = 0.69$
Bleeding/ asymptomatic	38 (74.5)	20 (76.9)	18 (72.0)	
Type of surgery				
Anterior resection	27 (52.9)	13 (50.0)	14 (56.0)	Anterior resection <i>vs</i> APR/ exenteration $P = 0.77$
APR	19 (37.3)	11 (42.3)	8 (32.0)	
Pelvic/exenteration	2 (3.9)	0	2 (8.0)	$P = 0.77$
None	3 (5.9)	2 (7.7)	1 (4.0)	
Pathologic response				
No response	15 (29.4)	8 (30.8)	7 (28.0)	No/ minimal/ Moderate resp <i>vs</i> Good/ complete $P = 0.015$
Minimal	7 (13.7)	4 (15.3)	3 (12.0)	
Moderate	15 (29.4)	3 (11.6)	12 (48.0)	
Good	5 (9.8)	5 (19.2)	0	
Complete	9 (17.6)	6 (23.1)	3 (12.0)	
Lymph nodes at surgery				
Negative	31 (60.8)	15 (57.7)	16 (64.0)	$P = 0.61$
Positive	16 (31.4)	9 (34.6)	7 (28.0)	
No surgery	3 (5.9)	2 (7.7)	1 (4.0)	

Pre-operative CEA not available in one patient. Lymph node evaluation was not available in the pathology report in one patient. APR: Abdomino-perineal resection; CEA: Carcinoembryonic antigen.

response (evident effect of treatment but significant tumor aggregates remaining), good response (only occasional scattered tumor cell aggregates remaining) and complete response (no evidence of tumor in the primary site or the lymph nodes).

OS was defined as the interval from the date of diagnosis to patient death or censored at the date of last contact. PFS was defined as the interval from the date of diagnosis until date of disease progression or censored at the date of last contact without evidence of recurrence. For the purpose of this study, patients with platelet counts of  $150\text{--}300 \times 10^9/L$  were included in the lower platelet count group. Patients with counts  $> 300 \times 10^9/L$  were included in the higher platelet count group. This value divided patients in two groups with almost equal numbers. Survival plots of the patients with lower and higher platelet counts were constructed using the Kaplan-Meier method and were compared using the Log-Rank test<sup>[11]</sup>. The  $\chi^2$  test was used to evaluate differences in clinical and biologic characteristics of the two groups<sup>[12]</sup>. The Student's *t*

test was used for comparison of means. All *P* values were considered to be significant at a level of  $P < 0.05$ . Statistical calculations were performed with online tools available from the Technical University of Denmark (<http://www.iscc-serv2.imm.dtu.dk/>) and a noncommercial site (<http://www.statpages.org/>). The study was approved by the Institutional Review Board of our institution. Due to the retrospective nature of the study, no patient consent was required or obtained.

## RESULTS

The median age of the patients was 58-year-old. From the 51 patients, 26 patients (51%) were included in the lower platelet ( $\leq 300 \times 10^9/L$ ) group and had mean platelet counts of  $232.5 \times 10^9/L$  (range, 167–297) at diagnosis of their disease (Table 1). Twenty-five patients (49%) were in the higher platelet ( $> 300 \times 10^9/L$ ) group and had mean platelet counts of  $347 \times 10^9/L$  (range, 303–693). The median age of the patients with lower platelet counts was 59-year-old (range, 32–79) and those with higher counts was 58-year-old (range, 24–74). In the lower platelet group 38.5% of patients were older than 60-year-old while in the higher platelet group 44% were older than 60-year-old ( $\chi^2$  test  $P = 0.69$ ). Forty-four patients in the series received neoadjuvant chemoradiation with continuous infusion of 5-FU or capecitabine as the chemotherapy part. Five additional patients (four in the higher platelet group and one patient in the lower platelet group) received 1–2 cycles of neo-adjuvant mFOLFOX before chemoradiation. Two patients (both in the higher platelet group) received neo-adjuvant radiation alone. No differences in the two groups were noted in the clinical stage at presentation, in the tumor marker CEA or patients' symptoms of presentation (Table 1). The type of surgery performed after neo-adjuvant therapy (whether an abdominal resection or abdomino-perineal resection (APR)/pelvic exenteration with permanent colostomy) was also not statistically different in the two groups (Table 1). All patients but two had negative pathologic surgical margins at surgery. Both patients with positive pathologic margins (one in the lower and one in the higher platelet group) underwent an APR, had minimal pathologic responses and had a recurrence 12 and 20 mo postoperatively respectively. All patients but three had post-operative 5-fluoropyrimidine-based chemotherapy. Three patients who had complete pathologic response (two in the lower platelet group and one patient in the higher platelet group) elected not to undergo surgery and were placed in close surveillance.

Overall about one third of patients in the series were lymph node positive on pathologic examination at the time of surgery and the percentage did not differ significantly between the two platelet groups ( $P = 0.61$ ) (Table 1). A complete pathologic response (defined as no pathologic evidence of tumor in either primary site or lymph nodes examined) was obtained after neo-adjuvant treatment in 9 patients (17.6%)

**Table 2** Comparison of characteristics of patients according to their pathologic response at surgery (*n* = 48) or at post-neoadjuvant treatment endoscopy (*n* = 3) *n* (%)

	No response/ minimal/ moderate	Good/ complete	$\chi^2$ <i>P</i> value
Age (yr)			
> 60	13 (35.1)	8 (57.1)	0.15
≤ 60	24 (64.9)	6 (42.9)	
Clinical stage			
II	16 (43.2)	9 (64.3)	0.18
III	21 (56.8)	5 (35.7)	
CEA ( <i>n</i> = 50)			
> 5	24 (64.9)	1 (7.7)	0.0004
< 5	13 (35.1)	12 (92.3)	
Symptoms			
Obstruction/pain	12 (32.4)	1 (7.1)	0.06
Bleeding/asymptomatic	25 (67.6)	13 (92.9)	
Platelets			
≤ 300	15 (40.5)	11 (78.6)	0.015
> 300	22 (59.5)	3 (21.4)	

Pre-operative CEA not available in one patient. CEA: Carcinoembryonic antigen.

in the series and an additional 5 patients (9.8%) had good pathologic responses. No response, minimal or moderate response were observed in 15 (29.4%), 7 (13.7%), and 15 (29.4%) patients respectively. Overall pathologic response differed between the groups. Eleven patients (42.3%) in the lower platelet group had a good or complete pathologic response while only three patients in the higher platelet group (12%) had such a response (*P* = 0.015). The mean platelet count at diagnosis of good and complete responders was 249.9 (SD = 69.6) while mean platelet count of no/minimal/moderate responders group was 327.0 (SD = 85.6) (*t* test *P* = 0.004). Among the 25 patients in the elevated platelet group, 16 patients had converted to a platelet count below  $300 \times 10^9/L$  after the neo-adjuvant treatment, in their pre-operative evaluation, while the remaining nine patients remained with a platelet count above  $300 \times 10^9/L$ . All three pathologic responders were among the 16 converted patients.

In the analysis for possible associated factors with a good or complete pathologic response a normal range (< 5 µg/L) CEA level at baseline (*P* = 0.0004) and lower platelet counts (*P* = 0.015) were associated with favorable pathologic response (Table 2). Patients that were asymptomatic at presentation (evaluated with a colonoscopy for anemia) or presented with bleeding had a trend towards a better pathologic response than patients presenting with obstruction or pain (*P* = 0.06). Age and clinical stage at presentation was not statistically associated with the degree of pathologic response. Logistic regression analysis with pathologic response as the outcome variable and platelet counts, CEA and symptoms at presentation as the predictor variables confirmed that lower platelet counts (*P* = 0.03, odds ratio 0.15, 95%CI: 0.02-0.85) and a normal CEA

**Table 3** Logistic regression analysis of pathologic response (complete or good *vs* moderate or minimal or no response) as the outcome variable and platelet counts (≤ *vs* >  $300 \times 10^9/L$ ), carcinoembryonic antigen (≤ 5 µg/L *vs* > 5 µg/L) and symptoms (obstruction or pain *vs* bleeding or asymptomatic) at presentation as the predictor variables

Variable	OR	95%CI	<i>P</i> value
Platelet count	0.15	0.02-0.85	0.03
CEA	0.04	0.004-0.41	0.006
Symptoms at presentation	4.7	0.39-55.8	0.20

CEA: Carcinoembryonic antigen.

(*P* = 0.006, OR = 0.04, 95%CI: 0.004-0.41) but not symptoms at presentation (*P* = 0.2, OR = 4.7, 95%CI: 0.39-55.89) were significantly associated with a good or complete pathologic response (Table 3).

The median overall survival of patients that had died in the whole cohort (11 of 51 patients) was 21 mo (range 5-68 mo). In the group of patients with lower platelet counts, 4 of the 26 patients (15.4%) had died with a median OS of 20 mo (range 5-24 mo). In the group of patients with higher platelet counts, 7 of the 25 patients (28%) had died with a median OS of 23 mo (range 7-68 mo). Kaplan-Meier survival analysis showed that there was not a statistically significant difference in the OS and PFS between patients in the two platelet groups (Log-Rank test *P* = 0.42 and 0.35 respectively) (Figure 1A and B). PFS of patients with a good or complete pathologic response was significantly better than that of patients with a lesser pathologic response (Log-Rank test *P* = 0.01) (Figure 2A). OS curve of pathologic responders clearly separated from lesser responders after 2 years but the difference did not reach statistical significance (Log-Rank test *P* = 0.15) (Figure 2B).

## DISCUSSION

Thrombocytosis is associated with several underlying pathologies among which cancer is included<sup>[2]</sup>. About two out of five patients (40%) with thrombocytosis had an occult cancer in one series<sup>[11]</sup>. Thrombocytosis or a higher platelet count defined with various cut-offs has been confirmed to be an adverse prognostic factor in several types of cancer including lung, breast, gynecologic and genitourinary<sup>[12-16]</sup>. It has also been studied and suggested to have prognostic relevance in virtually every type of gastrointestinal carcinoma including gastric, colon and rectal<sup>[4]</sup>.

In a series of Asian patients with colorectal carcinoma, thrombocytosis, defined as platelets counts greater than  $300 \times 10^9/L$ , similarly to our study, was a significant independent prognostic marker for survival<sup>[17]</sup>. This was confirmed in another large Japanese series and a smaller study of European patients<sup>[18,19]</sup>. An American study of 1513 patients with localized colorectal cancer that had undergone surgery also evaluated pre-operative thrombocytosis (defined in this study as more

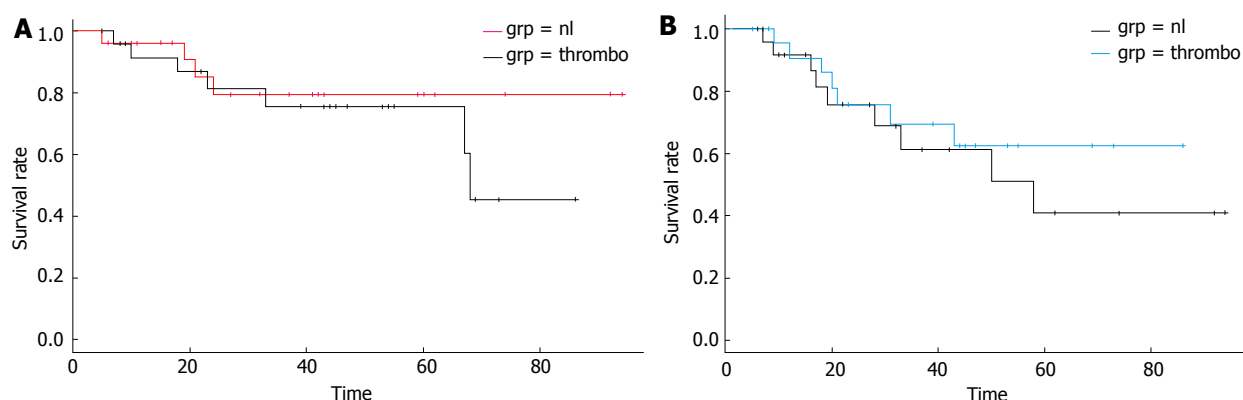


Figure 1 Kaplan-Meier overall survival (A) and progression free survival (B) curves in months from the diagnosis of rectal adenocarcinoma of patients with lower platelet counts ( $150\text{--}300 \times 10^9/\text{L}$ , labeled: nl) vs patients with higher platelet counts ( $> 300 \times 10^9/\text{L}$ , labeled: thrombo). Log-Rank tests  $P = 0.47$  (A),  $P = 0.35$  (B).

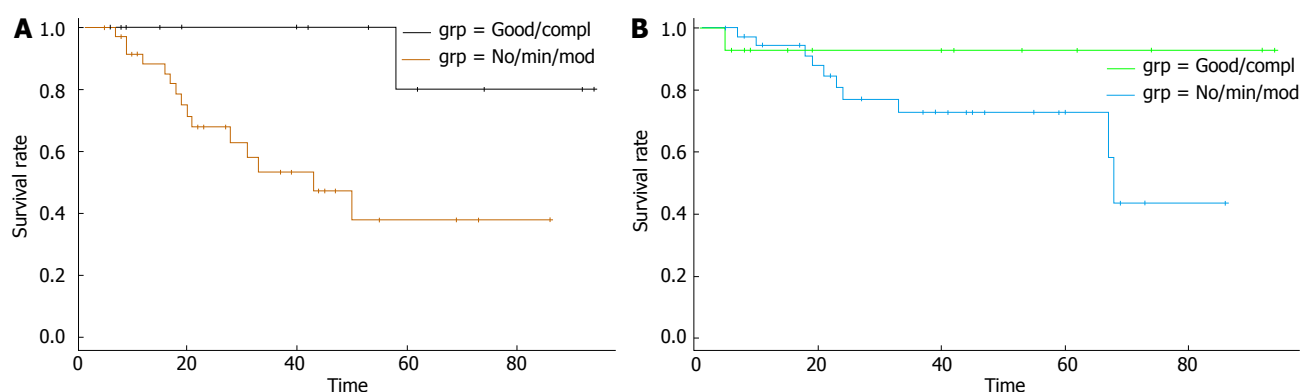


Figure 2 Kaplan-Meier progression-free survival (A) and overall survival (B) curves in months from the diagnosis of rectal adenocarcinoma of patients with a good or complete pathologic response (labeled: Good/compl) vs patients with no response or a minimal or moderate response (labeled: No/min/mod). Log-Rank tests  $P = 0.01$  (A),  $P = 0.15$  (B).

than  $400 \times 10^9/\text{L}$ ) as a prognostic factor for various survival outcomes<sup>[20]</sup>. Patients with thrombocytosis had a significantly worse overall survival than patients with normal platelets. Distant metastatic recurrence, but not overall recurrence rate or loco-regional recurrence rate, was also worse in patients with thrombocytosis. In contrast, another study of 630 patients showed no correlation of survival with thrombocytosis defined as platelets above  $450 \times 10^9/\text{L}$ <sup>[10]</sup>. Nevertheless this study included patients across stages which may have confounded results.

Two studies from the Far East have specifically examined the prognostic role of platelets in rectal cancer patients<sup>[9,21]</sup>. Both reports used a similar cut-off for thrombocytosis of  $365$  to  $370 \times 10^9/\text{L}$  and showed a similar percentage of thrombocytosis of about 20%. The authorship of the two publications is overlapping and is not clear if the patients of the smaller study<sup>[21]</sup> is a subset of the patients included in the larger study<sup>[9]</sup>. Both a lower platelet count, below the cut-off, and a normal CEA were associated with pathologic response<sup>[9]</sup>. These were the only predictors of such response in logistic regression analysis.

In order to further clarify the prognostic value of

platelets in rectal cancer in a predominantly white population, in this retrospective study we investigated the association between pre-treatment thrombocytosis and the effectiveness of neoadjuvant treatment in patients with stage II and III rectal adenocarcinoma. We also explored the relationship between thrombocytosis and both overall and progression free survival. In the current cohort of 51 patients we found no significant differences between the two groups with lower and higher platelet levels regarding the age of patients, pre-treatment tumor CEA, symptoms at presentation, clinical staging at presentation, and the presence of metastatic lymph nodes at the time of surgery. No difference was also observed in the type of surgery in the two groups. In contrast, a significant difference was present regarding the response to neoadjuvant treatment as patients with lower platelet counts were more likely to have a good or complete pathologic response to pre-operative treatment than patients with higher platelet counts ( $P = 0.015$ ). There was no statistical difference in the OS or PFS of the two platelet groups (Log-Rank test  $P = 0.47$  and  $0.35$  respectively).

The CEA tumor antigen at presentation was also a prognostic marker for pathologic response ( $P =$

0.0004). Patients with a CEA of less than 5 were more likely to have a good or complete response to neoadjuvant treatment than those that presented with a CEA greater than 5. A higher pre-treatment CEA has been associated with advanced locoregional disease and therefore may be linked to poorer local control<sup>[22]</sup>. Our data confirm that CEA is prognostic for PFS and predictive for pathologic response to neoadjuvant therapy, as suggested previously<sup>[22,23]</sup>.

The pathogenesis of thrombocytosis in cancer involves production of interleukin-6 (IL-6) at least in some malignancies. In ovarian carcinoma, for example, thrombocytosis was significantly correlated with plasma levels of IL-6<sup>[14]</sup>. This was investigated in mice bearing ovarian cancer xenografts of human origin, where human IL-6 was found to stimulate hepatocytes *via* the IL-6 receptor, producing thrombopoietin. Authors proposed that ovarian cancer cells produce IL-6, which functions by stimulating mice liver to produce thrombopoietin, which in turn positively regulates megakaryocyte progenitors in the bone marrow<sup>[14]</sup>. In renal carcinoma, most examined cases were positive for IL-6 by immunohistochemistry<sup>[24]</sup>. Serum levels of IL-6 were also elevated in prostate and breast cancer patients<sup>[25,26]</sup>. Thus cancer cell-derived IL-6 is a trigger of tumor-induced thrombocytosis across various cancers.

The pathophysiology of platelets' contribution to carcinogenesis involves a protective effect on circulating tumor cells from the attack of the immune system<sup>[27]</sup>. In addition platelets contribute to the attachment of tumor cells to endothelial cells at sites of metastases. Aggregates of platelets and tumor cells embolize in the microcirculation and facilitate the process of extravasation of tumor cells in metastatic sites. Platelets promote carcinogenesis by their normal function of promoting vascular integrity<sup>[28]</sup>. They protect the integrity of newly formed tumor vasculature which is prone to hemorrhage and prevent bleeding in tumor beds<sup>[29]</sup>. Platelets contain several types of active macromolecules and cytokines in their granules. These include vascular endothelial growth factor (VEGF), EGF, platelet-derived growth factor, hepatocyte growth factor, transforming growth factor  $\beta$  (TGF $\beta$ ), IL-1 $\beta$ , IL-8, CXC motif containing ligand 12 and Sphingosine-1-phosphate<sup>[30,31]</sup>. These factors have the potential to contribute to metastatic tumor establishment and progression. For example, platelet-derived TGF $\beta$  promotes epithelial to mesenchymal transition program in tumor cells through Smad and NF- $\kappa$ B signaling<sup>[32]</sup>. This program provides epithelial cells with a mesenchymal phenotype that promotes mobility and metastases while protecting them from apoptosis due to lack of adhesion (anoikis)<sup>[33]</sup>. It has also been found that platelets from cancer patients have a higher VEGF level than platelets from patients without cancer<sup>[34]</sup>. Interestingly, serum VEGF is not consistently elevated in cancer, with the exception of renal carcinoma, if methods are adequate in order to prevent platelet activation during venipuncture<sup>[34]</sup>. As a result, platelet levels could provide a better reflection of VEGF concentrations in the primary and

metastases sites micro-environment where they are activated and participate to tumor angiogenesis.

In conclusion, this retrospective analysis of patients with stage II and stage III localized rectal cancer patients shows that higher platelets counts (defined in the current study as platelets more than  $300 \times 10^9/L$ ) at the time of disease diagnosis has prognostic value regarding treatment effect outcome. Additionally, it was shown that pretreatment tumor CEA also has prognostic value regarding treatment effect outcome. Further study is needed in more extensive series to confirm these results, clarify survival prognostication value and to test whether thrombocytosis may be used as a predictive marker for specific therapies. It would be of particular interest to evaluate the role of thrombocytosis as a predictive element of anti-VEGF treatments. In this regard a study of metastatic renal cell cancer patients has shown that those with thrombocytosis have an increased probability for refractoriness to anti-VEGF treatments than patients with normal platelets counts<sup>[35]</sup>. Moreover, given the presumed role of platelets as protectors of circulating tumor cells from immune attack, an investigation into thrombocytosis as a predictive marker of response to the newly introduced immune checkpoint inhibitors may be worth pursuing.

## COMMENTS

### Background

Platelet counts are easily measurable laboratory values that are usually measured in all patients with a newly diagnosed cancer as part of a general evaluation.

### Research frontiers

This paper proposes the evaluation of this easily available laboratory evaluation as part of the prognostic armamentarium in better defining the therapeutic prospects of rectal cancer patients.

### Innovations and breakthrough

This is one of the first studies to specifically evaluate platelets as prognostic factors in neo-adjuvant treatment of newly diagnosed rectal cancer patients to be treated with neo-adjuvant therapy.

### Applications

Platelet counts measurement could be used in the clinic to predict the effectiveness of neo-adjuvant cancer treatment.

### Terminology

Platelet counts are part of the Complete Blood Count standard laboratory evaluation. Rectal cancers are adenocarcinomas of the terminal part of the colon below the peritoneal fold.

### Peer-review

The manuscript is concise, clear, and comprehensive. The purpose, results, and conclusion are clearly stated. The manuscript provides new information and it induce new research.

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## Anal intraepithelial neoplasia: A review of diagnosis and management

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lesion of the anal mucosa that is a precursor to anal cancer. Although anal cancer is relatively uncommon, rates of this malignancy are steadily rising in the United States, and among certain high risk populations the incidence of anal cancer may exceed that of colon cancer. Risk factors for AIN and anal cancer consist of clinical factors and behaviors that are associated with the acquisition and persistence of human papilloma virus (HPV) infection. The strongest HPV-associated risk factors are HIV infection, receptive anal intercourse, and high risk sexual behavior. A history of HPV-mediated genital cancer, which suggests infection with an oncogenic HPV strain, is another risk factor for AIN/anal cancer. Because progression of AIN to anal cancer is known to occur in some individuals over several years, screening for AIN and early anal cancer, as well as treatment of advanced AIN lesions, is reasonable in certain high-risk populations. Although randomized controlled trials evaluating screening and treatment outcomes are lacking, experts support routine screening for AIN in high risk populations. Screening is performed using anal cytological exams, similar to those performed in cervical cancer screening programs, along with direct tissue evaluation and biopsy *via* high resolution anoscopy. AIN can be treated using topical therapies such as imiquimod, 5-fluorouracil, and trichloroacetic acid, as well as ablative therapies such as electrocautery and laser therapy. Reductions in AIN and anal cancer rates have been shown in studies where high-risk populations were vaccinated against the oncogenic strains of HPV. Currently, the CDC recommends both high-risk and average-risk populations be vaccinated against HPV infection using the quadrivalent or nonavalent vaccines. It is important for clinicians to be familiar with AIN and the role of HPV vaccination, particularly in high risk populations.

**Key words:** Anal cancer; Anal intraepithelial neoplasia; Anal squamous cell carcinoma; Human papillomavirus vaccine; Human papillomavirus

### Abstract

Anal intraepithelial neoplasia (AIN) is a premalignant

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**Core tip:** Anal intraepithelial neoplasia (AIN) is the precursor lesion to anal squamous cell carcinoma. AIN incidence is low in the general population, but rivals colon cancer in high risk groups, particularly those with human immunodeficiency virus infection and men who have sex with men. Thus, screening for AIN and early anal cancer and treatment of these lesions at expert centers should be considered in high risk populations. Screening is performed using anal cytology and high resolution anoscopy, and treatment consists of either topical or ablative therapies. Finally, human papillomavirus vaccination appears to reduce the rate of AIN and possibly anal cancer.

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## INTRODUCTION

Anal intraepithelial neoplasia (AIN) is a clinically important premalignant lesion which can progress to squamous cell carcinoma of the anus. Despite this, AIN is frequently underappreciated by most gastroenterologists and other health care providers. With a predicted incidence of 8080 cases in the United States in 2016, equating to 1.8 cases per 100000 individuals, the incidence of anal cancer is dwarfed by colorectal cancer, which is predicted to affect roughly 135000 people in the United States in 2016<sup>[1,2]</sup>. Despite the lower rate of anal cancer, it is associated with significant morbidity and mortality, and has been steadily increasing in incidence, nearly doubling in the last 25 years<sup>[1]</sup>. The purpose of this review is to outline the burden of disease, risk factors, progression rates, and clinical consequences of AIN. We also address treatment and surveillance guidelines pertinent to practicing clinicians. Finally, special attention is given to the role of human papilloma virus (HPV) vaccination.

## DEFINITIONS

Anal cancer is defined as cancer arising from the squamous epithelium of the anus, making it distinct from colorectal cancer. The anal canal consists of stratified squamous epithelium originating outside the body and extending into the anus up to the dentate line, the point where it intersects the columnar epithelium of the rectum. Thus, the vast majority of anal cancer is squamous cell carcinoma (SCC), with a small minority consisting of adenocarcinoma or skin cancer variants<sup>[3]</sup>. The term "anal cancer" commonly refers to SCC and given the predominance of SCC, the two terms will be used interchangeably in this review.

**Table 1** Unification of terms relating to anal intraepithelial neoplasia

Normal	LSIL		HSIL	
	Condyloma or ASCUS	AIN grade I	AIN grade II	AIN grade III
	Mild dysplasia		Moderate dysplasia	Severe dysplasia

ASCUS: Atypical squamous cells of undetermined significance; LSIL: Low-grade squamous intraepithelial lesion; HSIL: High-grade squamous intraepithelial lesion; AIN: Anal intraepithelial neoplasia.

Anal cancer is preceded by intraepithelial neoplasia<sup>[4,5]</sup>, a premalignant lesion which has been described by a variety of different classification schemes throughout the literature owing to the evolving understanding of its underlying pathophysiology. Dysplastic lesions in the anal region were initially reported as "mild", "moderate", and "severe" by pathologists. However, as the connection between anal dysplasia and the HPV was established, new criteria borrowing from the cervical pathology classification system were developed, using the Bethesda System terminology AIN I, II, and III<sup>[6,7]</sup>. Unfortunately this led to uncertainty regarding the significance of AIN II lesions, which have been associated with poor interobserver agreement among pathologists, leaving clinicians unsure which lesions required close follow up and which could be followed expectantly<sup>[8]</sup>. Further clarification of the relationship of AIN with HPV, and the discovery that the oncogenic pathways of anal and genital cancers are closely related, has recently led to a simpler system consisting of a two-tiered approach of "low grade" and "high grade squamous intraepithelial lesions" (LSIL and HSIL, respectively; Table 1)<sup>[9]</sup>. Under this system, AIN I corresponds to LSIL and AIN II/III to HSIL. HSIL lesions are considered premalignant, whereas LSIL lesions are not felt to be premalignant, but do have the potential to progress to HSIL. Cytology reports will occasionally include the term "ASCUS", or "atypical squamous cells of undetermined significance", which can be generally included in the LSIL category.

## EPIDEMIOLOGY AND RISK FACTORS

Due to the low incidence of anal cancer, the changing nomenclature, the difficulties with validated testing, and the absence of large, population-based screening programs, it is difficult to estimate the true burden of AIN in the general population. However, as certain high risk groups have been identified and subsequently studied, it is possible to comment on disease prevalence within these groups. Table 2 summarizes the estimated risks for anal cancer among various populations, which will be explored in more detail below.

### High-risk sexual behavior

High-risk sexual behavior, most commonly defined as men who have sex with men (MSM), receptive anal



**Table 2** Rates of anal cancer among various populations compiled from various sources

Anal cancer rates among select populations, per 100000 person-years	
General population	2 <sup>[1]</sup>
General population, female	0.55-2.4 <sup>[13]</sup>
HIV positive women	3.9-30 <sup>[13]</sup>
HIV negative MSM	5.1 <sup>[12]</sup>
Solid organ transplant	10-15 <sup>[66]</sup>
Prior HPV related malignancy	0.8-63.8 <sup>[13]</sup>
HIV positive MSM	49.5 <sup>[12]</sup>
Colon cancer in general population	41 <sup>[2]</sup>

HIV: Human immunodeficiency virus; MSM: Sex with men; HPV: Human papilloma virus.

intercourse, or history of multiple sexual partners has been shown to be associated with higher rates of HSIL. One study of 1262 human immunodeficiency virus (HIV) negative MSM revealed 15% prevalence of LSIL and 5% for HSIL<sup>[10]</sup>. Another recent study of 203 individuals revealed elevated rates of HSIL, finding a prevalence of 20.9% of HIV negative MSM<sup>[11]</sup>. The largest meta-analysis to evaluate HIV negative MSM included 53 studies and determined a HSIL prevalence of 21.5%, and was able to provide an estimated anal cancer rate of 5 per 100000<sup>[12]</sup>. Less data is available regarding the rate of AIN in women; however, in a study of 251 HIV+ and 68 HIV- women, Holly *et al*<sup>[13]</sup> found rates of 8% for any type of AIN, and 2% for HSIL in HIV negative women. Receptive anal intercourse and concomitant abnormal cervical cytology were found to be statistically significant risk factors for AIN.

### HIV infection

The first clue that the HIV population was at particular risk for AIN came with the appreciation that anal cancer rates have notably increased in the HIV era as compared to previous eras<sup>[1]</sup>. Infection with HIV is associated with an increased risk for AIN in all infected persons, and the risk appears especially high in MSM populations. In the previously mentioned study by Holly *et al*<sup>[13]</sup> regarding risks of AIN in women, 26% and 6% of HIV positive women had any type of AIN or HSIL, respectively. Similarly, the aforementioned meta-analysis revealed an HSIL pooled prevalence of 29.1%, and found an anal cancer rate of 45.9 per 100000 in HIV positive MSM<sup>[12]</sup>.

This pattern of elevated risk for AIN in the HIV positive population has been demonstrated in several studies<sup>[14-16]</sup>, and is also seen in studies evaluating anal cancer. A 2012 study by Silverberg *et al*<sup>[17]</sup> reported rates of anal cancer in HIV+ MSM of 135 per 100000, HIV+ non-MSM of 45 per 100000, and HIV negative non-MSM with a rate of 2 per 100000. The latter figure reflects the risk of anal cancer in the general population. The same study also found anal cancer rates of 30 per 100000 for HIV positive women; there were no cases of anal cancer in the HIV negative women.

The reason that high-risk sexual behavior and HIV

infection is associated with an increased risk for AIN is likely due to the fact that both are associated with infection with HPV, inability to clear HPV infection, and for simultaneous infections with multiple strains of HPV<sup>[18]</sup>.

### HPV

The HPV family includes double-stranded DNA viruses that infect mucosal and cutaneous epithelia and induce cellular proliferation<sup>[19]</sup>. HPV is extraordinarily common, with most sources estimating a prevalence of 45% in the general population, with 75% of the general population acquiring an HPV infection at some point in their lifetime<sup>[20,21]</sup>. HPV has been shown to be causally associated with anogenital neoplasia, including AIN and anal cancer<sup>[22,23]</sup>. The association of HPV with anal cancer led to the realization that anal cancer shares features with genitourinary tract malignancies, such as cervical, vaginal and penile cancer, which are also SCCs closely linked to HPV infection<sup>[24,25]</sup>. It has been appreciated that the histologic transition zone in the anal canal, as in the cervix, is the most common site of the histopathologic changes associated with HPV infection<sup>[26]</sup>. The anus and cervix also share embryological origins and susceptibility to HPV infection, which might also explain the similarities between these malignancies.

Identification of a common disease pathway and causative agent prompted the adoption of the same pathological terminology across anogenital cancers as discussed above. The terms "LSIL" and "HSIL" are felt to be the most appropriate as they describe the histologic changes seen with transient vs chronic changes related to HPV infection; chronic HPV infection is the condition associated with anogenital cancer<sup>[27]</sup>.

### Other risk factors

A number of other risk factors for AIN have been described, though none appear to be as strongly associated with AIN as HIV and/or HPV infection and high risk sexual behavior. When considering an individual's risk for AIN and anal SCC, a personal history of CIN and gynecological cancers should be sought by clinicians, since a history of genital neoplasia is a risk factor for anal neoplasia.

Tobacco smoking has been consistently implicated as a risk for AIN<sup>[28,29]</sup>, despite a lack of clear understanding of the mechanism(s) involved. A recent study by Gautier *et al*<sup>[30]</sup> found that AIN regression after therapy failed to occur in any smoking patient ( $n = 14$ ; 30%), while in nonsmoking patients AIN regressed in  $n = 29$  (63%).

The presence of HPV-related dysplasia in other anatomical site in an individual is a well-established risk factor for AIN since the development of an HPV-related malignancy implies chronic infection with an oncogenic HPV strain, thus increasing the risk for other HPV-related malignancies<sup>[31]</sup>. Lastly, chronic immunosuppression has been implicated as a risk factor for the development

**Table 3** Progression rates of anal intraepithelial neoplasia to squamous cell carcinoma

Progression	No. patients	Rate of progression	Median or average progression time	Ref.
AIN II/III to SCC	72	11%	42 mo	[33]
AIN III to SCC	35	8.6%	53 mo	[34]
AIN I to AIN III	199	12.6% (8.1/100 person-years)	18 mo	[35]
ASCUS/AIN I to AIN II/III	556	24.5% (10.5/100 person-years)	36 mo	[36]
HSIL to SCC	138	19.6%	57 mo. w/prevalent HSIL; 64 mo. w/incident HSIL	[37]

ASCUS: Atypical squamous cells of undetermined significance; LSIL: Low-grade squamous intraepithelial lesion; HSIL: High-grade squamous intraepithelial lesion; AIN: Anal intraepithelial neoplasia; SCC: Squamous cell carcinoma.

of AIN and for the progression of AIN to cancer, presumably due to the increased HPV burden related to reduced viral clearance. This risk is best described in the post-transplant population<sup>[32]</sup>, but more data is needed to clarify the risks in other populations, particularly with the rapid development and employment of new biologic and other immunomodulatory therapies<sup>[30]</sup>.

## PROGRESSION TO ANAL CANCER

AIN is assumed to be the precursor lesion to anal cancer, in that some cases of LSIL progress to HSIL, and then to SCC. However, the rate and risk factors associated with AIN progression, as well as the factors associated with regression, are poorly characterized. Several trials support the concept that AIN progresses to SCC, with one trial reporting rates of progression of AINII/III (HSIL) to SCC of 11% over a median period of 42 mo in a cohort of 72 patients enrolled in an AIN surveillance program. Approximately one third of this population experienced a decrease in stage or regression of disease<sup>[33]</sup>. Another trial reported progression to SCC in 3 out of 35 (8.6%) of patients with AIN III followed for 53 mo; it is noteworthy that all three who progressed were being treated with systemic steroids for extended periods of time<sup>[34]</sup>.

Among high risk patients, Tong *et al.*<sup>[35]</sup> reported a progression rate from AIN I to AIN III in 25 of 199 (12.6%) male patients in an anal cancer screening clinic, equivalent to a rate of 8.1 per 100 person-years. HIV positive patients were at the greatest risk for progression, with a hazard ratio (HR) of 2.8 for AIN I to III progression. Interestingly, CD4 count was not a significant factor affecting progression rates. This study also reported spontaneous regression rates of AIN III, with 26 of 55 (47%) regressing from AIN III, equivalent to an incidence of 68.9 per 100 person-years. Among the 26 patients who spontaneously regressed, 11 (42%)

regressed to AIN II, 11 (42%) regressed to AIN I, and four (15%) regressed to no disease (negative biopsies). Similar progression rates were described by Burgos *et al.*<sup>[36]</sup> with progression occurring at 10.5/100 person-years among 556 HIV infected men followed for 649 person-years. This trial found that being on highly-active anti-retroviral therapy or in a stable personal relationship with another individual were protective, with progression rates of 2.8/100 person-years in these sub groups, while infection with HPV strains 16 or 18 were independent risk factors for progression. An additional retrospective study of patients with anal cancer found that 19.6% of individuals ( $n = 27/138$ ) had previously documented HSIL, with an average time from HSIL to cancer of 57 mo in prevalent HSIL, and 64 mo for incident HSIL<sup>[37]</sup>. Taken together, these studies suggest that while AIN can progress to SCC, the overall rates of progression are relatively low, are highest in high-risk populations, and that spontaneous regression from HSIL to LSIL and LSIL to normal will occur in some individuals (Table 3).

## HPV VACCINATION

Infection with HPV is now recognized to be responsible for nearly all cervical cancers, 95% of anal cancers, 65% of vaginal cancers, 50% of vulvar cancers, and 35% of penile cancers<sup>[38,39]</sup>, as well as a significant portion of head and neck cancers<sup>[40]</sup>. There are over 100 types of HPV that infect humans, with approximately 50% infecting the anogenital tract. Some of these subtypes are commonly found in anogenital cancers, whereas others never are, leading to the terminology of "high-risk", "intermediate-risk" and "low-risk" strains of HPV<sup>[19]</sup>. HPV types 16 and 18, and to a lesser extent 6 and 11, are the primary oncogenic strains found in cervical and anal cancer. These HPV strains are thought to drive oncogenesis primarily by inducing p53 degradation and upregulation of Rb, resulting in cellular proliferation<sup>[41,42]</sup>. The discovery of these strains led to the creation of vaccines targeting them. The first vaccine was designed against HPV types 16 and 18, and was soon followed by the "quadrivalent" vaccine targeting HPV types 6, 11, 16, and 18.

The quadrivalent vaccine was initially approved for the prevention of cervical cancer, but has since been shown to be efficacious in reducing rates of AIN. A recent study demonstrated 75% reductions in LSIL and HSIL in a population of 602 HIV negative MSM, with rates of persistent HPV infection reduced by 95%<sup>[43]</sup>. Vaccination also appears to be effective in preventing recurrent high grade AIN when administered after the diagnosis and treatment of high-grade AIN in HIV negative MSM. One study reported decreased rates of recurrent HSIL and anal cancer when vaccination was administered after diagnosis of HSIL, with 12/88 (13.6%) vaccinated patients and 35/114 (30.7%) unvaccinated patients developing recurrent high grade AIN during 340.4 person-years follow up<sup>[44]</sup>. Markov

modeling has demonstrated that vaccination with the quadrivalent vaccine for this indication was cost effective<sup>[45]</sup>.

More recently, a “nonavalent” vaccine has been developed, adding protection against HPV types 31, 33, 45, 52, and 58 to the previous four types, with rates of cervical and vulvar disease from these additional strains reduced from 1.6 per 1000 person-years in those receiving the quadrivalent vaccine to 0.1 per 1000 person-years in those receiving the nonavalent vaccine<sup>[46]</sup>. These trials provide strong evidence that HPV vaccination is effective at preventing progression of AIN and cancer, thus clinicians should be knowledgeable about HPV vaccination and advise their patients, particularly those with AIN or at high risk for AIN/anal cancer, to be vaccinated. Given the well-established similarities between cervical and anal HPV-related diseases, the CDC has recommended HPV vaccination for children of both genders to be given at age 11 or 12, and to men and women at high risk for AIN or cervical intraepithelial neoplasia (CIN), or anyone not previously vaccinated up to 26 years of age<sup>[47]</sup>.

## DIAGNOSIS

As discussed, anal and cervical cancer demonstrate similarities in tumor biology, and given the success of cervical cancer screening programs, a similar anal cancer screening program would seem reasonable<sup>[48]</sup>. Anal cancer is often detected in advanced stages, with local-regional spread, a 20%-40% rate of lymph node involvement, and a 10% rate of metastatic disease frequently present at the time of diagnosis based on SEER data<sup>[49]</sup>. Additionally, changes to colorectal cancer screening (CRC) guidelines in 1997 which eliminated digital rectal examination (DRE) as an appropriate screening test for CRC, along with the de-emphasis of DRE for prostate cancer screening since 2009, has possibly contributed to delayed anal cancer diagnosis<sup>[50]</sup>. Anorectal symptoms are commonly seen in individuals presenting to gastroenterologists<sup>[51]</sup>, thus an understanding of diagnosing AIN is important for gastroenterologists and other clinicians, particularly as many patients with AIN are symptomatic at presentation<sup>[33]</sup>.

### Anal cytology

Anal cytology is currently one mode of screening for AIN. The technique of anal cytology consists of inserting a water-moistened polyester fiber swab into the rectum until encountering the rectal wall, then removing the swab with a twisting motion while applying lateral pressure, allowing for sampling of the transitional zone and anal canal. The swab is then processed using a liquid cytology technique prior to Papanicolaou staining as with cervical specimens, and then analyzed by a pathologist (Figure 1). Sampling can be performed by either the clinician or the patient; the sensitivity has been shown to be slightly higher when the clinician

performs the procedure, though compliance may be improved when the patient performs the sampling<sup>[45]</sup>. Screening the general population with anal cytology has not been studied and is not currently recommended.

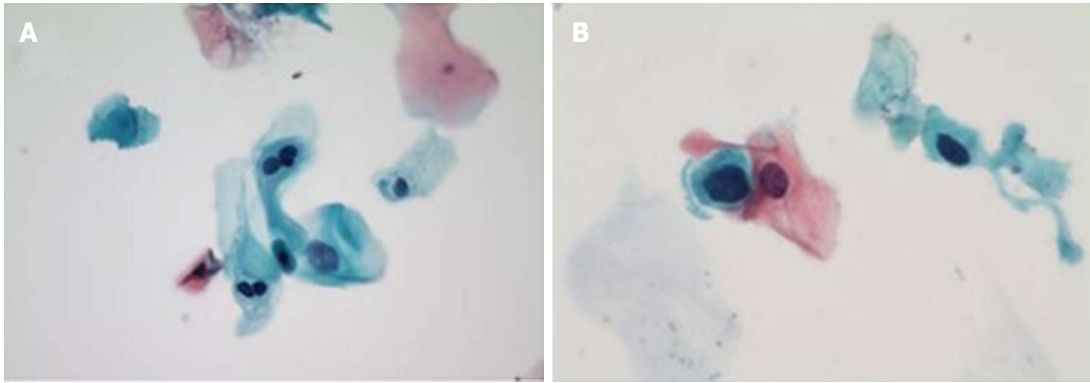
In studies of HIV negative MSM, the sensitivity of anal cytology reported in the literature varies, ranging from 47%-70% for the detection of AIN of any grade<sup>[52,53]</sup>. Clinicians need to be aware that while HSIL on anal cytology correlates well with high-grade AIN (*i.e.*, AIN II or III) obtained with a biopsy, findings of ASCUS and LSIL have been shown to have near equal distribution for low grade and high grade AIN on subsequent histological examination of a tissue biopsy. For this reason, it is recommended that direct anal examination and tissue biopsy be performed following any abnormal anal cytology result<sup>[54]</sup>. Finally, given the success of HPV testing in cervical cancer screening programs using PCR of cytology specimens<sup>[55]</sup>, HPV molecular testing on anal cytology specimens may provide improved diagnostic sensitivity. Initial studies comparing HPV testing to anal cytology have demonstrated equivalent sensitivity, but thus far combining cytology with HPV testing has not improved overall sensitivity, thus the roll of HPV molecular testing in the diagnosis of AIN is still under investigation<sup>[56]</sup>.

### High resolution anoscopy

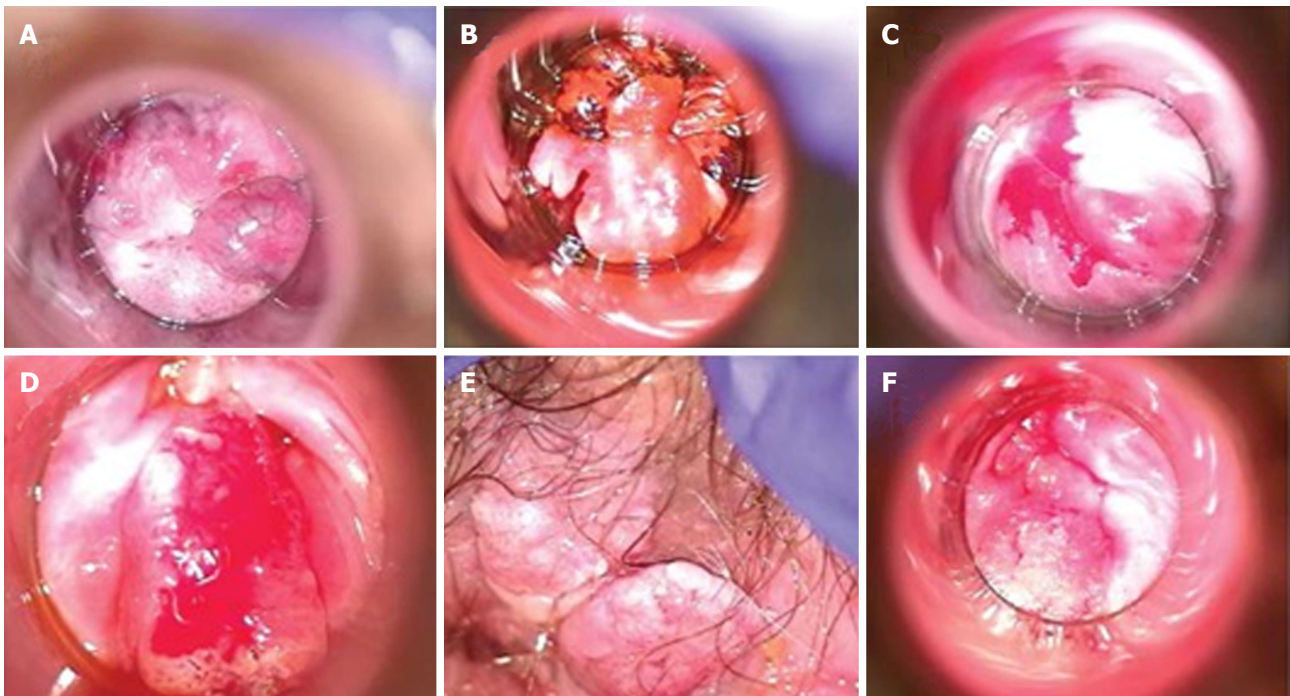
If abnormal cytology is detected with an anal cytological exam, the next step in management of AIN is HRA to attempt to localize the source of atypical cells. High resolution anoscopy (HRA) consists of examining the squamocolumnar junction, anal canal, and perianal skin under magnification using a colposcope in a procedure that is very similar to colposcopy of the cervix. During anoscopy, an anoscope is placed into the anus with lidocaine lubrication, and then a swab soaked in 3%-5% acetic acid solution is inserted into the anal canal while the anoscope is removed for several minutes. After acetic acid application, which causes an “acetowhite change” in areas of abnormal transitional epithelium, the mucosa is carefully inspected for changes characteristic of AIN, including flat or slightly raised areas of thickened mucosa with or without vascular pattern abnormalities<sup>[57]</sup>. Lugol’s iodine is then applied in similar fashion, but in this case concerning lesions fail to stain with iodine (“Lugol’s negative”) because iodine is glycophilic and dysplastic tissues lack glycogen and appear thick mustard colored (Figure 2). Any suspicious lesions, including condylomas, atypical surface configurations, punctuations, mosaicism, or atypical vessels, are then biopsied under direct visualization<sup>[58]</sup>. Areas with color changes seen on acetic acid staining that are subsequently found to be Lugol’s negative are highly suspicious for dysplasia, and are biopsied under direct visualization during HRA. Examples of H&E stained high-grade AIN lesions obtained from biopsies are shown in Figure 3.

The appearance of lesions under HRA with acetic acid staining is similar to those seen in cervical dysplasia





**Figure 1 Cytology of anal intraepithelial lesions.** A: LSIL, with representative binucleate hyperchromatic cells (koilocytes) and nuclear enlargement (Papanicolaou stain, original magnification  $\times 400$ ); B: HSIL, with representative markedly increased nuclear to cytoplasmic ratio as compared to LSIL at left (Papanicolaou stain, oil immersion, original magnification  $\times 1000$ ). Reproduced with permission<sup>[85]</sup>. LSIL: Low grade squamous intraepithelial lesions; HSIL: High grade squamous intraepithelial lesions.



**Figure 2 High-resolution anoscopy of representative examples of anal intraepithelial lesions.** A: Low grade AIN lesion after acetic acid application with representative acetowhitening; B: Low grade AIN lesion after application of Lugol's iodine with brown area representing normal uptake by glycogenated cells, and "mustard" colored area representing negative uptake and suggestive of dysplasia; C: High grade AIN seen after application of acetic acid and the dense acetowhite change; D: High grade AIN with concern for invasion; E: External/perianal high grade AIN after application of acetic acid; F: High grade AIN with concern for invasion. Reproduced with permission<sup>[85]</sup>. AIN: Anal intraepithelial neoplasia.

(Figure 2)<sup>[59]</sup>. HRA is considered superior to standard anoscopy as shown by Camus *et al.*<sup>[60]</sup> who reported that in a population of 102 patients [68% male; 57.3% HIV positive; mean 1.6 lesions (standard deviation 0.8) per patient] only 38.7% (65/168) of all lesions seen using HRA were visible with standard anoscopy<sup>[60]</sup>.

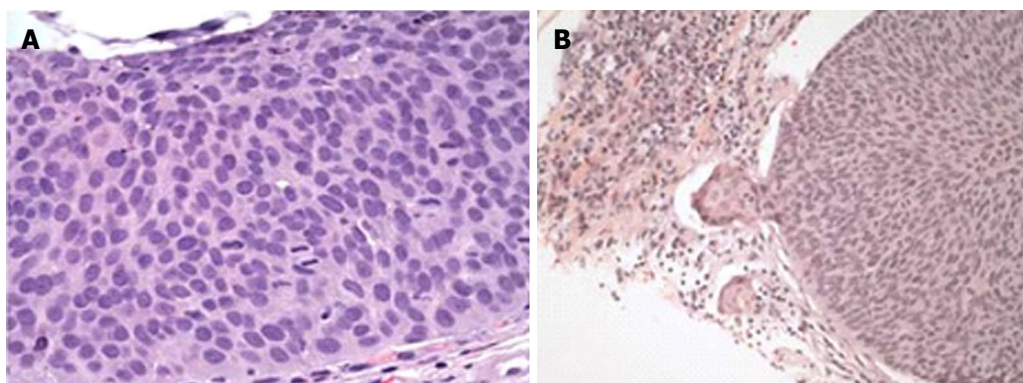
In addition to detection of AIN, HRA also facilitates the application of therapies targeting AIN, which are discussed in more detail below. Although HRA is generally considered safe for patients and not difficult for clinicians to perform, substantial training time is required in order to recognize anal lesions, which can

be subtle in appearance. Due to the limited number of patients with atypical findings associated with AIN in the general population, HRA is ideally performed at centers specializing in its use rather than at clinics lacking trained experts<sup>[61]</sup>.

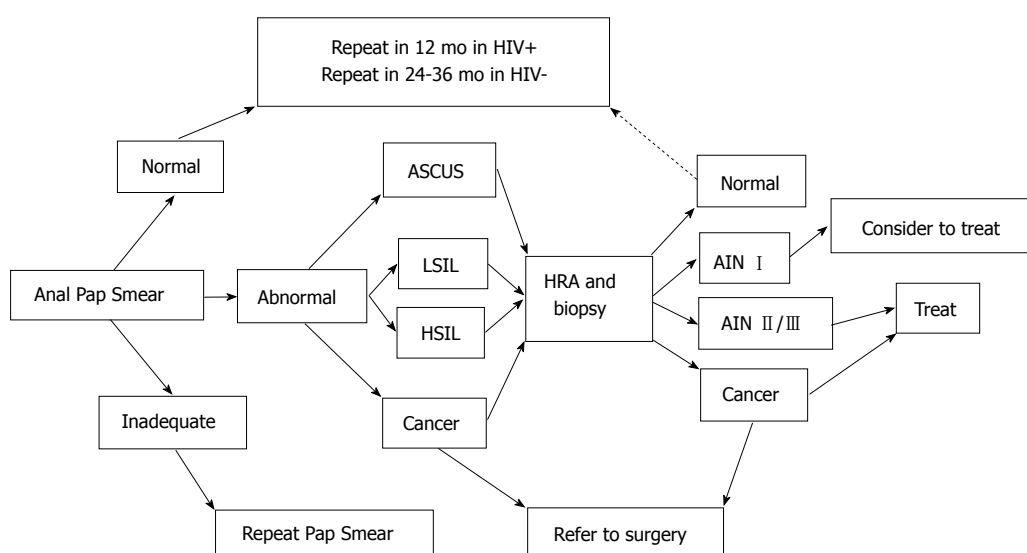
## SCREENING FOR AIN

The combination of anal cytology, possibly paired with anal HPV molecular testing, followed by HRA in individuals with positive results, represents a reasonable strategy to screen for AIN. However, at this time there





**Figure 3** Histologic examples of high grade anal intraepithelial neoplasia, hematoxylin and eosin stain. A: High grade AIN as demonstrated by nuclear pleomorphism, numerous mitoses and no maturation of the epithelium (original magnification  $\times 400$ ); B: Microinvasion of a high grade AIN demonstrated by a budding off of atypical cells with paradoxical maturation and a marked inflammatory response (original magnification  $\times 200$ ). Reproduced with permission<sup>[85]</sup>. AIN: Anal intraepithelial neoplasia.



**Figure 4** Algorithm for diagnosis, treatment and surveillance of anal intraepithelial neoplasia. Adapted from Palefsky and Rubin, 2009<sup>[86]</sup>. ASCUS: Atypical squamous cells of undetermined significance; LSIL: Low-grade squamous intraepithelial lesion; HSIL: High-grade squamous intraepithelial lesion; AIN: Anal intraepithelial neoplasia.

are no randomized clinical trials demonstrating the value of screening at risk populations or the general population. A randomized trial evaluating the effect of screening for AIN on anal cancer incidence and/or mortality would presumably be difficult given the low incidence of anal cancer, as the number of patients and duration of follow up necessary would likely be prohibitive. The rationale for AIN screening instead relies on the cervical cancer data given the similarities between the two malignancies, as well as the identification of certain high-risk populations that might benefit from screening. As mentioned previously, these at-risk populations with elevated rates of anal cancer development include HIV-positive individuals, MSM, women with a prior history of HPV related neoplasia (cervical, vulvar, etc.), and individuals with a history of solid organ transplants (Table 2)<sup>[62,63]</sup>. Notably, the incidence of anal cancer in HIV+ MSM is higher than the rate of colorectal cancer in the general population,

providing some support for screening this at-risk group for anal cancer. One possible screening algorithm for high-risk populations, *e.g.*, HIV+ MSM, is shown in Figure 4.

In a study analyzing cost, Goldie *et al.*<sup>[64]</sup> reported that screening for AIN in HIV+ MSM would be cost effective, with a trend towards cost effectiveness in post-transplant patients. Despite the elevated risk for anal cancer in these populations, the CDC, the American Society of Colon and Rectal surgeons, and most other organizations do not recommend screening high-risk groups, whereas the HIV Medicine Association of the Infectious Disease Society of America does recommend screening in the HIV+ MSM population<sup>[65]</sup>.

### Screening in the inflammatory bowel disease population

Another patient population that could conceivably benefit from AIN screening includes individuals diagnosed with inflammatory bowel disease (IBD),

particularly those taking potent immunosuppressive medications for their disease, given the generally accepted mechanism of chronic immunosuppression as a risk factor for anal cancer<sup>[34,66]</sup>. Research describing the risk of AIN in patients with IBD is limited; most of the literature is confined to case reports and case series. In one study, Ruel *et al*<sup>[67]</sup> performed a single-center retrospective review to assess rates of HPV infection and AIN in their cohort of IBD patients. They reported six cases of anal SCC, nine cases of HSIL and two cases of LSIL among their population, and demonstrated concomitant infection with HPV in 50% of SCC cases and all of the HSIL/LSIL cases. Of the six cases of SCC, one patient was on immunomodulatory therapy, defined only as "azathioprine/6-MP/methotrexate", two were not taking any immunosuppressive medications, and the immunosuppressive medications of the other three was reported as "unknown". Although this study suggests that individuals with IBD can develop AIN, the report is limited by the fact that the size of the IBD population was not noted, thus the prevalence rates of AIN cannot be determined.

Additional studies have suggested increased rates of HPV infection in IBD patients, particularly in those individuals who were taking immunosuppressive medications<sup>[68]</sup>. In an attempt to determine the prevalence of AIN in IBD patients, Shah *et al*<sup>[69]</sup> performed a cross-sectional study to determine the prevalence of AIN in 270 patients, including 100 with IBD on immunosuppression, 94 with IBD not on immunosuppression, and 76 healthy controls. This group found a non-statistically significant trend towards a higher prevalence of ASCUS in individuals with IBD regardless of immunosuppressive status compared to healthy controls ( $P = 0.10$ ), but no difference in IBD patients on vs off immunosuppressive medications ( $P = 0.90$ ). No patients in this study were diagnosed with LSIL, HSIL or SCC, which would suggest there is no increased risk of these conditions, at least in this relatively small population of IBD patients. Based on the lack of convincing data available, experts do not advocate screening patients with IBD for AIN on a routine basis, but it would be reasonable to consider HPV vaccination in this group.

## MANAGEMENT OF AIN

Because AIN and early anal cancer remain relatively rare conditions and require a level of expertise to diagnose and treat, it is recommended that individuals found to have positive anal cytology be referred to expert centers for HRA as well as any associated treatment. Additionally, because AIN can be misdiagnosed, and can progress to cancer in some yet regress in others, expert centers are best equipped to determine which individuals should be treated and what treatment modalities should be considered, particularly as randomized trials comparing treatment modalities are lacking<sup>[70]</sup>.

### Topical therapy

Topical therapy consists of direct application of a medication to either a specific lesion or to the entire anal canal. Medications, which can be applied in some cases by the patient, include trichloroacetic acid (TCA), 5-fluorouracil, or the immune modulator imiquimod. TCA is generally well tolerated, can be applied relatively simply without requiring specialized equipment, and is efficacious, with only 1-2 treatments necessary for most patients. TCA can be reapplied during further courses of treatment if necessary. Two retrospective studies of small populations of biopsy-confirmed high-grade AIN lesions reported rates of HSIL regressing to LSIL or complete resolution in 71%-79% of cases<sup>[71,72]</sup>. In a separate prospective pilot study, 46 patients with AIN were treated with 5-fluorouracil, with 39% having complete clearance, 17% experiencing a partial response, and 37% having no response. However, 50% of the complete responders had recurrence of AIN at 6 mo<sup>[73]</sup>.

Imiquimod therapy was evaluated in a double-blind, randomized controlled trial of 53 HIV+ MSM, with 28 patients on active drug and 25 patients on placebo. Of the 28 individuals on active drug therapy, 43% experienced either resolution or downgrading of their lesion, and 61% of imiquimod responders achieved sustained response at 36 mo<sup>[74]</sup>. Taken together, topical therapy appears to be generally well tolerated and has reasonable efficacy, although a substantial portion of patients will not respond and others will recur. For these reasons, topical therapy may best be utilized as an adjunct to local ablative therapy.

### Local ablative therapy

Local ablative therapy consists of targeted destructive therapy, most commonly radiofrequency ablation (RFA) or electrocautery, applied to anal lesions during an HRA examination. Electrocautery therapy has been studied more extensively than RFA. Chang *et al*<sup>[75]</sup> reported a somewhat favorable response to electrocautery therapy for high grade AIN, with 0% ( $n = 0/8$ ) of HIV negative patients having recurrence of disease, although 79% ( $n = 23/29$ ) of HIV positive patients experienced disease recurrence during a two-year follow up period. A more recent observational study of 83 HIV+ MSM with high grade AIN treated with electrocautery found that 32.5% ( $n = 27/83$ ) experienced complete response, 33.7% ( $n = 28/83$ ) partial response, and 33.7% ( $n = 28/83$ ) no response. Increased success was seen in those patients treated with two to four sessions compared to those treated with only one session. Similar to prior studies, the authors noted a recurrence rate of 25% in the responders after a median of 30 mo<sup>[76]</sup>.

RFA applied to the anal mucosa to treat AIN has been shown to be safe and tolerable, but evidence of treatment efficacy is limited at this time<sup>[77]</sup>. One retrospective clinical study of  $n = 74$  biopsy-proven high-grade AIN lesions in a population of 68 HIV+ MSM

treated with RFA found 64% ( $n = 47/74$ ) had resolution or downgrading to low-grade AIN at 140 d follow up<sup>[78]</sup>. Similar results were demonstrated by Goldstone *et al.*<sup>[79]</sup> who evaluated long term follow up of high grade AIN lesions treated by RFA in HIV- and HIV+ MSM. They reported 62% of HIV- MSM had recurrence during a mean time of 14 mo, while disease recurred in 91% of HIV+ MSM during a mean of follow up of 17 mo. Both electrocautery and RFA therapy are associated with minimal morbidity, particularly with a greater number of treatment sessions, but both are reasonably well tolerated<sup>[80]</sup>.

### Surgical treatment

With the wide availability of the local and targeted therapies discussed above, surgical therapy is largely historic in regards to AIN, although still a mainstay of therapy for anal cancer, which is beyond the scope of this review. Surgery for AIN is associated with significant morbidity, often requiring large excision of healthy tissue, occasionally necessitating rectal diversion, and yet is associated with a recurrence rate of 9%–63%<sup>[80,81]</sup>. As recurrence of AIN is presumably mediated by ongoing exposure to predisposing risk factors, notably ongoing HPV infection, and given the improvement in local therapy outcomes, surgical excision for AIN is not recommended as a routine treatment option.

### Surveillance of AIN

As discussed above, the recurrence rates of AIN are significant, particularly with high grade AIN, thus post treatment surveillance is essential. However, surveillance programs vary, due to uncertainties regarding the most appropriate surveillance interval as well as the best surveillance modality (*i.e.*, cytology alone, routine HRA, *etc.*). To investigate the potential role of surveillance, Crawshaw *et al.*<sup>[82]</sup> retrospectively reviewed 424 patients with biopsy proven AIN who were treated with topical or ablative therapy and then enrolled in a surveillance program. All patients underwent annual anal cytology and DRE, while 220 also received serial HRA examinations (the remaining 204 only underwent HRA if cytology or DRE were positive.) Overall, the five-year anal cancer rate was 6% for the expectant management group and 4.5% for the group also undergoing serial HRA, which was not significantly different ( $P = 0.37$ ). However, it is difficult to form conclusions based on differences in surveillance modalities because progression was rare overall: Only two patients in the expectant management group and one in the HRA group developed anal cancer, and all three of these patients were considered to be non-compliant with the recommended surveillance plan<sup>[82]</sup>. The authors of this study proposed that for highly compliant patients, active surveillance of AIN is effective regardless of the method, and that compliance with the recommended program is the most important factor in reducing progression or recurrence of AIN.

Given the limited data regarding the benefits of AIN surveillance, further research, ideally prospective

randomized trials, is needed. Currently, an active clinical study called the Anal Cancer HSIL Outcomes Research (ANCHOR) trial is enrolling patients across 12 United States sites. This trial aims to determine whether screening and treatment of HSIL is effective in reducing subsequent anal cancer in HIV+ men and women compared with active monitoring *via* regular exams (including anal cytology combined with HRA and biopsy of any concerning lesions) vs observation. Treatments can include imiquimod, fluorouracil, electrocautery, and laser therapy, and should provide further insight into the safety of these treatments<sup>[83]</sup>.

## CONCLUSIONS AND FUTURE

### DIRECTIONS

Anal intraepithelial neoplasia is the precursor lesion to squamous cell cancer of the anus. Although it is accepted that AIN progresses to SCC in a subset of patients, the actual risk of progression remains unclear. It has been shown, however, that the progression risk is elevated in certain high-risk groups, including: (1) those with persistent infection with high-risk HPV strains; (2) HIV-positive individuals, especially those with low CD4 counts; (3) MSM; and (4) individuals with a history of HPV-mediated genital cancers (particularly cervical cancer). Individuals in these groups will likely benefit from enrollment into formal AIN screening programs.

AIN can be challenging to diagnose and manage, thus referral to expert centers with the capability of interpreting cytology and pathology, performing HRA, and treating AIN is essential. The optimal treatment modalities and intervals have not been conclusively determined at this time, and recommendations are primarily based on expert consensus and driven by local expertise. Regardless of treatment modality, the recurrence rates of high-grade AIN remain high, and ongoing surveillance is recommended in patients with history of AIN.

Novel imaging technologies may identify high risk lesions without the need for tissue biopsy. Confocal laser microscopy, which has been shown to be at least as effective as tissue biopsy for detection of superficial esophageal squamous cell cancer, might be effective for the detection and grading of AIN<sup>[84–86]</sup>. Further studies are necessary to define the role and efficacy of confocal laser microscopy in AIN management.

In summary, AIN is a clinically important lesion that is frequently underappreciated by many clinicians. Although anal cancer remains relatively uncommon, the incidence of this malignancy is increasing. With the availability of effective HPV vaccines, it is important for clinicians to be aware of AIN, particularly in high-risk groups who might benefit most from vaccination.

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## Circular RNAs in colorectal cancer: Possible roles in regulation of cancer cells

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### Abstract

Colorectal cancer (CRC) is the third most commonly

diagnosed cancer in the world and the fourth principal cause of cancer deaths worldwide. Currently, there is a lack of low cost and noninvasive screening tests for CRC, becoming a serious health problem. In this context, a potential biomarker for the early detection of CRC has recently gained attention. Circular RNAs (circRNA), a re-discovered, abundant RNA specie, is a type of noncoding covalent closed RNAs formed from both exonic and intronic sequences. These circular molecules are widely expressed in cells, exceeding the abundance of the traditional linear mRNA transcript. They can regulate gene expression, acting as real sponges for miRNAs and also regulate alternative splicing or act as transcriptional factors and inclusive encoding for proteins. However, little is known about circRNA and its relationship with CRC. In this review, we focus on the biogenesis, function and role of these circRNAs in relation to CRC, including their potential as a new biomarker.

**Key words:** Circular RNA; Colorectal cancer; Gene regulation; CircRNA; Non-coding RNAs; Long non-coding RNA; Circularization

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**Core tip:** Circular RNAs (circRNAs) are noncoding RNAs, characterized for its circularized shape. These circRNAs are abundant and might play important roles in cancer. In particular, they exhibit altered expression in colorectal cancer, and its activity as miRNA sponge might be involved in the control of cancer progression. Moreover, owing to their stability, could serve as diagnostic or predictive biomarkers for colorectal cancer.

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## INTRODUCTION

Colorectal cancer (CRC) is the third most commonly diagnosed cancer in the world (1.4 million in 2012), and the fourth principal cause of cancer deaths worldwide (694000 deaths for both sexes)<sup>[1,2]</sup>. In terms of incidence, it represents almost 10% of global cancer diagnoses, and is the third most common cancer in males and second in females (746000 and 614000 respectively), with differences between developed and developing countries<sup>[2,3]</sup>. More diagnoses occur in developed regions, but less developed regions have higher death rates, reflecting a poorer rate of survival in these countries<sup>[1,3]</sup>. Sixty-five percent of the new cases occur in developed countries including the United States, Canada, Australia and Europe. In the United States in 2016, the American Cancer Society estimated over 134000 new diagnoses of CRC (95270 for colon, and 39220 for rectal cancer)<sup>[2,4]</sup>. Also in 2016, CRC is expected to cause over 49190 deaths in the United States<sup>[4]</sup>. Worldwide the mortality rates are lower in women than in men, except in the Caribbean. But this tendency seems less typical in countries with higher levels of human development<sup>[2]</sup>. In contrast, CRC incidence and mortality rates in Asia are continually increasing<sup>[5]</sup>. Besides Asia, in many regions of Europe and North America, the rates of CRC incidence and mortality have decreased for both men and women, due to new screening methods allowing for early diagnosis and treatment<sup>[4-6]</sup>. These screening tests can prevent the development of CRC, as some have the potential to detect polyps before they can progress into cancer. Not all polyps will progress into cancer tumors, but their removal can prevent the disease<sup>[6]</sup>.

There are several factors that contribute to the development of CRC, some of these are malleable, while others, such as genetic factors, personal medical history, age, racial and ethnic background, or pre-existing conditions (inflammatory bowel disease, Lynch syndrome, *MUTYH* associated polyposis and others) are not<sup>[6]</sup>. CRC is primarily considered a "lifestyle" disease because its incidence is high in countries with a sedentary population and high-fat diets from animal sources<sup>[2]</sup>. An association between diet, obesity and carcinogenesis is a very important issue as several studies have shown that obesity promotes inflammatory processes, triggering a cascade of critical mechanisms mediated by proinflammatory cytokines and tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ), resulting in CRC development<sup>[7-9]</sup>.

Three fundamental categories of genes have been identified in the carcinogenesis of CRC as follows: (1) *APC*, *DCC*, *TP53*, *SMAD2*, *SMAD4* and *p16INK4a* (tumor suppressor genes); (2) *K-ras* and *N-ras* (proto-oncogenes); and (3) *MMR* and *MUTYH* (DNA repair

genes). However, little is known about the role of circular RNAs (circRNAs) in the development of CRC. CircRNAs are a class of small group of competing endogenous RNAs (ceRNAs), in the family of the non-coding RNAs (ncRNAs) that function as a class of long non-coding RNA (lncRNA)<sup>[10-12]</sup>. These molecules have captured the interest of many in the scientific and medical communities, because hundreds of human genes are expressed in circRNA form<sup>[13-15]</sup>. CircRNA has several functionalities, including the ability to rearrange the order of genomic information, provide protection from exonucleases, and establish constraints on RNA folding<sup>[16]</sup>. CircRNAs can function as templates for viroid and viral replication, as intermediates in RNA processing reactions, and regulators of transcription in *cis*. Additionally, it is speculated that circRNAs serve as epigenetic microRNA (miRNA) sponges, negatively regulating miRNAs and thus contributing substantially to the ceRNAs network through RNA-binding protein (RBP) sequestering agents, or nuclear transcriptional regulators, which are frequent in cancer<sup>[16-19]</sup>. There exists evidence that most circRNAs in the eukaryotic cell are stable, cytoplasmic, lacking the 3' poly(A) tail and 5' end cap, arising from pre-mRNA back-splicing exons (downstream 3' splice donors are covalently linked to upstream 5' splice acceptors) and host noncoding transcripts generated from protein coding genes<sup>[10,12,20-22]</sup>. Another study has found evidence that circular isoforms of several human transcripts, exhibiting the non-orderly fashion in the splice junctions, are expressed in similar concentrations to the normal linear isoform<sup>[23]</sup>. They hypothesized that scrambled exons are a sign of local genomic rearrangements in cancer, and realized this hypothesis by polymerase chain reaction (PCR), where the exon scrambling detected in leukemia patients was also found in normal primary human cell and Hela cells, suggesting that most exon scrambling found in tumor samples are consequences of an active splicing process both in normal and malignant human cells types<sup>[23]</sup>. This new type of ncRNA can be secreted by cells to the extracellular environment, and can be identified and measured through non-invasive methods, such as stool, blood and other body fluid sampling, suggesting that circRNAs could serve as an effective and cost effective screening test for CRC<sup>[15]</sup>.

In this review, we discuss circRNAs, their biogenesis, possible functions, and implications in CRC.

## LITERATURE SEARCH

A systematic literature search was performed of the PubMed database, and the keywords "circRNA", "circRNA" and "cancer", "circRNA" and "colorectal cancer" as well other synonyms for circRNAs, with no limitations. The inclusion criteria were as follows: (1) reviews about CRC incidence, statistics, and features of this disease before October 2016; (2) initial studies about circRNAs, its biogenesis, biological functions



and techniques used for its identification; (3) studies showing the presence of circRNA in human and other species, detected using different approaches either mathematical, biochemical, biological and technological or a combination; (4) investigations showing the expression of circRNA in different types of biological and cell samples; (5) studies associating circRNA with biological functions and try to explain its biochemical pathway; (6) investigations relating circRNA and CRC; including past, present and future of the disease; and (7) studies associating circRNA with colorectal human samples or cells and discussing its potential use as biomarker for early diagnosis of the disease.

## PROPERTIES AND FUNCTIONS OF CIRC RNAS

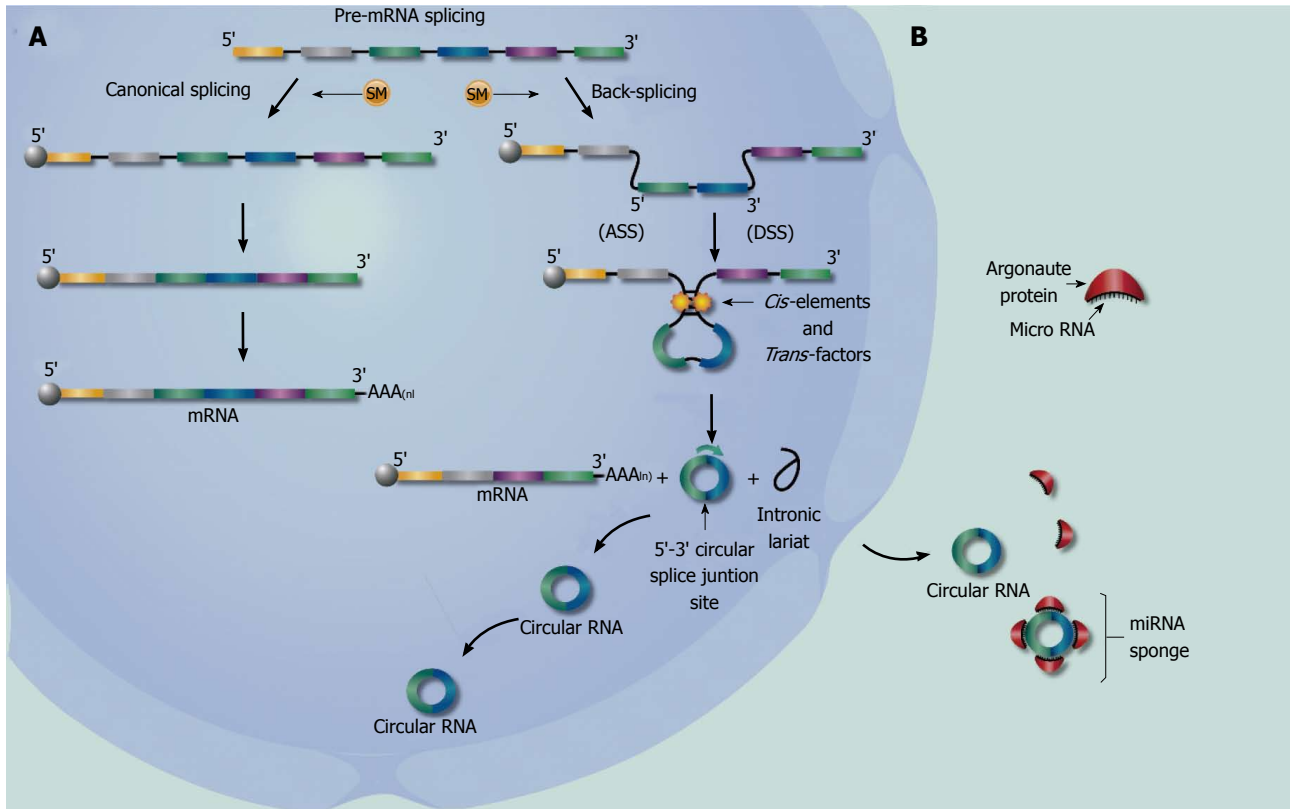
In the large family of non-protein-coding RNAs, circRNAs are a novel small group of ceRNAs, that could function as a new class of lncRNAs<sup>[10-12]</sup>. CircRNA is most often composed of exonic sequences containing one or more exons, in contrast to linear RNA which is generally formed by a covalently closed continuous loop containing a 5'-3' phosphodiester bond and without polyadenylation<sup>[10,12,24,25]</sup>. This new type of RNA is now known to be expressed in all tissues, at generally low levels but highly represented in the eukaryotic transcriptome<sup>[19,26]</sup>. Several studies have shown that the circular form of circRNAs gives this molecule a special role in gene expression, but their biological functions have yet to be determined<sup>[17,26-29]</sup>. In 2013 it was reported that circRNAs are a common, abundant and potentially developmentally regulated component of the gene expression programs in diverse animal species<sup>[25]</sup>. Salzman *et al.*<sup>[23]</sup> also demonstrated that circRNAs with scrambled exons compose a significant fraction of cellular RNA for many genes in cancer and normal human cells, suggesting also that the majority of scrambled exons detected in tumor samples were consequential of active splicing processes in both normal and malignant human cell types. Moreover, they subsequently reported that circRNA's relative abundance is regulated, in a gene and cell type specific manner, at levels comparable to those of canonical linear mRNA, and some of these circRNA species exhibited resistance to a 3'-5' exoribonuclease - RNase R<sup>[25]</sup>. Jeck *et al.*<sup>[12]</sup> demonstrated in the same year, that there exist up to 25000 different circRNAs in human cells, and in some cases are even considerably more abundant (> 10-fold) than associated linear transcripts. Although most circRNAs are expressed at low levels, some have proved to be more abundant than their linear counterparts<sup>[25]</sup>.

A distinctive property of circRNA is they differ structurally from other lncRNAs<sup>[19]</sup>. In order to create a linear RNA transcript with polarity 5' to 3', the eukaryotic cell uses spliceosomal machinery in conjunction with other biochemical process (5' capping and 3' polyadenylation) to create a canonical pre-mRNA,

removing introns and joint exons<sup>[10]</sup>. This splicing process is an ubiquitous feature of eukaryotic gene expression, and it is known that the Last Common Eukaryotic Ancestor was relatively intron-rich and had complex spliceosome machinery and splicing signals<sup>[21,22]</sup>. Conversely, most circRNAs in the eukaryotic cell are stable, cytoplasmic, lacking the 3' poly(A) tail and 5' end cap, come from pre-mRNA back-splicing exons and host noncoding transcripts generated from protein coding genes<sup>[10,12,20-22]</sup>. Guo *et al.*<sup>[11]</sup> examined the subcellular localization of circRNA, focusing on the 514 circRNAs detected in the K562 whole-cell samples, and found these circRNAs predominantly in the poly(A)-depleted cytoplasmic samples. Jeck *et al.*<sup>[12]</sup> demonstrated by FISH on Hs68 cells treated with Actinomycin D, against HIPK3; that circular forms of HIPK3 were preferentially localized in the cytoplasm as is consistent with prior studies of RNA circles. Apparently, endogenous circRNAs that are efficiently transported to the cytosol either undergo nuclear export or are released to the cytoplasm during mitosis, where they are extraordinarily stable<sup>[12,16,23,30]</sup>. This stability, or resistance, to debranching enzymes and RNA exonucleases<sup>[12]</sup> was also reported by Guo *et al.*<sup>[11]</sup>; they found that most circRNAs that span < 5 exons are RNase R-resistant. Jeck *et al.*<sup>[12]</sup> used a method described by Suzuki *et al.*<sup>[31]</sup> in 2006, using *E. coli* RNase R, which degrades linear RNAs with short 3' tails regardless of secondary structure, leaving circRNAs unaffected<sup>[12]</sup>. Salzman *et al.*<sup>[25]</sup> found that all the RNA species predicted to be circular are resistant to RNase R whereas all predicted linear sequences are highly sensitive to RNase R.

## BIOGENESIS AND REGULATION OF CIRC RNAS

CircRNAs are the product of a back-splicing process also catalyzed by canonical spliceosomal machinery, and it has been reported that this process is modulated by *cis*-regulatory elements and *trans*-acting factors, but it is still unclear under which conditions the spliceosomal machinery achieve discrimination between canonical splicing or back-splicing to give rise to a circRNA<sup>[10,16,19,20,26,32]</sup>. Ashwal-Fluss *et al.*<sup>[32]</sup> has provided evidence that circRNA are generated co-transcriptionally and their production rate is principally mediated by their flanking intronic sequences. They also demonstrated that canonical pre-mRNA splicing compete with circularization of exons, suggesting that a single pre-mRNA transcript can produce either a linear mRNA or a circular isoform (Figure 1A). Zhang *et al.*<sup>[33]</sup> indicated that the back-splicing process is generally coupled with canonical splicing, but process happens first depends on the specific spliceosomal machinery and still is under investigation. It is important to know that different types of circRNA molecules are generated by distinct mechanisms, independent of if they arise from intronic or exonic sequences<sup>[16,26]</sup>.



**Figure 1 Biogenesis and function of circular RNAs.** A: In the nucleus, a single pre-mRNA transcript can produce a linear mRNA and a circular isoform<sup>[32]</sup>. These two steps, canonical splicing and back-splicing are catalyzed by canonical spliceosomal machinery (Yellow circle, "SM"). In order to create a linear RNA transcript with polarity 5' to 3', the eukaryotic cell uses spliceosomal machinery SM together with other biochemical process (5' capping and 3' polyadenylation)<sup>[10]</sup>. Following, circular RNA is produced from a downstream 5'-DSS joined reversely to an upstream 3'-ASS, resulting in a covalently closed circRNA transcript with a 3', 5'-phosphodiester bond at the junction site (Figure 1)<sup>[16,22,26,33,34]</sup> and it seems to be modulated by *cis*-regulatory elements and *trans*-acting factors<sup>[10,16,19,20,26,32]</sup>. The final products of these processes are a linear mRNA, a circRNA, and an intron lariat; B: Endogenous circRNAs that are efficiently transported to the cytosol either undergo nuclear export or are released to the cytoplasm during mitosis, where they are extraordinarily stable<sup>[12,16,23,30]</sup>. In the cytoplasm circRNAs form a class of post-transcriptional regulators, acting as highly stable epigenetic miRNA sponges competing with the endogenous RNA network (sequestering miRNAs from binding mRNA targets), directly affecting the expression of any related gene<sup>[17,18,46]</sup>.

The process by which a pre-mRNA becomes a circRNA isoform begins with a start codon, and sometimes includes the canonical AUG of the associated linear transcript<sup>[12]</sup>. When the RNA Pol II recognizes the start codon, it transcribes the exonic circRNA; which is produced from a downstream 5'-DSS inversely joined to an upstream 3'-ASS, resulting in a covalently closed circRNA transcript with a 3', 5'-phosphodiester bond at the junction site<sup>[16,22,26,33,34]</sup>, or through 2'-5' linkages formed by a branch-point nucleophilic attack during splicing<sup>[35]</sup>. The specificity of this process still being studied, but is interesting to note that introns flanking mammalian circRNAs are longer than average<sup>[23]</sup>.

Two other mechanisms, direct back-splicing and lariat intermediate, have also been proposed as associated with circRNA formation from back-spliced exons<sup>[12,23]</sup>. This suggests that circRNA synthesis is evolutionarily dynamic, providing an alternative generation of circular transcripts<sup>[33]</sup>. Furthermore, some exons that generally are flanked by longer introns (upstream or downstream), result in a less efficient splicing process. Inverted repeat elements, such as ALU, can facilitate RNA circularization in humans<sup>[12,33,34]</sup>. Zhang *et al.*<sup>[33]</sup>

identified the phenomenon "Alternative Circularization" (AC), in which a number of multiple exon circularization events can be produced from a single gene loci. This novel process of splicing leads to formation of multiple circRNAs with different expression levels, and it has been associated with diverse biologic processes<sup>[12,36,37]</sup>. In some events, AC leads to circRNAs retaining introns, adding an additional layer of complexity to the circRNA transcriptome<sup>[22,25,26]</sup>. It has been suggested that the formation of these isoforms is derived from exon-skipping events. In these events, an exon-containing lariat is formed, which can then be internally spliced to create an exon circle<sup>[12,38]</sup>.

The back-splicing process appears to be regulated by the ratio of circular to linear transcripts, and the relative abundance of differentially spliced circular isoforms is cell-type specific<sup>[22,25]</sup>. In general, the efficiency of the back-splicing process seems to be much lower than canonical splicing, in steady-state levels of circRNAs; but this efficiency is dependent on the presence of canonical splice sites flanking the exons<sup>[20,26,32,33]</sup>. Besides, flanking intronic sequences are the main factor determining circularization efficiency of a given exon, and this

efficiency is related to the size of the intronic sequence. For example; circRNA flanked by longer introns are less efficiently spliced than other circRNA flanked by smaller introns<sup>[32]</sup>. Moreover, this circularization appears to have a negative effect on the splicing efficiency in the linear isoform and therefore on gene expression<sup>[32]</sup>. To overcome this inconvenience, most circRNAs have regulatory elements which reside in the flanking introns of circularized exons to continue with the back-splicing process and facilitate circRNA formation<sup>[10,26]</sup>. It has been shown that RBPs can regulate and promote circularization, after being recognized by regulatory elements such as short *cis* elements<sup>[26,32]</sup>.

Another way to facilitate the processing of circRNAs is by RNA base pairing. Complementary short repeat elements, such as inverted ALU repeats, in the flanking introns are used to circularize some intervening exons. This mechanism suggests that the promotion of back-splicing requires for exons to collaborate with intronic repeats and join the back splice sites, thereby facilitating catalysis through base pairing<sup>[12,39]</sup>. However, in the case of base pairing, the simple presence of these inverted repeats does not necessarily mean that a circRNA will be produced with similar efficiency in all cell types<sup>[39]</sup>. It is suggested that RBPs, base pairing, and any splicing factor (*cis* regulatory elements and *trans* factors), might determine the balance between canonical RNA splicing and back-splicing processes, but whether circRNA is produced co-transcriptionally or post-transcriptionally is still to be determined.

## FUNCTIONS OF CIRCULAR RNAS

Little is known about circRNA biology, and a large number of possible functions have been attributed to this circular isoform. In human cells<sup>[40]</sup>, another class of nuclear circRNAs has been identified by Gardner *et al.*<sup>[41]</sup>: Circular intronic RNAs (ciRNAs) or stable intronic sequences RNA (sisRNA). In human cells (HeLa and human embryonic stem cells hESCs-H9), exon-intron circRNAs were reported, finding that these circRNAs, associated with the elongation Pol II machinery, act as positive regulators of Pol II transcription, suggesting a *cis*-regulatory role of noncoding intronic products in the efficient transcription of their parent coding genes<sup>[42]</sup>. CiRNAs do not function only as microRNA sponges, but, some abundant ciRNAs may be able to regulate gene expression in *trans* under certain circumstances.

A wide variety of studies have demonstrated that abnormal expressions of circRNAs are closely associated with various diseases including CRC<sup>[43-45]</sup>. It has been speculated that circRNAs form a class of post-transcriptional regulators, acting as epigenetic, highly stable miRNA sponges to compete with the endogenous RNA network, directly affecting the expression of any related gene (Figure 1B)<sup>[17,18,46]</sup>. CircRNA could be sequestering specific miRNA complexes and releasing them after cleavage<sup>[17]</sup>. The miRNA sponges ciRS-7 and miR-7, highly expressed in brain, interact to

form a miR-7 inhibitor/sponge complex. It is possible that the competition between ciRS-7 and miR-7 effects oncogenesis<sup>[18,47]</sup>. Similarly, the testis-specific circRNA (*Sry*) regulates activity of miR-138, a tumor suppressor<sup>[17,48]</sup>. Therefore, ciRS-7 and *Sry* complex with miR-7 and miR-138, respectively, to form microRNA sponges blocking functionality, and reducing the invasiveness, metastasis and proliferation of several cancers<sup>[47]</sup>.

CRC shows a negative correlation between global reduction of circRNA and cell proliferation, suggesting that the back-splice machinery responsible for RNA circularization, is dysfunctional in tumor cells<sup>[46]</sup>. Recently, Xie *et al.*<sup>[49]</sup> found an association between miR-145 and hsa\_circ\_001569 and the regulation of CRC progression.

Another circRNA, cir-ITCH acts as a miRNA sponge with an inhibitory effect on esophageal squamous cell carcinoma. Cir-ITCH stimulates ITCH levels, provoking an ubiquitin-mediated Dvl2 degradation, and inhibition of the canonical Wnt/ $\beta$ -catenin pathway, finally, contributing to cancer tumor advancement<sup>[50]</sup>. The expression of hsa\_circ\_001988 is downregulated in CRC, and contributes to differentiation and perineural invasion<sup>[51]</sup>. A study in breast carcinoma cells (MDA-MB-231)<sup>[52]</sup> shows the possible functions of circ-Foxo3 (hsa\_circRNA\_104170), which binds to eight miRNAs (miR-22, miR-136\*, miR-138, miR-149\*, miR-433, miR-762, miR-3614-5p and miR-3622b-5p), as well as Foxo 3 Protein (Foxo3P) and Foxo3 mRNA. Circ-Foxo3 has a sponging effect on these miRNAs, promoting Foxo3 mRNA translation, suppressing both tumor growth and cancer cell proliferation<sup>[52]</sup>.

## CIRC RNAS IN CRC

Recent reviews have reported that in CRC cell lines and CRC tissues, a global reduction of circRNA abundance is observed, in comparison to healthy tissue, therefore allowing for the proliferation of CRC cells<sup>[53,54]</sup>.

CircRNAs were associated for the first time with CRC in transcripts of DCC (Deleted in CRC)<sup>[30]</sup>. In 2015, Bachmayr-Heyda *et al.*<sup>[46]</sup> reported a global reduction of circRNA abundance in CRC cell lines and tumor samples, as compared to normal mucosa in patients with CRC. Using RNA-seq and the algorithm described by Memczak *et al.*<sup>[21]</sup> and found by RT-qPCR, 39 circRNAs differentially expressed in the normal colon mucosa and CRC samples, in which 11 of the circRNAs were upregulated and 28 were downregulated. Interestingly, the Bachmayr's group found that the expression of circRNAs was reduced in tumor samples (circRNA expression 27.8%) compared to normal colon mucosa samples (circRNA expression 78.1%). Similar evidence was found in 11 CRC cell lines, with even wider gaps in expression ratios<sup>[46]</sup>. In order to validate reduced circRNA expression in CRC compared to normal mucosa samples, they enriched circRNAs through RNase R digestion and subsequent deep sequencing, reporting 21653 distinct

Table 1 Circular RNA found to date in colorectal cancer

Name	Location	Length [nt]	Tissue/Cell line	Dysregulation	Gene	miRNA associated	Ref.
DCC circ0817	chr18q21.2 chr11	253, 948 653	HCT116 CRC tissue and CRC cell lines	None Downregulated	DCC CUL5	None None	[30] [46]
circ3204	chr15	706	"	Downregulated	USP3	None	[46]
circ6229	chr14	629	"	Downregulated	METTL3	None	[46]
circ7374	chr17	288	"	Upregulated	TNS4	None	[46]
circ7780	chr7	317	"	Downregulated	--	None	[46]
circ_001988	chr4q3	-	CRC tissue	Downregulated	CDR1as	miRNA-7	[21,51]
circ_001569	chr16q13.1	-	CRC tissue	Upregulated	ABCC1	miRNA-145	[49]

CRC: Colorectal cancer.

back spliced junctions. This method revealed that it is impossible to ensure the detection of all circRNA, and that the actual number of circRNA species is much higher. This was also corroborated in other cell lines in a non-cancerous neo-proliferative disease, showing a similar negative correlation as the circRNA index and proliferation observed in colon tissues and cell lines<sup>[46]</sup>. Finally, the authors hypothesized that the back-splice machinery responsible for RNA circularization is dysfunctional in tumor cells due to an increased degradation by oncomiRNAs<sup>[15,46]</sup>. Recently, Wang *et al.*<sup>[51]</sup> reported similar findings concerning the correlation of hsa\_circ\_001988 abundance and CRC in tumor tissue and adjacent normal mucosa from 62 CRC patients. By RT-qPCR they verified the presence of hsa\_circ\_001988 in these tissues, and it was found to be significantly downregulated in tumor tissue compared to healthy samples. The expression level of hsa\_circ\_001988 was significantly related to differentiation ( $P < 0.05$ ) and perineural invasion ( $P < 0.05$ ). Perineural invasion is a predictor of outcome in CRC and negatively associated with survival time and local recurrence in CRC patients. They concluded that hsa\_circ\_001988 may play a profound role in differentiation and perineural invasion and could be a potential target to regulate cytological behaviors<sup>[51]</sup>. Recently Xie *et al.*<sup>[49]</sup> provided information about an up-regulated circRNA (circ\_001569) complexing with the tumor suppressor miR-145. MiR-145 has been related with patient survival after CRC diagnosis<sup>[55-57]</sup>. They explored the expression pattern of circ\_001569 by real-time PCR in 30-paired samples of CRC patients, and found expression of circ\_001569 was significantly higher in the CRC tissues and found correlation with aggressive characteristics of CRC, including distant metastasis and poor differentiation. Then to detect the function of circ\_001569 in the progression and invasion of CRC, they proceeded to over-express and silence the circular isoform in four CRC cell lines (over-expressed in SW480 and HCT116 and silenced in SW620 and LOVO). Accordingly, over-expression increased proliferative and invasive ability in circ\_001569 expressing cells, while a sharp reduction in proliferation and invasion rates was shown in circ\_001569 silenced cells. In addition, they

found the level of miR-145 was significantly lower in CRC tissues<sup>[49]</sup>. Furthermore, using the bioinformatic algorithms (TargetScan, Pictar and miRANDA), along with Luciferase action, RT-qPCR and Western Blotting assays, they found that circ\_001569 increased the protein levels of E2F5, BAG4 and FMNL2 in SW480 and HCT116 cells, and knockdown of the circRNA in SW620 and LOVO cells had the opposite effect. Here, they provided evidence showing tumor promoting functions (proliferation and invasion) of circ\_001569 in CRC cells, directly inhibiting the regulatory activity of miR-145, and subsequently up-regulating its protein targets E2F5, BAG4 and FMNL2. Finally, they concluded that this interaction between miR-145 and hsa\_circ\_001569 in regulating CRC progression may provide new insights and therapeutic strategies for CRC prevention and treatment<sup>[49]</sup>.

CircRNAs that have been related with CRC are shown in Table 1.

## CONCLUSION

The information reviewed is the beginning of a better understanding of mechanisms and functions of this recently re-discovered circular isoform. CircRNAs can act as competing endogenous RNAs or miRNA sponges, regulating alternative splicing or transcription, modulating the expression of parental gene, managing local concentration of RBPs and RNAs through RNA transport. In cancer these molecules complex with miRNAs as inhibitor/sponge, with profound effects on oncogenesis and regulation of cancer pathways. It is possible that more important functions will be associated to this circular transcript in the near future. Still, the molecular mechanisms concerning the positive or negative relationship between circRNAs and miRNAs in CRC are poorly understood.

The search for a suitable circRNA biomarker for CRC is an important topic requiring further study. It is probable that the principal role of circRNAs in the progression of cancer is the modulation of the gene expression of oncogenes and or tumor suppressor genes, through the control of miRNA targets.



Summarizing, we conclude that microRNAs expression levels are regulated by circRNAs in a cell-type specific way, playing an important role in oncogenesis and the malignant behavior of cancer. Nevertheless, the function between circRNA and cancer, as well as the regulatory mechanisms is still elusive and needs to be further explored. Someday this circular isoform might become an important biomarker with a potential use in the diagnosis, prognosis and therapy of CRC.

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Retrospective Study

# Surgical management of hepato-pancreatic metastasis from renal cell carcinoma

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## Abstract

### AIM

To investigate the outcomes of liver and pancreatic resections for renal cell carcinoma (RCC) metastatic disease.

### METHODS

This is a retrospective, single centre review of liver and/or pancreatic resections for RCC metastases between January 2003 and December 2015. Descriptive statistical analysis and survival analysis using the Kaplan-Meier estimation were performed.

### RESULTS

Thirteen patients had 7 pancreatic and 7 liver resections, with median follow-up 33 mo (range: 3-98). Postoperative complications were recorded in 5 cases, with no postoperative mortality. Three patients after hepatic and 5 after pancreatic resection developed recurrent disease. Median overall survival was 94 mo (range: 23-94) after liver and 98 mo (range: 3-98) after pancreatic resection. Disease-free survival was 10 mo

(range 3-55) after liver and 28 mo (range 3-53) after pancreatic resection.

## CONCLUSION

Our study shows that despite the high incidence of recurrence, long term survival can be achieved with resection of hepatic and pancreatic RCC metastases in selected cases and should be considered as a management option in patients with oligometastatic disease.

**Key words:** Renal cell carcinoma; Metastasis; Pancreas; Liver; Surgery

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**Core tip:** The evidence on the role of surgery in management of renal cell carcinoma (RCC) metastatic disease to the liver and pancreas remains limited due to the rare nature of the disease. We have treated 13 patients in our institution, achieving median overall survival of 94 mo (range: 23-94) after liver and 98 mo (range: 3-98) after pancreatic resection. Disease-free survival was 10 mo (range: 3-55) after liver and 28 mo (range: 3-53) after pancreatic resection. Long term survival can be achieved with resection of hepatic and pancreatic RCC metastases in selected cases and should be considered in patients with oligometastatic disease.

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## INTRODUCTION

Renal cell carcinoma (RCC) is one of the commonest causes of cancer related mortality, with 5-year survival ranging between 10%-90% depending on the stage of the disease<sup>[1]</sup>. Metastatic disease is present at the time of diagnosis in almost a third of patients, while 20%-50% of patients with localised disease will develop metastases after nephrectomy<sup>[1,2]</sup>. The most common site of metastatic disease is lung, followed by lymph nodes, bones and liver<sup>[3-6]</sup>. Hepatic metastasis has been reported in 41% of patients on autopsy<sup>[7]</sup>, while pancreatic metastases are rarer, representing less than 5% of RCC metastases<sup>[1,8,9]</sup>.

Current management strategies of metastatic RCC include surgical resection, chemotherapy [including newer agents such as tyrosine kinase inhibitors (TKI)], radiotherapy and hormonal therapy. However, the results are still poor with a median 5-year survival 5%-20%<sup>[3,4,10-14]</sup>. The role of surgery is still not clear

as published data are limited. Nonetheless, a survival benefit has been reported in selected cases rendering surgical resection a management option in cases of oligometastatic disease.

We present our centre's experience in the surgical management of hepato-pancreatic RCC metastases.

## MATERIALS AND METHODS

This is a retrospective review of all the patients who had liver and/or pancreatic resection for RCC metastasis in our institution, a tertiary regional referral centre for the surgical management of hepatic and pancreatic malignancies, between January 2003 and December 2015. Data from the pre- and post-operative period were collected from the hospital's electronic records and histopathological database. Follow-up data were obtained based on the most recent oncology clinic or electronic medical entry. It is our institution's policy for all patients to provide informed consent for research at the time of the operation.

All patients with suspected RCC metastatic disease to the liver or pancreas were managed by the hepatopancreatobiliary multidisciplinary team. Pre-operative staging was based on imaging with contrast enhanced computer tomography (ceCT) of the chest, abdomen and pelvis. During the later part of the study (after 2011), positron emission tomography CT was used in the staging of all patients and magnetic resonance imaging of the liver in all cases with suspected liver metastasis. Tissue diagnosis was obtained in all cases of suspected pancreatic metastasis by endoscopic ultrasound-guided fine needle aspiration (EUS-FNA). The diagnosis of liver metastatic disease was predominantly based on three dimensional imaging, with half of the cases ( $n = 3$ ) also undergoing CT- or ultrasound-guided guided core biopsy.

All patients received standard peri-operative care and data regarding the post-operative complications were recorded. Pancreatic fistula was defined and graded using the recommendation of the International Study Group on pancreatic fistula, as drain output of any measurable volume of fluid on or after post-operative day 3 with amylase content greater than 3 times the serum amylase activity<sup>[15]</sup>.

No patient received any neoadjuvant therapy. The post-operative follow-up consisted of standard imaging surveillance with ceCT of the chest, abdomen and pelvis. No patient received any adjuvant treatment. Patients diagnosed with disease recurrence received appropriate individualised care (radiation, chemotherapy or palliative care). Survival analysis was performed using the Kaplan-Meier estimation (software package SPSS for Windows version 13.0; SPSS Inc., Chicago, IL, United States).

## RESULTS

Thirteen patients were treated by our team for meta-



**Table 1** Descriptive cohort characteristics

Patient demographics and primary disease (RCC)	
Age	63 yr (range: 44-80)
Gender: male:female	7:6
Nephrectomy: right:left	6:7
Hepatic and pancreatic metastatic disease	
Total cases	14
Synchronous	3 (2 liver, 1 pancreas)
Metachronous	11
Time between nephrectomy and diagnosis of metachronous disease	57 mo (range: 4-292)
Liver	29 mo (range: 4-129)
Pancreas	80 mo (range: 10-292)
Resection of metastases	
Total cases	14
Hepatic resections	7
Right hepatectomy	1 (with IVC resection)
Non-anatomical hepatectomy	6 (1 with en block diaphragmatic resection)
Pancreatic resections:	7
PD	3
TP + S	2
DP + S	2
Post-operative complications	5
Hepatic resections	1
Chylous ascites	1 (managed with parenteral nutrition)
Pancreatic resections	4
Pancreatic fistula	2 (grade B)
Fluid collection	2 (managed with radiological-guided drainage and antibiotics)
Bleeding	2 (operative management)

RCC: Renal cell carcinoma; IVC: Inferior vena cava; PD: Pancreaticoduodenectomy; TP + S: Total pancreatectomy and splenectomy; DP + S: Distal pancreatectomy and splenectomy.

static RCC to the liver or pancreas over the 13-year period. The majority of the primary tumours ( $n = 12$ ) were of the clear cell histological type, while 1 was adenocarcinoma. Median follow-up was 33 mo (range: 3-98). Cohort descriptive characteristics, as well as the individual case characteristics can be found on Tables 1 and 2 respectively. Fourteen resections for metastatic disease were performed, 7 pancreatic and 7 liver resections. One patient had a laparoscopic distal pancreatectomy and splenectomy followed by a laparoscopic non-anatomical liver resection. In 2 cases the patients were found to have low volume pulmonary metastases during pre-operative staging. In both cases the multidisciplinary team decision was to proceed with the major abdominal operation first and consider subsequent management of the pulmonary metastases after the patients were fully recovered. All resections were histologically R0 with the exception of one total pancreatectomy which had a clear histological margin of less than 1 mm (R1). In only one pancreatectomy case nodal metastasis was confirmed histologically. The overall median length of stay was 15 d (range: 4-28);

18 d (range: 8-28) for pancreatic and 9 d (range: 4-27), for liver resections.

Eight patients developed recurrent disease after hepatic ( $n = 3$ ) and pancreatic ( $n = 5$ ) resection, with only 1 case of local recurrence (Table 2). The median overall survival and the 1-, 3- and 5-year rates for the whole cohort, as well as separately after liver and pancreatic resection, are shown on Figure 1. The median disease-free survival and the 1-, 3- and 5-year rates for the whole cohort, as well as separately after liver and pancreatic resection, are shown on Figure 2.

## DISCUSSION

The published evidence on the management of RCC metastatic disease is scarce. Long-term survival with targeted treatment, including surgical resection, has been reported. Differences have also been noted in the literature with regards to the management approach and outcomes of RCC metastases to the liver and the pancreas, while results from the use of the novel chemotherapy agents TKIs in liver metastatic disease are non-existent. Data on the role of systemic therapy in combination with operative management in a neoadjuvant and/or adjuvant setting are scarce and limited to case series. Following liver resection, overall survival has been reported between 16 and 142 mo after liver resection and disease-free survival between 7 and 30 mo (Table 3). Our experience demonstrates comparable results, with 94 mo (range: 23-94) median overall survival and 10 mo (range: 3-55) median disease free survival after hepatectomy.

With regards to pancreatic metastases, initial data from published case series (Table 3) and literature reviews<sup>[1,16,17]</sup> suggested that surgical management offers superior results with median overall survival between 22-120 mo. Survival rates have been reported at 67%-91% at 3 years and 61%-88% at 5 years. On the contrary, non-surgical management resulted in significantly worse 3-year and 5-year survival of 21% and 0%-47% respectively<sup>[16-18]</sup>. However, more recent data suggest that the use of TKI-based chemotherapy conferred similar overall survival to surgery<sup>[6]</sup>. Even though the median survival achieved with resection was about 1 year longer compared to TKI-based chemotherapy (86 mo vs 103 mo), it did not reach statistical significance ( $P = 0.201$ ). Since no complete disease response was observed with chemotherapy, the study concluded that surgery may be a better option in the subgroup of patients with good prognostic criteria and no other concomitant metastases as it can radically treat the disease. In our series, after pancreatic resection the median overall survival was 93 mo (range: 3-98) and 3- and 5-year rates 71.4%, which is comparable to the published data. Likewise, we recorded median disease-free survival at 28 mo (range: 3-53), which is comparable to the published reports of median disease-free survival between 16 and 65 mo after pancreatic resection for RCC metastases (Table 3).

Table 2 Individual case characteristics

Patient	Age, gender	Site of metastasis	Time nephrectomy to metastasis (mo)	Other metastasis pre-operatively	Operation	Post-operative complications	Follow-up (mo)	Status at last follow-up	Site of recurrence	Time from resection of metastasis to recurrence (mo)
1	50, female	Pancreas	73	Lungs (resected)	PD	Bleeding, pancreatic fistula	50	Alive, disease-free	-	-
2	61, female	Pancreas	110	-	PD	Collection	66	Alive, recurrence	Right iliac bone (radiation)	28
3	64, male	Pancreas	93	-	TP + S	-	98	Alive, recurrence	Pancreatic bed recurrence and abdominal LN (Pazopanib)	53
4	66, male	Pancreas	292	-	TP	-	4	Death from disease	Brain (radiation)	3
5	63, male	Pancreas	0	-	Laparoscopic DP + S	-	61	Alive, disease-free	Liver	17
		Liver	28		Laparoscopic non-anatomical				-	-
6	63, female	Pancreas	10	-	DP + S	Pancreatic fistula	16	Alive, recurrence	Right kidney and lung (Pazopanib)	5
7	80, female	Pancreas	26	-	PD	Bleeding, collection	3	Death, no evidence of disease	-	-
8	65, male	Liver	29	-	Non-anatomical	-	94	Death, no evidence of disease	Lungs (resected)	55
9	44, female	Liver	0	-	Non-anatomical	Chylous scites	23	Death, recurrence	Liver (Pazopanib)	6
10	70, male	Liver	4	Right kidney	Non-anatomical + right partial nephrectomy	-	33	Death, recurrence	Lung and spine (radiation and Sunitinib)	3
11	61, female	Liver	0	Right adrenal, lungs (resected)	Non-anatomical	-	36	Alive, disease-free	-	-
12	59, male	Liver	40	Adrenal (in specimen of RCC resection)	Non-anatomical + diaphragm	-	32	Alive, recurrence	Liver (Pazopanib)	10
13	65, male	Liver	129	Lungs	Right hepatectomy + IVC resection	-	26	Alive, with disease, no recurrence	-	-

RCC: Renal cell carcinoma; IVC: Inferior vena cava; PD: Pancreaticoduodenectomy; TP + S: Total pancreatectomy and splenectomy; DP + S: Distal pancreatectomy and splenectomy; LN: Lymph nodes.

Liver and pancreatic resections carry a significant risk for perioperative morbidity and a small risk for mortality. Morbidity after resection of RCC liver metastases has been reported between 13% and 55% and after pancreatic resection as high as 47% (Table 3). Mortality has been reported between 0% and 5.3% after liver and between 0% and 6.4% after pancreatic resections for RCC metastases. Therefore, a careful patient selection for surgical management should be employed. Patients

with disseminated or very aggressively progressing disease are not considered good surgical candidates for liver or pancreas metastasectomies, as this may substantially impact their quality of life without adding any survival benefit. Similarly, patients with significant medical history and/or poor performance status, that would not tolerate a major resection, should be considered for non-surgical management. In our cohort, all patients had Karnofsky performance score 80-100

**Table 3** Reports on surgical management of renal cell carcinoma metastasis to the liver and pancreas

Ref.	Patient No. and site	Syn-chronous (n and %)	Time nephrectomy to metastasis (mo, median and range)	Pre-op treatment (n and %)	Post-op treatment (n and %)	Mortality (%)	Morbidity (%)	OS (mo; median and range)	1-yr OS (%)	3-yr OS (%)	5-yr OS (%)	DFS (mo; median and range)	1-yr DFS (%)	3-yr DFS (%)	5-yr DFS (%)
Ruys <i>et al</i> <sup>[41]</sup>	33, liver	10 (30)	50 (7-360)	12 (36)	-	0	54.5	33 (4-224)	78	47	43	10 (1-54)	49	18	11
Hatzaras <i>et al</i> <sup>[51]</sup>	43, liver	9 (21)	17 (2-189)	5 (12)	25 (58)	2.3	23.3	NR	94.2	62.1	-	15.5 (3-76)	79.5	27.3	-
Staehler <i>et al</i> <sup>[10]</sup>	68, liver	19 (28)	-	-	54 (80)	0	20.1	142 (115-169)	-	-	62.2	-	-	-	-
Aloia <i>et al</i> <sup>[22]</sup>	19, liver	5 (26)	53 (9-137)	-	-	5.3	32	36 (-)	-	52	26	13 (-)	-	25	25
Langan <i>et al</i> <sup>[23]</sup>	10, liver	5 (50)	-	4 (40)	4 (40)	0	30	24 (3-254)	79	45	34	7.2 (-)	-	-	-
Alves <i>et al</i> <sup>[24]</sup>	14, liver	-	-	-	-	-	-	26 (-)	69	26	-	-	-	-	-
Karavias <i>et al</i> <sup>[25]</sup>	6, liver	5 (83)	-	0	2 (33)	0	-	NR	NR	NR	NR	NR	66.7	66.7	66.7
Kawata <i>et al</i> <sup>[26]</sup>	4, liver	2 (50)	4 (2-6)	2 (50)	4 (100)	0	-	30 (12-40)	75	37.5	NR	30 (12-30)	50	50	NR
Thelen <i>et al</i> <sup>[27]</sup>	31, liver	6 (19)	-	-	-	3.2	12.9	48 (-)	82.2	54.3	38.9	27 (-)	76.2	38.1	26.1
Yezhelyev <i>et al</i> <sup>[28]</sup>	13, liver	13 (100)	0	-	-	-	-	16 (1-34)	-	-	-	-	-	-	-
Santoni <i>et al</i> <sup>[6]</sup>	44, pancreas	1 (2)	-	6 (14)	-	-	-	103 (-)	-	-	-	27 (3-167)	-	-	-
Bassi <i>et al</i> <sup>[8]</sup>	17, pancreas	-	126 (-276)	-	-	0	47	22 (9-35)	-	-	-	16 (3-30)	-	-	-
Schwartz <i>et al</i> <sup>[9]</sup>	62, pancreas	2 (3)	120 (0-300)	3 (5)	-	6.4	-	-	-	72	63	26 (5-166)	-	54	35
Konstadinidis <i>et al</i> <sup>[29]</sup>	20, pancreas	1 (5)	104 (0-264)	-	-	-	-	104 (15-144)	-	-	61	-	-	-	-
Eidt <i>et al</i> <sup>[30]</sup>	7, pancreas	0	169 (108-240)	-	12 (100)	0	-	65 (5-86)	85.7	85.7	85.7	65 (5-86)	85.7	85.7	85.7
Ghavamian <i>et al</i> <sup>[31]</sup>	11, pancreas	0	108 (18-295)	-	-	-	-	120 (5-120)	90.9	90.9	80.8	NR	90.9	90.9	80.8
Reddy <i>et al</i> <sup>[32]</sup>	21, pancreas	3 (14)	112 (-)	-	-	0	-	58 (4-220)	-	-	-	-	-	-	-
Tanis <i>et al</i> <sup>[17]</sup>	10, pancreas	0	107 (5-228)	-	1 (10)	-	-	69 (6-69)	88.9	66.7	66.7	35 (6-69)	100	58	38.9
Zerbi <i>et al</i> <sup>[20]</sup>	23, pancreas	0	-	-	-	0	47.8	NR	100	88	88	44 (33-52)	-	-	-
Kassabian <i>et al</i> <sup>[21]</sup>	5, pancreas	0	12 (4-15)	-	-	-	-	-	-	-	67	-	-	-	-
Untch <i>et al</i> <sup>[33]</sup>	27, pancreas	-	-	-	-	-	-	96 (-)	-	-	-	-	-	-	-

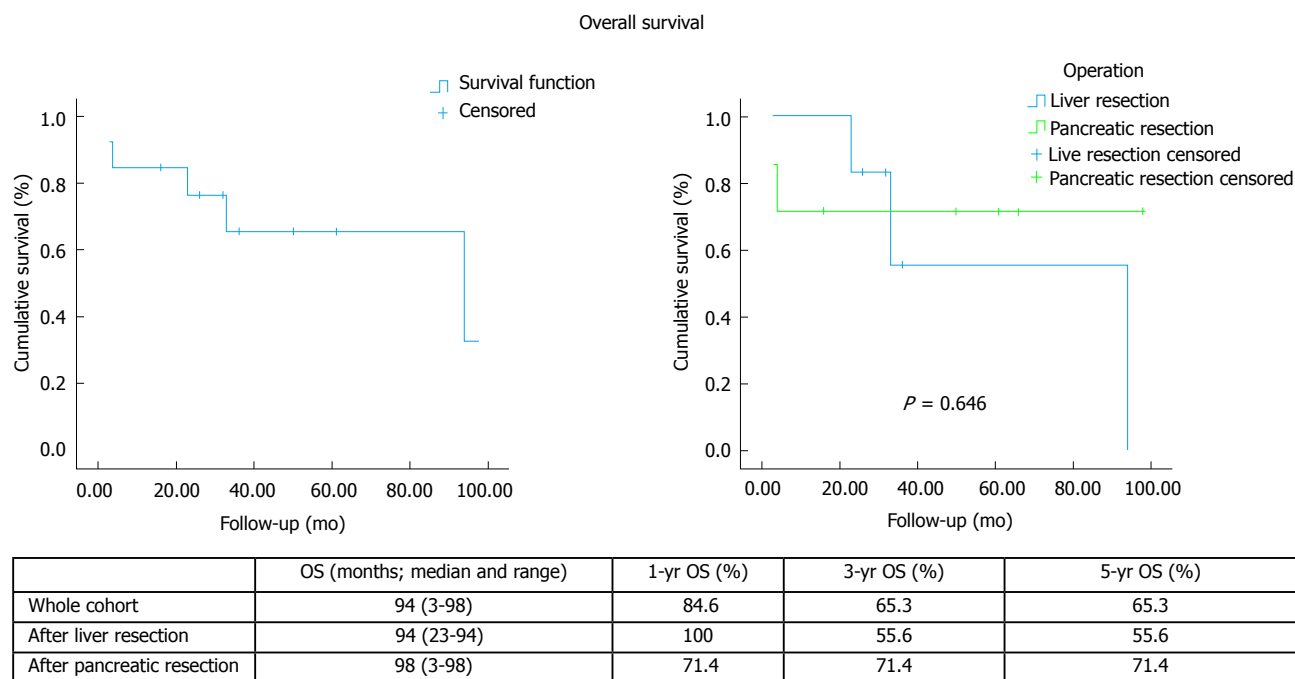
OS: Overall survival (median and range); DFS: Disease-free survival (median and range in months); NR: Not reached; Pre- and post-op treatment: Treatment modalities before and after surgical resection of renal cell carcinoma metastasis, including chemotherapy, immunotherapy, tyrosine kinase inhibitors.

and were deemed fit for major surgery after clinical and laboratory assessment by the multidisciplinary team. Postoperative complications were recorded in 5 cases (one liver and 4 pancreatic resections), with no postoperative mortality.

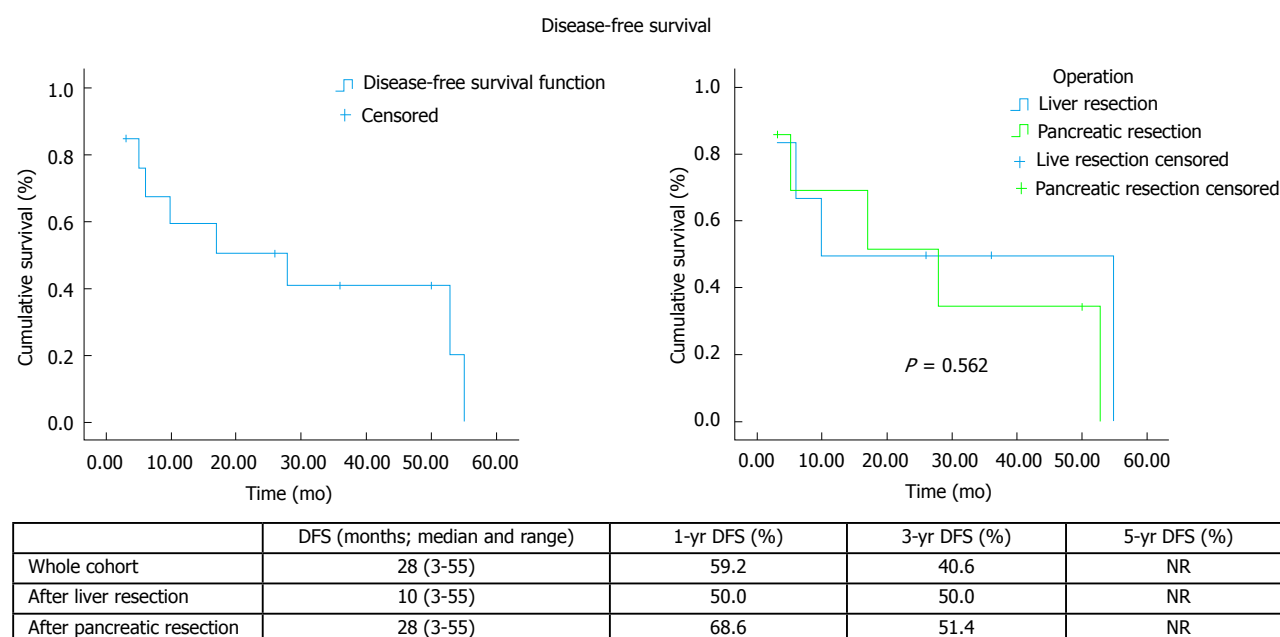
In this study, the median time interval between nephrectomy for the primary tumour and the diagnosis of metastatic disease was 57 mo (range: 4-292) (Table 1). Specifically for liver metastasis, with the exception of the 2 cases of synchronous metastatic disease, this period was of 29 mo (range: 4-129), which is comparable to reports in the literature of a median 4-53 mo (Table 3). For pancreatic metastasis this period was 80 mo (range: 10-292), somewhat shorter than most published case series (Table 3) and reviews<sup>[1,16,17]</sup>, reporting a period between 104-169 mo after nephrectomy. A possible explanation could be the implementation of more intensive follow-up protocols

for these patients in the recent years after the resection of the primary tumour. Furthermore, due to the rare nature of RCC metastasis to the pancreas all cases were confirmed with EUS-FNA. The need for tissue diagnosis was further supported by the fact that in almost all of our patients (6 out of 7) the pancreatic metastasis was the first and only site of metastatic disease. The use of immunohistochemistry (e.g., cytokeratin, vimentin, CD10, synaptophysin, CK7, CK20 and CA19) in addition to the standard histopathological stains can be useful in facilitating the diagnosis and is strongly advisable<sup>[19,20]</sup>.

The evidence on the management of RCC metastatic disease to the liver and pancreas remains inconclusive. The rare nature of the disease precludes randomised studies and therefore current evidence is drawn from published case series. Regardless of the small size of our cohort, our data show that despite the high incidence of recurrence, long term survival can be achieved with



**Figure 1** Kaplan Meier overall survival analysis for the whole cohort (left) and stratified according to type of resection (right). Comparison of survival curves performed with the use of Log-Rank test.



**Figure 2** Kaplan Meier disease-free survival analysis for the whole cohort (left) and stratified according to type of resection (right). Comparison of survival curves performed with the use of Log-Rank test.

resection of hepatic and pancreatic RCC metastases in selected cases and should be considered as a management option in patients with oligometastatic disease.

## COMMENTS

### Background

Renal cell carcinoma (RCC) is one of the commonest causes of cancer related mortality. Metastatic disease is present at the time of diagnosis in almost a

third of patients, while 20%-50% of patients with localised disease will develop metastases after nephrectomy. The role of surgery in the management of metastatic RCC is still not clear as published data are limited.

### Research frontiers

This is a retrospective single centre experience in the surgical management of patients with metastatic RCC to the liver and pancreas.

### Innovations and breakthroughs

Surgical management of liver and pancreas RCC metastases can be performed



with low morbidity and mortality. In our series, postoperative complications were recorded in 5 cases (out of a total of 14), with no postoperative mortality. Median overall survival was 94 mo (range: 23-94) after liver and 98 mo (range: 3-98) after pancreatic resection. Disease-free survival was 10 mo (range 3-55) after liver and 28 mo (range 3-53) after pancreatic resection.

## Applications

The authors' study shows that despite the high incidence of recurrence, long term survival can be achieved with resection of hepatic and pancreatic RCC metastases in selected cases and should be considered as a management option in patients with oligometastatic disease.

## Peer-review

The authors have well described these cases and the review of the literature about this topic has been conducted sufficiently in depth.

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## Observational Study

# Prognostic significance of vascular endothelial growth factor polymorphisms in colorectal cancer patients

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## Abstract

### AIM

To investigate the associations of the genetic polymor-

phisms of vascular endothelial growth factor A (*VEGF-A*) -1498C>T and -634G>C, with the survival of patients with colorectal cancer (CRC).

## METHODS

A prospective cohort consisting of 131 Brazilians patients consecutively operated on with a curative intention as a result of sporadic colorectal carcinoma was studied. DNA was extracted from peripheral blood and its amplification and allelic discrimination for each genetic polymorphism was performed using the technique of polymerase chain reaction (PCR) in real-time. The real-time PCR technique was used to identify the *VEGF-A* -1498C>T (rs833031) and -634G>C (rs2010963) polymorphisms. Genotyping was validated for *VEGF-A* -1498C>T polymorphism in 129 patients and for *VEGF-A* -634G>C polymorphism in 118 patients. The analysis of association between categorical variables was performed using logistic regression, survival by Kaplan-Meier method and multivariate analysis by the Cox regression method.

## RESULTS

In the univariate analysis there was a significant association (OR = 0.32;  $P = 0.048$ ) between genotype CC of the *VEGF-A* -1498C>T polymorphism and the presence of CRC liver metastasis. There was no association between *VEGF-A* -1498C>T polymorphism and *VEGF-A* -634G>C polymorphism with further clinical or anatomopathologic variables. The genotype CC of the *VEGF-A* -1498C>T polymorphism was significantly correlated with the 5-year survival ( $P = 0.032$ ), but not significant difference ( $P = 0.27$ ) was obtained with the *VEGF-A* -634G>C polymorphism with the 5-year survival in the univariate analysis. The genotype CT (HR = 2.79) and CC (HR = 4.67) of the polymorphism *VEGF-A* -1498C>T and the genotype CC (HR = 3.76) of the polymorphism *VEGF-A* -634C>G acted as an independent prognostic factor for the risk of death in CRC patients.

## CONCLUSION

The CT and CC genotypes of the *VEGF-A* -1498C>T and the CC genotype of the *VEGF-A* -634C>G polymorphisms are prognostic factors of survival in Brazilians patients with sporadic colorectal carcinoma.

**Key words:** Colorectal cancer; Genetic polymorphisms; Vascular endothelial growth factor-A; Colorectal surgery; Genetic variation

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**Core tip:** Vascular endothelial growth factor A (*VEGF-A*) affects the tumor biological behavior and phenotype. An applied research with relevant achievement that will possibly be favored by such information is the pharmacogenetics impact of *VEGF-A* polymorphisms. *VEGF-A* is a significant goal in the anticancer therapy and results about *VEGF-A* polymorphisms may enhance

the targeted therapies. This approach will be of great help to the suitability of individual therapies and improve the quality of post operative treatment. Moreover, since polymorphisms often show a discrepancy between ethnic groups, more studies are also warranted to clarify the association between the *VEGF-A* polymorphisms and the CRC in diverse ethnic populations.

do Espírito Santo GF, Galera BB, Duarte EC, Chen ES, Azis L, Damazo AS, Saba GT, de Sousa Gehrke F, Guerreiro da Silva IDC, Waisberg J. Prognostic significance of vascular endothelial growth factor polymorphisms in colorectal cancer patients. *World J Gastrointest Oncol* 2017; 9(2): 78-86 Available from: URL: <http://www.wjgnet.com/1948-5204/full/v9/i2/78.htm> DOI: <http://dx.doi.org/10.4251/wjgo.v9.i2.78>

## INTRODUCTION

Colorectal cancer (CRC) is one of the most common malignancies in the world. Despite advances in diagnostic and treatment modalities, patients still face a poor prognosis, and a more individualized treatment approach appears necessary<sup>[1-4]</sup>.

Unlike genetic mutations, polymorphisms represent variations of the naturally occurring DNA sequence. Polymorphisms are found in at least 1% of the healthy population<sup>[5]</sup>. The vast majority (90%) of DNA polymorphisms is single nucleotide (SNPs)<sup>[6,7]</sup> and functionally neutral. However, certain polymorphisms have effects on the regulation of gene expression or on the function of the encoded protein<sup>[8]</sup> and thereby influence the susceptibility and severity of the disease<sup>[9,10]</sup>. Thus, the polymorphisms may modify the route of angiogenesis and the susceptibility and severity of malignancies<sup>[11]</sup>.

Angiogenesis is a sequence of processes starting with vessel dilatation and pericyte recruitment in the pre-existing vessels, followed by endothelial cell proliferation, formation of new vessels and recruitment of perivascular cells<sup>[9]</sup>. In the progress toward malignancy, the normal cells must switch to an angiogenic phenotype to attract the nourishing vasculature that they depend on for their growth<sup>[9,11]</sup>.

Malignant tumors depend on angiogenesis for their growth and metastasis<sup>[9,11]</sup>. It is generally assumed that microvessel formation around the tumor is stimulated by various angiogenic growth factors secreted by the tumor cells<sup>[12]</sup>. Among them, the vascular endothelial growth factor (VEGF), one of the most potent endothelial cell mitogens, is considered one of the strongest promoters of angiogenesis in CRC<sup>[11,12]</sup>. The VEGF is vital for the invasion and metastasis of neoplasms through the formation of new blood vessels from mature endothelial cells<sup>[13,14]</sup>. Moreover, studies have shown that the genetic polymorphisms can be used to predict the clinical outcomes of gastrointestinal<sup>[14,15]</sup>, breast<sup>[16]</sup>, ovary<sup>[17]</sup> and pancreatic cancer<sup>[18]</sup>.

The cancer targeted therapy with the use of antiangio-



genic agents is aimed at inhibition of angiogenic function and tumor dissemination since neoangiogenesis stimulates the growth and invasion of adjacent tissues by tumor cells<sup>[9,12]</sup>. On the other hand, the inhibition of VEGF limits the tumor growth<sup>[9,13]</sup>. In colorectal carcinoma, VEGF levels are increased and they are related to further spread and poor prognosis<sup>[12,14]</sup>. The antiangiogenic agent bevacizumab is a humanized monoclonal immunoglobulin G antibody against recombinant VEGF activity and it is used in the treatment of metastatic CRC<sup>[13,14]</sup>.

Clinical studies have shown that an association between the level of VEGF expression and increased microvessel density in tumors is correlated with an advanced stage of CRC, and with shorter survival. Therefore, it is important to determine the presence of metastasis, and patients with CRC and VEGF overexpression have higher tumor progression and poor prognoses<sup>[19-21]</sup>. Increased VEGF expression in CRC may predict the risk of multiple liver metastasis and play a role in the spread of CRC cells to the lymph nodes<sup>[22]</sup>. Due to these properties, VEGF has been used as a therapeutic target for the creation of anticancer drugs and is considered a potential prognostic marker of CRC<sup>[13,23]</sup>.

The VEGF-A gene is located on chromosome 6p21.3 and consists of 8 exons separated by 7 introns that exhibit alternative splicing to form a family of proteins<sup>[24]</sup>. This gene is a member of the platelet-derived growth factor/VEGF family and encodes a protein that is often found as a disulfide-linked homodimer.

The human VEGF-A gene is highly polymorphic, with more than 15 SNPs described<sup>[24,25]</sup>, thus enabling wide variation in its expression between individuals from different ethnic groups, and there are few studies involving Latinos, Hispanics<sup>[20]</sup>, and particularly Brazilians.

VEGF-A is a dimeric glycoprotein and is considered to be the main, dominant inducer of the growth of blood vessels. VEGF-A is essential for adults during organ remodeling and diseases that involve blood vessels in wound healing, tumor angiogenesis, diabetic retinopathy and age-related macular degeneration.

The -634G>C genetic polymorphism in the promoter region and the -1498C>T genetic polymorphism in the 3'-untranslated region were found to be associated with variations in VEGF-A protein synthesis<sup>[24,26,27]</sup>. Actually, the VEGF-A -634G/C polymorphism appears to be associated with a higher VEGF-A expression<sup>[28,29]</sup>.

The participation of common genetic polymorphisms of VEGF-A in the prognoses of patients with CRC is not yet clearly established<sup>[19,20,23,25,29]</sup>. Furthermore, studies investigating the association between VEGF-A genetic polymorphisms and CRC risk report conflicting results<sup>[30]</sup> and the specific associations still remain controversial<sup>[31]</sup>.

Since VEGF-A is known to be a potent pro-angiogenic factor, we evaluated the potential association of two VEGF-A genetic polymorphisms (-634G>C and -1438C>T) with the clinicopathologic variables and its

possible implication for prognosis in a population of Brazilian patients operated on CRC.

## MATERIALS AND METHODS

### Study design and sample

The present study was conducted according to the ethical principles of the Declaration of Helsinki from the World Medical Association and has been approved by the Institutional Research Ethics Committees.

The study was conducted as a prospective cohort study (observational study) of 131 adult patients of both genders with CRC, without a distinction of ethnicity and operated on consecutively with a curative intention in the period from 2008 to 2011.

The patients were 80 (61.1%) males and 51 (38.9%) females, with a mean age of  $58.3 \pm 12.5$  years (20 to 85 years) and median age of 58 years. Regarding the ethnicity, 121 (92.4%) patients were white and 10 (7.6%) afrodescendants.

The following patients were excluded from the study: Those with familial adenomatous polyposis, colorectal neoplasia other than carcinoma, inflammatory bowel disease, other hereditary CRC syndromes, submitted to neoadjuvant treatment or with synchronous/metachronous tumors elsewhere, except for basal cell carcinoma of skin, and those who were impossible to contact, whether the patients or the patient's relatives, to obtain the necessary information.

### Collection and processing of biological material

Peripheral blood samples were collected by venipuncture into tubes containing 0.1% EDTA and kept in the refrigerator at 4 °C for up to 48 h. If DNA extraction did not take place within this period, the samples were frozen for a maximum of seven days at a temperature of -20 °C. DNA extraction was performed using the method of salting out and storing the material in a freezer at -80 °C.

### Surgery data

Regarding the anatomical distribution of the tumors, 98 (74.8%) was found in the colon: 41 (31.3 %) in the right colon, 7 (5.3%) in transverse and 50 (38.2%) in the left colon. In the remaining 33 (25.2%) patients, the tumors were located in the rectum. A right colectomy was performed in 42 (32.1%) cases, a left colectomy in 45 (34.3%), rectosigmoidectomy in 41 (31.3%) and an abdominoperineal resection in 3 (2.3%).

### Genotyping

The real-time PCR technique was used to identify genetic polymorphisms in VEGF-A (rs833061 and rs2010963) genes. DNA was amplified using Taqman assays C\_10 (rs833061) and C\_10 (rs2010963) (Applied Biosystems, Life Technologies Corporation, Foster City, CA, United States) and VIC/FAM dyes (FAM<sup>TM</sup>/ROX<sup>TM</sup> and VIC<sup>®</sup>/ROX<sup>TM</sup> Dye Normalization Plates, Applied Biosystems, Life Technologies Corporation, Foster City, CA,

United States). The dye was used as ROX™ passive reference. In each PCR reaction, the following were used: 10 µL of TaqMan Universal Master Mix II (Applied Biosystems, Life Technologies Corporation, Foster City, CA, United States), 1.0 µL of TaqMan Pre-Designed SNP Genotyping Assays 20 × solution (Applied Biosystems, Life Technologies Corporation, Foster City, CA, United States), 30–50 ng of genomic DNA and a final volume of 20 µL of Nuclease-Free Water (Promega, Madison, WI, United States). The equipment used for amplification and allelic discrimination was the Fast ABI-7500 (Applied Biosystems, Life Technologies Corporation, Foster City, CA, United States). Protocols for genotyping were performed according to the manufacturer involving the cycling amplification for 10 min at 95 °C and then 40 cycles for 15 s at 92 °C and for 1 min at 60 °C. At that time, there was the post-run for 1 min allele determination. Negative controls (no template control – NTC) were used, results were analyzed and 10% of all samples were genotyped more than once to ensure there was no contamination. Genotyping was validated for *VEGF-A* -1498C>T polymorphism in 129 patients and for *VEGF-A* -634G>C polymorphism in 118 patients.

### Statistical analysis

The estimation of the sample power, calculated as the ability to detect a hazard ratio (HR)  $\geq 1.7$  with an alpha value of 0.05 and a statistical power of 80% provided a minimum sample size of 112 individuals. The estimation was performed using the Stata 11.0 (Stata Statistical Software, StataCorp LP, College Station, TX, United States).

The variable survival was well-defined as the time elapsed between the date of the surgery and the event of interest represented by the patient's death by CRC. Operationally, this variable is composed of the duration of the follow-up after the operation that was previously established with a maximum duration of 60 mo or the occurrence of death. The clinical variables analyzed were gender, age and presence of metastasis and/or relapse after the operation.

The analyzed histopathological variables were the anatomical site of the CRC in the large intestine, adjacent invasion, degree of cellular differentiation, venous/lymphatic/perineural invasion, and staging of the neoplasia according to the 2010 TNM staging system<sup>[32]</sup>.

Patients who survived until the end of the study follow-up (60 mo) were considered "censored". In cases in which the event of interest has not occurred after exceeding the maximum observation period of the study (60 mo), loss of observation or occurrence of death were also considered "censored".

The univariate analysis was made by means of a log-rank test. The significant statistical level was considered as 5% ( $P < 0.05$ ).

The Cox regression analysis was used to identify the independent effect of the prognostic factors (independent variables). For the selection of independent variables to be included in the multivariate models of Cox regression,

**Table 1** Frequency of vascular endothelial growth factor A -1498C>T and vascular endothelial growth factor A -634G>C genetic polymorphisms in blood samples of patients with resected colorectal carcinoma

Polimorphism	Genotype	n (%)
<i>VEGF-A</i> -1498C>T (n = 129)	CC	27 (20.9)
	CT	58 (44.9)
	TT	44 (34.1)
<i>VEGF-A</i> -634G>C (n = 118)	CC	26 (22.0)
	CG	42 (35.6)
	GG	50 (42.4)

VEGF-A: Vascular endothelial growth factor A.

each individual variable in the Cox model was tested and the following criteria for inclusion in the multivariate model were verified: Variables with a descriptive level of significance lower than 20% ( $P < 0.20$ ) in the univariate model and the genetic variations (polymorphisms) used in this study. For the selection of a final model, the automatic selection backwards in Stata 11.0 (Stata Statistical Software, StataCorp LP, College Station, TX, United States) was used.

## RESULTS

The follow-up period of 131 patients ranged from 1.8 to 60 mo, with a mean of 33.8 ( $\pm 21.9$ ) mo and a median of 34.0 mo. Liver metastasis was found in 26 (19.1%) patients. The involvement of regional lymph nodes occurred in 63 (48.1%) patients and the invasion of adjacent organs was found in 26 (19.8%) cases. At the end of this follow-up period, 70 (53.4%) patients were alive without the disease, 14 (10.7%) were alive with the disease, 42 (32.1%) died with CRC, 3 (2.3%) had died of causes unrelated to CRC and 2 (1.5%) were lost in the follow-up. Thus, censored occurred in 84 (67.9%) patients with a complete observation after 60 mo. The estimate of overall survival at 5 years was 60.7%, with an average of 45.2% (95%CI: 41.5 to 49.0) and a median of 33.0%.

The results of the genotypes' frequency of the *VEGF-A* -1498C>T and *VEGF-A* -634G>C genetic polymorphisms are shown in Table 1. The stages of the CRC and their respective polymorphisms of *VEGF* -634G>C and the *VEGF* -1498C>T genes are described in Tables 2 and 3.

In the univariate analysis there was a significant association (OR = 0.32;  $P = 0.048$ ) between genotype CC of the *VEGF-A* -1498C>T polymorphism and the presence of CRC liver metastasis. There was no association between *VEGF-A* -1498C>T polymorphism with further clinical or anatomopathologic variables (Table 4). The *VEGF-A* -634G>C polymorphism showed no significant association between clinical or anatomopathologic variables in the univariate analysis.

Following the previously established criteria, the genotype CC of the *VEGF-A* -1498C>T polymorphism was significantly correlated with the 5-year survival ( $P$

**Table 2** TNM staging of colorectal tumors and their respective polymorphisms of vascular endothelial growth factor -634G>C gene (*n* = 118)

Stage ( <i>n</i> )	C/C <i>n</i> (%)	G/C <i>n</i> (%)	G/G <i>n</i> (%)
I + II (55)	10 (18.2)	22 (40.0)	23 (41.8)
III + IV (63)	16 (25.4)	20 (31.7)	27 (42.9)
Total	26 (22.0)	42 (35.6)	50 (42.4)
<i>P</i>	-	0.27	0.53
OR (95%CI)	1	0.56 (0.21-1.53)	0.73 (0.27-1.92)

Cox multiple regression test. OR: Odds ratio.

**Table 3** TNM staging of colorectal tumors and their respective polymorphisms of vascular endothelial growth factor -1498C>T gene (*n* = 129)

Stage ( <i>n</i> )	C/C <i>n</i> (%)	G/C <i>n</i> (%)	G/G <i>n</i> (%)
I + II (61)	20 (32.8)	9 (14.8)	32 (52.5)
III + IV (68)	24 (35.3)	18 (26.5)	26 (38.2)
Total	26 (22.0)	42 (35.6)	50 (42.4)
<i>P</i>	-	0.31	0.33
OR (95%CI)	1	1.66 (0.61-4.51)	0.67 (0.30-1.48)

Cox multiple regression test. OR: Odds ratio.

= 0.032) (Table 5), but not significant difference (*P* = 0.27) was obtained in relation to the *VEGF-A* -634G>C polymorphism with the 5-year survival in the univariate analysis.

In the multivariate analysis, the genotypes CT (HR = 2.79; 95%CI: 1.01-7.66) and CC (HR = 4.67; 95%CI: 1.51-14.43) of *VEGF-A* -1498C/T polymorphism and the genotype CC (HR = 3.76; 95%CI: 1.29-10.93) of *VEGF-A* -634C>G polymorphism were associated with a reduced 5-year survival (Table 6; Figures 1 and 2). The *VEGF-A* -1498C/T and the *VEGF-A* -634G>C polymorphisms showed no significant association between clinical or anatomopathologic variables in the multivariate analysis.

## DISCUSSION

The selection of studies was carried out by publications on the participation of these SNPs in CRC<sup>[25-30]</sup>. Due to involvement of angiogenesis in neoplasms, *VEGF-A* may influence the biology and the phenotype of the CRC<sup>[33-36]</sup>. The results regarding the association of polymorphisms of *VEGF-A* with CRC prognosis are controversial<sup>[13,29,36-38]</sup>.

In the present analysis, we examined whether two common *VEGF-A* -1498C>T and *VEGF-A* -634G>C polymorphisms were related to prognoses and clinico-pathologic features of CRC patients whose tumors had been surgically resected with curative intent.

We used blood samples of patients with colorectal carcinoma because the majority of polymorphism analyses have been carried out on germline DNA extracted from peripheral blood as it is easily obtained

**Table 4** Univariate analysis of vascular endothelial growth factor A -1498C>T genetic polymorphism and the presence of liver metastasis in patients with resected colorectal carcinoma (*n* = 129)

	Liver metastasis	Genotype TT <i>n</i> (%)	Genotype CC <i>n</i> (%)	Genotype CT <i>n</i> (%)
	Yes (25)	7 (28.0)	10 (40.0)	8 (32.0)
	No (104)	37 (35.6)	17 (16.3)	50 (48.1)
<i>P</i>		0.34	0.048	0.765
OR (95%CI)		1	0.32 (0.10-0.98) <sup>1</sup>	1.18 (0.39-3.55)

<sup>1</sup>Significant. OR: Odds ratio.**Table 5** Univariate analysis of vascular endothelial growth factor A -1498C>T genetic polymorphism and 5-year survival in patients with resected colorectal carcinoma (*n* = 129)

Polymorphism	Genotype	<i>n</i> (%)	5-yr survival (%)	<i>P</i>
<i>VEGF-A</i> -1498C>T ( <i>n</i> = 129)	CC	27 (20.9)	46.4	0.032 <sup>1</sup>
	CT	58 (44.9)	61.7	
	TT	44 (34.1)	67.1	

<sup>1</sup>Significant Mantel-Cox log-rank test.**Table 6** Multivariate analysis of vascular endothelial growth factor A -1498C>T genetic polymorphism (*n* = 129) and vascular endothelial growth factor A -634C>G (*n* = 118) genotypes and 5-year survival in patients with resected colorectal carcinoma

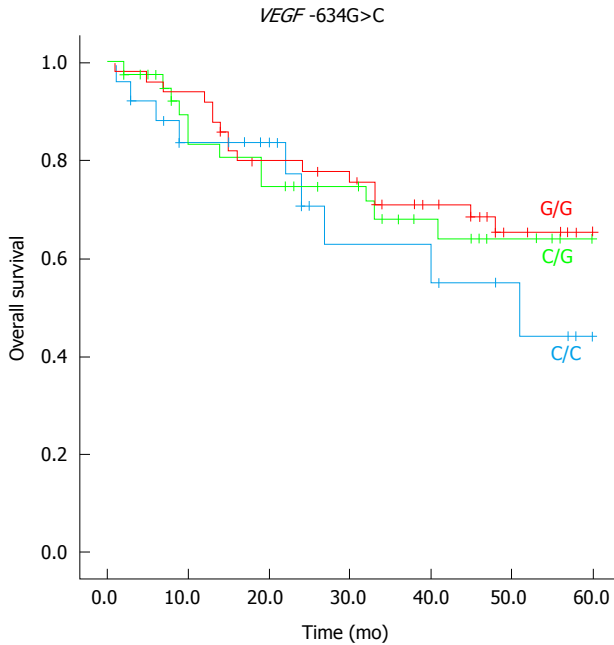
Variables	Category	HR (95%CI)	<i>P</i>
<i>VEGF-A</i> -1498C>T	TT	1 (reference)	-
	CT	2.79 (1.01- 7.66)	0.047 <sup>1</sup>
	CC	4.67 (1.51-14.43)	0.007 <sup>1</sup>
<i>VEGF-A</i> -634C>G	GG	1 (reference)	-
	CG	1.44 (0.58-3.55)	0.433
	CC	3.76 (1.29-10.93)	0.015 <sup>1</sup>

<sup>1</sup>Significant Cox multiple regression test.

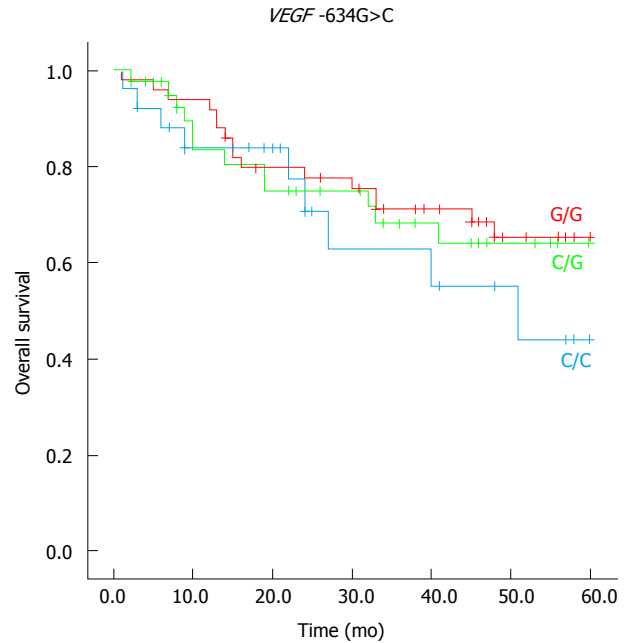
and generates large amounts of high quality DNA. Fixation in paraffin-embedded tissue can cause cross-linking and damage of DNA isolated damaging the amplification reaction and primer pattern recognition, besides mutation artifacts, e.g., artificial C-T or G-A transitions<sup>[39]</sup>.

The results of the present study did not support an association of the *VEGF-A* -1498C>T and *VEGF-A* -634G>C polymorphisms with tumor size, histological grading, tumor stage, lymph node metastasis and age at diagnosis in CRC cases. In the same way, Hofmann *et al.*<sup>[28]</sup> and Dassoulas *et al.*<sup>[36]</sup> found no correlation between the *VEGF-A* -634G>C and *VEGF-A* -1498C>T polymorphisms and the tumor characteristics. On the other hand, Chae *et al.*<sup>[40]</sup> showed that the T allele was related to the advanced stage of CRC.

Kim *et al.*<sup>[29]</sup> reported that -634GC and -634CC genotypes were associated with a favorable prognosis compared to -634GG genotype *VEGF-A* polymorphism



**Figure 1** The Kaplan-Meier survival curves as a function of vascular endothelial growth factor A-1498C>T genetic polymorphism genotypes in colorectal cancer patients. VEGF: Vascular endothelial growth factor.



**Figure 2** The Kaplan-Meier survival curves as a function of vascular endothelial growth factor A -634G>C genetic polymorphism genotypes in colorectal cancer patients. VEGF: Vascular endothelial growth factor.

in CRC patients. Particularly, these authors reported that -634G>C *VEGF-A* polymorphism is an independent prognostic factor for CRC. Watson *et al.*<sup>[24]</sup> observed that there is a significant correlation between production of VEGF-A protein from peripheral blood mononuclear cells and *VEGF -634G-A>C* polymorphism. These authors also reported the decreased production of VEGF-A protein in patients with homozygotes CC *VEGF-A* gene and increased production of VEGF-A protein in homozygotes GG *VEGF-A* genes. In the present series, the CC genotype of *VEGF-A -634C>G* polymorphism was significantly related to survival in patients with resected colorectal carcinoma. In accordance with Kim *et al.*<sup>[29]</sup>, our results pointed out that 634C>C polymorphism was an independent prognostic factor and it was associated with a worse 5-year survival rate compared to the *VEGF-A* GC genotype. Dassoulas *et al.*<sup>[36]</sup> analyzed DNA extracted from paraffin-embedded tissue from 312 Greek patients with CRC in all stages and evaluated the prognostic value of five *VEGF-A* polymorphisms, including the -634G>C and -1498C>T polymorphisms. They reported that -634 CC genotype was associated with a poor prognosis in the Greek population, which agree with the results we found with Brazilian patients in the present series. Dassoulas *et al.*<sup>[36]</sup> concluded that, in Greek patients with CRC, *VEGF-A -634G>C* and -1498C>T, polymorphisms were independent markers of prognosis. Hansen *et al.*<sup>[41]</sup> demonstrated obvious relationships between genetic variations in the *VEGF-A* gene and response to first-line capecitabine in patients with metastatic colorectal cancer, which translated to a significant difference in progression-free survival.

Chae *et al.*<sup>[40]</sup> analyzed the associations of *VEGF-A -634G>C* polymorphism in patients with CRC. These

authors observed that there was no significant correlation between the genotype GC with TNM stage III/IV, lymph node involvement and distant metastasis in CRC. On the other hand, Jang *et al.*<sup>[42]</sup> genotyped the *VEGF-A -634G>C* polymorphism in 350 CRC cases from the Korean population. The results suggest that this genetic polymorphism variant is not a potential genetic marker for CRC prognosis.

However, Hansen *et al.*<sup>[41]</sup> reported opposite results. The authors studied CRC in Danish patients with stages II and III and found that -634GC heterozygote genotype exhibited lower free disease and survival rates compared to the corresponding wild-type homozygote genotypes. This result was the opposite of what we found in the present study.

Kjaer-Frifeldt *et al.*<sup>[43]</sup> found in a multivariate analysis that *VEGF-A -1498C>T* and *VEGF-A -634G>C* polymorphisms were independent prognostic factors for the risk of death of patients by CCR, as we observed in our cases.

The difference between the results of these studies is not sufficiently clear. The discrepancy between the studies of *VEGF-A* polymorphism and CRC prognosis can be attributed to the differences in disease status, race and size of the sample studied<sup>[29,36]</sup>. Another possible explanation for these results is the DNA sequence variations in the *VEGF-A*, variation in the gene *locus*, action by several other genes and environmental characteristics. All these variables may alter VEGF-A production and/or activity, thereby causing inter-individual differences in the lymphangiogenesis and lymphatic tumor spread and, thus, in the development and progression of the tumors<sup>[29,36,39]</sup>. The differential role that individual polymorphisms of *VEGF-A* may play



in the biological activity of VEGF-A protein secreted by intratumoral variability of VEGF-A genetic expression is enhanced by these findings. In a large meta-analysis, involving 27 studies Des Guez *et al.*<sup>[19]</sup> demonstrated that VEGF-A overexpression is significantly correlated with poor overall survival and with an increased risk of relapse in CRC patients.

The differences between the results of published studies can be attributed to the different sources of DNA, the different ethnic backgrounds of the patients studied, the number of patients tested, the different designs of the studies, a lack of prospective randomized trials, laboratory tests, numerous genetic polymorphisms and errors in the interpretation of results<sup>[44-46]</sup>.

In summary, in the univariate analysis we found an association between genotype CC of the VEGF-A -1498C>T polymorphism and the occurrence of hepatic metastasis of CRC. In the multivariate analysis, genotypes CT and CC of VEGF-A -1498C>T polymorphism and genotype CC of VEGF-A -634C>G genetic polymorphism are independent prognostic factors for the risk of death in Brazilian patients with sporadic CRC.

The study of VEGF-A polymorphisms can bring new impacts on pharmacogenetics as VEGF-A is an important target in antineoplastic therapy, and the results of the VEGF-A polymorphisms may enhance the targeted therapies. This approach will be of great help to physicians in terms of tailoring individual therapies and enhancing the quality of patients' postoperative treatment. Moreover, since genetic polymorphisms often show a discrepancy between ethnic groups, more studies are also warranted to clarify the association between the VEGF-A polymorphisms and the CRC in diverse ethnic populations.

In conclusion, our data suggested that the CT and CC genotypes of the VEGF-A -1498C>T polymorphisms and the CC genotype of the VEGF-A -634C>G polymorphisms are independent prognostic factors for the risk of death in Brazilian patients with sporadic colorectal carcinoma.

## COMMENTS

### Background

Polymorphisms are naturally occurring DNA sequence variations, which differ from the gene mutations. The functional polymorphisms could contribute to the difference between individuals according to the susceptibility and severity of diseases. Polymorphisms alone or in combination with environmental factors may affect the angiogenic pathway and, thereby, the susceptibility and severity of cancer. The vascular endothelial growth factor (VEGF), one of the most potent endothelial cell mitogens, is considered one of the strongest promoters of angiogenesis in colorectal cancer (CRC). Given these characteristics, VEGF is a potential marker for determining the prognosis of CRC and it has also been used as a therapeutic target for the new molecular anticancer drugs such as bevacizumab. VEGF-A is considered to be the main, dominant inducer of the growth of blood vessels. The VEGF-A -634G/C polymorphism appears to be associated with a higher VEGF-A expression.

### Research frontiers

Studies have shown that the genetic polymorphisms can be used to predict the clinical outcomes of gastrointestinal, breast, ovary and pancreatic cancers. The

human VEGF-A gene is highly polymorphic, with more than 15 single nucleotide polymorphisms (SNPs) described, thus enabling wide variation in its expression between individuals from different ethnic groups, and there are few studies involving Latinos, Hispanics, and particularly Brazilians.

### Innovations and breakthroughs

The contribution of common VEGF-A genetic polymorphisms to the CRC prognosis remains unclear. Furthermore, studies investigating the association between VEGF-A genetic polymorphisms and CRC risk reporting conflicting results and the specific associations still remain controversial. Because VEGF-A is known to be a potent proangiogenic factor, the authors evaluated the potential association of two VEGF-A genetic polymorphisms (-634G>C and -1438C>T) with the clinicopathologic variables and its possible implication for prognoses in a population of Brazilian patients who had surgical procedures to remove CRC. The authors found an association between genotype CC of the VEGF-A -1498C>T genetic polymorphism and the occurrence of hepatic metastasis of CRC. Moreover, genotypes CT and CC of VEGF-A -1498C>T genetic polymorphism and genotype CC of VEGF-A -634C>G genetic polymorphism were independent prognostic factors for the risk of death in Brazilian patients with sporadic CRC.

### Applications

An important translational research field that will benefit from this knowledge is the pharmacogenetics field, in which researches study the impact of VEGF-A polymorphisms. VEGF-A protein is an important target in anticancer therapy and findings about VEGF-A polymorphisms may enhance the targeted therapies. This approach will be of great help to physicians in terms of tailoring individual therapies and enhancing the quality of patients' postoperative treatments.

### Terminology

Polymorphism is the occurrence of two or more clearly different forms or alternative phenotypes in the population of a species. Genetic polymorphism is the occurrence together in the same population of two or more genetically determined phenotypes in such proportions that the rarest of them cannot be maintained merely by recurrent mutation. Most genetic polymorphisms are functionally neutral, but some have effects on the regulation of the gene expression or on the function of the coded protein. SNPs are a genetic polymorphism between two genomes that is based on substitution, deletion, insertion, or exchange of a single nucleotide. Angiogenesis is a sequence of processes starting with vessel dilatation and pericyte recruitment in the pre-existing vessels, followed by endothelial cell proliferation, formation of new vessels, and recruitment of perivascular cells.

### Peer-review

This is an excellent article and the findings will definitely add to the existing knowledge.

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## Observational Study

**Bayesian adjustment for over-estimation and under-estimation of gastric cancer incidence across Iranian provinces**

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**Abstract****AIM**

To correct the misclassification in registered gastric cancer incidence across Iranian provinces in cancer registry data.

**METHODS**

Gastric cancer data is extracted from Iranian annual of national cancer registration report 2008. A Bayesian method with beta prior is implemented to estimate the rate of misclassification in registering patient's



permanent residence in neighboring province. Each time two neighboring provinces with lower and higher than 100% expected coverage of cancer cases are selected to be entered in the model. The expected coverage of cancerous patient is reported by medical university of each province. It is assumed that some cancer cases from a province with a lower than 100% expected coverage are registered in their neighboring province with more than 100% expected coverage.

## RESULTS

The condition was true for 21 provinces from a total of 30 provinces of Iran. It was estimated that 43% of gastric cancer cases of North and South Khorasan provinces in north-east of Iran was registered in Razavi Khorasan as the neighboring facilitate province; also 72% misclassification was estimated between Sistan and balochestan province and Razavi Khorasan. The misclassification rate was estimated to be 36% between West Azerbaijan province and East Azerbaijan province, 21% between Ardebil province and East Azerbaijan, 63% between Hormozgan province and Fars province, 8% between Chaharmahal and bakhtyari province and Isfahan province, 8% between Kogiloye and boyerahmad province and Isfahan, 43% Golestan province and Mazandaran province, 54% between Bushehr province and Khuzestan province, 26% between Ilam province and Khuzestan province, 32% between Qazvin province and Tehran province (capital of Iran), 43% between Markazi province and Tehran, and 37% between Qom province and Tehran.

## CONCLUSION

Policy makers should consider the regional misclassification in the time of programming for cancer control, prevention and resource allocation.

**Key words:** Cancer incidence registry; Misclassification; Bayesian correction; Gastric cancer; Iran

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**Core tip:** Due to the lack of equipped health facilities in some provinces of the country, patients seek health care in neighboring full-featured provinces and they do not mention their own permanent residence. It makes misclassification error in cancer registry data. This error flaws planning for resource allocation to different regions of the country for cancer control and prevention. The aim of this study is to use a Bayesian method to estimate the rate of misclassification in cancer incidence registry among neighboring provinces of Iran and re-estimating the rate of gastric cancer in each province.

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## INTRODUCTION

Gastric cancer was 4<sup>th</sup> most common cancer among men and 5<sup>th</sup> among women diagnosed in 2012 in the world<sup>[1]</sup>. Generally, its incidence is about twice in males as in females. Eastern Asia, Eastern Europe and South America have the highest rates of gastric cancer incidence and the lowest incidence rates are belong to North America and most parts of Africa<sup>[2]</sup>. As a result of population aging and growth, and also adoption of cancer-related lifestyle like westernized diets and physical inactivity, the burden of cancer is increasing in developing countries<sup>[3]</sup>. Gastric cancer is the first common cancer among Iranian men and the 3<sup>rd</sup> common cancer (after breast cancer and colorectal cancer) among Iranian women<sup>[4]</sup>.

Nowadays, information about incidence and mortality is one of the most important needs for making effective medical decisions. Having information regarding chronic diseases, especially cancers as one of the major causes of human mortality, has a particular importance<sup>[5]</sup>. Considering different diseases with different etiologies, just incidence rate is not enough to be an index of burden in the population. The different incidence rates of cancer diseases in different regions, races and occupational groups emphasize the importance of comprehensive information about it. Identifying the distribution of disease between different populations is a good manner for finding causations and quantifying the potentials for disease prevention<sup>[6,7]</sup>.

Incidence, the number of new cases occurring, is produced by population-based cancer registries. Cancer registries, which originated in the twentieth century, have expanded in the last 20 years<sup>[8,9]</sup> and is known as the main source of epidemiological data for all cancer fighting sectors at the local and international level. These registries collect information regarding burden of cancers by recording the incidence, mortality, prevalence and survival for different cancers in a systematic manner<sup>[10-12]</sup>. Their role has expanded into the planning and evaluation of cancer screening programs, detecting the impact of interventions and treatments to cancer control, improvement in patient care and specifying future needs for material and manpower resources. Accurate information of incidence and mortality rate is the most essential requirement to control cancer which is related to the correctness of information for individual cancer patients which is included the patient's residence, date of diagnosis, primary site and histological type of tumor, and date of death<sup>[9]</sup>.

Most cancer patients throughout the country seek diagnostic and medical treatment in Tehran (capital of Iran) or neighboring facilitate provinces either due to lack of proper facilities in their own residence or to obtain better-quality treatment and diagnostic

services<sup>[13]</sup>, and they don't mention their permanent residence address. It makes misclassification error in cancer registry data. Misclassification error is the disagreement between the observed and the true value. As the evidence of existence of misclassification error, the expected coverage of cancer incidence in different provinces can be noted; that the observed rate of incidence is more than expected rate in some provinces, and on the other hand, it is much less than expected rate in neighboring provinces<sup>[14]</sup>. However it happens while it is expected that the rate of cancer incidence be about the same in neighboring provinces that are similar in lifestyle and environmental conditions.

There are two approaches to reduce the effects of misclassification error; the first is using a small validation sample<sup>[15]</sup> and the second is Bayesian method which provides subjective prior information for some of the parameters for estimating misclassification parameter and correcting the statistic<sup>[16-18]</sup>. Bayesian models also can accommodate unobserved variables like individual's true information in the presence of Misclassification error. Bayesian method is a flexible method in which the prior information from previous studies can be incorporate with observed data in the analysis and even if vague priors are specified, Bayesian Monte Carlo Markov Chain method can be used to fit highly realistic models. The aim of this study is to use a Bayesian method to estimate misclassification rate in neighboring provinces in Iran using gastric cancer incidence registry data of 2008.

## MATERIALS AND METHODS

Gastric cancer incidence data is extracted from Iranian annual of national cancer registration report in 2008<sup>[14]</sup>. Firstly, the age standardized rate (ASR) for gastric cancer [coded according to the 10<sup>th</sup> revision of the International Classification of Diseases (ICD-10; C16)] is calculated for all provinces of Iran using direct standardization method and the standard population in WHO 2000 for both genders and four age groups (0-14 years, 15-49 years, 50-69 years and over than 70 years old) in order to compare statistics on cancer in Iran with those for the rest of the world<sup>[19]</sup>. Then expected coverage of cancer cases is calculated for medical university of each province. In the process of cancer incidence registry, all new diagnosed cancer cases by pathology centers and other diagnostic centers in the country are entered in software which is made by ministry of health. Medical university of each province sends this information recorded in this temporary data bank to the ministry of health. Ministry of health after coding the recorded cancers based on 10<sup>th</sup> revision of international coding of disease and removing duplicates, makes a permanent data bank of cancer cases and sends it back to medical university of each province. So each medical university has an observed number of cancer cases and also has an expected coverage of cancer cases that is considered to

be 113 per 100000. By dividing the observed number to the expected number of cancer cases, the percent of expected coverage for each province is calculated. For entering the data to the Bayesian model two vectors  $y_1$  and  $y_2$  were used. Vector  $y_1 = [y_{11}, y_{21}, \dots, y_{r1}]'$  for the province that has an expected coverage less than 100% and vector  $y_2 = [y_{12}, y_{22}, \dots, y_{r2}]'$  for a neighboring province with a more than 100% expected coverage. Subscript  $r$  shows the number of covariate patterns that is made by age group and sex group combinations. A Poisson distribution was considered for count data  $y_1$  and  $y_2$ <sup>[20-22]</sup>. An informative beta prior distribution was assumed for  $\theta$  as the probability of registering a data in misclassified group; so  $\theta \sim \text{beta}(a, b)$ <sup>[23-25]</sup>. For selecting prior value for the parameters of beta distribution, the calculated expected coverage for the medical university which has a lower than 100% expected coverage was used as  $b$  and  $a$  was calculated with subtracting  $b$  from 100. Thus  $a/(a + b)$  which is the expectation of beta distribution converges to the misclassified rate. Since  $\theta$  is unknown, a latent variable approach was employed to correct the misclassification effect<sup>[20,21]</sup>. The latent variable  $U$  with binomial distribution, i.e.,  $U_i | \theta, y_1, y_2 \sim \text{Binomial}(y_{i2}, P_i)$  that  $P_i = (\lambda_{i1}\theta) / (\lambda_{i1}\theta + \lambda_{i2})$ , was considered as the number of events from the first group that are incorrectly registered in the misclassified group. Finally by using a Gibbs sampling algorithm, the posterior distribution appears in the following form;  $\theta | U_i, y_1, y_2 \sim \text{Beta}(\sum_i U_i + a, \sum_i y_{i1} + b)$ <sup>[20,26-28]</sup>. After estimating the misclassification rate between each two neighboring provinces, the rates of gastric cancer incidence for each province were re-estimated. Analyses were carried out using R software version 3.2.0.

## RESULTS

All incidence records due to gastric cancer that have registered at Iranian annual of national cancer registration report in 2008 included in this study.

Twenty-one provinces from a total of 30 provinces of Iran are selected to be included in this study based on their expected coverage of cancer cases. In the other nine provinces, the number of cancer cases was about the same as their expectation from the number of cancerous patients that are registered in those provinces.

For example the reported percent of expected coverage of cancer incidence for Razavi Khorasan province in north-east of Iran was 155.5%. It means that Razavi Khorasan province have covered 55.5% more new cancer cases than its expectation, whereas the South and North Khorasan provinces that are in neighborhood of Razavi Khorasan have just covered respectively 41.4% and 34.8% of their expected coverage of cancer incidence; that is a clear indication of existence of misclassification error. The expected coverage for other provinces is mentioned in Table 1.

After implementation of the Bayesian method, it was found that there was 43% misclassification in

**Table 1** Expected coverage of cancer cases for Iranian provinces in 2008

Medical University	Percent of expected coverage
Razavi Khorasan	155.5
North Khorasan	34.8
South Khorasan	41.4
Sistan and balochestan	25
East Azerbaijan	123.6
West Azerbaijan	69
Ardebil	63
Isfahan	107.5
Kohgiluyeh and boyer-ahmad	25.1
Chaharmahal and bakhtyari	38
Fars	120.8
Hormozgan	19
Khozestan	101.19
Bushehr	25
Ilam	39.4
Mazandaran	338.4
Golestan	50.8
Tehran	155.62
Qom	53.9
Qazvin	66.3
Markazi	69.6

gastric cancer incidence registry from North and South Khorasan provinces in Razavi Khorasan province, 72% misclassification from Sistan and balouchestan province located in south-west of Iran in Razavi Khorasan that is one of most facilitate provinces of Iran with equipment health care services, 8% misclassification from Kohgilouye and boyer-ahmad province in Isfahan province that is one of the biggest provinces in central region of Iran, 8% misclassification from Chaharmahal and bakhtyari province in Isfahan, 36% misclassification from West Azerbaijan province in East Azerbaijan province that is its neighboring facilitate province in north west of Iran, 21% misclassification from Ardebil province in East Azerbaijan, 43% misclassification from Golestan province in Mazandaran province in the margin of Caspian sea, 26% misclassification from Ilam province in Khozestan province in the south of the country that has more health facilities proportional to its neighboring provinces, 54% misclassification from Bushehr province in Khozestan, 63% misclassification from Hormozgan province in Fars province that is either one of the most facilitate provinces of Iran, 32% misclassification from Qazvin province in Tehran that is the capital of Iran in the central region of the country, 43% misclassification from Markazi province in Tehran and 37% misclassification from Qom province in Tehran. Number of gastric cancer incidence and ASR of gastric cancer before and after Bayesian correction in each province for total population and also separately for male and female appeared in Tables 2 and 3.

## DISCUSSION

According to this study, incidence of gastric cancer in Iran's provinces is registered with misclassification

error and some patients are diagnosed and registered in their neighboring provinces; especially the provinces that don't have equipment health centers with enough diagnostic devices and experts.

Knowledge about spatial pattern of diseases is useful for assessing the influencing factors on disease incidence and planning for disease prevention and control<sup>[29,30]</sup>. Geographic variations in the incidence of certain cancers (especially gastric cancer) have been investigated before<sup>[31]</sup>. High-risk areas for gastric cancer, with age-adjusted incidence rates over than 20 in 100000 person-years include Japan, China, South Korea, Brazil and Costa Rica. Areas with a moderate risk and standardized incidence of between 10 and 20 per 100000 persons include England, Germany, New Zealand and Turkey. Areas with low risk and adjusted incidence of less than 10 include United States, Denmark and Sweden<sup>[32]</sup>. Iran is one of the countries with moderate risk of gastric cancer. Furthermore, different areas of the country has obvious geographical differences in gastric cancer incidence rate<sup>[33]</sup>. Clustering of gastrointestinal cancer incidence in counties of Iran at the margin of the Caspian Sea for 2008 has been reported a significant difference in the rate of gastrointestinal cancer incidence in different parts of this area<sup>[34]</sup>. A study in Ardebil province in north-west of Iran also showed that this province is a high risk area in terms of global statistics<sup>[35]</sup>. Another study investigated the clustering status of the gastric cancer incidence among provinces of Iran from 2004 to 2009 and showed that gastric cancer had a significant clustering status in northern, north-western and western provinces<sup>[36]</sup>. When a cluster of high incidence is not occurred by chance, we need to ask what could be the underlying causal mechanism. It is natural to look first at the known or hypothesized risk factors<sup>[34]</sup>. Several factors such as genetics, infection of *Helicobacter pylori*, excessive use of tobacco (especially cigarettes), high salt consumption, high intake of nitrates and inadequate intake of antioxidants, low social economic status and environmental factors were among the known risk factors for gastric cancer<sup>[37-39]</sup>. But major difference in gastric cancer incidence rate in neighboring provinces that are almost the same in environmental factors, dietary habits and lifestyle is only justifiable with existence of misclassification error in registering permanent residence of cancerous patients that are diagnosed and treated in neighboring facilitate provinces or Tehran as the capital of the country.

The study of Geographical spread of gastrointestinal tract cancer incidence in the Caspian Sea region also noted that a number of residents of Mazandaran and Golestan seek medical care outside the region, especially in Tehran, and occasionally in neighboring provinces<sup>[34]</sup>. In a study in Fars province (in the south of Iran) with aim of measuring the completeness of coverage of cancer registry information recorded between 2000 and 2009, actual registry data compared with the expected incidence rate. The maximum

**Table 2** Number of gastric cancer incidence and the percent of change before and after Bayesian correction in Iranian provinces 2008

Province	Before bayesian correction			After bayesian correction			Change%
	Male	Female	Both	Male	Female	Both	
Fars	199	102	301	105	54	158	-47.37
Isfahan	211	86	297	188	77	264	-10.97
Razavi Khorasan	550	194	744	371	131	502	-32.55
East Azerbaijan	399	148	547	237	88	325	-40.53
Mazandaran	338	150	488	274	122	396	-18.81
Khozestan	233	114	347	173	85	258	-25.69
Tehran	1131	567	1698	1019	511	1530	-9.88
West Azerbaijan	191	80	271	291	122	412	52.17
Kohgiluyeh and Boyer-Ahmad	38	18	56	50	24	74	31.88
Hormozgan	26	17	43	112	73	186	331.58
Chaharmahal and Bakhtiari	46	24	70	56	29	85	21.06
Sistan and Baluchistan	38	9	47	147	35	182	288
Ilam	37	13	50	61	22	83	65.98
North Khorasan	42	15	57	94	34	127	123.56
South Khorasan	22	13	35	45	27	71	103.86
Golestan	104	35	139	173	58	231	66.04
Qom	58	26	84	98	44	142	68.64
Ardebil	172	69	241	229	92	321	33.33
Bushehr	17	9	26	54	28	82	216
Qazvin	80	33	113	119	49	168	48.27
Markazi	63	27	90	102	44	146	61.78

**Table 3** Age standardized rate of gastric cancer incidence before and after Bayesian correction in Iranian provinces 2008

Province	Before Bayesian correction			After Bayesian correction		
	Male	Female	Both	Male	Female	Both
Fars	12.21	7.01	9.61	6.43	3.69	5.06
Isfahan	0.94	5.56	3.25	0.84	4.95	2.89
Razavi Khorasan	10.24	24.18	17.21	6.91	16.31	11.61
East Azerbaijan	26.61	12.09	19.35	15.82	7.19	11.51
Mazandaran	28.53	15.57	22.05	23.16	12.64	17.9
Khozestan	13.82	7.78	10.8	10.27	5.78	8.02
Tehran	20.61	12.39	16.5	18.57	11.17	14.87
West Azerbaijan	17.26	8.68	12.97	26.27	13.21	19.74
Kohgiluyeh and Boyer-Ahmad	14.61	7.71	11.16	19.27	10.17	14.72
Hormozgan	4.71	4.02	4.37	20.33	17.35	18.85
Chaharmahal and Bakhtiari	13.46	8.6	11.03	16.29	10.41	13.36
Sistan and Baluchistan	4.16	0.97	2.56	16.14	3.76	9.95
Ilam	17.16	6.33	11.75	28.48	10.51	19.5
North Khorasan	12.62	5.37	9	28.21	12.01	20.12
South Khorasan	9.7	7.62	8.66	19.77	15.53	17.66
Golestan	16.86	6.27	11.56	28	10.41	19.2
Qom	13.87	7.96	10.92	23.39	13.42	18.41
Ardebil	35.18	17.49	26.33	46.91	23.32	35.11
Bushehr	4.21	3.29	3.75	13.3	10.4	11.84
Qazvin	17.76	8.43	13.1	26.33	12.5	19.42
Markazi	11.67	5.75	8.71	18.88	9.3	14.09

acceptable error rate was 5% for deficiencies in personal and demographic information (gender, age, father's name and area of residence) and encoding the cancers. At the beginning of their program, the address of patients was not recorded with detail and in a systematic manner. Although the rate of deficiencies was decreased as a result of staff training, the error rate in address information (7.87%) remained above the 5% threshold by the end of the study period in 2009<sup>[8]</sup>.

These findings confirm the existence of misclassification error and incompleteness of address-related information in Iran's cancer registry that results under-

estimation of cancer incidence rate in some provinces and overestimation in some others.

In conclusion, despite international efforts to standardize cancer incidence data collection processes, the quality of data from many countries remains poor. Low quality of cancer incidence data leads to a misclassified registered data<sup>[40]</sup>. Iran as a developing country is not an exception. So, there are provinces with higher or lower incidence of gastric cancer than the registered rates, and policy makers employ these data to allocate the facilities and resources. Regionally misclassified cancer incidence data leads to misallocation of resources. So



authorities should notice that low incidence rate of gastric cancer in some provinces, do not mean that they are in a good health condition and gastric cancer incidence is really low in these provinces, but quite the contrary, this may be the effect of misclassification error and it is needed to allocate them more health facilities, equipped health centers, and improve the quality of registration system.

Enhancing hardware and software resources, training and increasing the number of educated and motivated staff in all public and private sectors involved in the cancer registry program in order to complete the cancer case registry forms accurately and remit them to the appropriate center, expert researchers in medicine and cancer knowledge, computer science and biostatistics, and greater attention to epidemiological research are needed to qualify the cancer registry program and increasing its completeness; specially completeness of address-related information<sup>[41,42]</sup>. So, the better the quality of cancer registry data, better the possibility of effective use of these data in planning and prevention.

## COMMENTS

### Background

Most patients from low facility provinces, seek medical treatment in their neighboring facilitate provinces. Some of them do not mention their permanent residence. It makes misclassification error in cancer registry data and misleads health policy makers who use cancer registry data for resource allocation and cancer control programs. The aim of this study is to use Bayesian method for estimating the rate of misclassification between neighboring provinces and re-estimating the rate of gastric cancer incidence in each province of Iran.

### Research frontiers

Information about geographic spread of cancers is so important for cancer control and prevention. Iran's cancer registry data is subject to misclassification in patient's permanent residence that leads to under-estimation of cancer risk in some provinces and consequently over-estimation in other provinces. Cancer registry data is usually used in spatial analysis to determine the high risk areas without considering the existence of misclassification error. The hotspot of this study is using the Bayesian method for accounting and correcting for misclassification in registering cancer incidence.

### Innovation and breakthroughs

By using the Bayesian method, having prior information about the misclassification rate is enough and it is not needed to use valid data for estimating the misclassification rate. Bayesian method for correcting the misclassification is faster and more cost effective in comparison to data validation which is time consuming and in many cases is not achievable.

### Applications

Since cancer registry data is used by health policy makers for allocating medical resources to different provinces, it is important to correct for misclassification in registering patient's permanent residence, in order to have more accurate estimates from the rates of gastric cancer incidence and consequently better planning for cancer control and prevention.

### Terminology

Misclassification is a measurement error which defines as disagreement between the observed value and the true value in categorical data. Bayesian method is a statistical approach that assigns a prior distribution to events or parameters, based on expert idea or previous knowledge and modifies those distributions after obtaining the data by using Bayes' theorem.

## Peer-review

In this manuscript, authors were aimed to correct the misclassification of gastric cancer incidence across Iranian provinces in registry data.

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## Helical tomotherapy for duodenal adenocarcinoma in an elderly patient: A case report

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### Abstract

To evaluate the efficacy and feasibility of external beam radiotherapy (EBRT) for duodenal adenocarcinoma in an 84-year-old female who underwent EBRT (2.2 Gy/d for a total dose of 46.2 Gy) using helical tomotherapy (HT). Toxicity was evaluated on the National Cancer Institute's common toxicity criteria (CTCAE 3.0). The patient completed the treatment without G3-G4 toxicity. After 22-mo follow-up, she is alive and well, in complete remission with no late side effects. HT seems to be feasible and effective for duodenal adenocarcinoma in old to very old patients.

**Key words:** The elderly; Duodenal carcinoma; Helical tomotherapy; Toxicity

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**Core tip:** Radiotherapy is now effective and safe for old to very old patients with duodenal adenocarcinoma, thanks to better dose conformity and tissue sparing with helical tomotherapy.

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Zucchetti C, Falcinelli L, Aristei C. Helical tomotherapy for duodenal adenocarcinoma in an elderly patient: A case report. *World J Gastrointest Oncol* 2017; 9(2): 94-97 Available from: URL: <http://www.wjgnet.com/1948-5204/full/v9/i2/94.htm> DOI: <http://dx.doi.org/10.4251/wjgo.v9.i2.94>

## INTRODUCTION

Duodenal adenocarcinoma accounts for about 0.3% of gastrointestinal carcinoma<sup>[1]</sup>. Treatment consists of complete surgical resection (segmental resection, pancreaticoduodenectomy and pancreas-preserving duodenal resection)<sup>[2-4]</sup>. As symptoms are often non-specific, the disease is frequently diagnosed at advanced stage when bypass surgery or stenting ± chemotherapy play a palliative role<sup>[2-5]</sup>. When radical surgery is feasible, the role of adjuvant chemotherapy or radiochemotherapy is unclear since duodenal carcinoma is so rare<sup>[6-8]</sup>. They are recommended for patients at high-risk of recurrence (high grade, nodal metastases, margin positivity, advanced stage disease) as disease recurrence and metastases are common<sup>[9]</sup>. Although external beam radiotherapy (EBRT) was claimed to be unsuitable for duodenal adenocarcinoma as the small bowel poorly tolerates radiation<sup>[10-12]</sup>, it was reported to improve local disease control but not overall survival<sup>[11]</sup>. With advanced radiotherapy techniques like intensity-modulated radiotherapy, volumetric radiotherapy and tomotherapy, dose distribution is now homogeneous and exposure of organs at risk (OARs) to high dose irradiation is now limited, thus decreasing the risk of complications<sup>[13]</sup>.

This case report shows EBRT with helical tomotherapy (HT) was efficacious and safe in an elderly female with duodenal adenocarcinoma who was ineligible for surgery.

## CASE REPORT

An 84-year-old female presented in hospital with abdominal pain, itching and cholestasis.

Clinical examination confirmed abdominal pain. Blood were counts normal with glutamic oxaloacetic transaminase 56UI/L (Normal range (nr) 40-45 U/L), glutamic pyruvic transaminase 46 UI/L (nr 5-35 U/L), alkaline phosphatase 241 UI/L (nr 55-142 U/L), Lactate Dehydrogenase 256Mu/MI (nr 122-222 U/L), total bilirubin 1.48 mg/dL (nr 0.3-1.0 mg/dL).

Endoscopic retrograde cholangiopancreatography (ERCP) revealed a mass involving the entire thickness of the duodenal wall mid-portion with extension of 12 mm without ampulla of Vater involvement. Findings were confirmed by contrast-enhanced computed tomography (CT) which did not detect any nodal metastases. Biopsy findings were duodenal adenocarcinoma which was staged cT3N0M0 (AJCC Cancer Staging Manual 7<sup>th</sup> edition)<sup>[14]</sup>.

**Table 1 Planning target volume coverage**

HT PTV	
D90%	49.27 Gy
D95%	48.76 Gy
D50%	50.61 Gy
D2%	51.5 Gy
V107	0 Gy
HI	0.96

HT: Helical tomotherapy; PTV: Planning target volume; HI: Homogeneity index; Gy: Gray.

As surgery and systemic chemotherapy were excluded because of age and comorbidities (high blood pressure and ischaemic heart disease) the patient was not investigated further. Instead of palliative support we opted for radical treatment with EBRT. To visualize the lesion a CT without contrast medium was performed after having inserted a metal stent and a clip about 0.5 cm below the distal margin during a repeated ERCP.

CT images were acquired with 0.25 cm slice thickness and transmitted to the Pinnacle<sup>3</sup> TPS V9.8. One radiation oncologist (VL) contoured the clinical target volume (CTV), *i.e.*, the diseased duodenal tract. CTV was expanded 5 mm in all directions except for the cranial and caudal which were expanded 7 mm to provide the planning target volume (PTV). The radiation oncologist contoured the liver and kidneys as OARs.

The HT plan was generated using the Tomotherapy HD System commercial planning software. The dose prescription was 46.2 Gy to PTV in 21 fractions.

HT provided good PTV coverage as shown in Table 1. Table 2 showed dosimetric results for the OARs, which were markedly lower than pre-defined dose constraints.

Treatment was associated with G2 acute abdominal toxicity (diarrhoea), as defined by CTCAE 3.0 criteria.

Follow-up included a clinical examination and complete blood work-up every three months, and abdominal ultrasound or CT scans every six months.

After 22-mo follow-up, the patient is alive and well, in complete remission without late side effects.

## DISCUSSION

Radiotherapy was the only option for this patient as surgery and chemotherapy were ruled out on account of age and comorbidities. However, despite the patient's age and advanced stage disease, her performance status was good (80% Karnofsky), she did not have cognitive and/or motor deficits and was compliant with treatment. These positive factors influenced our decision to opt for radical treatment and no palliative support. Even though a 22 mo follow-up is relatively short, our very old patient has achieved a complete response.

A mild hypofractionated schedule seemed appropriate for overcoming duodenal carcinoma's putative radioresistance. Indeed, duodenal adenocarcinoma may be chemo-radiation-sensitive as neo-adjuvant radio-



**Table 2** Organs at risk dosimetry for helical tomotherapy

	Constraints	HT
Right kidney		
V12 Gy	< 55%	5.6%
V20 Gy	< 32%	1.3%
V23 Gy	< 30%	0.7%
V28 Gy	< 20%	0.2%
Dmean	15-18 Gy	6.10 Gy
Left kidney		
V12 Gy	< 55%	22%
V20 Gy	< 32%	0%
V23 Gy	< 30%	0%
V28 Gy	< 20%	0%
Dmean	15-18 Gy	8.9 Gy
Liver		
V30 Gy	< 30%	0%
Dmean	< 28 Gy	5.32 Gy

HT: Helical tomotherapy; Dmean: Mean dose; Gy: Gray.

chemotherapy was associated with an overall survival benefit in 11 patients with a R0 resection (5-year 83% vs 53%)<sup>[15]</sup>. Onkendi *et al*<sup>[16]</sup> also showed that neo-adjuvant chemo- and/or chemoradio-therapy as well as intra-operative radiotherapy could potentially prolong survival.

HT provided good target cover and a homogeneous dose to the target volume. Furthermore, it limited exposure of OARs, keeping the kidney and liver doses as low as possible and circumvented the small bowel's poor tolerance of radiation<sup>[13]</sup>. In fact irradiation of a limited target area, as our patient had no nodal metastases, may have made a major contribution to low toxicity as she was affected by only G2 acute toxicity and suffered no late irradiation-related side effects. Finally, daily imaging-guided radiotherapy with megavoltage CT served to monitor treatment administration. Images were co-registered automatically with planning CT images using the bone and tissue technique and, if needed, the treatment position was adjusted.

Treating duodenal adenocarcinoma with radiotherapy alone, as in this case report, appears to be innovative since we have been unable to find any other reports of this approach. Data deriving from small single-institution, retrospective reports or biased case reports are available on adjuvant radiotherapy with or without chemotherapy but outcomes are divergent. A prospective, randomized European study<sup>[17]</sup> showed no difference in overall survival in patients with pancreatic or peri-ampullary tumours, when compared with surgery alone. It did not, however, distinguish between duodenal cancer and other tumours and did not describe the radiotherapy techniques and doses that were used. On the other hand, Swartz *et al*<sup>[11]</sup> reported local control rates of 93% and longer median survival (41 vs 21 mo) in 14 patients who received concurrent fluorouracil-based chemotherapy, a median of 50 Gy radiotherapy and maintenance chemotherapy compared with a control group who underwent only surgery. Confirming these findings, Overman *et al*<sup>[18]</sup> showed that adjuvant

chemo-radiotherapy improved disease-free survival and overall survival in patients at high-risk of relapse.

The present study has the usual case report limitations. It does, however, propose an option for the management of duodenal adenocarcinoma when surgery and chemotherapy are precluded and offers hope that surgery is no longer the only means of cure, particularly in old to very old patients<sup>[10]</sup>. Given the rarity of duodenal adenocarcinoma, future studies are needed to establish the most suitable treatment.

## COMMENTS

### Case characteristics

An 84-year-old female presented abdominal pain, itching and cholestasis.

### Clinical diagnosis

Clinical examination confirmed abdominal pain.

### Differential diagnosis

Ampullary carcinoma or other duodenum disease.

### Laboratory diagnosis

Blood counts with glutamic oxaloacetic transaminase, glutamic pyruvic transaminase, alkaline phosphatase, LDH, total bilirubin.

### Imaging diagnosis

The patient underwent to the endoscopic retrograde cholangiopancreatography, contrast-enhanced computed tomography.

### Pathological diagnosis

Biopsy findings were duodenal adenocarcinoma.

### Treatment

The patient was treated with external beam radiotherapy. The dose prescription was 46.2 Gy to PTV in 21 fractions.

### Related reports

Radiotherapy can be an option for the management of duodenal adenocarcinoma when surgery and chemotherapy are precluded.

### Term explanation

Helical tomotherapy provided a homogeneous dose distribution and limited exposure of organs at risk to high dose irradiation.

### Experiences and lessons

Given the rarity of duodenal adenocarcinoma, future studies are needed to establish the most suitable treatment.

### Peer-review

This manuscript provides very important clinical information of effectiveness of helical tomotherapy of duodenal cancer in the elderly. This may certainly be of use for treatment of duodenal cancer. The case presentation and discussions are well described and organized. The conclusion derived is consistent and sound.

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*WJGO* covers topics concerning carcinogenesis, tumorigenesis, metastasis, diagnosis, prevention, prognosis, clinical manifestations, nutritional support, molecular mechanisms, and therapy of benign and malignant tumors of the digestive tract. The current columns of *WJGO* include editorial, frontier, diagnostic advances, therapeutics advances, field of vision, mini-reviews, review, topic highlight, medical ethics, original articles, case report, clinical case conference (Clinicopathological conference), and autobiography. Priority publication will be given to articles concerning diagnosis and treatment of gastrointestinal oncology diseases. The following aspects are covered: Clinical diagnosis, laboratory diagnosis, differential diagnosis, imaging tests, pathological diagnosis, molecular biological diagnosis, immunological diagnosis, genetic diagnosis, functional diagnostics, and physical diagnosis; and comprehensive therapy, drug therapy, surgical therapy, interventional treatment, minimally invasive therapy, and robot-assisted therapy.

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## Can molecular biomarkers replace a clinical risk score for resectable colorectal liver metastasis?

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### Abstract

In resectable colorectal liver metastasis (CRLM) the role and use of molecular biomarkers is still controversial. Several biomarkers have been linked to clinical outcomes in CRLM, but none have so far become routine for clinical decision making. For several reasons, the clinical risk score appears to no longer hold the same predictive value. Some of the reasons include the ever expanding indications for liver resection, which now increasingly tend to involve extrahepatic disease, such as lung metastases (both resectable and non-resectable) and the shift in indication from "what is taken out" (*e.g.*, how much liver has to be resected) to "what is left behind" (that is, how much functional liver tissue the patient has after resection). The latter is amenable to modifications by using adjunct techniques of portal vein embolization and the associating liver partition and portal vein ligation for staged hepatectomy techniques to expand indications for liver resection. Added to this complexity is the increasing number of molecular markers, which appear to hold important prognostic and predictive information, for which some will be discussed here. Beyond characteristics of tissue-based genomic profiles will be liquid biopsies derived from circulating tumor cells and cell-free circulating tumor DNA in the blood. These markers are present in the peripheral circulation in the majority of patients with metastatic cancer disease. Circulating biomarkers may represent more readily available methods to monitor, characterize and predict cancer biology with future implications for cancer care.

**Key words:** Colorectal cancer; Liver metastasis; *KRAS*; Disease-free survival; Circulating tumor cell; Liver surgery; Overall survival; Molecular biomarkers

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**Core tip:** As a general rule, "good" colorectal liver metastasis (CRLM) cases amenable for surgery have fewer bad

genetic traits, such as less likelihood for *BRAF* mutations or *KRAS* mutations. *KRAS* mutation in patients with resectable CRLM suggests a more aggressive disease with shorter progression free and overall survival. Emerging evidence suggest that tumors change during the course of treatment and, thus giving way to new clones that may be of a different genetic makeup and have a different resistance pattern. Thus, new ways of monitoring disease and markers of progression is needed, including circulating cancer biomarkers and tissue-based genetic profiles.

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## INTRODUCTION

Colorectal cancer (CRC) is a leading cause of cancer deaths in the western world. For patients with non-metastatic disease at diagnosis, the prognosis for disease-free as well as overall survival (OS) is very good and, currently, exceeding 60% for both colon and rectum cancer<sup>[1]</sup>. Yet, still, some 40% will develop metastasis and die from the disease. Furthermore, about 20%-25% present with metastasis at the time of diagnosis, of which only a minority will be amenable to attempt at curative resection for both primary and metastatic disease. The liver is the most frequent site of metastasis in both situations, followed by the lungs and peritoneum. The current use of the TNM system as a guide of adjuvant therapies and prognosis is imperfect at best and is heavily debated<sup>[2]</sup>, emphasised by the need for continuous updates (now in its 8<sup>th</sup> edition). Notably, there is a strong need for better understanding of which tumours will develop metastasis and how cancer cells are able to invade, escape, colonize and grow as distant metastasis. Further, when metastases are present, better knowledge of what therapy can be used and how the cancer biology can be influenced, is direly needed.

For unresectable metastatic CRC disease the OS has dramatically changed over the past few decades. The improved survival is due to changes in chemotherapy and targeted drugs. A median survival historically reported around 6 mo for best supportive care alone is now approaching 24 mo and above with currently available chemo-regimens and targeted therapy<sup>[3]</sup>. Importantly, RAS profiling has emerged as an important predictive and prognostic factor, with *KRAS* and *BRAF* mutants displaying poor prognosis. In stage IV disease, targeted therapy (EGFR directed drugs<sup>[4]</sup>) is implemented in clinical practice and knowledge of mutated pathways is actively used to shape design of new trials, with recent guidelines for extended RAS testing being launched<sup>[5]</sup>.

Conversely, in resectable colorectal liver metastasis (CRLM) the role and use of molecular biomarkers is

more controversial. Several biomarkers have been linked to clinical outcomes in CRC, but none have so far become important in classification of cancer stage or in determining oncological or surgical treatment of the tumour or metastasis. Notably, as knowledge of tumor biology has increased, so has the emergence of molecular markers also come of age.

Currently, 5-year survival rates in patients with resectable CRLM ranges from 25% to 40% dependent on inclusion criteria and selection of cohorts. Several past studies have been published in an attempt to identify risk factors and predict survival. The scoring systems vary in terms of its clinical use, but risk factors include synchronous liver disease, primary tumor node status and histology, number and size of liver metastases, CEA level, disease-free interval and presence of extrahepatic disease<sup>[6-8]</sup>. The most widely used clinical scoring system is that proposed by Fong *et al*<sup>[8]</sup>, as depicted in Table 1. For scores 1-2, surgery for CRLM was clearly recommended, but for patients with scores of 5, the benefit was deemed questionable. Notably, the authors argued in their seminal paper, that to make the scoring system widely applicable, the additional inclusion of cellular or genetic markers was not reasonable. The latter prediction may have changed with more widespread molecular laboratories and considerable reduction in unit costs for molecular analyses.

For several reasons, the clinical risk score appears to no longer hold the same predictive value in current evaluation and management of CRLM. Some of the reasons include the ever expanding indications for liver resection, which now increasingly tend to involve extrahepatic disease, such as lung metastases<sup>[9]</sup> (both resectable and non-resectable) and the shift from "what is taken out" (*e.g.*, how much liver has to be resected) towards "what is left behind" (that is, how much functional liver tissue is the patient left with), the latter for which adjunct techniques of portal vein embolization<sup>[10]</sup> and the associating liver partition and portal vein ligation for staged hepatectomy (ALPPS) techniques<sup>[11]</sup> have continued to expand indications for liver resection. Added to this complexity is the increasing number of molecular markers, which appear to hold prognostic and predictive information, for which some will be discussed here.

## ROLE OF *KRAS* IN RESECTED CRLM

Up to 30%-40% of patients with CRC have mutated *KRAS*. The frequency for mutated *KRAS* in CRLM corresponds well with that of the primary tumour<sup>[12]</sup>. The incidence of *KRAS* mutation in resectable CRLM is variable, and in one meta-analysis reported a frequency between 15% and 37%<sup>[12]</sup>, likely indicating differences in selection criteria for CRLM surgery among the studies<sup>[13]</sup>. The meta-analysis included 14 studies with a total of 1809 patients<sup>[12]</sup>. Eight studies reported OS after resection of CRLM in 1181 patients. The mutation rate was 27.6%, and *KRAS* mutation was negatively associated with OS [hazard ratio (HR) = 2.24, 95%CI:



**Table 1** The clinical risk score (as suggested by Fong *et al.*<sup>[8]</sup>)

Score	0	1	Predicted 5-yr survival
Nodal status of primary tumor (pN0 vs pN+)	-	+	
Disease-free interval <sup>1</sup>	> 12 mo	< 12 mo	
Number of tumors	≤ 1	> 1	
Pre-operative CEA level	≤ 200 ng/mL	> 200 ng/mL	
Size of largest tumor	≤ 5 cm	> 5 cm	
Score			
0			60%
1			44%
2			40%
3			20%
4			25%
5			14%

<sup>1</sup>From primary tumor to discovery of liver metastasis. CEA: Carcino-embryonic antigen.

1.76-2.85]. Seven studies reported recurrence-free survival (RFS) after resection of CRLM in 906 patients. The mutation rate was 28.0%, and *KRAS* mutation was negatively associated with RFS (HR = 1.89, 95%CI: 1.54-2.32). Thus, there was an overall consistent poorer overall- and RFS for patients with mutated *KRAS* among the studies.

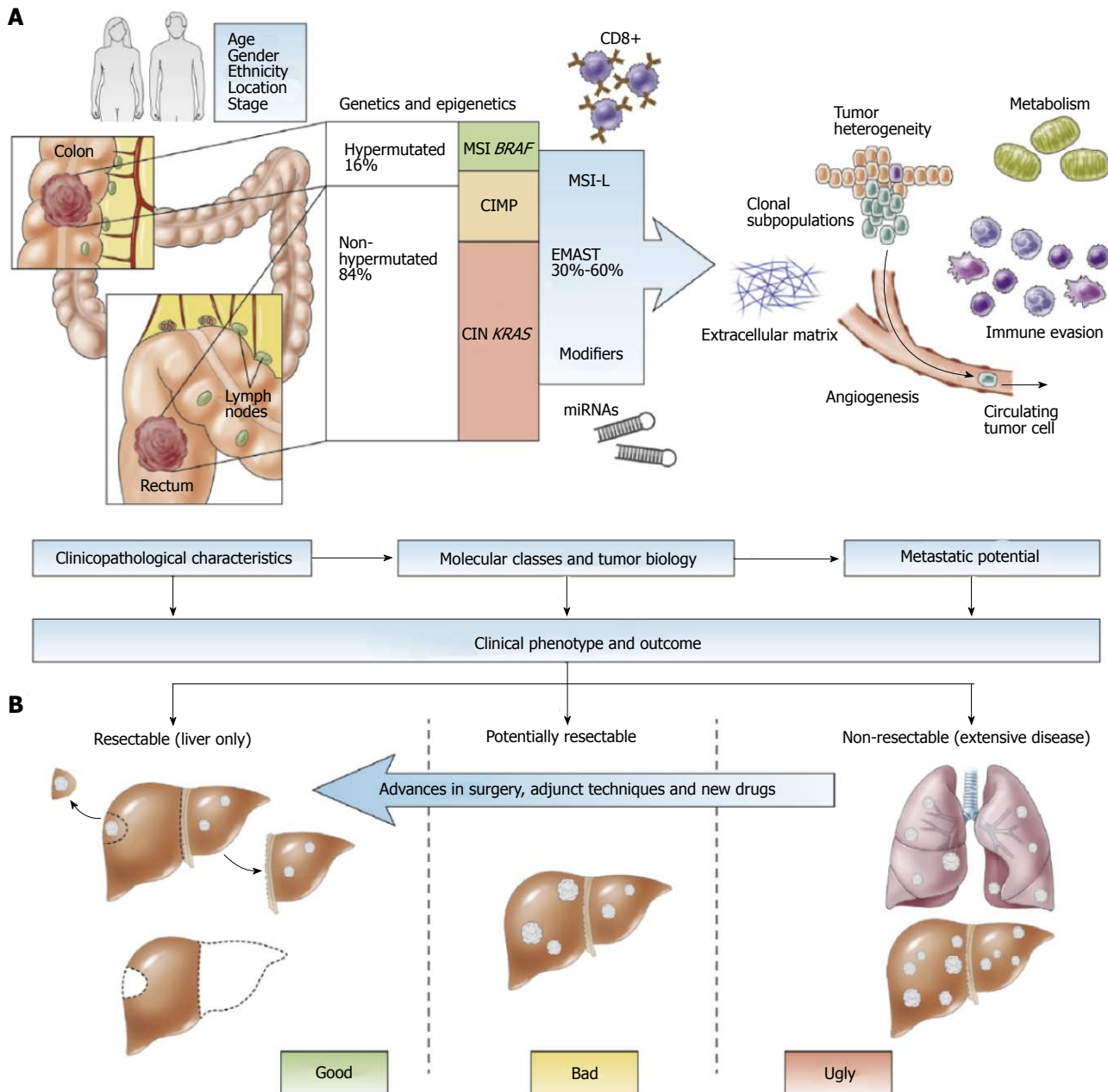
Still, the overall prognostic role of *KRAS* mutations is not clear. It seems that there is a higher rate of *KRAS* mutation in patients with extrahepatic metastasis and non-resectable CRLM<sup>[12,14]</sup>, that there is a higher risk of subsequent recurrence in all sites (brain, bone, liver and lungs) for patients with *KRAS* mutations<sup>[15,16]</sup> and, that *KRAS* mutation in patients with resectable CRLM suggests a more aggressive disease with shorter progression free and OS<sup>[12]</sup>. Indeed, as shown<sup>[17]</sup>, the difference in survival after liver resection was attributed to having either wild type *KRAS* or mutant *KRAS*, rather than achieving an R0 or R1 situation. This emphasizes the role of inherent cancer biology rather than resection margins. Factors that are associated with aggressive or advanced tumor biology (e.g., bilobar disease, multiple metastasis, large metastasis, and metastasis in difficult locations) are also associated with technically complex cases and are as such being at higher risk for a potential R1 resection. These data therefore suggests that it is the cancer biology, and not the R1 resection, that is related to worse survival<sup>[17]</sup>. Similar results were showed in a study were recurrence-free and OS were examined after treatment for CRLM with liver resection followed by adjuvant hepatic arterial infusion and chemotherapy. Positive surgical resection margins (R1) were not found to significantly predict RFS<sup>[15]</sup>, but rather, again, a decreased RFS occurred for *KRAS* mutant CRLMs. Furthermore, down-stream *BRAF* mutations in the RAS-pathway<sup>[4]</sup> signify a particularly poor prognosis in resected CRLM<sup>[18]</sup>. Thus, the clinical role of *KRAS* in resectable CRLM is slowly becoming clearer. In one recent study from the MD Anderson Cancer Center<sup>[19]</sup>, the investigators found that patients with poor prognostic features, such as node-positive primary tumor (pN+), largest liver metastasis > 3 cm and who had > 7 cycles of preoperative chemotherapy in addition to *KRAS*

mutation had a particularly poor prognosis. The authors conclude that major hepatectomy may be ill advised in such patients and that other therapeutic alternatives should be considered<sup>[19]</sup>.

## MOLECULAR MARKERS TO DEFINE "GOOD" FROM "BAD" BIOLOGY IN CRLM

In addition to mutations in the RAS-pathway, a plethora of known and new markers are considered as predictive and prognostic, yet few have found their way to clinical use. As a general rule, "good" CRLM cases amenable for surgery have fewer bad genetic traits, such as less likelihood for *BRAF* mutations or *KRAS* mutations. Adding to the complexity in understanding the role of genetic mutations and targeted therapy is the findings from the "new EPOC" study<sup>[20]</sup> of adjuvant chemotherapy with or without cetuximab (an EGFR inhibitor) to patients with resectable CRLM and *KRAS* wt. In theory, the drug should have a beneficial effect on outcomes, but to the investigators surprise, the group who received cetuximab actually had a worse RFS<sup>[20]</sup>. While the study has received critique for its design, conduct and analysis<sup>[21,22]</sup>, the uncertainty linked to these results await further exploration and clarification. The jury is still out regarding the role of cetuximab for resectable CRLM in the adjuvant setting.

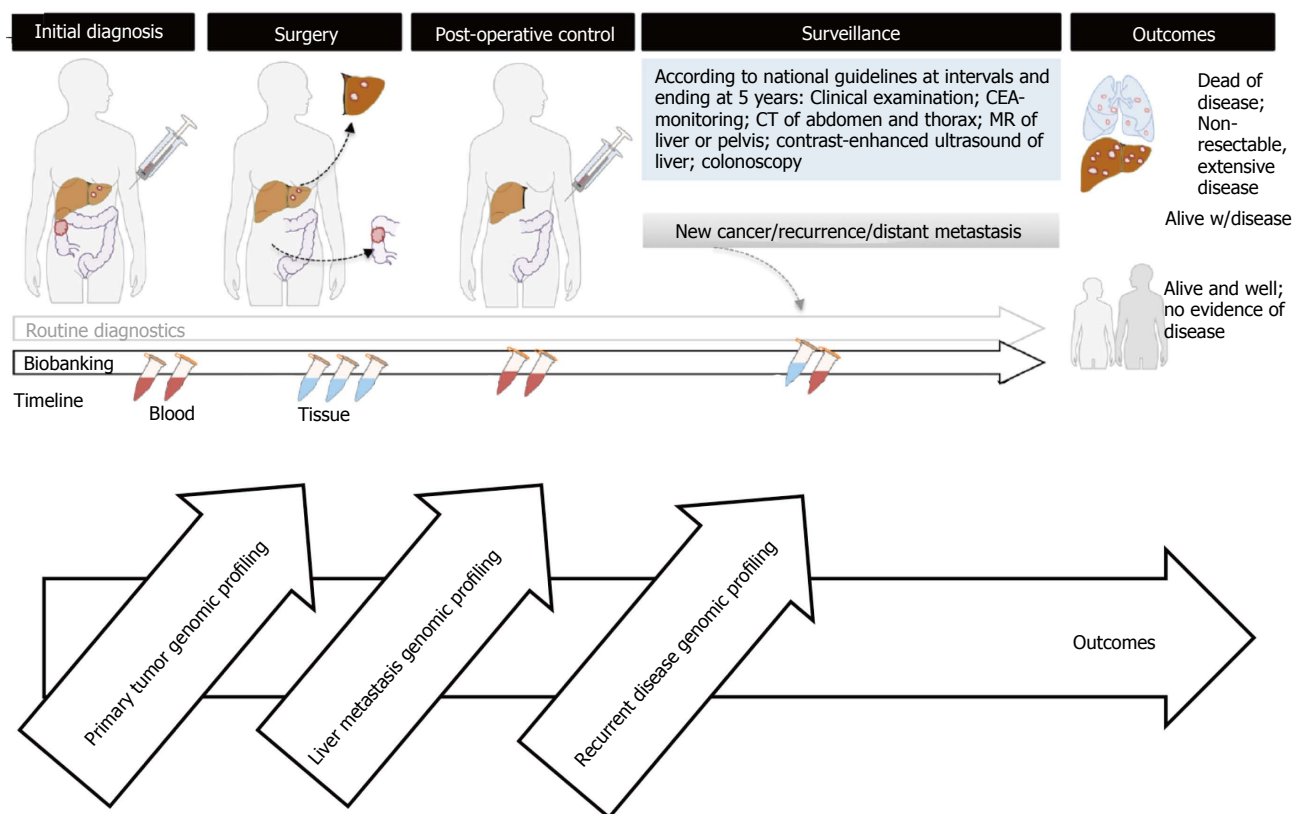
In CRC, presence of microsatellite instability (MSI) is known as a favourable genetic trait<sup>[23]</sup>, yet with an emerging role for subtypes of such microsatellite alterations, including elevated alterations at selected tetranucleotide repeats (EMAST)<sup>[24]</sup>. One recent study<sup>[25]</sup> found that CRLM with a favourable outcome are more likely to have EMAST and low-frequency MSI (MSI-L). How this relates to other markers need to be further explored and validated in external series, but proves that molecular markers can aid in deciphering the cancer biology and thus possibly help predict outcomes<sup>[26]</sup>. Patients with concomitant liver and lung metastases have an "ugly" tumor biology and are more likely to have high frequencies



**Figure 1 Clinical and molecular influence on cancer biology in colorectal liver metastases.** A: Clinical behaviour of colorectal cancer is determined by several factors, including demographic data (age, gender, race) and tumor presentation (location, stage) and timing of presentation of metastasis (synchronous or metachronous). Embedded in the cancer cells are the molecular pathways, which follows distinct forms of genomic instability yet with partly overlapping areas. Hypermutated cancers belong to the microsatellite instable (MSI) cancers and in part the CpG-island methylator phenotype (CIMP) cancers. Non-hypermutated cancers follow in large parts the chromosomal instability (CIN)-driven pathways, often involving *KRAS* mutations from an early stage. The propensity to develop metastasis may possibly be modified through the elevated microsatellite alterations at selected tetranucleotide repeat (EMAS) and associated mechanisms, such as regulation of microRNAs or activity and numbers of CD8<sup>+</sup> immune cells. Finally, the microenvironment contains numerous factors that may facilitate or propagate metastasis to invade, spread and settle in a new organ sites, particularly the liver and the lungs; B: Determined by the clinical presentation, the genetic traits and molecular mechanisms, the prognosis in colorectal liver metastasis is related to resectability for long-term survival. Reprinted from Søreide K, Watson MM, Hagland HR. Deciphering the Molecular Code to Colorectal Liver Metastasis Biology Through Microsatellite Alterations and Allelic Loss: The Good, the Bad, and the Ugly. *Gastroenterology* 2016 Apr; **150** (4): 811-814, Copyright (2016), with permission from Elsevier.

of both *KRAS* and *BRAF* mutations and respond poorly to any line of treatment (Figure 1). The “bad” cases are considered as “in between” - where the current shift from “nonresectable” to “resectable” experiences a drift with changing practice in surgical strategy, novel techniques

and use of conversion chemotherapy regimens to detect responders and improve outcomes. Novel biomarkers may aid in understanding aggressiveness of CRLM, assist in clinical decision-making and help to find new and more efficient therapies.



**Figure 2 Translational cancer research design for investigation of cancer biology.** The illustration is based on the ACROBATICC (Assessment of Clinically Related Outcomes and Biomarker Analysis for Translational Integration in Colorectal Cancer) project flow, see main article for details. Reproduced with permission from Søreide *et al.*<sup>[39]</sup>. *J Transl Med* 2016; **14** (1): 192. © 2016 Søreide *et al.* CEA: Carcino-embryonic antigen; CT: Computed tomography; MR: Magnetic resonance.

## LIQUID BIOPSIES: CIRCULATING TUMOR CELLS AND CIRCULATING TUMOR DNA

While several of the genetic markers may bear prognostic info and may be a valuable source for further decision-making after resection, there is a problem with having to explore tissues after surgery is first done. Emerging evidence suggest that tumors change during the course of treatment and, thus giving way to new clones that may be of a different genetic makeup and have a different resistance pattern<sup>[27-29]</sup>. Consequently, finding methods where disease determinants can be found prior to resection would be beneficial. Further, being able to base such info on "liquid biopsies" (e.g., blood test, serum samples or the like) rather than tissue biopsy is an attractive approach.

Circulating tumor cells (CTCs) are cells present in the peripheral circulation in the majority of patients with metastatic cancer disease. Similarly, most cancers shed cell-free circulating tumor DNA (ctDNA) in the blood<sup>[30]</sup>. ctDNA can be analyzed to generate molecular profiles which capture the heterogeneity of the disease more comprehensively than tumor tissue biopsies. This approach commonly called "liquid biopsy" can be applied to monitor response to therapy, to assess minimal residual disease and to uncover the emergence of drug resistance. However, technological shortcomings and difficulty in finding the perfect markers to identify such CTCs or ctDNA

have resulted in few studies of any clinically valuable difference in terms of survival outcomes or prediction<sup>[31]</sup>. Other studies appear promising, including one recent meta-analysis on the prognostic role of ctDNA<sup>[32]</sup>, also for disease prediction but are small and need further validation<sup>[33-35]</sup>.

What appears essential though for tumor biology is that in the majority of the patients, CTCs reflected the molecular characteristics of metastatic cells better than the primary tumors<sup>[36]</sup>. Also, metastases appear to shed new cells of an invasive type, thus giving further rise to the metastatic tumor phenotype<sup>[37]</sup>. Remaining challenges is the isolation and characterization of CTCs and the sensitivity and specificity in detection of ctDNA<sup>[38]</sup>. Thus, CTC-, and ctDNA-based liquid biopsies may not be widely adopted for routine cancer patient care until the suitability, accuracy, and reliability of these tests are validated and more standardized protocols are corroborated in large, independent, prospectively designed trials. As technology is refined and better and more accurate markers validated, there is likely to be an increasing role for circulating markers in the future.

## CONCLUSIONS AND WAY FORWARD

Currently, clinicians will still heavily rely on the clinical features and disease presentations of patients with CRLM. However, as aggressive treatment regimens progress, new

technology make more patients amenable for resection and as an increasing number of patients are diagnosed and considered in a synchronous setting, the need for better predictors of outcome becomes increasingly important. There is a continued need for better studies, with proper design for biomarker research, with findings of interest and importance that need to be evaluated in test-sets and validation cohorts. External validation in cohorts derived outside the index institution should be sought in order to explore and define generalizability and validity. Biobanking and biopsies should preferably include the course of disease, from primary tumor to metastatic disease to recurrence, with samples including recurrence-free intervals or samples taken during change in chemoregimens. Only then can the natural course and clonal evolution of cancer be explored and proper therapy initiated. However, most studies do not have the opportunity to do this at the moment, most often restricted by logistics, funding and investigator initiatives. In our own prospective translational cancer cohort<sup>[39]</sup> we seek to obtain blood samples and tissue samples from all CRC and CRLM resected within a defined population (Figure 2). This is done with the hopes of having samples that can identify tissue- or serum-based markers of disease-specific outcomes. Hopefully, this may in the near future move us away from clinical risk scores alone, to more precise molecular markers in the genomic era. Truly, to overcome cancer as a disease, the key to success lies in better understanding of the cancer biology. To paraphrase the surgeon oncologist Blake Cady<sup>[40]</sup>: "Biology is King; selection of cases is Queen, and the technical details of surgical procedures are Princes and Princesses of the realm who frequently try to overthrow the powerful forces of the King and Queen, usually to no long-term avail, although with some temporary apparent victories".

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## CpG island methylator phenotype in adenocarcinomas from the digestive tract: Methods, conclusions, and controversies

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### Abstract

Over the last two decades, cancer-related alterations in DNA methylation that regulate transcription have been reported for a variety of tumors of the gastrointestinal tract. Due to its relevance for translational research, great emphasis has been placed on the analysis and molecular characterization of the CpG island methylator phenotype (CIMP), defined as widespread hypermethylation of CpG islands in clinically distinct subsets of cancer patients. Here, we present an overview of previous work in this field and also explore some open questions using cross-platform data for esophageal, gastric, and colorectal adenocarcinomas from The Cancer Genome Atlas. We provide a data-driven, pan-gastrointestinal stratification of individual samples based on CIMP status and we investigate correlations with oncogenic alterations, including somatic mutations and epigenetic silencing of tumor suppressor genes. Besides known events in CIMP such as *BRAF V600E* mutation, *CDKN2A* silencing or *MLH1* inactivation, we discuss the potential role of emerging actors such as Wnt pathway deregulation through truncating mutations in *RNF43* and epigenetic silencing of *WIF1*. Our results highlight the existence of molecular similarities that are superimposed over a larger backbone of tissue-specific features and can be exploited to reduce heterogeneity of response in clinical trials.

**Key words:** CpG island methylator phenotype; CpG island; Promoter; DNA methylation; Hypermethylation; Gastrointestinal cancer

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**Core tip:** Awareness of the CpG island methylator phenotype (CIMP) is growing for all adenocarcinomas. Here, we summarize previous work on the topic and discuss unanswered questions regarding commonalities and differences of CIMP tumors from esophageal, gastric, and

colorectal adenocarcinomas, where data has been made available from the Cancer Genome Atlas. Our analysis includes a review of our pan-cancer method to stratify tumors based on CIMP and addresses the most frequent mutations found in those samples. We include new data implicating truncating mutations in RNF43 and silencing of WIF1I. We also describe in detail the methylation of CpG sites within the MLH1 promoter across these tumor types.

Sánchez-Vega F, Gotea V, Chen YC, Elnitski L. CpG island methylator phenotype in adenocarcinomas from the digestive tract: Methods, conclusions, and controversies. *World J Gastrointest Oncol* 2017; 9(3): 105-120 Available from: URL: <http://www.wjgnet.com/1948-5204/full/v9/i3/105.htm> DOI: <http://dx.doi.org/10.4251/wjgo.v9.i3.105>

## INTRODUCTION

Aberrant patterns of DNA methylation occur in human cancers<sup>[1-3]</sup>, with the most notable being a widespread and pronounced gain of methylation at CpG islands in tumor cells<sup>[4]</sup>. A prominent increase in global levels of CpG island methylation observed across multiple samples was first reported in a subset of patients with colorectal cancer (CRC) and it is now a clinically recognized characteristic of many types of tumor, referred to as the CpG island methylator phenotype (CIMP)<sup>[5]</sup>. In this commentary, we discuss the classification and functional ramifications of CIMP across four types of gastrointestinal adenocarcinomas (esophageal, gastric, colon and rectal), using data from The Cancer Genome Atlas (TCGA) to address lingering questions and identify novel areas of inquiry to spur future investigation. Finally, we explore CIMP's potential application to cancer diagnostics and subtyping, while emphasizing that much remains unknown regarding the molecular mechanisms of tumor-associated DNA methylation, including CIMP generation and maintenance.

CpG islands play a crucial biological role in development and disease by acting as transcriptional regulatory elements in the genome and controlling the expression of ubiquitously expressed genes. Approximately 50% of all CpG islands are located within promoter regions<sup>[6]</sup>, and approximately 70% of all annotated promoters are associated with a CpG island<sup>[7]</sup>. Hypermethylation of CpG dinucleotides within these regions results in the establishment or reinforcement of repressive chromatin and the steric occlusion of transcription factor binding<sup>[4,8]</sup>, reducing gene expression. When promoters of tumor suppressor genes are methylated, repression can represent a critical "hit", in the terminology of the double-hit theory of gene inactivation, conferring a selective advantage to affected cancer cells<sup>[9]</sup>. For example, the heterozygous silencing of *BRCA1* via DNA methylation plays a critical role in breast cancer oncogenesis and tumor proliferation<sup>[10]</sup>. Other well-known examples of silencing involve *MLH1* in CRCs<sup>[5,11]</sup> and *MGMT* silencing in gliomas<sup>[12]</sup>. In the

case of *MLH1*, methylation-derived silencing inhibits DNA repair<sup>[11,13,14]</sup>, which leads to microsatellite instability (MSI) and cascades into many other downstream functional consequences.

Researchers have identified reproducible, tissue-specific patterns of CpG island promoter hypermethylation in various types of tumors<sup>[15]</sup>. The specificity of hypermethylation appears to result from the precise targeting of CpG islands by polycomb repressors<sup>[16]</sup>, resulting in the preferential deposition of DNA methyl groups during oncogenesis<sup>[17-19]</sup>. Because these patterns are frequently occurring in cancer patients, they have been used as novel, clinically relevant molecular markers for cancer diagnosis and prognosis<sup>[20]</sup>. To cite two examples, hypermethylation of the *GSTP1* promoter in more than 90% of prostate adenocarcinomas has been used to improve diagnosis of this disease<sup>[21]</sup>, whereas hypermethylation of SET pseudogene 9 allows researchers to differentiate among different stages of CRC<sup>[22]</sup>.

The demonstration that tumors exhibiting CIMP represent a distinct clinical subtype of CRC<sup>[5]</sup> provided the first evidence that, by subdividing cancers into methylation subclasses, clinicians could potentially refine treatment outcomes. Numerous studies have since demonstrated the presence of CIMP in additional cancer types<sup>[23-25]</sup>. However, little overlap has been detected among these CIMP incarnations, indicating the tissue-specific nature of the effect. Current models indicate that tumorigenesis affects DNA methylation at CpG islands where repressive H3K27me3 modifications are already present<sup>[26]</sup>, providing a more permanent layer of suppression in differentiated cells and explaining the origin of tissue-specific patterns. According to such models, aberrant DNA methylation is not a stochastic outcome, but a targeted, albeit abnormal, process. In this light, it becomes reasonable to speculate that distinct tumor types could use similar cellular pathways to target their own characteristic CpG islands for DNA methylation. Mechanistic congruity among different tumor types would allow us to understand multi-cancer and pan-cancer processes from a unified molecular perspective. However, testing this hypothesis requires us to use consistent methods to assess DNA methylation across tumor types and to analyze large numbers of samples to provide statistical power. In the rest of this article, we provide examples of such analyses.

## EVALUATING CIMP: FROM GENE PANELS TO GENOME-WIDE METHYLATION PROFILES

A quick overview of important milestones in the study of CIMP within the context of gastrointestinal cancers is provided in Table 1. Given the diversity of methods for assessing DNA methylation, profiling has been performed over a wide range of technical depths and breadths. Initially, the implementation challenges of wide-scale methylation profiling limited the scope of CIMP evaluation. Researchers working on CRC employed panels of genes

**Table 1** Overview of previous studies of CpG island methylator phenotype in tumors from the gastrointestinal track

Year	Event	Ref.
1999	CIMP is first reported in a set of CRC patients	[5]
2004	Nature Reviews paper discussing CIMP in a variety of tumors besides CRC	[23]
2006	Refined molecular subtyping includes CIMP-low and CIMP-0 categories in CRC, with associations to <i>KRAS</i> mutations	[47]
	New insights are gained about the interplay between <i>BRAF</i> V600E mutations, MSI status, <i>MLH1</i> promoter methylation and CIMP in CRC	[14]
2006-2012	High throughput DNA methylation arrays become widely available, enabling the use of larger gene panels for CIMP characterization	[45,46]
2014	TCGA marker paper on gastric cancer highlights the biological relevance of CIMP for molecular subtyping, exploring associations with EBV infection	[64]
	A better mechanistic understanding of CIMP in CRC is gained through elucidation of the role of <i>MAFG</i> in the context of <i>MLH1</i> silencing and <i>BRAF</i> V600E mutations	[76]
2015	Pan-cancer stratification of solid tumors reveals similarities in CIMP across a wide variety of cancer types	[51]

CIMP: CpG island methylator phenotype; CRC: Colorectal cancer; MSI: Microsatellite instability; TCGA: The Cancer Genome Atlas.

using a low-throughput approach, such as methylation-specific PCR. These panels varied in size from four<sup>[27]</sup> to several dozen genes<sup>[28]</sup>, and invariably included subsets of the sequences originally employed by Toyota *et al.*<sup>[5,29]</sup>. Although other CIMP-tumor characterizations have emerged, CRC remains the most heavily investigated tumor type with respect to CIMP subtypes. A variety of gene panels are still in use<sup>[30]</sup>, some of which include *MLH1*<sup>[31-35]</sup> due to its aforementioned connections to MSI<sup>[36,37]</sup>.

Following an increase in the scope of methylation studies, individual CpG sites started being used to detect aberrant methylation across multiple cancer types. For example, *CDKN2A* profiling has been used in at least 10 cancer types<sup>[24]</sup>, and *MLH1* profiling has been extended to pancreatic cancer<sup>[38]</sup>, leukemia<sup>[39]</sup>, ovarian cancer<sup>[40]</sup>, endometrial cancer<sup>[41]</sup>, gastric cancer<sup>[42]</sup>, and lung cancer<sup>[43]</sup>. Although these sites are consistently differentially methylated in multiple tumor types, none of them are informative enough to classify samples as CIMP in an independent manner.

The limitations of these early ascertainment methods and lack of extensive overlaps across tumor types, coupled with a variable range of methylation at any given CpG site, fueled a debate over the relevance of CIMP in cancer<sup>[44]</sup>. The advent of array-based platforms for measuring DNA methylation, such as the Illumina Infinium HumanMethylation27 and HumanMethylation450 arrays<sup>[45]</sup>, helped end this debate<sup>[46]</sup>. Recent genome-wide experiments using high-throughput data have not only corroborated the biological relevance of CIMP to CRC diagnostics and survival rates, but have led to finer subdivisions of methylation levels, such as CIMP-low and CIMP-zero<sup>[47-49]</sup>. These classifications better reflect global patterns of hypermethylation, which often fail to fit within "high" or "low" classes in colorectal and other cancers. For example, our early studies of gynecological tumor epigenomes showed a finely increasing signal of CpG island hypermethylation among ovarian and endometrial tumors, rather than a binary methylation signature<sup>[50]</sup>. This signature represented an intermediate ranking between the fully methylated and unmethylated states, where the CIMP intermediate group corresponded to the

serous subtype with TP53 mutations. This observation weighed heavily into our recently demonstrated method to stratify DNA methylation patterns of most cancer types collected by TCGA at the time for CIMP classification. Categories that we defined include CIMP+, CIMP-intermediate (CIMPi), and CIMP-<sup>[51]</sup>. Such broad-scale analyses provide a means of subtyping individual tumor collections into relatively homogenous methylation subgroups, notwithstanding the fact that each subgroup can contain a gradient in methylation levels. The absence of a highly dichotomous methylation pattern suggests that a complex interplay of factors determines CIMP status, including tumor heterogeneity and clonality<sup>[52]</sup>, multiple somatic/germline mutations<sup>[53]</sup>, copy number variation, and mutation heterozygosity<sup>[54]</sup>.

Within the ongoing effort to better the understanding of cancer biology, we argue that evaluating methylation on an epigenome-wide scale should be favored over the analysis of a few, select loci. For example, large-scale analyses have revealed the now widely recognized phenomenon that DNA methylation occurs at genes with a role in early development and morphogenesis, leading to the discovery that polycomb binding is a precursor to aberrant DNA methylation<sup>[25,55,56]</sup>. Also, a number of recent studies have highlighted important similarities in terms of somatic alterations and epigenetic patterns across cancers of different organs and tissues<sup>[51,57-59]</sup>. This type of multi-cancer or pan-cancer approach benefits from increased statistical power compared with smaller studies of individual cancer types, which, however, are better suited to capturing tissue-specific features. Researchers can harness the advantages of both approaches by studying related cancer types that occur in tissues derived from common cell lineages. A good example of this approach is provided by previous multi-cancer analyses of tumors of the gastrointestinal tract<sup>[60]</sup>.

## GENOMIC CHARACTERISTICS ASSOCIATED WITH CIMP IN GASTROINTESTINAL TUMORS

TCGA has used patterns of mutation to classify colo-



**Table 2** Gastrointestinal adenocarcinoma types, sample sizes, probe set sizes, and CpG island methylator phenotype status

Cancer type	Differentially methylated probes	Control samples	Tumor samples	CIMP-	CIMPi	CIMP+
EAC	6717	11	87	26	31	30
STAD	1110	2	260	109	95	56
COAD	2656	38	274	96	92	86
READ	1255	7	96	31	39	26

CIMP: CpG island methylator phenotype; CIMPi: CIMP intermediate; COAD: Colon adenocarcinoma; EAC: Esophageal adenocarcinoma; READ: Rectal adenocarcinoma; STAD: Stomach adenocarcinoma.

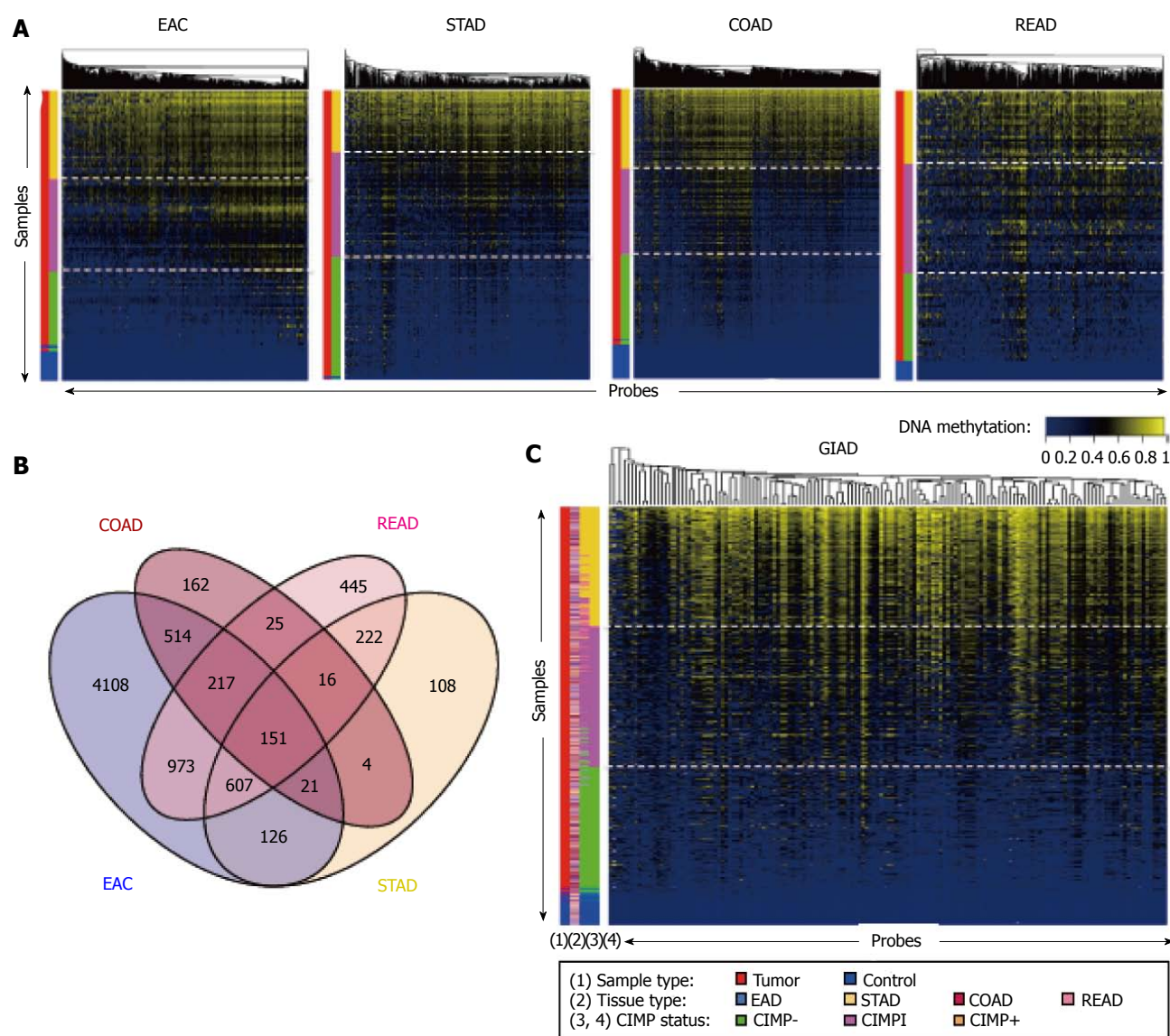
rectal sample genomes into two large groups, non-hypermethylated and hypermethylated<sup>[61]</sup>. Colon and rectal tumor samples in the former class largely possess CIMP-low phenotypes and have almost indistinguishable molecular signatures in terms of copy number variation, mRNAs, and miRNAs. By contrast, hypermethylated samples are predominantly tumors of the colon. Roughly three-quarters exhibit CIMP-high status, as well as *MLH1* silencing and MSI, whereas the other quarter are characterized by mutations in other mismatch repair genes such as *MLH3* and mutations in *POLE*. The contrast between samples exhibiting high chromosomal instability (CIN) and samples exhibiting large mutational load is not unique to CRCs, as it has been described in other cancer types, including the endometrioid vs serous subtypes of both ovarian and endometrial cancers<sup>[50,62]</sup>. Consistent with these observations, the importance of CIMP as a mutually exclusive alternative to CIN has been underscored in describing dysfunctional events in tumor genomes<sup>[63]</sup>. As we have reported previously<sup>[51]</sup>, the MSI vs CIN duality largely corresponds to a CIMP+ vs CIMP- dichotomy. This can be extrapolated to a pan-cancer dichotomization of tumors into a “mutator” class, characterized by a large number of somatic mutations, closer to CIMP+, and a “copy number” class, characterized by an abundance of copy-number alterations but lacking excessive mutations, closer to CIMP-. This duality has been previously referred to as the cancer genome hyperbola<sup>[57]</sup>.

Even if it is conceptually helpful, the simple high-level dichotomy assessed by mutations or copy number alterations fails to adequately represent all of the mechanisms of diversity in gastric tumors. For example, a comprehensive molecular study carried out by TCGA subdivided gastric tumors into four distinct subgroups<sup>[64]</sup>. Two distinct CIMP-high tumor subgroups were identified: One associated with Epstein-Barr virus (EBV), and one associated with MSI. Among 10 different cancer types analyzed by TCGA, the EBV-CIMP subgroup exhibited the highest frequency of DNA hypermethylation at gene promoters, highlighting the interplay, causative or correlational, between environmental exposures such as viral infection and DNA methylation of the tumor genome. Studies involving other infectious agents also suggest potentially relevant associations between presence of pathogens, gastric cancer prognosis and CIMP status. For example, in patients infected with *Helicobacter pylori*, CIMP+ tumors exhibit higher rates of recurrence and metastasis than CIMP- tumors<sup>[65]</sup>.

Of the four types of gastrointestinal cancer examined in the present article, esophageal cancers have been the least thoroughly studied with regards to CIMP stratification. However, CIMP and its associated driver mutations have been investigated in the context of some esophageal tumor subtypes<sup>[66]</sup>. In particular, subsets of tumors exhibiting high levels of methylation have been reported in both esophageal adenocarcinoma and Barrett's esophagus, a precursor lesion to esophageal adenocarcinoma<sup>[67]</sup>. Moreover, the overall amounts of DNA hypermethylation in Barrett's esophagus predict progression to esophageal adenocarcinoma<sup>[68,69]</sup>. Genes such as *CDKN2A*, *APC*, *CDH1*, *TAC1* and *MGMT* have been reported to exhibit increased methylation in esophageal adenocarcinomas, esophageal squamous cell carcinomas and Barrett's esophagus when compared to normal esophageal DNA<sup>[70]</sup>. By contrast, *MLH1* promoter methylation has been reported in esophageal squamous cell carcinomas, but not adenocarcinomas<sup>[70,71]</sup>, confirming differences in methylation profiles between esophageal subtypes.

## ANALYSES OF CIMP IN GASTROINTESTINAL CANCERS

Here, we investigated CIMP in four types of gastrointestinal adenocarcinoma (GIAD) samples provided by TCGA: Esophageal adenocarcinoma (EAC), which is a subset of esophageal carcinoma (or ESCA, using the TCGA nomenclature); stomach adenocarcinoma (STAD); colon adenocarcinoma (COAD); and rectal adenocarcinoma (READ). Using a previously described approach<sup>[51]</sup>, we assessed mean methylation levels in tumor and healthy adjacent tissues and ranked samples using unsupervised clustering. Specifically, we measured DNA methylation levels at a set of informative probes (*i.e.*, sets of loci that were differentially methylated between tumor and normal samples at statistically significant levels) using statistical selection criteria applied independently for each tumor collection (Table 2; see research). We then evaluated CIMP status by classifying samples according to average methylation levels across the set of informative probes. This type of CIMP stratification, in which samples with similar methylation intensity levels are grouped together, reduces heterogeneity within the full tumor collection and facilitates the identification of functional somatic alterations that may play a shared role across different cancer types (and subtypes).



**Figure 1** CpG island methylator phenotype analysis of gastrointestinal adenocarcinoma samples from the Cancer Genome Atlas. A: CIMP analysis for EAC, STAD, COAD, and READ samples. Each row represents a sample, and each column represents a probe. The two-color side bar shows tumor samples (red) and normal samples (blue). The four-color side bar indicates CIMP status: CIMP+ (gold), CIMP intermediate (CIMPi; magenta), CIMP- (green), and normal (blue). Samples were ranked vertically according to mean methylation levels across all of the probes shown in the heat map; B: Venn diagram showing the intersection of the selected, informative probes with regard to CIMP status across the four cancer types; C: CIMP analysis for the combined GIAD data set, in which samples from the four individual tumor types were pooled together. The side bars show (1) sample type (tumor vs adjacent tissue); (2) cancer type (EAC, STAD, COAD, or READ); (3) CIMP status based on the individual analyses shown in panel A; and (4) CIMP status based on the analysis using the pooled data set. CIMP: CpG island methylator phenotype; EAC: Esophageal adenocarcinoma; STAD: Stomach adenocarcinoma; COAD: Colon adenocarcinoma; READ: Rectal adenocarcinoma; GIAD: Gastrointestinal adenocarcinoma.

After clustering based on average methylation levels across the probes, samples were categorized into three distinct groups: CIMP+, CIMPi, and CIMP-. CIMP- samples had CpG island methylation profiles that were closer to those observed in normal samples, whereas CIMP+ samples showed a reproducible pattern of DNA hypermethylation with respect to non-cancer controls (Figure 1A). CIMPi samples displayed methylation levels that fell between the CIMP+ and CIMP- groups. In subsequent analyses, we compared CIMP- and CIMP+ samples and excluded the intermediate group, to avoid borderline cases and to guarantee that the tumors being compared were sufficiently different from a molecular

point of view.

In a previous study, we showed that our CIMP+ and CIMP- assignments largely coincided with independent assignments by the TCGA for an overlapping sample set of CRC tumors<sup>[51]</sup>. Here, we compared our CIMP classification with the four molecular subtypes defined by TCGA for gastric tumors: (1) EBV+; (2) MSI; (3) genomically stable (GS); and (4) CIN<sup>[64]</sup> (Table 3). We observed a significant association between CIMP+ status and the EBV+ and MSI subtypes, in agreement with the extreme CIMP reported for these subtypes by TCGA. Highlighting the previously mentioned incompatibility of CIMP and CIN, CIN samples were significantly skewed

**Table 3** Comparison between our CpG island methylator phenotype classification of stomach adenocarcinomas and the four subtypes defined by The Cancer Genome Atlas Research Network<sup>1</sup>

	CIN	EBV +	GS	MSI	Total
CIMP+	<sup>2</sup> 12 (26.6)	<sup>3</sup> 13 (5.4)	<sup>2</sup> 2 (11.3)	<sup>3</sup> 27 (10.7)	-54
CIMPi	43 (45.75)	12 (9.4)	20 (19.5)	18 (18.4)	93
CIMP-	<sup>3</sup> 67 (49.68)	<sup>2</sup> 0 (10.2)	<sup>3</sup> 30 (21.2)	<sup>2</sup> 4 (20.0)	101
Total	122	25	52	49	248

<sup>1</sup>Numbers outside parentheses correspond to actual sample counts, whereas numbers in parentheses show expected counts under the null model of independent classification; <sup>2</sup>Indicates under-represented counts; <sup>3</sup>Indicates cells with significantly over-represented counts ( $P < 0.05$ , based on Fisher's exact test). CIMP: CpG island methylator phenotype; CIMPi: CIMP intermediate; CIN: Chromosomal instability; EBV+: Epstein-Barr virus positive; GS: Genomically stable; MSI: Microsatellite instability.

toward CIMP- status. However, other samples also occupied the CIMP- category, including GS samples, which displayed few alterations in DNA methylation and lacked MSI.

In addition to evaluating CIMP in each of the four cancer types independently, we combined all of the data into a single set. Here, the intersection of the loci selected in the four previous, independent analyses was considered informative ( $n = 151$ , Figure 1B). In this new classification of samples (Figure 1C), CIMP labels remained largely consistent with the previously assigned labels. Importantly, when samples in the pooled data set were ranked according to their average level of DNA methylation across the set of selected probes, they tended to cluster by CIMP status rather than tissue of origin. This novel finding implies commonalities in the underlying generation of aberrant methylation across cancer types.

## CIMP AND *MLH1* PROMOTER HYPERMETHYLATION

Early studies of CIMP established that the *MLH1* promoter is consistently hypermethylated in CRC<sup>[5]</sup>. This observation has since been extended to other cancer types<sup>[72]</sup>, and its importance is highlighted by the inclusion of *MLH1* in many gene panels used to evaluate CIMP. The strong association between CIMP and *MLH1* promoter hypermethylation continues to be reinforced by recent studies with large sample sizes, such as a pan-cancer analysis performed by our group<sup>[51]</sup> using a catalog of 479 somatic functional events (Ciriello *et al.*<sup>[57]</sup>, 2013). In this previous work, we investigated a cohort of 3299 samples that spanned 9 different cancer types and found that *MLH1* promoter silencing was the single genomic functional event that displayed the strongest statistical association with CIMP.

Since promoter hypermethylation is usually associated with gene silencing<sup>[4,8]</sup>, one could compare the effects of *MLH1* promoter hypermethylation and disabling gene mutations, addressing parallels with loss-of-function. Indeed, *MLH1* promoter silencing replicates the phenotype of *MLH1* loss-of-function mutations in hereditary nonpolyposis colon cancer, which displays dinucleotide repeat instability<sup>[73]</sup>. Moreover, research in cell lines demonstrates that reversing *MLH1* promoter

hypermethylation increases transcription of the gene and restores mismatch repair capacity<sup>[11,74]</sup>. It is therefore tempting to hypothesize that *MLH1* promoter hypermethylation, which is strongly associated with CIMP and displays the functional hallmarks of a loss-of-function mutation, is a causal event in the onset of CIMP. However, previous studies, including our own, have shown that CIMP can be observed in the absence of *MLH1* promoter hypermethylation or mutation<sup>[51,61,75]</sup>, implying either a relationship that is correlational but not causal, or multiple mechanisms underlying CIMP development.

Only recently has experimental evidence emerged to help elucidate the role of *MLH1* promoter hypermethylation in CIMP. In CRC, Fang *et al.*<sup>[76]</sup> have shown that the common BRAF V600E mutation leads to elevated levels of the protein MAFK. In turn, MAFK binds to the promoter of *MLH1* and other genes, where it recruits a heterodimeric partner, BACH1; a chromatin remodeling factor, CHD8; and a DNA methyltransferase, DNMT3B - ultimately resulting in increased methylation at the target sites. These results suggest that mutations such as BRAF V600E orchestrate aberrant methylation patterns; therefore, *MLH1* promoter hypermethylation might be thought of as part of the CIMP onset process rather than an initiating event.

Many interesting genes may fit into a model in which, following the onset of somatic mutations, a cascade of downstream methylation events occurs. For instance, *CDKN2A* promoter hypermethylation is also linked to BRAF mutations, through increased expression of the DNA methyltransferase DNMT3B<sup>[77]</sup>. Similarly, hypermethylation and silencing of the *INK4-ARF* locus (also known as *CDKN2A* and *CDKN2B*) occurs through KRAS activation of ZNF304, which recruits the DNA methyltransferase, DNMT1<sup>[78]</sup>.

## *MLH1* PROMOTER METHYLATION IN GASTROINTESTINAL TUMOR DATA FROM TCGA

We analyzed GIAD data supplied by TCGA to learn more about the relationship between *MLH1* promoter methylation and CIMP. First, we identified 41 probes from the Illumina Infinium HumanMethylation450 array



We also examined the association between mutations that disable *MLH1* and the presence of CIMP. First, we collected all somatic mutations mapped to *MLH1* in samples whose CIMP status had been determined (Table 4 and Figure 2C). The most detrimental somatic alterations in *MLH1* are frameshift mutations, which render large fractions of the protein product nonfunctional. We observed frameshift mutations in all three CIMP classes (CIMP+, CIMP<sub>i</sub> and CIMP-), without a significant bias toward CIMP+ samples. In fact, several truncating mutations within the DNA mismatch repair functional domain of the protein occurred in CIMP<sub>i</sub> and CIMP- samples. These data suggest that loss of function alterations at *MLH1* might not be sufficient for the onset of CIMP.



**Table 4** Somatic mutations found in the tumor suppressor gene *MLH1* in gastrointestinal adenocarcinoma samples

Sample	Cancer type	CIMP class	Mutation	Mutation type	AA pos.	Aff. AAs	VEST score <sup>1</sup>
TCGA-A6-6780-01	COAD	CIMP+	chr3:37038192.G>A	Missense substitution	67	1	0.994
TCGA-CA-6719-01	COAD	CIMP+	chr3:37067243.G>A	Missense substitution	385	1	0.701
TCGA-CM-6171-01	COAD	CIMP+	chr3:37070349.C>-	Frameshift deletion	495	262	-
TCGA-EI-6917-01	READ	CIMP+	chr3:37058999.C>T	Missense substitution	265	1	0.981
TCGA-BR-6452-01	STAD	CIMP+	chr3:37107356.A>G	3' UTR	-	-	-
TCGA-FP-A4BE-01	STAD	CIMP+	chr3:37090086.C>T	Nonsense substitution	659	98	-
TCGA-A6-6138-01	COAD	CIMPi	chr3:37035084.G>A	Missense substitution	16	1	0.901
TCGA-AD-6889-01	COAD	CIMPi	chr3:37053348.->A	Frameshift insertion	195	562	-
TCGA-AZ-6601-01	COAD	CIMPi	chr3:37067242.C>T	Missense substitution	385	1	0.952
TCGA-CM-4746-01	COAD	CIMPi	chr3:37059062.A>-	Frameshift deletion	286	471	-
TCGA-EI-6884-01	READ	CIMPi	chr3:37058995.A>G	Acceptor splice site	264	493	-
TCGA-BR-6802-01	STAD	CIMPi	chr3:37053348.A>-	Frameshift deletion	195	562	-
TCGA-F1-6874-01	STAD	CIMPi	chr3:37050312. ACCTTTTACAAACATAGCC>-	Frameshift deletion	154	603	-
TCGA-A6-6781-01	COAD	CIMP-	chr3:37053348.A>-	Frameshift deletion	195	562	-
TCGA-CM-6674-01	COAD	CIMP-	chr3:37058999.C>-	Frameshift deletion	265	492	-
TCGA-F4-6856-01	COAD	CIMP-	chr3:37089123.GAA>-	In-frame deletion	615	1	-
TCGA-R6-A6KZ-01	EAC	CIMP-	chr3:37034874.T>C	5' UTR	-	-	-
TCGA-CG-5723-01	STAD	CIMP-	chr3:37053550.G>-	Frameshift deletion	213	544	-

<sup>1</sup>Computed using the VEST tool<sup>[100]</sup>, which evaluates only the effect of missense substitutions. AA pos.: Amino acid position; Aff. AAs: Number of affected amino acids (the MLH1 protein contains 756 residues); CIMP: CpG island methylator phenotype; CIMPi: CIMP intermediate; COAD: Colon adenocarcinoma; EAC: Esophageal adenocarcinoma; READ: Rectal adenocarcinoma; STAD: Stomach adenocarcinoma; VEST: Variant effect scoring tool.

## CIMP AND PROMOTER HYPERMETHYLATION OF TUMOR SUPPRESSOR GENES

The evidence pointing to *MLH1* inactivation as a corollary to the appearance of CIMP suggests that other tumor suppressor genes could potentially be silenced through promoter hypermethylation and result in comparable functional vulnerabilities as well; moreover, the silencing of these genes could represent actionable clinical targets. We explored this concept by searching for known tumor suppressor genes that exhibited concerted promoter hypermethylation in all four GIAD cancer types. Using the TSGene database<sup>[79]</sup>, we found that 26 of 634 tumor suppressor genes (4.1%) contained at least one probe site in the promoter region that exhibited methylation levels significantly different between CIMP+ and CIMP- samples across all four cancer types (Table 5). These genes included *ERBB4*, *WT1*, *WIF1*, and *RASSF2*. By contrast, only 2.4% of genes not included in the TSGene database exhibited concordant differential methylation in CIMP+ samples across the four cancer types ( $P = 0.007$ , hypergeometric test).

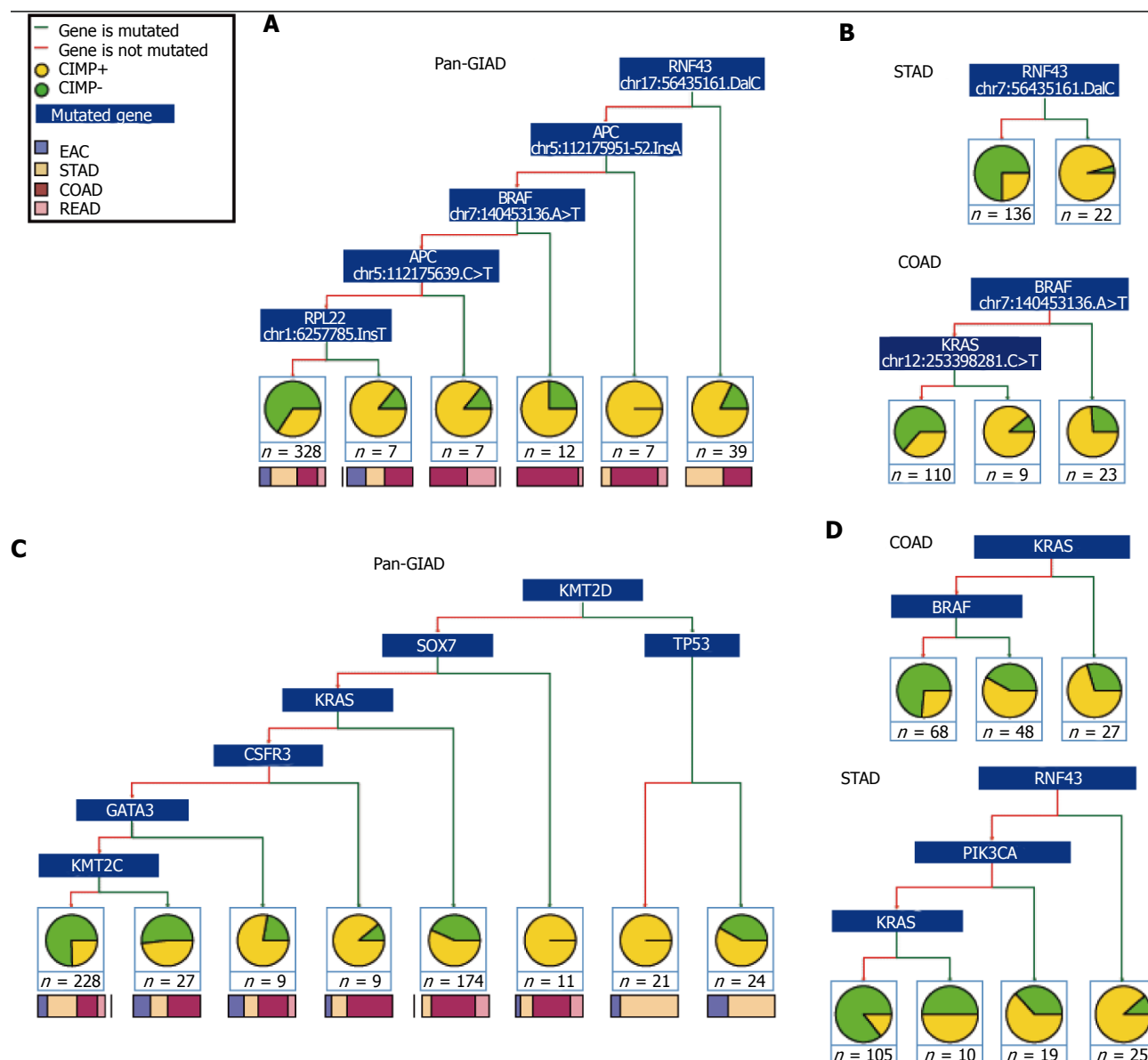
Furthermore, in affected tumor suppressor genes, such as *DNFA5*, *RASSF2* and *WIF1*, promoter methylation was significantly negatively correlated with mRNA expression across several tumor types (Table 5), which is indicative of epigenetic silencing. *DNFA5* is a tumor suppressor gene involved in apoptosis and response to DNA damage<sup>[80,81]</sup>. Its hypermethylation has been reported in colorectal and gastric cancer, where it is associated with EBV-positive status<sup>[82,83]</sup>. In addition, *WIF1* and *RASSF2*, whose methylation and expression levels were significantly correlated across all four cancer types in our study, have been described in the

context of CIMP in gastrointestinal adenocarcinomas<sup>[60,65,84-86]</sup>. These data suggest that, in a subset of genes, selective pressure may favor loss-of-function events caused by DNA methylation, facilitating tumor growth.

## CIMP AND ASSOCIATED SOMATIC MUTATIONS

An outstanding question that remains is the causal connection between somatic mutations and the onset of CIMP. Over the years, extensive association analyses in colon and rectal cancers have been performed to address this problem<sup>[30,87]</sup>. The results have highlighted the diverse mutation spectrum across tissues, which refutes the hypothesis of a universal driver mutation being responsible for altered DNA methylation levels<sup>[51]</sup>. Mutations associated with CIMP have been found in *CDKN2A*, *IDH1/2*, *TET2* and *RB1*, among other genes<sup>[25]</sup>. In addition, as discussed, mutations in *BRAF* directly lead to hypermethylation at specific loci<sup>[76,77]</sup>, and their effects probably extend to myriad targets across the genome.

We further explored the association between somatic mutations and DNA methylation using data from TCGA. For this purpose, we compared the recurrence of somatic mutations in CIMP+ and CIMP- samples across the entire GIAD cohort. A decision tree analysis pointed to several alterations associated with CIMP+ status (Figure 3A and B). This approach ranks mutations in descending order of statistical significance based on their presence or absence in CIMP+ samples. The top-scoring mutation was a 1-bp deletion at chr17:56,435,161 (Figure 3A), which was present in 21 of 22 STAD CIMP+ samples (Figure 3B). This mutation causes a frameshift in the last exon of *RNF43*, a tumor suppressor that encodes a RING-type



**Figure 3** Binary decision trees for separating gastrointestinal adenocarcinomas into CpG island methylator phenotype categories. Recursive partitioning of GIADs from TCGA using binary classification trees based on CIMP status and mutational profiles. Results are provided for A: The combined GIAD data set at the individual mutation level; B: The STAD and COAD data sets at the individual mutation level; C: The combined GIAD data set at the mutated gene level; D: The STAD and COAD data sets at the mutated gene level. Red and green branches illustrate whether a specific mutation is present or absent (or whether a given gene is mutated or not) in the corresponding subset of tumors. Terminal nodes show the number of samples and the associated CIMP+ vs CIMP- fractions, as well as the proportion of different cancer types represented in each subset. GIADs: Gastrointestinal adenocarcinomas; TCGA: The Cancer Genome Atlas; CIMP: CpG island methylator phenotype.

E3 ubiquitin ligase (p.G659fs\*41). *RNF43* is upregulated in colon cancer<sup>[88]</sup> and inhibits Wnt/ $\beta$ -catenin signaling in pancreatic cancer cells<sup>[89]</sup>. Two other top-scoring mutations affect *APC*, a tumor suppressor whose inactivation is associated with the onset of colon cancer. One was a nonsynonymous C-to-T substitution at chr5:112,175,639, and the second was an AA insertion at chr5:112,175,951. Although these alterations were present in a relatively small number of samples (14 in total), they were observed almost exclusively in CIMP+ tumors (13 out of 14). Not surprisingly, we also found a BRAF V600E mutation (A-to-T change at chr7:140,453,136) that was significantly associated with CIMP+ status (Figure 3A). Together with a common *KRAS* mutation (C-to-T change

at chr12:2,539,281; p.G13D), these represent the only two mutations significantly associated with CIMP+ in COAD samples; this is consistent with their already characterized presence in COAD<sup>[14,78]</sup>. Finally, a T insertion at chr1:6257785 affecting *RPL22* was also significantly associated with CIMP+ status across GIAD samples, although the number of affected samples was relatively small (6 out of 7 were CIMP+). In the future, these associations may be explored further to investigate their potential functional role in the context of aberrant DNA methylation.

We also compared mutations in CIMP+ and CIMP- samples by aggregating point mutations at the gene level (Table 6). Amid the top scorers in this analysis, we found

**Table 5** Association between methylation and gene expression in tumor suppressor genes with significantly hypermethylated promoters in CpG island methylator phenotype + samples across four gastrointestinal adenocarcinoma types<sup>1</sup>

Gene symbol	Promoter probes	Differential methylation				Correlation with expression							
		Significant probes per cancer type				EAC		STAD		COAD		READ	
		COAD	EAC	READ	STAD	cor	p-val	cor	p-val	cor	p-val	cor	p-val
TP73	24	18	3	2	23	-0.34	4.E-01	-0.24	1.E-01	-0.10	1.E+00	-0.20	1.E+00
MAL	8	6	5	2	7	-0.37	3.E-01	-0.46	2.E-07	-0.47	7.E-09	-0.45	3.E-02
C2orf40	8	5	3	1	7	-0.51	2.E-03	-0.57	5.E-13	-0.39	2.E-05	-0.24	6.E-01
TMEFF2	7	7	7	7	7	-0.54	2.E-03	-0.49	2.E-08	-0.41	2.E-03	-0.32	5.E-01
ERBB4	6	7	7	2	7	-0.26	5.E-01	-0.15	5.E-01	NA	1.E+00	-0.30	6.E-01
TWIST2	5	5	4	1	4	-0.26	3.E-01	-0.33	3.E-04	-0.38	2.E-06	-0.37	3.E-02
LRRC3B	13	9	7	1	12	-0.36	5.E-01	-0.38	2.E-04	-0.41	4.E-03	-0.11	1.E+00
HTRA3	10	6	3	1	6	0.02	1.E+00	-0.03	1.E+00	-0.10	1.E+00	-0.07	1.E+00
UNC5C	13	13	13	8	13	-0.38	7.E-02	-0.34	5.E-04	-0.43	4.E-08	-0.40	3.E-02
FAT4	13	13	9	2	13	-0.33	2.E-01	-0.44	4.E-07	-0.34	4.E-05	-0.28	4.E-01
IRX1	5	3	4	3	4	-0.37	2.E-01	-0.37	3.E-04	NA	1.E+00	NA	1.E+00
SCGB3A1	9	9	4	2	9	-0.27	4.E-01	-0.37	3.E-05	-0.22	3.E-01	-0.12	1.E+00
AKAP12	10	9	5	1	10	-0.19	1.E+00	-0.42	1.E-06	-0.42	7.E-08	-0.27	6.E-01
DFNA5	12	10	8	1	9	-0.75	0.E+00	-0.56	1.E-12	-0.36	1.E-05	-0.34	1.E-01
TFPI2	15	22	14	19	22	-0.49	3.E-03	-0.54	2.E-11	-0.38	4.E-06	-0.22	1.E+00
NRCAM	7	6	1	1	6	-0.52	3.E-04	-0.47	2.E-08	-0.19	8.E-02	-0.17	1.E+00
CNTNAP2	14	14	11	1	14	-0.14	1.E+00	-0.27	2.E-02	-0.14	1.E+00	-0.12	1.E+00
PAX6	12	12	5	3	11	-0.22	1.E+00	-0.18	6.E-01	-0.04	1.E+00	-0.32	3.E-01
WT1	12	12	12	3	12	-0.25	1.E+00	-0.04	1.E+00	-0.26	8.E-03	-0.23	1.E+00
PHOX2A	11	11	6	5	11	-0.26	1.E+00	-0.13	1.E+00	-0.38	3.E-03	-0.26	1.E+00
WIF1	8	5	5	3	7	-0.57	2.E-03	-0.32	5.E-03	-0.44	9.E-07	-0.56	2.E-04
SLC5A8	9	11	10	4	12	-0.26	1.E+00	-0.28	2.E-01	-0.17	1.E+00	-0.33	9.E-01
TBX5	17	11	7	1	16	-0.32	5.E-01	-0.09	1.E+00	0.03	1.E+00	-0.16	1.E+00
ATP8A2	8	5	4	2	5	-0.28	1.E-01	-0.37	2.E-05	-0.24	5.E-03	-0.24	3.E-01
ADAMTS18	8	7	5	3	7	-0.27	3.E-01	-0.36	6.E-05	-0.34	5.E-05	-0.30	2.E-01
GALR1	29	27	5	8	27	-0.17	1.E+00	-0.47	2.E-04	0.00	1.E+00	-0.14	1.E+00
RASSF2	5	6	4	3	6	-0.52	5.E-04	-0.31	1.E-03	-0.41	1.E-07	-0.40	2.E-02
CDH4	3	2	2	2	4	-0.17	6.E-01	-0.09	8.E-01	-0.16	3.E-01	-0.32	1.E-01

<sup>1</sup>Promoter regions were designated as 2-kb regions encompassing 1.5 kb upstream and 0.5 kb downstream of the transcription start site. For each cancer type, probes were considered significant if the *P*-value after a one-sided Mann-Whitney *U* test with Bonferroni correction for multiple testing was < 0.05. The total number of probes considered across all four cancer types was 395814. COAD: Colon adenocarcinoma; EAC: Esophageal adenocarcinoma; READ: Rectal adenocarcinoma; STAD: Stomach adenocarcinoma.

chromatin remodeling genes such as *ARID1A*, which is an important member of the SWI/SNF complex, and histone methyltransferase genes such as *KMT2D* (*MLL2*) and *KMT2C* (*MLL3*). These two MLL complexes are involved in H3K27 demethylation and H3K4 methylation, which regulate the transcription of many developmental genes, including the *HOX* gene family<sup>[90]</sup>. The list of genes whose mutation levels were associated with CIMP status was also significantly enriched for genes from the RTK/RAS/PI(3)K signaling pathway ( $FDR < 4 \times 10^{-8}$ ), including *ERBB2*, *ERBB3*, *ERBB4*, *KRAS*, *PIK3CA*, *NRAS*, and *PTEN*. These results suggest that the cumulative signal of somatic mutations in coding genes could contribute to CIMP.

Finally, we applied binary decision trees to identify combinations of mutated genes that correlate with CIMP+ or CIMP- status (Figure 3C and D). Using the pooled GIAD data set, our tree shows that *KMT2D* mutations recur in gastroesophageal (*i.e.*, STAD and EAC) samples (Figure 3C). In fact, tumors with mutated *KMT2D* and wild-type *TP53* consist exclusively of CIMP+ samples ( $n = 21$ ). We observed a second set of samples (including representatives from all four histologies) that contained *SOX7* mutations and lacked *KMT2D* mutations; all 11 of these tumors were CIMP+. Our trees from individual

cancers (Figure 3D) show that *KRAS* and *BRAF* mutations in COAD, as well as *RNF43*, *PIK3CA*, and *KRAS* mutations in STAD, are associated with CIMP+ status.

## CONFOUNDING FACTORS IN THE EVALUATION OF CIMP

Basing CIMP classification on mean methylation levels in tumor vs normal tissues allows us to separate cancer-related features from tissue-of-origin signals, but it also makes stratification vulnerable to a number of potential technical and biological artifacts. For example, our classification algorithm relies on the assumption of having a sufficiently large and sufficiently heterogeneous set of controls for each individual tumor type in order to guard against potentially confounding variables such as age, gender, race or anatomic location. Since only two non-tumor control samples were available for STAD, we may have encountered false positives in the probe selection process for this cancer type<sup>[51]</sup>. Another confounding effect may come from tumors' stimulation of the immune response, leading leukocytes (including T cells, NK cells, and macrophages) to infiltrate cancerous tissues and skew the methylation signature<sup>[91]</sup>. Additionally, tumor

**Table 6** Genes differentially mutated between CpG island methylator phenotype+ and CpG island methylator phenotype- gastrointestinal adenocarcinoma samples<sup>1</sup>

Gene	Count CIMP +	% CIMP +	Count CIMP-	% CIMP-	P% Diff	P-value	FDR	Pathway
KMT2D	35	20.30%	10	4.30%	16.00%	6.22E-07	2.24E-05	Chromatin
ARID1A	60	34.90%	32	13.90%	21.00%	1.15E-06	2.24E-05	Chromatin
RNF43	42	24.40%	17	7.40%	17.10%	3.04E-06	3.79E-05	Wnt
CSF3R	19	11.00%	2	0.90%	10.20%	4.19E-06	3.79E-05	ERK
SOX7	14	8.10%	0	0.00%	8.10%	4.86E-06	3.79E-05	ERK
PIK3CA	48	27.90%	26	11.30%	16.70%	2.62E-05	1.70E-04	PI3K/RAS
PAX6	17	9.90%	2	0.90%	9.00%	3.96E-05	2.21E-04	Differentiation
ATM	37	21.50%	17	7.40%	14.20%	5.05E-05	2.46E-04	DNA damage
KRAS	52	30.20%	32	13.90%	16.40%	1.04E-04	4.53E-04	PI3K/RAS
EGR1	15	8.70%	2	0.90%	7.90%	1.63E-04	6.37E-04	Differentiation
GATA3	19	11.00%	5	2.20%	8.90%	2.22E-04	7.87E-04	NF-KB
KMT2C	38	22.10%	22	9.50%	12.60%	6.15E-04	2.00E-03	Chromatin
ALDH2	10	5.80%	1	0.40%	5.40%	1.18E-03	3.30E-03	Metabolic
CDK12	18	10.50%	6	2.60%	7.90%	1.18E-03	3.30E-03	PI3K/RAS
SAFB	15	8.70%	4	1.70%	7.00%	1.44E-03	3.73E-03	Chromatin
BCOR	19	11.00%	7	3.00%	8.00%	1.68E-03	4.09E-03	Chromatin
PTEN	24	14.00%	11	4.80%	9.20%	1.97E-03	4.32E-03	PI3K/RAS
AXIN2	21	12.20%	9	3.90%	8.30%	2.00E-03	4.32E-03	Wnt
CTCF	14	8.10%	4	1.70%	6.40%	2.73E-03	5.41E-03	Chromatin
PALB2	11	6.40%	2	0.90%	5.50%	2.77E-03	5.41E-03	DNA repair
ERBB3	18	10.50%	7	3.00%	7.40%	2.96E-03	5.49E-03	PI3K/RAS
ERBB4	29	16.90%	17	7.40%	9.50%	4.05E-03	6.97E-03	PI3K/RAS
FBXW7	32	18.60%	20	8.70%	9.90%	4.11E-03	6.97E-03	Notch
CIC	23	13.40%	12	5.20%	8.20%	6.55E-03	1.06E-02	Proliferation
HLA.A	17	9.90%	8	3.50%	6.40%	1.13E-02	1.71E-02	Immune
MSH6	19	11.00%	10	4.30%	6.70%	1.14E-02	1.71E-02	MMR
ERBB2	15	8.70%	8	3.50%	5.30%	2.98E-02	4.21E-02	PI3K/RAS
CASP8	13	7.60%	6	2.60%	5.00%	3.02E-02	4.21E-02	Apoptosis
SMAD4	27	15.70%	20	8.70%	7.00%	4.05E-02	5.45E-02	Wnt
TFE3	6	3.50%	1	0.40%	3.10%	4.53E-02	5.90E-02	Wnt
APC	82	47.70%	87	37.70%	10.00%	5.24E-02	6.60E-02	Wnt
NRAS	10	5.80%	5	2.20%	3.60%	6.55E-02	7.74E-02	PI3K/RAS
SMARCB1	10	5.80%	5	2.20%	3.60%	6.55E-02	7.74E-02	Chromatin
IGFBP7	3	1.70%	0	0.00%	1.70%	7.70E-02	8.65E-02	DNA Damage
TBL1XR1	6	3.50%	2	0.90%	2.60%	7.76E-02	8.65E-02	Wnt

<sup>1</sup>Results are based on a combined set of 179 CIMP- and 154 CIMP+ gastrointestinal adenocarcinoma samples. *P*-values were computed using a two-tailed Fishers' exact test. Only genes with FDR < 10% are shown. CIMP: CpG island methylator phenotype.

samples often consist of a heterogeneous mixture of cancer cells and non-cancer cells from adjacent tissues, the latter unwittingly included as a result of some biopsy collection procedures. As of today, there are no universally accepted methods to correct for tumor heterogeneity in DNA methylation studies; however, estimates of tumor heterogeneity can be computed from molecular data, such as copy number changes and mRNA expression, and these estimates can be used to discard problematic samples or to eliminate potential biases in downstream analyses<sup>[92,93]</sup>. As an alternative, future studies may benefit from improved sample collection requirements (*e.g.*, tumor microdissection) that lead to enhanced tumor purity and lower stromal contamination.

## ASSESSING THE IMPACT OF TUMOR HETEROGENEITY ON CIMP CLASSIFICATION

We examined our CIMP classifications using the measure

of tumor purity calculated with ABSOLUTE, a computational method based on the analysis of somatic DNA alterations<sup>[92]</sup>. As a proof of principle, we reclassified CIMP status for the STAD data set using only high-purity (*i.e.*,  $\geq 50\%$ ) samples, as determined by the purity estimates available through TCGA<sup>[64]</sup>. We then compared sets of selected probes and CIMP designations before and after filtering for purity. After removing low-purity samples, the number of differentially methylated probes increased from 1110 to 1610. This result is consistent with the removal of samples that added background noise and masked the methylation signal of tumor cells. Since the new set of differentially methylated probes encompassed the original probe set, the inclusion of low-purity samples does not appear to have appreciably impacted our precision for feature selection, although it may have impoverished recall due to an increased number of false negatives. After using the new probe set, only five samples changed status from CIMP+ to CIMP<sub>i</sub>, and 11 samples changed status from CIMP<sub>i</sub> to CIMP-. However, no sample changed from CIMP+ to CIMP- or



*vice versa*. Thus, our CIMP classification system is robust in the presence of varying sample purity.

## CONCLUSIONS AND PERSPECTIVES

Ever since the original study in CRC by Toyota *et al.*<sup>[5,29]</sup>, evaluation of CIMP status in cancer has been an active area of research. CIMP stratification has direct implications for patient treatment<sup>[24]</sup>. Because DNA methylation is potentially reversible, it represents an attractive target for therapies that can be tailored to individual cancer epigenomes<sup>[20,94]</sup>. Nucleoside analogs, such as 5-azacytidine, can be incorporated into DNA to reversibly block DNA methylation, and their effectiveness is being tested in numerous clinical trials.

In this commentary, we have provided evidence that supports refining the molecular profiles of gastrointestinal tumors based on CIMP status, to look beyond traditional tissue-of-origin interpretations. Our analysis of four types of gastrointestinal tumors not only confirms known CIMP associations but also leads to several new observations relevant to current models of DNA methylation and cancer. For example, we report recurrence of a frameshift mutation in *RNF43* that is significantly associated with CIMP status in stomach and, to a lesser extent, colon tumors. A recent study linked *RNF43* mutations to MSI in colorectal and endometrial tumors, which are Wnt-dependent<sup>[95]</sup>. The tumor suppressor function of this gene qualifies its mutations to be potential drivers of STAD, although mechanistic links to DNA methylation remain inconclusive. In addition, *RNF43* mutations had been identified in endometrioid and mucinous ovarian carcinomas<sup>[96]</sup>; we have shown the former tumor subtype is largely CIMP+<sup>[50]</sup>. The *RNF43* frameshift mutation that we highlighted in STAD samples in this paper is located within a 7-bp, CG-rich tract, and it may be created by the mismatch repair deficiency responsible for the MSI phenotype. Thus, the mutation's connection to CIMP status may occur downstream of MSI. However, *RNF43*-truncating mutations, which are common in MSI+ colorectal tumors, display mutual exclusivity with inactivating *APC* mutations<sup>[95]</sup>, suggesting a more direct role in oncogenesis. Furthermore, our results point to additional events that could target the Wnt signaling pathway, such as epigenetic silencing of *WIF1*, which is consistently observed across the four GIAD types, or several of the somatic mutations highlighted in Table 6.

We believe that subdividing samples according to CIMP status has the potential to reduce heterogeneity within cancer subtypes and lead to more uniform molecular and phenotypic characteristics, thus producing more uniform response rates in clinical trials. Whether employed within cohort analyses or individual-level assessments, CIMP profiles have the potential to orient researchers and clinicians toward the biological properties of a tumor through their associations with MSI phenotypes, specific mutational profiles, and the repression of important tumor suppressor genes. Each of these avenues could potentially

identify complementary therapeutic modalities. Guided in this way, researchers may identify new candidates for synthetic lethal therapeutic targeting, in which bottlenecks in pathways necessary for tumor cell survival can be targeted, resulting in more precise interventions than many of the current standard-of-care regimens.

## RESEARCH

### Data

We downloaded level 3 DNA methylation data from TCGA's data portal (<https://tcga-data.nci.nih.gov/tcga/>). Data had been acquired using the Illumina HumanMethylation 450K platform and pre-processed following TCGA standard protocols. We further normalized the data from each sample using the BMIQ method<sup>[97]</sup>, which corrects for technical differences between type I and type II probes in the Illumina HumanMethylation platform. We also downloaded level 3 RNA-Seq data from the Broad Institute TCGA Genome Data Analysis Center (standard run dated 06/01/2015, <http://dx.doi.org/10.7908/C1251HBG>). For EAC, COAD, and READ, we used log<sub>2</sub>, normalized RSEM RNA-SeqV2 values. For STAD, we used log<sub>2</sub> RPKM RNA-Seq values, since RSEM estimates were not available. In addition, somatic mutation data for all four cancer types were downloaded through the bulk download interface of the TCGA portal (<https://tcga-data.nci.nih.gov/tcga/findArchives.htm>). Finally, CpG island and transcript annotation data were downloaded from the UCSC genome browser (cpgIslandExt track for CpG islands, and reflat and knownGene tracks for transcripts).

### Algorithms and statistical analysis

All statistical analyses were done using the R statistical package. We used CpG island annotations from UCSC for hg19 and gene annotations provided by Illumina for the HumanMethylation 450K platform. Promoter regions were defined as 2-kb regions encompassing the TSS of protein-coding loci (1.5 kb upstream of the TSS and 500 bps downstream of the TSS). Our DNA methylation analysis was restricted to probes located within CpG islands. Within each individual cancer type, we discarded probes with low variance across samples (SD < 0.1, based on normalized  $\beta$  values between 0 and 1), as well as probes located on the X and Y chromosomes.

Discriminative probes were selected by requiring minimal methylation in control samples (average methylation in controls < 0.05) and increased methylation in tumor samples (average methylation in tumors > 0.25). After a set of discriminative probes had been chosen separately for each tumor type, samples were classified into CIMP categories using *k*-means clustering on the vector of average methylation values computed across the set of selected probes (*k* = 3, initial centroids chosen to match population quartiles). Binary decision trees were computed using the R package "partykit"<sup>[98-100]</sup>.

Probe selection, CIMP classification, and decision tree analysis were performed as published in our previous

pan-cancer study<sup>[51]</sup>. We computed Spearman correlation values between expression values for each of the 28 genes in Table 5 and methylation values for probes in the corresponding TSSs. *P*-values were adjusted using the Bonferroni correction to account for the multiple probes associated with each gene.

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## Retrospective Cohort Study

# Gastric peritoneal carcinomatosis - a retrospective review

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**Author contributions:** Tan HL conceptualized the study, collected the data, analyzed the data and wrote the manuscript; Chia CS, Tan GHC and Teo MCC conceptualized the study and critically revised the manuscript; Choo SP, Tai DWM, Chua CWL and Ng MCH assisted in data collection and critically revised the manuscript; Soo KC critically revised the manuscript.

**Institutional review board statement:** This study involved an electronic medical record review of the included human study subjects. Ethics approval was obtained from the Singhealth Centralised Institutional Review Board.

**Informed consent statement:** No informed consent was deemed necessary as majority of the patients we studied have passed away at the point of the study. Furthermore, the study involved a retrospective electronic medical record review, with no patient identifiers included in the results presented, hence bearing minimal risk to the patients included in the study.

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## Abstract

### AIM

To characterize patients with gastric peritoneal carcinomatosis (PC) and their typical clinical and treatment course with palliative systemic chemotherapy as the current standard of care.

### METHODS

We performed a retrospective electronic chart review of all patients with gastric adenocarcinoma with PC diagnosed at initial metastatic presentation between January 2010 and December 2014 in a single tertiary referral centre.

### RESULTS

We studied a total of 271 patients with a median age of 63.8 years and median follow-up duration of 5.1 mo. The majority ( $n = 217$ , 80.1%) had the peritoneum as the only site of metastasis at initial presentation. Palliative systemic chemotherapy was eventually planned for 175 (64.6%) of our patients at initial presentation, of which 171 were initiated on it. Choice of first-line regime was in accordance with the National Comprehensive Cancer Network Guidelines for Gastric Cancer Treatment. These

patients underwent a median of one line of chemotherapy, completing a median of six cycles in total. Chemotherapy disruption due to unplanned hospitalizations occurred in 114 (66.7%), while cessation of chemotherapy occurred in 157 (91.8%), with 42 cessations primarily attributable to PC-related complications. Patients who had initiation of systemic chemotherapy had a significantly better median overall survival than those who did not (10.9 mo *vs* 1.6 mo,  $P < 0.001$ ). Of patients who had initiation of systemic chemotherapy, those who experienced any disruptions to chemotherapy due to unplanned hospitalizations had a significantly worse median overall survival compared to those who did not (8.7 mo *vs* 14.6 mo,  $P < 0.001$ ).

## CONCLUSION

Gastric PC carries a grim prognosis with a clinical course fraught with disease-related complications which may attenuate any survival benefit which palliative systemic chemotherapy may have to offer. As such, investigational use of regional therapies is warranted and required validation in patients with isolated PC to maximize their survival outcomes in the long run.

**Key words:** Peritoneal carcinomatosis; Gastric cancer

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**Core tip:** We present a retrospective review of the clinical course and treatment outcomes of patients with gastric peritoneal carcinomatosis. It carries a poor prognosis with a clinical course fraught with disease-related complications which disrupts planned systemic palliative chemotherapy in the majority of patients. Such disruptions attenuate the benefits of systemic chemotherapy and decrease overall survival. Patients with isolated peritoneal disease may as such benefit from investigational loco-regional therapies pending further studies and validation.

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## INTRODUCTION

Gastric cancer is the fifth most common cancer across the world, accounting for 723000 deaths per year, the third most frequent cause of cancer-related deaths<sup>[1-3]</sup>. In Singapore, gastric cancer ranks as the seventh and ninth most common cancers, but accounts for the fourth and fifth most frequent cancer deaths amongst males and females respectively<sup>[4]</sup>. The poor prognosis of gastric cancer has in part been attributed to the high incidence of advanced disease at presentation, with up to 39% harboring disseminated disease at diagnosis<sup>[5]</sup>. Metastatic gastric cancer carries a grim prognosis

with a median overall survival of approximately four months and five-year survival rates of 3%-6%<sup>[1,6,7]</sup>. Although palliative systemic chemotherapy has been demonstrated in numerous trials to improve survival amongst patients with metastatic gastric cancer to a median of 7.5-12.3 mo<sup>[8-11]</sup>, whether such a benefit accrues equally to all sites of gastric cancer metastases is unclear.

Peritoneal carcinomatosis (PC) is recognized as an independent poor prognostic factor and is known to have a penchant for causing a wide range of troubling clinical symptoms including symptomatic ascites, intestinal obstruction, perforation and obstructive uropathy<sup>[12]</sup>. This can result in repeated hospitalizations, therapeutic interventions and rapid deterioration of a patient's performance status, which may serve to interrupt and prematurely terminate any planned palliative systemic chemotherapy regime a patient might be on. There is a paucity of literature on the characteristics of patients with gastric PC<sup>[5]</sup>, with virtually no studies examining the clinical and treatment course of these patients. The advent of studies examining the role of cytoreductive surgery (CRS) and hyperthermic intraperitoneal chemotherapy (HIPEC) for the treatment of gastric PC was borne from the concept of peritoneal metastases being a loco-regional disease extension rather than a true systemic dissemination of gastric cancer<sup>[13-18]</sup>. Should patients with gastric PC be less likely to complete planned courses of palliative systemic chemotherapy and hence perform relatively poorly, it would further bolster the case for studying CRS and HIPEC for a select group of patients with gastric PC to maximize their survival.

As such, we aim to characterize patients with gastric PC and their typical clinical and treatment course to glean a better understanding of how well this subset of metastatic gastric cancer patients are doing with palliative systemic chemotherapy as the current standard of care.

## MATERIALS AND METHODS

We performed a retrospective review of all patients with metastatic gastric cancer managed at the National Cancer Centre Singapore, the largest tertiary referral centre for cancer treatment locally, over a 5-year period between January 2010 and December 2014. All patients with gastric adenocarcinoma with peritoneal metastasis diagnosed at initial metastatic presentation, with or without other concomitant distant sites of metastasis, were included in our study. Patients with isolated positive peritoneal cytology were excluded. Electronic records were reviewed for various patient characteristics including patient demographics, gastric cancer characteristics, treatments administered and subsequent clinical course through each patient's follow-up duration.

All patients included in the study had computed tomography scans of the thorax, abdomen and pelvis performed for initial staging following the diagnosis of

**Table 1** Baseline patient demographics

Characteristic	n (%)
Median age (range) (yr)	63.8 (26.9-89.0)
Ethnicity	
Chinese	203 (74.9)
Indian	13 (4.8)
Malay	20 (7.4)
Others	35 (12.9)
Gender	
Male	134 (49.4)
Female	137 (50.6)
ECOG status	
0	89 (32.8)
1	135 (49.8)
2	28 (10.3)
3	11 (4.1)
4	8 (3.0)
ASA score	
1	107 (39.5)
2	128 (47.2)
3	36 (13.3)
Comorbidities	
Diabetes mellitus	56 (20.7)
Hypertension	124 (45.8)
Cardiac comorbidities	43 (15.9)
Respiratory comorbidities	15 (5.5)
Chronic renal impairment	10 (3.7)
Central nervous system comorbidity	19 (7.0)
Previous cancer	27 (10.0)
Cigarette smoking	58 (21.4)
Regular alcohol use	31 (11.4)

ECOG: Eastern Cooperative Oncology Group; ASA: American Society of Anaesthesiology.

gastric adenocarcinoma. Patients without radiological evidence of metastatic disease then underwent staging laparoscopy with intra-operative frozen section assessment of peritoneal deposits.

Palliative systemic chemotherapy was offered as the standard of care in our institution to all patients with metastatic gastric cancer in close discussion with each patient. The first-line palliative systemic chemotherapy regime for each patient was chosen in accordance to the National Comprehensive Cancer Network Guidelines for Gastric Cancer Treatment with consideration to each patient's performance status and preferences, favoring two to three-agent chemotherapy combinations over single-agent chemotherapy where possible<sup>[19]</sup>. Trastuzumab was additionally offered in cases which were positive for Her2/Neu overexpression. Chemotherapy regime was switched or discontinued based on clinician discretion during the course of follow-up if patients experienced unacceptable levels of toxicity or had clinical evidence of disease progression. Chemotherapy was also put on hold or stopped entirely in the event of acute deteriorations in patients' functional and/or medical conditions. Other therapeutic interventions including surgery, endoscopic therapy and radiotherapy were also undertaken where clinically indicated. Follow-up duration of each patient is calculated in months beginning from initial diagnosis till the last follow-up or death at the point

of data collection.

### Statistical analysis

Statistical analysis was performed using SPSS Statistics Version 19.0 (Armonk NY: IBM Corp). Continuous and categorical variables and survival data were analyzed using the Student's *t*-test,  $\chi^2$  test and Kaplan-Meier analysis respectively, with a statistical significance level of 5% used.

## RESULTS

### Demographics and baseline characteristics

We studied a total of 271 patients with gastric adenocarcinoma with PC diagnosed at initial metastatic presentation with a median follow-up duration of 5.1 mo (IQR: 2.2-11.7). Patient characteristics are summarized in Table 1. The median age was 63.8 years (range 26.9-89.0), with relatively equal gender proportions (49.4% male) and a predominant Chinese ethnicity (74.9%). The majority of patients had good functional and medical conditions at the point of diagnosis, with Eastern Cooperative Oncology Group (ECOG) performance status ratings of 0-1 in 82.6% and American Society of Anaesthesiology (ASA) scores of 1-2 in 86.7%.

The bulk of our patients ( $n = 258$ , 95.2%) had peritoneal metastasis diagnosed at initial gastric cancer diagnosis, while the remaining 13 (4.8%) cases of PC were diagnosed as a metastatic recurrence of previously treated gastric cancer. In our cohort, 217 (80.1%) patients had the peritoneum as the only site of metastasis at initial presentation, while 54 (19.9%) had other concomitant distant site(s) of metastasis. Approximately half of the diagnosis of PC was made radiologically ( $n = 134$ , 49.4%) while the remainder was made intra-operatively. Other gastric cancer-related characteristics of our cohort are summarized in Table 2. Of note, when comparing patients with peritoneal metastasis only to patients with other concomitant distant sites of metastasis, there was a higher proportion of females (53.9% vs 37.0%,  $P = 0.026$ ) and diffuse histology (57.1% vs 33.3%,  $P = 0.002$ ), and a lower proportion of HER2/Neu overexpression (16.4% vs 33.3%,  $P = 0.029$ ).

### Palliative systemic chemotherapy

Palliative systemic chemotherapy was offered as standard of care in all patients with metastatic gastric cancer in our institution in close discussion with each patient, with 175 (64.6%) patients eventually planned for systemic chemotherapy following initial metastatic presentation. The subsequent chemotherapy-related clinical course of these patients is summarized in Figure 1. Expectedly, the baseline functional and medical status of patients planned for systemic chemotherapy were significantly better than those who opted for best supportive care upfront, with ECOG ratings 0-2 in 98.9% vs 82.3% ( $P < 0.001$ ) and ASA scores of 1-2 in 92.0% vs 77.1% ( $P = 0.001$ ). Four patients planned for systemic chemotherapy



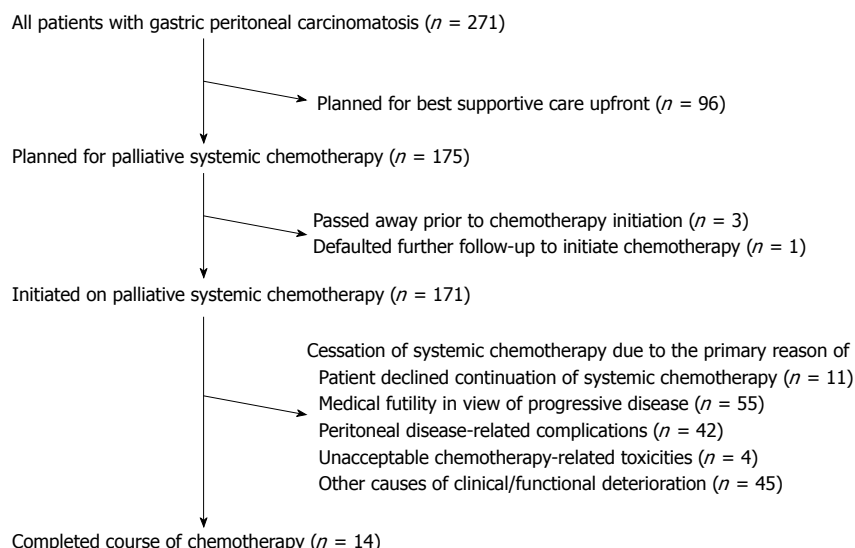


Figure 1 Chemotherapy-related clinical course.

Table 2 Baseline gastric cancer-related characteristics

Characteristic ( $n = 271$ )	$n$ (%)
Presentation of peritoneal metastases	
At initial gastric cancer diagnosis	258 (95.2)
Recurrence of treated gastric cancer	13 (4.8)
Site of metastases	
Peritoneal only	217 (80.1)
Peritoneal and distant site(s)	54 (19.9)
Primary gastric cancer location	
Gastroesophageal junction	23 (8.5)
Proximal gastric	16 (5.9)
Gastric body	101 (37.3)
Distal gastric	103 (38.0)
Linitis plastica	28 (10.3)
Lauren's classification	
Intestinal	129 (47.6)
Diffuse	142 (52.4)
c-erb-B2 receptor status ( $n = 195$ )	
Positive	37 (19.0)
Negative	158 (81.0)

Table 3 First-line chemotherapy regime

Chemotherapy regime ( $n = 171$ )	$n$ (%)
Anthracycline + platinum-based agent + nucleotide analogue	13 (7.6)
Examples	
epirubicin + cisplatin + 5-fluorouracil	
epirubicin + oxaliplatin + 5-fluorouracil	
Platinum-based agent + nucleotide analogue	97 (56.7)
Examples	
cisplatin + 5-fluorouracil	
oxaliplatin + capecitabine	
cisplatin + S-1	
Nucleotide analogue monotherapy	30 (17.5)
Examples	
5-fluorouracil	
capecitabine	
S-1	
FOLFOX (5-fluorouracil + leucovorin + oxaliplatin)	24 (14.0)
Other regimes (e.g., Docetaxel + cisplatin + 5-fluorouracil)	7 (4.2)

did not eventually initiate chemotherapy as three passed away prior to chemotherapy initiation due to cancer-related complications while one defaulted further follow-up for chemotherapy initiation.

The most common first-line chemotherapy regimes utilized in our cohort are summarized in Table 3. Notably, of the 171 patients who eventually initiated chemotherapy, 138 (80.7%) patients received two or three-agent chemotherapy regimes as first-line therapy. Our patients underwent a median of one line of chemotherapy (IQR 1-2), completing a median of six cycles in total (IQR 3-11). Chemotherapy was disrupted in 114 (66.7%) cases due to unplanned hospitalizations, with a median duration of disruption of two weeks (IQR 1-2.25) each time. Chemotherapy-related toxicity was documented in 81 (47.4%) of cases, most commonly affecting the gastrointestinal ( $n = 28$ , 34.6%), neurological ( $n = 23$ , 28.4%) and hematological ( $n = 22$ , 27.2%) systems.

Cessation of systemic chemotherapy occurred in

157 (91.8%) of patients due to a variety of reasons as delineated in Figure 1. Of note, slightly over a quarter ( $n = 42$ , 26.8%) of cessations were primarily attributable to peritoneal disease-related complications. Eventually, only 14 (8.2%) patients completed their courses of chemotherapy with subsequent close clinical surveillance.

### Clinical course and therapeutic interventions

Through the course of follow-up, 201 (74.2%) patients required unplanned hospitalizations (median number of hospitalizations = 2, IQR 1-3) following initial diagnosis for various disease and/or treatment-related complications including symptomatic ascites, sepsis, gastric outlet obstruction and gastrointestinal tract bleeding (Table 4). 242 (89.3%) patients required some form of therapeutic intervention in an outpatient and/or inpatient setting including surgery, endoscopic stenting, feeding tube insertion and radiotherapy (Table 5). Median overall survival of our patient cohort was 8.7 mo (95%CI: 7.3-10.1) with a trend towards longer survival amongst

**Table 4** Reasons requiring unplanned hospitalizations

Reason ( <i>n</i> = 201)	<i>n</i> (%)
Symptomatic ascites	64 (31.8)
Sepsis	64 (31.8)
Gastric outlet obstruction	60 (29.9)
Bleeding GIT	60 (29.9)
Intestinal obstruction	59 (29.4)
Chemotherapy-related toxicity	23 (11.4)
Obstructive jaundice	17 (8.5)
Obstructive uropathy	17 (8.5)
Tumour perforation	7 (3.5)

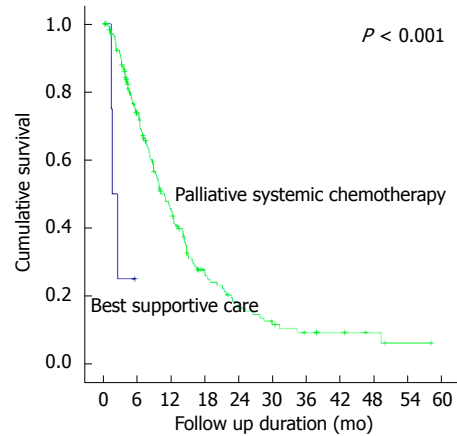
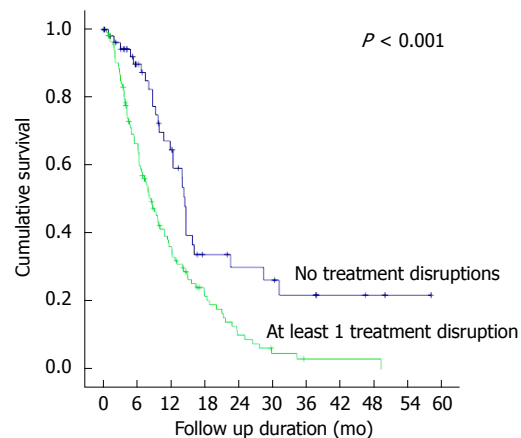
**Table 5** Therapeutic interventions required

Treatment category	Treatment	<i>n</i> (%)
Surgery <i>n</i> = 89 (32.8%)	Palliative gastrectomy	32 (36.0)
	Surgical bypass	46 (51.7)
	Open gastrostomy	2 (2.2)
	Feeding jejunostomy	5 (5.6)
	Cytoreductive surgery and hyperthermic intraperitoneal chemotherapy	1 (1.1)
	Others	3 (3.3)
Endoscopic intervention <i>n</i> = 57 (21.0%)	Feeding tube insertion only	45 (78.9)
	Stenting only	8 (14.0)
	Feeding tube insertion and stenting	4 (7.0)
Radiotherapy <i>n</i> = 35 (12.9%)	Radiotherapy to gastric tumour	29 (82.9)
	Radiotherapy to other sites	6 (17.1)

patients with peritoneal metastasis only as compared to those with other concomitant distant sites of metastasis (median survival 8.9 mo vs 7.0 mo,  $P = 0.061$ ). The 171 patients who initiated systemic chemotherapy, when compared to the rest of the patients who received best supportive care upfront, had a significantly better median overall survival of 10.9 mo vs 1.6 mo ( $P < 0.001$ ) (Figure 2). Of these patients who initiated systemic chemotherapy, the ones who experienced any disruptions to chemotherapy due to unplanned hospitalizations had a significantly worse median survival compared to those without chemotherapy disruptions (8.7 mo vs 14.6 mo,  $P < 0.001$ ) (Figure 3).

## DISCUSSION

Existing literature on gastric cancer has largely evaluated metastatic gastric cancer as a homogeneous, undifferentiated entity, with clinical features, prognostic factors, treatment outcomes and overall survival examined as a single patient group<sup>[1-3]</sup>. In a similar vein, treatment for metastatic gastric cancer has also been predominated by palliative systemic chemotherapy and supportive care<sup>[20]</sup>. There has been a growing interest in the treatment of PC with the advent of CRS and HIPEC especially in colorectal and appendiceal malignancies. The idea of peritoneal metastasis representing loco-regional disease extension as opposed to systemic

**Figure 2** Overall survival of patients initiated on palliative systemic chemotherapy vs patients who received best supportive care upfront.**Figure 3** Overall survival of patients initiated on palliative systemic chemotherapy who had treatment disruptions vs without treatment disruptions.

dissemination in other sites of metastasis has likewise encouraged ventures at examining the benefit of CRS and HIPEC in the treatment of PC of other primaries, including gastric cancer<sup>[21]</sup>. Our understanding of the clinical characteristics of this subgroup of metastatic gastric cancer patients with PC and how they fare with the current gold standard of treatment - palliative systemic chemotherapy - remains limited, a knowledge gap we sought to address through this study.

Gastric PC was reported by Thomassen *et al*<sup>[5]</sup> to account for a sizeable 35.0% of metastatic disease at presentation, with PC as the only site of metastasis in 68.6% of cases, comparable to 80.1% of cases in our cohort. It similarly represents 36.0%-45.9% of metastatic recurrences after previous curative treatment for gastric cancer<sup>[22,23]</sup>. Several studies have reported clinical characteristics predictive of peritoneal metastasis at presentation or recurrence including a younger age, female gender, serosal involvement of primary tumor and a diffuse histology. We found the female gender, diffuse histology and absence of HER2/Neu overexpression to be associated with PC as the sole site of metastatic disease in gastric cancer<sup>[5,23]</sup>.

Palliative systemic chemotherapy has been well established as the current standard of care for patients with metastatic gastric cancer. A recent meta-analysis of 35 trials involving 5726 patients demonstrated that, in terms of overall survival, systemic chemotherapy achieves superior outcomes compared to best supportive care, and that combination chemotherapy is superior to single-agent chemotherapy with the trade-off of increased incidence of chemotherapy-related toxicity<sup>[20]</sup>.

Despite the fact that the subgroup of our patient cohort planned for systemic chemotherapy had highly optimal baseline functional and medical statuses (98.9% with ECOG ratings of 0-2 and 92.0% with ASA scores of 1-2) with a large proportion (80.7%) undergoing two to three-agent combination chemotherapy regimes upfront, only a mere 14 (8.2%) patients eventually completed their chemotherapy regimes with subsequent close clinical surveillance. This could be attributed to several reasons.

Firstly, a significant proportion of patients who initiated chemotherapy had clinical evidence of progressive disease in spite of treatment, and even after a trial of second, third or fourth-line regimes in a handful of patients, 32.2% eventually opted for supportive care in view of treatment futility. The poor response of PC to conventional systemic chemotherapy could in part be accounted for by the poor penetration of peritoneal deposits by chemotherapeutic agents administered systemically<sup>[24]</sup>. This has spurred efforts at studying the efficacy of a combined bidirectional intravenous and intraperitoneal route of chemotherapy administration for PC, which has been proven to confer survival benefit in ovarian cancer, and has been tested in several phase 2 trials for gastric PC with encouraging results<sup>[25,26]</sup>.

Secondly, a large proportion of our cohort (74.2%) required unplanned hospitalizations due to disease and/or treatment-related complications, each of which could significantly accelerate the process of clinical deterioration. The median number of hospitalizations each of these patients required was 2, for a variety of complications including those attributable to PC including symptomatic ascites, bowel obstruction, obstructive uropathy and obstructive jaundice. Besides resulting in unforeseen breaks in systemic chemotherapy occurring in 66.7% of our patients who underwent chemotherapy, these acute clinical events also directly contributed to significant clinical and functional decline necessitating premature cessation of chemotherapy in a quarter of cases (Figure 1). Considering that a sizeable proportion of patients who ceased chemotherapy due to other causes of clinical/functional deterioration were attributable to nosocomial infections contracted during admissions for peritoneal disease-related complications, the inadvertent impact of PC on cessation of systemic chemotherapy in these patients may indeed be even greater. The consequent impact on survival outcomes is evident from our finding of a significantly worse overall survival amongst patients on systemic chemotherapy who experienced treatment disruptions (Figure 3).

We report an overall survival of 8.7 mo in our cohort, comparable to survival outcomes of 7.5-12.3 mo achieved by patients on systemic chemotherapy in several trials<sup>[8-11]</sup>. Stratified analysis revealed a trend towards improved survival in patients with isolated peritoneal metastasis, in keeping with findings of Thomassen *et al*<sup>[5]</sup> which reported median survival of 4.6 and 3.3 mo in patients with isolated peritoneal metastasis and peritoneal plus other concomitant distant sites of metastasis respectively. This is consistent with proposed theories of isolated PC as a loco-regional disease extension rather than a true systemic dissemination of metastatic disease, which further lends support to the investigational use of aggressive loco-regional treatment with CRS and HIPEC in at least selected cases to maximize survival outcomes.

While we recognize the limitations inherent to the retrospective nature of our study, it is, to the best of our knowledge, the only study after Thomassen *et al*<sup>[5]</sup> to examine the demographic and disease characteristics of gastric PC, and the first study to examine the clinical and treatment course of these patients.

Looking ahead, further studies could examine and compare the clinical course and outcomes of gastric cancer patients with different groups of metastatic sites (e.g., peritoneal metastasis vs isolated liver metastasis vs other distant sites of metastasis). Additionally, delving further into gastric PC, further work could be put into determining if the extent of peritoneal involvement affects clinical course and outcomes, which could in turn help better define a patient subgroup which may best benefit from aggressive loco-regional treatment options.

## Conclusion

Gastric PC carries a grim prognosis with a clinical course fraught with disease-related complications which may attenuate any survival benefit palliative systemic chemotherapy has to offer. As such, investigational use of regional therapies is warranted and required validation in patients with isolated PC to maximize their survival outcomes in the long run.

## COMMENTS

### Background

Systemic palliative chemotherapy is the current standard of care for all metastatic gastric cancers, including cases with peritoneal carcinomatosis (PC). Gastric PC is known to cause symptoms requiring repeated hospitalizations which may interrupt and terminate planned palliative systemic chemotherapy. There exists a paucity of literature examining the clinical course of patients with gastric PC. The authors aimed to characterize patients with gastric PC and their typical clinical and treatment course with palliative systemic chemotherapy as the current standard of care.

### Research frontiers

While systemic palliative chemotherapy has been established as the standard of care through several randomized controlled trials demonstrating survival benefit, the overall prognosis of metastatic gastric cancer remains poor. Investigational loco-regional treatment options such as intraperitoneal chemotherapy and cytoreductive surgery are currently being studied and validated as alternative treatment of patients with gastric PC.

## Innovations and breakthroughs

This is, to the best of the knowledge, the only study after Thomassen *et al* to examine the demographic and disease characteristics of gastric PC, and the first study to examine the clinical and treatment course of these patients.

## Applications

The authors demonstrated that patients with gastric PC have a grim prognosis, with frequent disease-related complications requiring unplanned hospitalizations which disrupt and terminate planned palliative systemic chemotherapy. As such, patients with gastric PC may benefit from investigational loco-regional treatment options as an alternative.

## Peer-review

The authors present a retrospective study of a cohort of patients with gastric cancer and peritoneal metastases treated in a single oncology center. The rationale for the study is important as the prognosis remains poor in this group of patients. The study derives a lot of clinical data describing patients' baseline characteristics and their course during palliative therapy. The results are consistent with those presented in previous studies.

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## Lung cancer metastasis to the gastrointestinal system: An enigmatic occurrence

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### Abstract

Adenocarcinoma of the lung infrequently metastasizes to the gastrointestinal tract. We report a rare case of a 65-year-old male with no respiratory symptoms diagnosed with adenocarcinoma of the lung by histopathological examination of metastatic sites which included an ulcer in the gastric body and a mass in the rectum. Metastatic disease also involved the liver as well. Patient was treated with systemic chemotherapy but unfortunately expired five months after the diagnosis was made.

**Key words:** Lung cancer; Gastrointestinal metastasis

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**Core tip:** It is an extremely uncommon finding to discover lung cancer on gastric ulcer and rectal mass biopsy. Also, this patient did not have a pre-existing cancer diagnosis. Computerized tomography did reveal liver lesions as well. With increasing use of endoscopy and colonoscopy in the current era, physicians should be mindful of the uncommon differentials as well.

Badipatla KR, Yadavalli N, Vakde T, Niazi M, Patel HK. Lung cancer metastasis to the gastrointestinal system: An enigmatic occurrence. *World J Gastrointest Oncol* 2017; 9(3): 129-134 Available from: URL: <http://www.wjgnet.com/1948-5204/full/v9/i3/129.htm> DOI: <http://dx.doi.org/10.4251/wjgo.v9.i3.129>

## INTRODUCTION

Gastrointestinal metastasis of a primary lung cancer although previously reported in literature is a rare presentation. When metastasis does occur, the small bowel appears to be the most common site<sup>[1]</sup>. Clinical presentation may vary from being asymptomatic to non-specific abdominal pain and life threatening complications like massive bleeding and perforation requiring emergent surgical interventions<sup>[2]</sup>. Our case is first of its kind where in adenocarcinoma of lung is diagnosed by histology from a mass lesion in the rectum and gastric ulcer, in a person with no prior respiratory symptoms.

## CASE REPORT

A 65-year-old man presented to our hospital emergency room (ER) with complaints of bilateral flank pain. He denied nausea, vomiting and change in bowel habit. He reported decrease in appetite and loss of 15-pound (lbs.) weight in one month.

His medical history was significant for mild intermittent bronchial asthma, benign essential hypertension, major depressive disorder and prostate cancer treated with radiation therapy 5 years prior to current hospital admission which is currently in remission. He did not undergo any surgical procedures in the past. There were no gastrointestinal malignancies diagnosed in his immediate or distant family members. He never used tobacco products, alcohol or recreational drugs. He was not allergic to any medications.

On initial evaluation he was afebrile with heart rate of 75 beats per minute, respiratory rate of 18 per minute and blood pressure of 150/70 millimeters of mercury (mm of Hg). His oxygen saturation was 95% on room air. Abdomen was non-distended, soft and non-tender to palpation. On auscultation bowel sounds were noted to be normoactive. Digital rectal examination (DRE) revealed a hard palpable, non-mobile mass on the posterior rectal wall. Cardiorespiratory and neurological examination was within normal limits.

Laboratory results were significant for microcytic anemia with hemoglobin of 8.5-g percent with normal white cell counts and platelets. Coagulation parameters were within normal limits. Liver function tests showed elevated alkaline phosphatase of 482 units per liter, with remaining liver parameters being unremarkable. Tumor markers showed mildly elevated carcinoembryonic antigen level of 38.2 nanogram/milliliter and Cancer antigen-125 (CA-125) of 682.8 units/milliliter. Serum prostate specific antigen (PSA) level was 0.12 nanogram/



Figure 1 Computed tomography chest showing lung opacity, pleural effusion and lymph nodes.

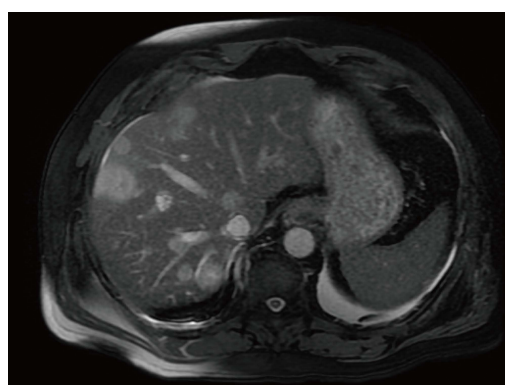


Figure 2 Magnetic resonance imaging showing multiple metastatic liver lesions.

milliliter. Computerized tomography of chest, abdomen and pelvis showed an area of opacity measuring 8.8 cm × 4.6 cm × 6.3 cm in the left upper lobe of the lung, diffuse mediastinal adenopathy and moderate to large left sided pleural effusion (Figure 1). There was diffuse osteo-sclerosis of multiple bones suspicious for osteoblastic metastatic disease. Also, noted were several intrahepatic masses suspicious for metastatic disease and shotty mesenteric and portocaval adenopathy. Magnetic resonance imaging (MRI) confirmed computed tomography (CT) findings of liver metastases (Figure 2).

Gastroenterology consultation was obtained in view of anemia and possible liver lesions. Patient underwent upper gastrointestinal endoscopy, colonoscopy and subsequent endoscopic ultrasound under monitored anesthesia care. Upper gastrointestinal endoscopy showed a 15-millimeter cratered gastric ulcer without any stigmata of recent bleeding which was biopsied (Figure 3). Colonoscopy showed a large mass in the rectum (Figure 4) and four polyps in the cecum, all of which were biopsied. Subsequent endoscopic ultrasound (EUS) of the rectal lesion revealed irregular hypoechoic lesion causing thickening of the submucosal layer and irregular out-borders suggestive of malignant nature of the lesion. Patient also underwent CT guided left thoracentesis and liver biopsy.

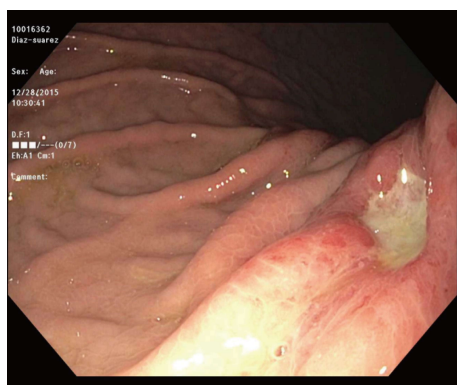


Figure 3 Gastric ulcer in the body.

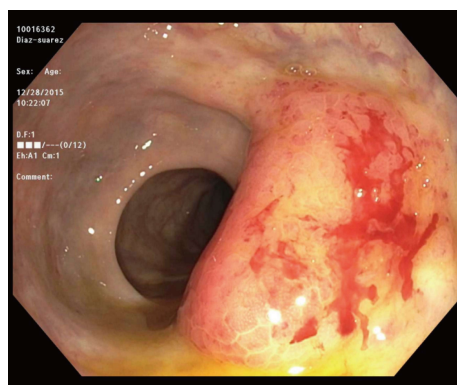


Figure 4 Rectal mass.

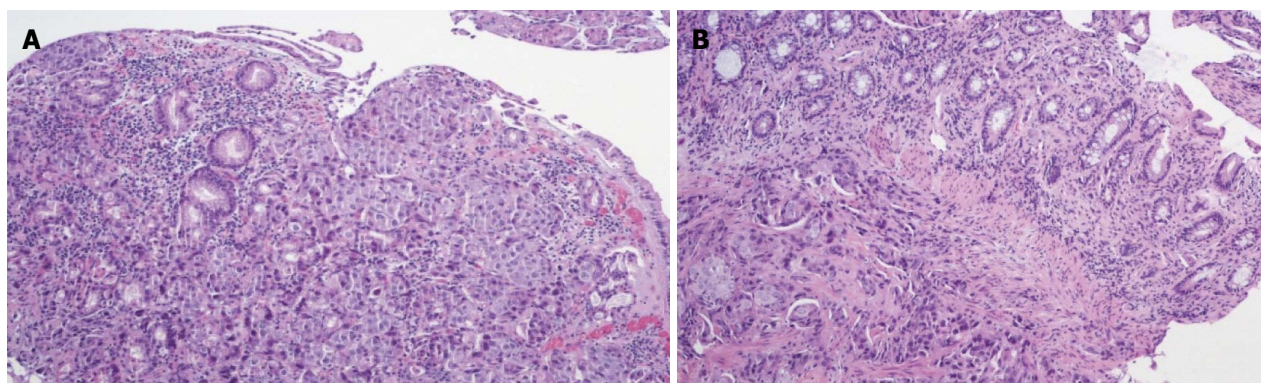


Figure 5 Pathology from both rectal mass and gastric ulcer showed metastatic adenocarcinoma, consistent with lung primary. A: Gastric mucosa with metastatic adenocarcinoma; B: Rectal mass showing submucosa and deep mucosa with metastatic adenocarcinoma.

Table 1 Reported cases of metastatic lung cancer to the colon

Ref.	Histology	Prior diagnosis of lung malignancy	Presenting clinical scenario
Jevremovic <i>et al</i> <sup>[13]</sup> , 2016	Adenocarcinoma	New diagnosis	Iron deficiency anemia
Miyazaki <i>et al</i> <sup>[15]</sup> , 2015	Squamous cell	Known case	Abdominal pain and anemia
Kaswala <i>et al</i> <sup>[14]</sup> , 2013	Adenocarcinoma	Known case	Surveillance colonoscopy
Sakai <i>et al</i> <sup>[16]</sup> , 2012	Squamous cell	Known case	Abdominal pain
Hirasaki <i>et al</i> <sup>[17]</sup> , 2008	Squamous cell	Diagnosed at the same time	Asymptomatic with positive fecal occult blood testing.
Yang <i>et al</i> <sup>[2]</sup> , 2006	Squamous cell	Known case	Bloody stools
Stinchcombe <i>et al</i> <sup>[18]</sup> , 2006	Squamous cell	Diagnosed at the same time	Asymptomatic with positron emission tomography computer tomography scan done showing increased colonic uptake
Habeşoğlu <i>et al</i> <sup>[19]</sup> , 2005	Squamous cell	Cancer naive	Bowel obstruction
Carroll <i>et al</i> <sup>[20]</sup> , 2001	Squamous cell	Cancer naive	Weight loss and diarrhea
Bastos <i>et al</i> <sup>[21]</sup> , 1998	Squamous cell	Known case	Abdominal pain, diarrhea and bloody stools
Gitt <i>et al</i> <sup>[22]</sup> , 1996	Squamous cell	Known case	Bowel perforation
Gateley <i>et al</i> <sup>[23]</sup> , 1993	Squamous cell	Known case	Gastrointestinal bleeding
Brown <i>et al</i> <sup>[24]</sup> , 1980	Anaplastic carcinoma	Diagnosed at the same time	Abdominal pain, weight loss
Smith <i>et al</i> <sup>[25]</sup> , 1978 (2 cases)	Histology not known	Not known	Intermittent obstruction, bleeding or anemia

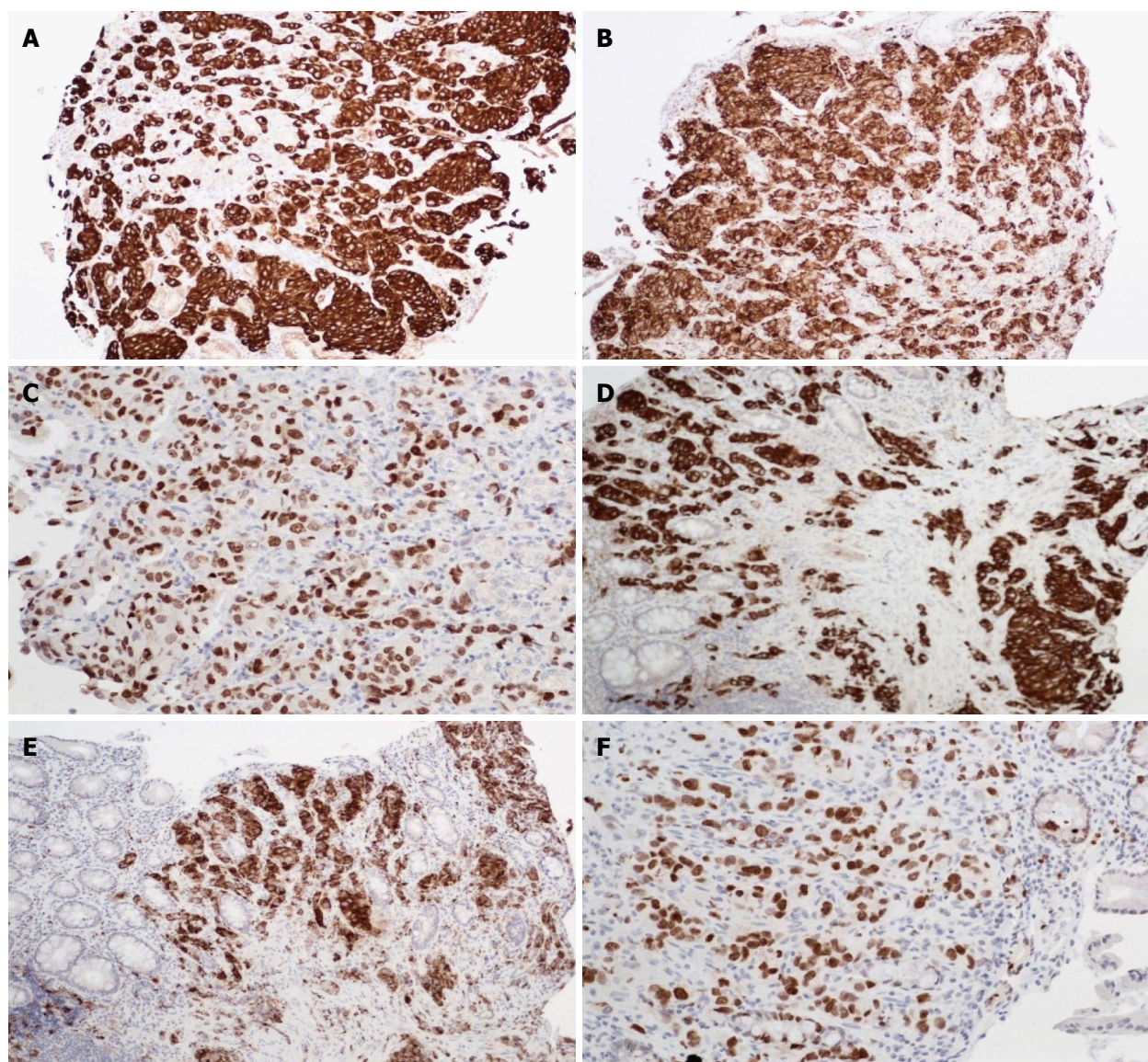
Pathology from both rectal mass and gastric ulcer showed metastatic adenocarcinoma, consistent with lung primary (Figure 5). Immuno-histochemical staining was positive for cytokeratin 7 (CK 7), thyroid transcription factor-1 (TTF-1) and napsin-A antibodies (Figure 6). It was negative for PSA, prostate specific acid phosphatase, CK 20, CDX-2, cancer antigen 19-9 (CA 19-9) and P504 antibodies consistent with lung primary. Results from thoracentesis and liver biopsy yielded similar results showing

metastatic adenocarcinoma of the lung origin. Patient was started on chemotherapy with combination of Carboplatin and Paclitaxel. After receiving two cycles of chemotherapy, patient and family opted for palliative care and he expired five months after the diagnosis was made.

## DISCUSSION

Lung cancer is the most common cancer worldwide





**Figure 6 Immunohistochemical staining.** A: Gastric biopsy showing positivity to CK 7; B: Gastric biopsy showing positivity to Napsin-A; C: Gastric biopsy showing positivity to TTF-1; D: Rectal biopsy showing positivity to CK 7; E: Rectal biopsy showing positivity to Napsin-A; F: Rectal biopsy showing positivity to TTF-1. CK 7: Cytokeratin 7; TTF-1: Thyroid transcription factor-1.

accounting for 19.4% of all the cancer related deaths<sup>[3]</sup>.

Adenocarcinoma of the lung is known to metastasize to liver, lung, brain and bone with half of the patients harboring metastasis at the time of presentation<sup>[4]</sup>. Gastrointestinal tract is an infrequent site of metastasis. In a large retrospective study done by Kim *et al.*<sup>[5]</sup> gastrointestinal metastasis was found in 0.19% of all the cases with small bowel being the most common site, although autopsy studies revealed higher rates of metastatic disease<sup>[1]</sup>. Metastatic lung cancer has known to spread to any location from the oral cavity to the anus<sup>[6]</sup> with lymphatic and hematogenous routes being the possible modes of spread<sup>[7]</sup>.

Symptomatology spectrum ranges from being totally asymptomatic to bleeding<sup>[8]</sup>, pain and dysphagia in case of esophageal involvement<sup>[9]</sup>. Peritonitis, perforation<sup>[10]</sup> and bowel obstruction are among the acute complications that were reported<sup>[11]</sup>. Laboratory analysis may reveal iron deficiency anemia.

Diagnosis is based on endoscopy with biopsies. On gastrointestinal endoscopy variable endoscopic appearances have been described including ulcerated lesion, nodularity, diffuse mucosal involvement, polyp or mass lesions<sup>[12]</sup>. Small bowel endoscopy (SBE) may be needed for evaluation of small bowel lesions. Histological examination of post-surgical specimens usually reveals diagnosis. On colonoscopy lesions, may vary from sub-centimeter lesions to more larger mass lesions as in our case. So far, review of literature shows 15 cases of metastatic lung cancer to the colon<sup>[2,13-25]</sup> (Table 1). The most common reported histology appears to be squamous cell carcinoma followed by adenocarcinoma being the less common variant<sup>[13,14]</sup> (Table 1).

Immuno-histochemical staining of the tissue is useful in streamlining the diagnosis<sup>[26]</sup> with TTF-1 and napsin-1 being specific for lung adenocarcinoma<sup>[27]</sup>.

Prognosis appears to be poor in patients with gastro-

intestinal metastasis. Palliative resection has been described as treatment option especially in small bowel lesions to prevent further complications.

In summary, our case describes an extremely rare occurrence of synchronous metastasis of adenocarcinoma of lung presenting as gastric ulcer and rectal mass in an asymptomatic patient. To the best of our knowledge, our case is the first case described in literature of such a presentation. This again throws light that metastasis to the gastrointestinal tract may be considered among the differential while encountering such lesions in the gastrointestinal tract and appropriate diagnosis and prompt treatment may be helpful in these cases.

## COMMENTS

### Case characteristics

A 65-year-old man with anemia, weight loss and liver lesions noted to have gastric ulcer on endoscopy and rectal mass on colonoscopy.

### Clinical diagnosis

Lung cancer presenting as metastatic gastric ulcer and rectal mass in a cancer naïve patient.

### Differential diagnosis

Metastatic lung cancer to the gastrointestinal system.

### Laboratory diagnosis

Laboratory results were significant for microcytic anemia with hemoglobin of 8.5 g percent. Alkaline phosphatase was 482 units per liter. Tumor markers showed mildly elevated carcinoembryonic antigen levels of 38.2 units/milliliter and cancer antigen -125 of 682.8 units/ milliliter.

### Imaging diagnosis

Computed tomography revealed lung lesion with mediastinal adenopathy and metastasis to liver.

### Pathological diagnosis

Histopathology from gastric ulcer and rectal mass revealed adenocarcinoma of lung.

### Treatment

Chemotherapy.

### Related reports

Prior reports of gastrointestinal metastasis from lung cancer included mostly autopsy series with small bowel being the most common site. There have been no reports of synchronous metastasis of lung cancer to stomach and rectum as in the case.

### Term explanation

Adenocarcinoma of the lung is one the types of lung cancer with malignant potential.

### Experiences and lessons

This is a unique presentation of lung cancer metastasis.

### Peer-review

The case is well drafted and references are adequate.

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## Heavily calcified gastrointestinal stromal tumors: Pathophysiology and implications of a rare clinicopathologic entity

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### Abstract

Gastrointestinal stromal tumors (GISTs) are the most common mesenchymal neoplasms of the gastrointestinal tract, and are characterized by a broad spectrum of clinical, histological and molecular features at presentation. Although focal and scattered calcifications are not uncommon within the primary tumor mass, heavy calcification within a GIST is rarely described in the literature and the clinical-biological meaning of this feature remains unclear. Cases with such an atypical presentation are challenging and may be associated with diagnostic pitfalls. Herein, we report a gastric GIST with the unusual presentation of prominent calcifications that was identified incidentally on imaging during a post-trauma diagnostic work-up. The patient underwent laparoscopic surgery with a radical resection of the mass, which was subsequently characterized by histological analysis as spindle-shaped tumor cells, positive for CD117/c-KIT, CD34 and DOG1, and with calcified areas. Given the intermediate risk of recurrence, no adjuvant therapy was recommended and



the patient underwent regular follow-up for 22 mo, with no evidence of relapse. Our case can be considered of interest because of the rarity of clinical presentation and the uniquely large size of the GIST at diagnosis (longest diameter exceeding 9 cm). In closing, we discuss the pathophysiology and clinical implications of calcifications in GISTs by reviewing the most up-to-date relevant literature.

**Key words:** Gastrointestinal stromal tumor; Calcification; Stomach; Atypical presentation; Computed tomography

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**Core tip:** Gastrointestinal stromal tumors (GISTs) are heterogeneous neoplasms that may present with a wide range of clinicopathologic characteristics upon diagnosis; among these, massive calcification is an infrequently occurring feature of GIST presentation. By reporting on such a rare clinical case and reviewing the available literature, this manuscript discusses major diagnostic challenges as well as the pathophysiologic and clinical implications related to a heavily calcified mass diagnosed as GIST.

Salati M, Orsi G, Reggiani Bonetti L, Di Benedetto F, Longo G, Cascinu S. Heavily calcified gastrointestinal stromal tumors: Pathophysiology and implications of a rare clinicopathologic entity. *World J Gastrointest Oncol* 2017; 9(3): 135-141 Available from: URL: <http://www.wjgnet.com/1948-5204/full/v9/i3/135.htm> DOI: <http://dx.doi.org/10.4251/wjgo.v9.i3.135>

## INTRODUCTION

Gastrointestinal stromal tumors (GISTs) are the most common type of mesenchymal neoplasm of the gastrointestinal tract (GIT), where they account for roughly 0.1% to 3.0% of all tumors<sup>[1]</sup>. GISTs were only recently recognized as distinct clinicopathologic entities. Postulated to arise from the interstitial cells of Cajal throughout the entire GIT, the reported GISTs have mainly involved the stomach (60%-70%), with involvement of the small intestine (20%-30%), colon and rectum (5%) and esophagus (< 1%) in fewer cases<sup>[2]</sup>.

The discovery that virtually all GISTs stain positively for c-KIT and/or DOG1 has facilitated their differential diagnosis from other mesenchymal tumors (*i.e.*, leiomyoma, leiomyosarcoma)<sup>[3,4]</sup>. Due to their slow-growing behavior, GISTs remain asymptomatic for a long period, having in some cases reached a huge size by the time of diagnosis. The most frequently reported symptoms are vague, depending on tumor size and location, and include nausea, vomiting, abdominal discomfort and early satiety; however, an acute abdomen caused by tumor rupture into the peritoneal cavity or gastrointestinal obstruction may represent the first dramatic presentation of a GIST<sup>[5]</sup>.

When studied by imaging, a GIST typically appears as a large, well-defined soft tissue mass with heterogeneous enhancement, reflecting the frequent occurrence of hemorrhagic necrosis and cystic degeneration within.

Although the majority of GISTs present as localized masses amenable to radical surgical resection, at least 50% of them recur locally or spread diffusely throughout the peritoneum and/or the liver, and the malignant potential of GIST varies greatly. Hence, determining the risk of disease recurrence, as assessed by available risk-stratification tools, is helpful in identifying patients most likely to benefit from an adjuvant treatment<sup>[6-8]</sup>. Size, mitotic activity and primary tumor size are the most reliable predictive factors for risk of relapse after complete surgery.

Discovery of the crucial role played by the oncogenic activating mutations of c-KIT (80%) and PDGFA-R (5%-10%) in the molecular pathogenesis of GISTs<sup>[9,10]</sup>, along with the clinical availability of multitarget tyrosine kinase inhibitors such as imatinib, sunitinib and regorafenib<sup>[11]</sup>, led to dramatic improvement in the outcome of a tumor which, until recently, was regarded as resistant to conventional chemotherapies. Current reports put the disease control rate at up to 85% and the median overall survival of nearly 5 years for patients with unresectable or metastatic GIST receiving frontline imatinib<sup>[12]</sup>. Moreover, studies of imatinib in the adjuvant setting have shown that it improves the rate of relapse-free survival, as compared with placebo, and that 3 years of therapy gives better rates of relapse-free survival and overall survival than 1 year of therapy in high-risk patients<sup>[13,14]</sup>.

## CASE REPORT

An otherwise healthy 60-year-old woman was admitted to the emergency department with complaint of right-sided chest pain following a car accident in September 2014. The patient was hemodynamically stable and, with the exception of tenderness on the right hemithorax, had unremarkable findings upon physical examination. A total body contrast-enhanced computed tomography (CT) scan was promptly performed and revealed a 7<sup>th</sup> and 10<sup>th</sup> right rib fracture, without signs of visceral injury.

Surprisingly, a large mass, measuring 6.5 cm, arising from the external wall of the gastric antrum and projecting into the abdominal cavity, was incidentally identified on imaging (Figure 1). The mass appeared hyperdense, well-circumscribed, heterogeneously enhanced and with prominent calcifications within; there was neither evidence nor loco-regional nor of distant spread. Hence, laparoscopic surgery was ordered to perform a radical resection of the mass. Gross pathology examination of the resected specimen showed it to be exophytic, lobulated, yellowish-gray and extensively calcified, with necrotic-cystic zones measuring 9.3 cm × 5.5 cm (Figure 2).

Microscopic pathology examination revealed a population of spindle-shaped tumor cells (Figure 3A and B) and calcified areas (Figure 3C and D), with positive

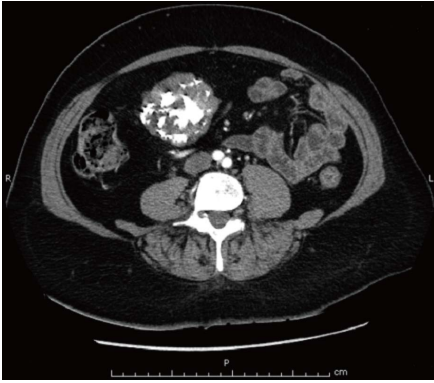


Figure 1 Axial contrast-enhanced computed tomography scan showing a well-defined heterogeneously enhanced gastric mass with coarse and diffuse calcifications.

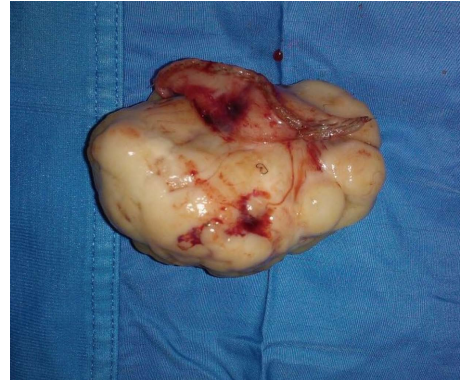


Figure 2 Macroscopic presentation of the resected mass, consisting of an exophytic and yellowish-gray tumor.

staining for CD117/c-KIT, CD34 and DOG1 (Figure 3E). In addition, Ki67-MIB1 immunostaining indicated a low proliferative rate (count rate < 1%) (Figure 3E). Molecular testing identified the c-KIT gene as wild-type but the PDGFRA gene as mutated (an exon 18 point mutation). The mass was diagnosed accordingly as GIST of gastric origin.

Recovery from the surgical resection was uneventful and prompt. Based on the estimated intermediate risk of recurrence, made according to the modified National Institutes of Health Criteria<sup>[15]</sup> (gastric site, size of 5-10 cm, mitosis count of < 5 per 50 high power fields, no tumor rupture), only regular follow-up was recommended. To date, the patient has been followed-up for 22 mo and shown no evidence of relapse.

## DISCUSSION

Circumscribed patchy calcifications may occur within the primary mass of large GISTs, and the reported series have indicated a wide variability among these (10% to 50%)<sup>[16]</sup>. Contrariwise, coarse and extensive calcifications represent an uncommon radiological finding, and only a few cases of these have been reported in the literature<sup>[17-23]</sup>. Indeed, our search of the MEDLINE/PubMed database identified only 8 cases of extensively calcified GISTs (Table 1).

From a clinical standpoint, when facing a calcified intra-abdominal juxta-gastric mass, various differential diagnoses should be ruled out. For example, diffuse and punctate calcifications may accompany mucin-producing adenocarcinoma of the stomach<sup>[24,25]</sup>. In the pancreas, solid pseudopapillary tumors and mucinous cystic neoplasms may develop peripheral curvilinear wall calcification, whereas serous cystadenomas may have central calcification within the central fibrous scar<sup>[26]</sup>. Finally, an ingested foreign body in the stomach may mimic gastric calcification.

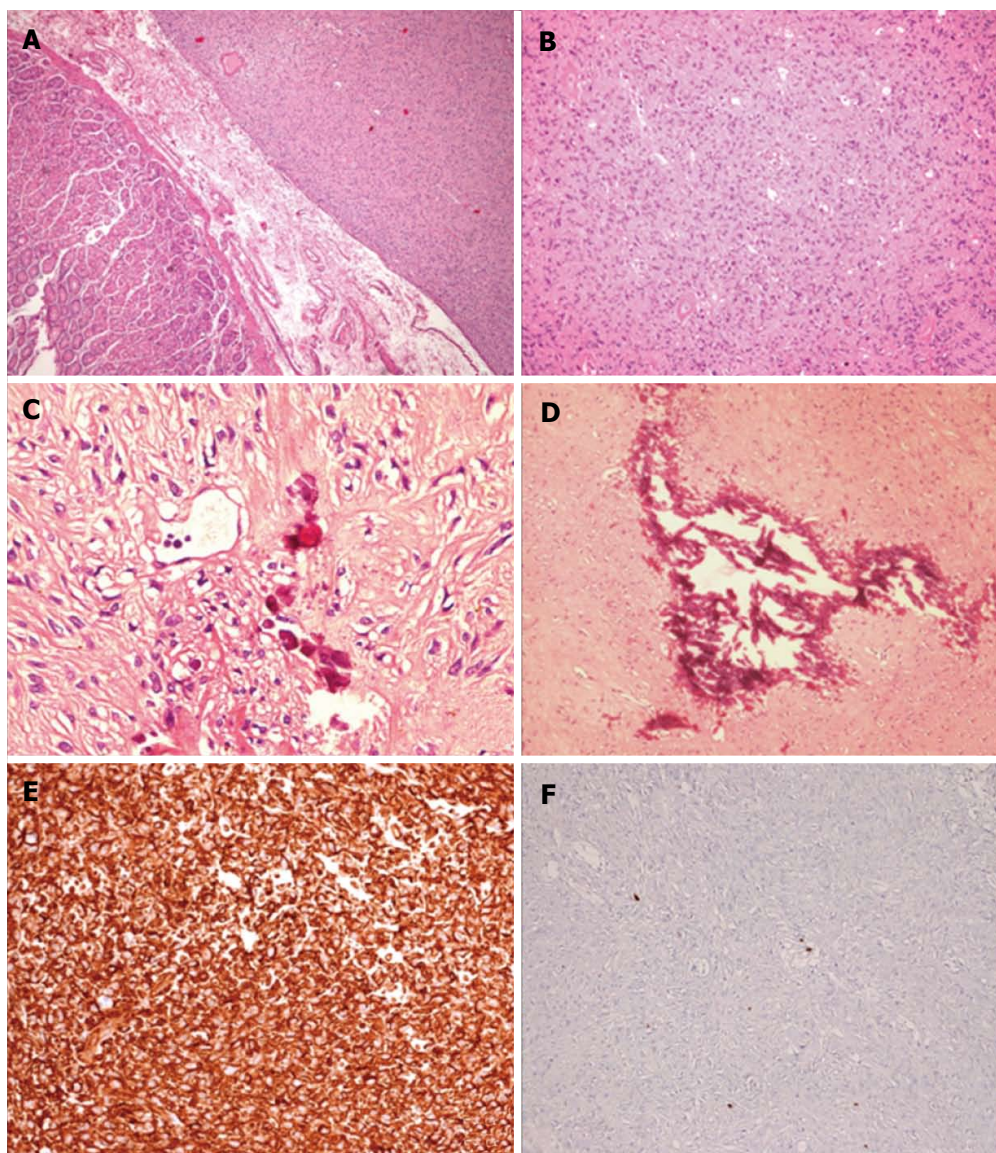
From a pathogenetic standpoint, several mechanisms have been implicated in calcific deposition. The most acknowledged process is dystrophic calcification, which typically involves degenerated tissues that are characterized by necrosis, inflammation and hemorrhage, as

frequently occurs in GISTs. In such a relatively alkaline background, the binding of denatured proteins to both phosphate and calcium ions is ultimately responsible for the formation of calcium phosphate precipitates. On the other hand, the diffuse punctate calcification pattern that is typical of mucin-forming gastric adenocarcinomas seems to be linked instead to calcium salt precipitates with glycoprotein in an alkaline matrix<sup>[27]</sup>. Metastatic calcifications occurring in the context of malignant hypercalcemia, as well as granulomatous tissue engulfed by the tumor, represent rarer causes of calcification. More recently, a unique case of osseous metaplasia with mature bone formation was described in a newly diagnosed gastric GIST<sup>[28]</sup>.

Among the proposed theories, however, the production of multiple cell mediators—such as PDGF, the receptor of which is frequently mutated in GISTs, and the bone morphogenic protein, which is produced by pluripotent stromal cells committed towards the osteoblastic lineage—are thought to play a crucial role in the regulation of ectopic bone formation<sup>[28]</sup>. Intriguingly, several clinical reports have demonstrated the appearance of calcification in metastatic sites of GIST following treatment with imatinib<sup>[29]</sup>. The underlying mechanism, which is still far from being fully elucidated, is likely to be different from that occurring in the primary mass, as is suggested by the absence of inflammation or necrosis in the specimen obtained from liver metastasis<sup>[30]</sup>. It is also interesting that this morphologic change has been hypothesized as predictive of tumor response. Finally, examples of osseous differentiation have also been observed in cases of treated GIST, suggesting that under imatinib suppression, GIST cells may become able to mediate the cell cycle and to express a gene signature associated with other differentiated cell phenotypes, thereby providing a mechanism of acquired drug resistance<sup>[31]</sup>.

Following early evidence that indicated solitary calcification could be an aggressive feature<sup>[32]</sup>, researchers continue to investigate the potential relevance of calcification in predicting the malignant potential of GISTs. However, a study by Kim *et al.*<sup>[33]</sup> found no CT features, other than size, to be correlated with biological behavior and prognosis of GIST. This finding was confirmed very recently by Chen *et*





**Figure 3** Histologic appearance of the tumor. A and B: Interlacing bundles of spindle cells with unremarkable mitotic activity (H and E stain; magnification  $\times 10$ ); C and D: Calcified areas (H and E stain; magnification  $\times 20$ ); E: Intense immunoreactivity for DOG1 (Immunohistochemistry stain; magnification  $\times 20$ ); F: Inconspicuous Ki67-MIB1 label index (count rate  $< 1\%$ ; immunohistochemistry stain; magnification  $\times 20$ ).

*et al.*<sup>[34]</sup>; specifically, that retrospective analysis of 110 patients who underwent endoscopic ultrasonography identified tumor size as an independent risk factor for malignancy ( $P \leq 0.0001$ ) but not for calcification ( $P = 0.667$ ).

Although no conclusions can be drawn regarding the predictive value of calcification in GIST because of the limited cases reported and the retrospective nature of the analyses, heavily calcified tumors appear to have a less aggressive course, as shown by their low mitotic rate (Table 1). With regard to our case, a relatively indolent behavior and the alleged long history of disease probably contributed to the development of calcification.

In conclusion, to the best of our knowledge, we have presented herein the largest ( $> 9$  cm) heavily calcified GIST ever described in the literature. Our case is noteworthy because of the rarity of clinical presentation in general and the unique size at diagnosis for this patient

in particular. Finally, our case and review of the literature indicates that a diagnosis of GIST should be considered when large calcified masses are identified in the GIT, and even more so when in the stomach.

## COMMENTS

### Case characteristics

An otherwise healthy 60-year-old woman was admitted to the emergency department with complaint of right-sided chest pain following a car accident.

### Clinical diagnosis

Upon admission, the patient was hemodynamically stable and only showed tenderness on the right hemithorax upon physical examination, the findings of which were otherwise unremarkable.

### Differential diagnosis

Mucin-producing adenocarcinoma of the stomach; pancreatic solid pseudo-

**Table 1** Selected features of the reported cases of extensively calcified gastrointestinal stromal tumors

Ref.	Year	Age, sex	Site	Size (mm)	Mitotic index (HPF)	IHC pattern	Calcification pattern	Clinical presentation	Risk <sup>1</sup>
Izawa <i>et al</i> <sup>[20]</sup>	2012	61 yr, female	Stomach	32 × 23 × 23	< 5/50	KIT+, CD34+, S-100-, desmin-, SMA-	Peripheral ring	Epigastric discomfort	Low
Kim <i>et al</i> <sup>[17]</sup>	2012	53 yr, male	Stomach	80 × 75 × 50	< 5/50	KIT+, CD34+, S-100-, desmin-, SMA-	Extensively calcified	Epigastric pain	Intermediate
		69 yr, female	Stomach	62 × 55 × 42	< 5/50	KIT+, CD34+, S-100-, desmin-, SMA-	Extensively calcified	Epigastric pain	Intermediate
Yu <i>et al</i> <sup>[21]</sup>	2011	81 yr, female	Stomach	60	< 5/50	KIT+, CD34+, S-100-, desmin-	Semicircular plate	Abdominal pain, hematemesis	Intermediate
Ong <i>et al</i> <sup>[18]</sup>	2006	94 yr, female	Rectum	25 × 15 × 15	< 5/50	KIT+, CD34+, S-100-, desmin-	Entirely	Rectal bleeding	Low
Yoshida <i>et al</i> <sup>[22]</sup>	2005	77 yr, male	Stomach	28 × 26 × 26	< 5/50	KIT+, CD34+, vimentin+, S-100-, desmin-, SMA-	Patchy	Chest discomfort, loss of consciousness	Low
Testroote <i>et al</i> <sup>[23]</sup>	2007	53 yr, male	Rectum	31 × 23 × 18	< 5/50	KIT+, CD34+, SMA focally positive	Entirely	Abdominal pain, constipation	Low
Rana <i>et al</i> <sup>[19]</sup>	2006	62 yr, female	Colon	39 × 56	NA	KIT+, CD34+	Multiple shadowing	Abdominal pain	NA

<sup>1</sup>Risk is expressed according to the modified NIH classification system<sup>[15]</sup>. HPF: High-power field; IHC: Immunohistochemistry; N/A: Not applicable.

papillary tumor and mucinous cystic neoplasm; pancreatic cystadenoma; ingested foreign body in the stomach.

### Laboratory diagnosis

All laboratory findings were within normal limits.

### Imaging diagnosis

In addition to a 7<sup>th</sup> and 10<sup>th</sup> right rib fracture, computed tomography (CT) scan revealed a large mass, measuring 6.5 cm, arising from the external wall of gastric antrum and projecting into the abdominal cavity.

### Pathological diagnosis

Gastrointestinal stromal tumor (GIST) of gastric origin.

### Treatment

Complete laparoscopic surgical excision of the mass.

### Related reports

Circumscribed patchy calcifications may occur within the primary mass of large GISTs, and the reported series have indicated a wide variability among these (10% to 50%). Contrariwise, coarse and extensive calcifications represent an uncommon radiological finding, and only a few cases of these have been reported in the literature.

### Term explanation

Gastrointestinal stromal tumors are the most common mesenchymal neoplasms of the gastrointestinal tract (GIT), where they account for roughly 0.1% to 3.0% of all tumors. GISTs were only recently recognized as distinct clinicopathologic entities. Postulated to arise from the interstitial cells of Cajal throughout the entire GIT, the reported GISTs have mainly involved the stomach (60%-70%), with involvement of the small intestine (20%-30%), colon and rectum (5%) and

esophagus (< 1%) in fewer cases.

### Experiences and lessons

The authors' case is noteworthy because of the rarity of clinical presentation of calcified GISTs and the unique large size of the tumor at diagnosis. A diagnosis of GIST should be considered when large calcified masses are identified in the GIT, and even more so when in the stomach.

### Peer-review

This article offers an interesting overview of calcified GISTs, discussing the pathophysiology and clinical implication of calcifications in GISTs by reviewing the most up-to-date relevant literature. While the case reported is not the first that describes a massively calcified GIST, the incidental diagnosis and unique size of the mass are peculiar and noteworthy.

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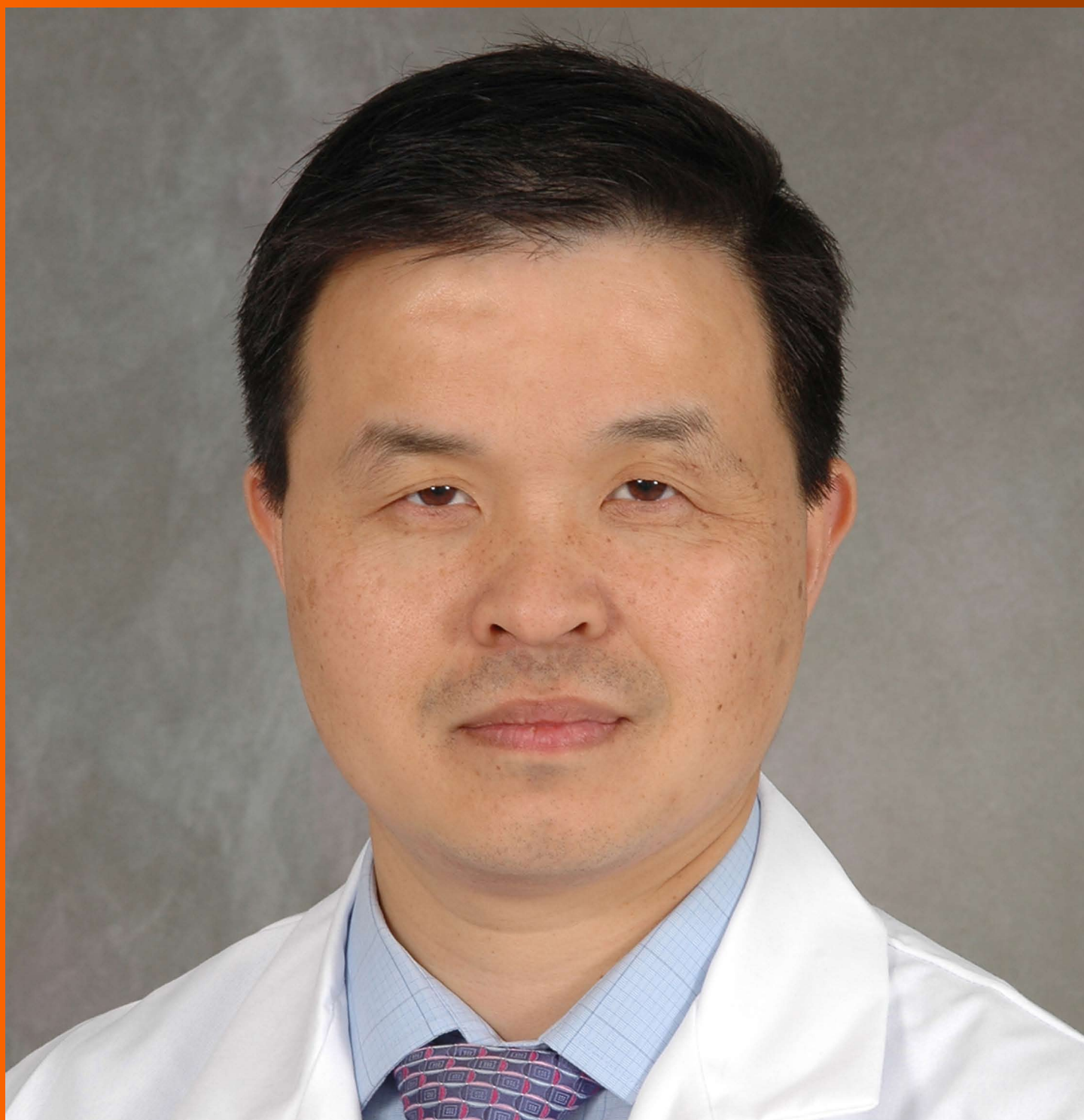
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## Abnormal DNA methylation as a cell-free circulating DNA biomarker for colorectal cancer detection: A review of literature

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### Abstract

Colorectal cancer (CRC) is one of the most prevalent malignancies in the world. CRC-associated morbidity and mortality is continuously increasing, in part due to a lack of early detection. The existing screening tools such as colonoscopy, are invasive and yet high cost, affecting the willingness of patients to participate in screening programs. In recent years, evidence is accumulating that the interaction of aberrant genetic and epigenetic modifications is the cornerstone for the CRC development and progression by alternating the function of tumor suppressor genes, DNA repair genes and oncogenes of colonic cells. Apart from the understanding of the underlying mechanism(s) of carcinogenesis, the aforementioned interaction has also allowed identification of clinical biomarkers, especially epigenetic, for the early detection and prognosis of cancer patients. One of the ways to detect these epigenetic biomarkers is the cell-free circulating DNA (circDNA), a blood-based cancer diagnostic test, mainly focusing in the molecular alterations found in tumor cells, such as DNA mutations and DNA methylation.

In this brief review, we epitomize the current knowledge on the research in circDNA biomarkers - mainly focusing on DNA methylation - as potential blood-based tests for early detection of colorectal cancer and the challenges for validation and globally implementation of this emergent technology.

**Key words:** Colorectal cancer early detection; Colorectal cancer screening; Circulating free DNA; Colorectal cancer blood-based biomarkers; DNA methylation blood biomarkers

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**Core tip:** Colorectal cancer (CRC) is one of the most prevalent malignancies in the world. CRC-associated morbidity and mortality is continuously increasing, in part due to a lack of early detection. The main aim of this article is the brief description of the basic screening modalities and their efficacy for CRC detection, the process of colorectal carcinogenesis and how the molecular pathways of CRC (focusing on epigenetic modifications) influence the clinical application of new blood-based biomarkers such as circDNA. Then we will focus on the most recent findings concerning the studies on circDNA, mainly related to DNA methylation and the challenges for validation and globally implementation of this emergent technology.

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## INTRODUCTION

Colorectal cancer (CRC) is the most common malignancy presented in gastrointestinal (GI) tract and the third most frequent cancer globally, with an incidence approximately approaching 1.5 million cases per year<sup>[1,2]</sup>. Likewise, it is considered that over 600000 deaths occur each year by neoplasms of the large bowel making them, the third commonest cause of cancer-related deaths<sup>[2]</sup>. It is thought that the gradual adoption of Westernized lifestyle and dietary habits by the majority of the countries in association with aging of the population are responsible for the increase in morbidity and mortality rates from CRC. This is in accordance with the World Health Organization which estimates that a substantial increase in the number of newly diagnosed CRC cases and a 80% rise in deaths from CRC are expected by 2030<sup>[3]</sup>. It should also be pointed out that colorectal adenocarcinomas are distinctive for their relatively fast progression and late clinical presentation, characteristics that are fairly preventable if identified at an early stage.

Nevertheless, the currently available screening tests for the early detection of CRC need improvement enough in order to increase their cost-effective status. Thus, it is conceivable that, there is a significant interest in using noninvasive blood biomarkers which could be of low cost and high sensitivity and specificity to help reduce the predicted surge in the incidence of CRC by identification and removal of a larger number of polyps that potentially could lead to CRC over time<sup>[3]</sup>. These biomarkers are designed to detect molecular indicators in the plasma or serum such as DNA, RNA or protein in order to expand the existing list of CRC screening modalities<sup>[4]</sup>.

Epigenetic phenomena contribute to colorectal neoplasia<sup>[5]</sup>. This term refers to the mechanisms that alter gene expression without changing their DNA sequence. Epigenetic phenomena may include DNA methylation, histone modification and chromatin regulation through non-coding RNAs (microRNAs, lncRNAs, etc.)<sup>[6]</sup>. Since DNA methylation and DNA mutations detected in tumor cells, it is reasonable to assume that these alterations are reflected in circDNA released from neoplastic tissue into blood circulation. Testing for circDNA in the peripheral blood could serve as an important candidate biomarker for the detection of CRC at early stages. An existing paradigm of commercial blood test for CRC detection in the circDNA is the monitoring of methylation of the septin 9 gene (*SEPT9*) promoter region.

Therefore, the aim of this article is the brief description of the basic screening modalities and their efficacy for CRC detection, the process of colorectal carcinogenesis and how the molecular pathways of CRC (focusing on epigenetic modifications) influence the clinical application of new blood-based biomarkers such as circDNA. Then we will focus on the most recent findings concerning the studies on circDNA, mainly related to DNA methylation and the challenges for validation and globally implementation of this emergent technology.

## EXISTING SCREENING MODALITIES TO CRC

### Screening modalities

There are various strategies for screening nowadays; the most accepted being the colonoscopy, and the combination of sigmoidoscopy and fecal occult blood test (FOBT). The high sensitivity and specificity has established the colonoscopy the cornerstone for the early identification of colonic malignancies in the average-risk population<sup>[7,8]</sup>. There are some drawbacks that limit the desired wide acceptance. As an invasive examination, complications may be unavoidable, the most common being cardiovascular events during the procedure and the post-polypectomy bleeding and perforation<sup>[7]</sup>. Other disadvantages could be a significant miss rate of lesions even for large colonic abnormalities, its high cost and the low acceptance level by the population<sup>[9]</sup>.

Compared to colonoscopy, sigmoidoscopy has quite few disadvantages due to low cost, less preparation time



and no need for sedation<sup>[10]</sup>. The main problem is the ability to detect only the lesions of distal colon making the decision of performing colonoscopy a subject for controversy even to date.

The third and most frequently applied screening test is FOBT<sup>[11]</sup>. Although these tests are easier to perform than colonoscopy or sigmoidoscopy, they are associated with false positive and false negative results due to diet, other conditions like colitis and hemorrhoids and the effect of temperature on the samples<sup>[12]</sup>. Moreover, FOBT cannot be used as solo screening test, as a positive results lead to colonoscopy performance<sup>[13]</sup>.

Thus, there is an emergent need for new screening tests such as blood-based test which could detect CRC earlier, increase patient participation with minimal risks, costs, and false positive and negative results.

## MOLECULAR PATHWAYS IN CRC AND DNA METHYLATION

### CRC molecular pathways

Colorectal cancer is a multifarious disease. The comprehension of the molecular pathways involved in its development, will help to optimize the screening procedure based on distinctive pathologic and molecular features of the malignancies. Three basic pathways of colorectal carcinogenesis have been recognized since 1990 that is, Chromosomal Instability (CIN), Microsatellite Instability (MSI) and CpG island methylator phenotype (CIMP) pathway<sup>[14]</sup>.

Chromosomal instability, also entitled "the suppressor pathway", was first introduced in 1990 by Fearon *et al.*<sup>[14]</sup> and is the most frequent etiology for gene alteration in colorectal neoplasia. Its main characteristic is the modification of whole chromosome or some of its regions, affecting important genes leading to carcinogenesis. These genomic defects provoke suppressor genes inactivation as Deleted in Colon Carcinoma (*DCC*), SMAD family member 2 (*SMAD2*), SMAD family member 4 (*SMAD4*), Adenomatosis polyposis coli (*APC*) and tumor protein p53 (*TP53*) and oncogene activation such as the human homolog of the Kirsten rat sarcoma-2 virus oncogene (*KRAS*)<sup>[15]</sup>. The accumulation of these modifications seems to play the most crucial role for cancer to develop and not the sequence of their presentation as once considered. The second model which involved in normal intestinal mucosa transformation to malignancy is the microsatellite instability. MSI is another type of genomic instability which refers to deletions or insertions of a few nucleotides in genes responsible for repair during DNA replication, the DNA mismatch repair (MMR) genes<sup>[16]</sup>. This aberrant genomic region mainly segregates in repetitive DNA nucleotide unit (microsatellites) throughout the genome resulting in the inactivation of MMR genes (*i.e.*, *MSH2*, *MLH1*, *MSH6*, *PMS1-2*, *MLH3*, *MSH3*, *ExoI*). It is well-known that this route of carcinogenesis is involved in Lynch syndrome and for a notable proportion of sporadic CRC (15%-20%)<sup>[17]</sup>. The third model involved in the

CRC development and progression, is CIMP which refers to the presence of simultaneous hypermethylation of multiple genes. It belongs to the epigenetic mechanisms leading to silence gene function after methylation at the 5'-CG-3'(CpG) dinucleotide in the promoter region of many genes (*APC*, *MCC*, *MLH1*, *MGMT*), resulting therefore, in inactivation of tumor suppressor genes<sup>[18]</sup>. CIMP is accountable for 15%-20% of sporadic CRC and according to the study of Jass<sup>[19]</sup>, we are able to classify CRC according to the presence of MSI and CIMP as Figure 1 shows.

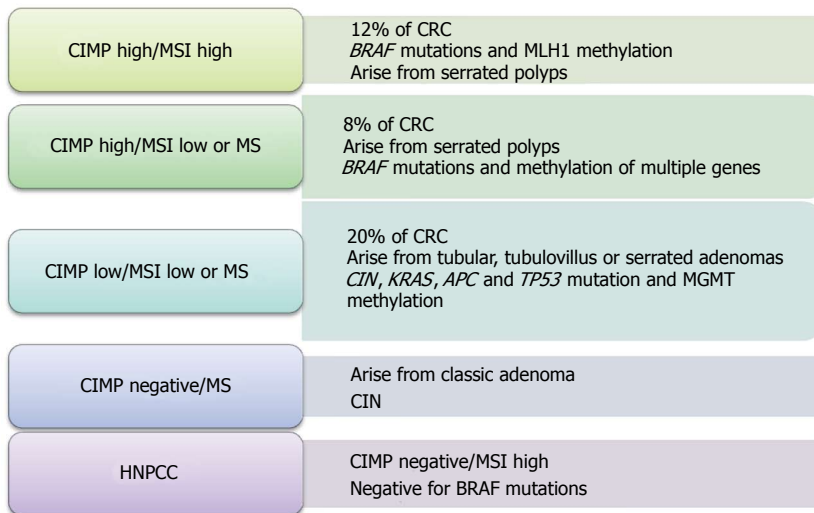
### DNA methylation and its role in CRC carcinogenesis

DNA methylation mainly occurs in specific parts of the genome called, as we have seen previously, CpG islands. Considering the stability of DNA methylation compared to mutations, we may presume that methylation is a favorable area for biomarker exploration.

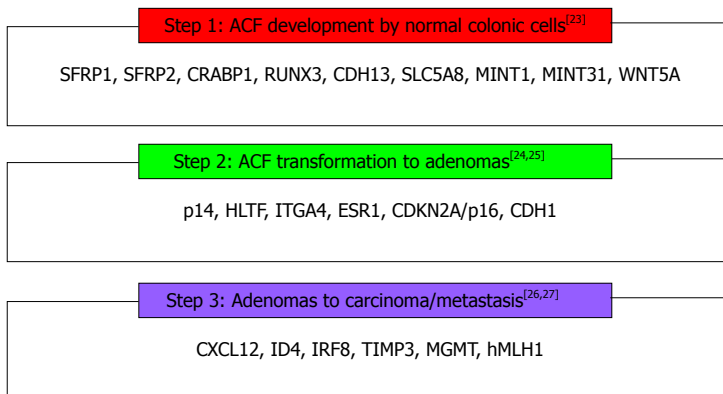
The concept that genome methylation may play a critical role in specific steps in the CRC carcinogenesis has been expressed in 1983 by Feinberg and Vogelstein<sup>[20]</sup> who showed that at early stages of CRC there is a DNA hypomethylation, mainly located at CpG islands. They also demonstrated that this loss of methylation was combined by hypermethylation and inactivation of tumor suppressor or DNA repair genes<sup>[21]</sup>. This epigenetic modification has recently been associated with the normal mucosa-aberrant crypt focus (ACF)-adenoma-carcinoma sequence, playing an important role in CRC development<sup>[22]</sup>. Consequently, DNA methylation appears to be one of the cornerstones of carcinogenesis because it occurs at the first steps of CRC process; involves CIMP pathway with MSI, as hypermethylation of MMR genes results in MSI sporadic CRC; through CIMP, it has been linked with CIN in colon malignancy (promoter methylation of *GATA4*, *GATA5*, *p16* resulted in chromosomal loss or gain); and finally it is implicated in each of these paths through many abnormally methylated genes as recently studies have revealed<sup>[23-28]</sup> (Figure 2). The design of genetic and epigenetic biomarkers, especially those related to detection of aberrant methylated genes able to offer the maximum coverage of intestinal neoplasia, seems to be a reasonable approach. Accordingly, several studies have been performed the last decade, for the potential use of DNA markers in different biologic fluids as strategies for colorectal carcinoma early detection<sup>[29]</sup>.

## CELL-FREE CIRCULATING DNA AS A BLOOD-BASED BIOMARKER

Mandel and Metais<sup>[30]</sup> in 1948, were the pioneers who discovered the existence of cell-free nucleic acids (cfNAs) in blood, leading to "discrimination" of affected patients from healthy controls, even though the first reported presence of cfNAs was in 1869<sup>[31]</sup>. Since then, several studies have been made, especially related to cancer pathogenesis, showing that malignant-nucleic acids could be present in different "body fluids" (*i.e.*,



**Figure 1 Molecular classification of colorectal cancer.** The figure shows the different molecular profile and clinic-histopathological characteristics of each classification. CIMP: CpG island methylator phenotype; MSI: Microsatellite instability; MS: Microsatellite stability; CRC: Colorectal cancer; CIN: Chromosomal instability; HNPCC: Hereditary nonpolyposis colorectal cancer; MGMT: O-6-methylguanine-DNA methyltransferase; BRAF: v-raf murine sarcoma viral oncogene homolog B1; MLH1: MutL homolog 1; KRAS: Kirsten rat sarcoma 2 viral oncogene homolog; APC: Adenomatous polyposis coli; TP53: Tumor protein p53.



**Figure 2 The figure exhibits the most frequent methylated genes/loci involved in step-by-step adenoma-carcinoma process in the context of colorectal cancer development.** SFRP1: Secreted frizzled-related protein 1; CRABP1: Cellular retinoic acid binding protein 2; RUNX3: Runt-related transcription factor 3; CDH13: Cadherin 13; SLC5A8: Sodium solute symporter family 5 member 8; MINT1: Methylated in tumor locus 1; WNT5A: Wingless-type MMTV integration site family, member 5A; p14: Tumor protein 14; HLF: Helicase-like transcription factor; ITGA4: Integrin, alpha 4; ESR1: Estrogen receptor 1; CDKN2A/p16: Cyclin-dependent kinase inhibitor 2A; CDH1: E-cadherin; CXCL12: Chemokine (C-X-C) ligand 12; ID4: Inhibitor of DNA binding 4; IRF8: Interferon regulatory factor 8; TIMP3: Tissue inhibitor of metalloproteinase 3; MGMT: O-6-methylguanine-DNA methyltransferase; hMLH1: MutL homolog 1.

stool, blood, urine). Therefore, it was a matter of time the establishment of the potential advantages using cfNA as noninvasive neoplasia detection<sup>[32,33]</sup>. The first report referring to detection of “abnormal” cfNAs in CRC patients took place in 1992, when Vogelstein *et al.*<sup>[34]</sup> discovered *KRAS* gene mutation in stools samples of CRC-affected individuals. Since then, a great number of studies have performed evaluating other than *KRAS*, genome modifications directly expressed by cfNAs. These modifications are characterized mainly by analysis of high mutation frequency genes (*KRAS*, *TP53*, and *APC*), MSI, Loss of Heterozygosity (LOH), DNA, and microRNA methylation changes. Except the latter one, all the others are key factors for colorectal carcinogenesis which could be expressed by increased circDNA concentrations in blood of CRC patients compared with healthy controls, first mentioned by Leon *et al.*<sup>[35]</sup> in 1977, followed by many other studies<sup>[35-37]</sup>.

Thus, since circDNA in blood reflects significant genome alterations emerging during CRC carcinogenesis, it could be used systematically as a potential biomarker for early detection of colonic malignant tumors, especially after the recent advances in next generation sequencing (NGS) technology<sup>[38]</sup>. Here follows a discussion of methods to detect circDNA-based markers in blood and the studies focusing on description of these markers namely, aberrant DNA methylation and mutations, microsatellite

alterations, DNA modifications in mitochondria, integrity and quantification of DNA.

### Methods to detect methylation-related circDNA markers in blood

Methodologies suitable to investigate and detect in serum or plasma frequently methylated genomic regions, offering high detection rate is more than an appealing aim. These ideal markers should have low levels of background methylation so as to avoid the decreased specificity and increased false-positive results. Several molecular approaches are currently performed to identify circDNA in blood. Conventional methylation-specific PCR, though very sensitive method, presents high levels of false positive and negatives results and its qualitative method to interpret the findings, limits its clinical utility<sup>[39]</sup>. On the other hand, methods based on quantitative methylation-specific PCR (*i.e.*, MethyLight, SMART-methylation-specific PCR) offers the opportunity to select more easily the methylation thresholds and avoid false positive results identifying incomplete bisulfite conversion<sup>[40]</sup>. Other approaches include Methylation Array, DNA array, Surface-Enhanced Raman Scattering (SERS), and Restriction Fragment Length Polymorphism (RFLP). Another interesting and “more digital” methodology, called “Methyl-BEAMing”, was demonstrated by Li *et al.*<sup>[41]</sup> in order to digitally quantify cancer-derived vimentin DNA, confronting the issues with

**Table 1** DNA integrity index in colorectal cancer patients

Ref.	Increased DNA integrity	Decreased DNA integrity
Umetani <i>et al</i> <sup>[45]</sup> , 2006	Yes	-
Da Silva Filho <i>et al</i> <sup>[46]</sup> , 2013	Yes	-
Leszinski <i>et al</i> <sup>[47]</sup> , 2014	Yes	-
Mouliere <i>et al</i> <sup>[48]</sup> , 2011	-	Yes
Mead <i>et al</i> <sup>[49]</sup> , 2011	-	Yes
Mouliere <i>et al</i> <sup>[50]</sup> , 2014	-	Yes
Yörüker <i>et al</i> <sup>[51]</sup> , 2015	-	Yes

the small fraction of blood circDNA.

Given the great concerns arouse by previous approaches concerning the inability for reproducibility and high sensitivity, promising results revealed by the study of Leary *et al*<sup>[42]</sup>. The use of NGS approach showed encouraging results, distinguishing CRC patients at advanced stage from healthy controls. Thus, NGS could provide high sensitivity, covering large regions of genome for CRC early detection.

### Cell-free circulating DNA-based markers

During the last two decades, circDNA has become a potential biomarker for diagnosis of malignant tumors, exhibiting their genetic and epigenetic modifications. It exists in the plasma or serum, being source of apoptotic, necrotic cancer cells or even living cells. It can appear as unbound DNA molecule; as histone part in nucleosome; or as portion of apoptotic cells. As already mentioned, there are several methods of assessing circDNA as a potential biomarker for detection of CRC at early stages. Herein, we would try to summarize the main characteristics of each method, noting presentative studies reflecting the potential clinical use of these circDNA-based modalities.

One of these methods is the quantification of circDNA levels in blood, studied thoroughly in CRC patients since the research by Leon *et al*<sup>[35]</sup> in 1977 who proved that concentration of circDNA was higher than that of healthy persons. Additional studies, such as the ones performed by Frattini, Schwarzenbach *et al*<sup>[43]</sup> respectively, verified the elevated circDNA levels in the plasma of CRC individuals compared with the non-cancerous controls<sup>[43]</sup>. Although, patients with malignancies may present greater levels of circDNA than normal persons, it should be emphasized that circDNA in plasma may also be observed in other clinical entities like trauma, inflammatory disorders even in healthy individuals<sup>[35]</sup>.

In the recent years, it is well-established that the manner, with which the circDNA is released in blood-stream, reflects its size and morphology. The exact mechanism is yet to be clarified but it is believed that circDNA entered the blood by apoptosis and then it is fragmented by the action of nucleases or phagocytes, into small particles of 185 to 200 bp in length<sup>[44]</sup>. The measure of the ratio of long circDNA fragments to short ones mirrors circDNA integrity. A great number of studies

have been performed, demonstrating inconsequent results (Table 1)<sup>[45-51]</sup> as concerns the sensitivity and specificity of circDNA integrity index for CRC early detection. Interestingly, a recent research by Hao *et al*<sup>[52]</sup> showed that the combination of DNA integrity index (ALU247/115 and ALU115 index) and carcinoembryonic antigen (CEA) detection may be efficient and reproducible method for early diagnosis of CRC. Therefore, larger clinical studies should be performed in order to limit the inconsistencies that circDNA integrity method exhibits.

Microsatellite alterations is another investigation field related to tumorigenesis of CRC and consist of MSI and LOH. Due to their presence in circDNA, it is assumed that they could be potential CRC biomarkers for early diagnosis of affected individuals. As we already have mentioned, *MSI* refers to deletions or insertions of a few nucleotides (1-6 bp in length) in genes responsible for repair during DNA replication, the MMR genes<sup>[16]</sup>, while *LOH* analysis emphasizes the loss of chromosomal parts carrying tumor suppressor genes. These somatic alterations have been detected in blood, nearly in 35% of all CRC patients. The existence of MSI-related circDNA fragments is known since the ending of 20<sup>th</sup> century followed by many studies focusing on the presence of MSI and LOH in circDNA<sup>[53]</sup>. One of these by Hibi *et al* showed in 1999 that, although *LOH* and *MSI* found in 80% CRC patients when examined their microsatellite alterations, these shifts weren't verified upon the corresponding serum-based circDNA. Therefore, the available data reveals the relatively low sensitivity and specificity in diagnosing CRC at early stages when microsatellite alterations are investigated.

Similar disappointing results have been arisen from the study of circulating mitochondrial DNA (mtDNA) as potential circDNA-based biomarker for premature diagnosis of colonic neoplasia. Mitochondria are the cornerstone in energy metabolism, aging, and apoptosis, playing a crucial role in shifting the cell from scheduled death to abnormal cell growth, thus having potential contribution to the carcinogenesis<sup>[54]</sup>. An important part of mtDNA is its D-loop region, a noncoding region which involves the expression and organization of the mitochondrial genome. It is hypothesized that this part of mtDNA is a hotspot of mutations leading to DNA instability, opinion that has been verified in several types of cancers such as, head and neck, colorectal, stomach, prostate, breast<sup>[55]</sup>. Despite the initial encouraging signs, mtDNA shows reduced detection rate of early stage CRC, as the study by Hibi *et al*<sup>[56]</sup> revealed, where the discovery of mtDNA modifications (somatic mutation in D-loop region) in tissues of early CRC patients haven't been noted in their circDNA.

As it is stated previously, the blood-based circDNA in CRC patients is composed by important molecules which have implicated in tumorigenesis process. Since 1992 when Vogelstein *et al*<sup>[34]</sup> discovered *KRAS* gene mutation in stools samples of CRC-affected individuals, high mutation frequency genes, as *KRAS*, *TP53*, and *APC*

have been used as potential markers in circDNA analysis for early diagnosis of colonic malignant lesions<sup>[57,58]</sup>. The results were discouraging due to low concentration of tumor circDNA (based on the somatic mutations analysis of *KRAS/TP53/APC* genes modification) in CRC patients compared with the wild-type circDNA in non-CRC individuals<sup>[58]</sup>. Moreover, it should be noted that even the use of NGS circDNA detecting method, hasn't offered any improvement in detection of these aberrant tumor DNA mutations<sup>[59]</sup>. Finally, as aforementioned, genes such as *APC*, *TP53*, and *KRAS* are mutated in a great degree of CRC cases, spreading over different parts of genome, making mutational assessment difficult. Thus, it is reasonable to assume that very large genomic regions would need to be evaluated in order to obtain a respectable sensitivity and in combination of the unique presentation of modified genes in each patient, it is still difficult enough to use somatic mutations for CRC early detection.

### **Abnormal DNA methylation as a cell-free circulating DNA biomarker**

As it has been already highlighted, the critical role of abnormal DNA methylation to specific steps in the CRC carcinogenesis has been expressed since 1983 from Feinberg and Vogelstein<sup>[20]</sup>. Since then and during the recent years, many studies have revealed that this epigenetic modification has been associated with the normal mucosa-ACF-adenoma-carcinoma sequence, playing an important role in CRC development, mainly, at early stages<sup>[22-27]</sup>. It is known that during DNA methylation, DNA methyl transferases (DNMTs) catalyze the addition of a methyl group (-CH<sub>3</sub>) to the fifth carbon position on cytosines within CpG dinucleotides. The latter, although spread over throughout the human genome, they are frequently discovered in the promoter regions of nearly 70% of genes, usually named as "CpG-islands"<sup>[60]</sup>. Furthermore, it is well-established by now that hypermethylation of tumor suppressor promoters genes could induce transcriptional gene silencing, resulting on aberrant cellular signaling and therefore potential initiation of tumorigenesis process<sup>[61]</sup>. Moreover, it is interesting that methylation could happen in CpG sites throughout the genomic body and not necessarily only in promoter regions leading though to transcriptional activation<sup>[62]</sup>. On the other hand, global hypomethylation which frequently presented prematurely during carcinogenesis, exhibits loss of DNA methylation throughout the genome, resulting on CIN and cell mutation<sup>[63]</sup>. Consequently, the significance of aberrant DNA methylation led to investigation and discovery of blood-based mainly, due to its noninvasiveness and cost-effectiveness, CRC detection biomarkers.

One of the most investigated genes is the *SEPT9* gene involved in cellular proliferation control. The methylation of v2 promoter region of *SEPT9* has been demonstrated in CRC biopsy lesions compared with normal tissues. According to Grützmann *et al.*<sup>[64]</sup>, its detection in plasma of CRC patients

exhibited a sensitivity of 72% and specificity of 90%, something that was validated by the study of Warren *et al.*<sup>[65]</sup>. Nevertheless, a recent prospective trial performed by Church *et al.*<sup>[66]</sup> investigated the *SEPT9* methylation in 7941 asymptomatic individuals during screening with available assay showing a CRC detection rate up to 48.2% and specificity up to 91.5%. Obviously, the need of further researches upon this commercially available test is indispensable not only to improve its detection rate but also to discover new assays for *SEPT9* methylation detection. Furthermore, researchers understanding the usefulness of *SEPT9* have assessed potential combinations with other methylation biomarkers. Tänzer *et al.*<sup>[67]</sup> have shown that methylated DNA from advanced premalignant intestinal lesions could be discovered using the panel of aristaless-like homeobox 4 (*ALX4*), and *SEPT9* markers. Similarly, Kostin *et al.*<sup>[68]</sup> compared the methylation status of *SEPT9*, Helicase-like transcription factor (*HLTF*) and *ALX4* genes in macroscopically findings compatible with colorectal cancer ( $n = 55$ ) and morphologically intact areas of the large bowel ( $n = 71$ ), showing that this panel of biomarkers characterized by a sensitivity nearly to 74%-88% and a specificity 90%-96% for CRC early identification. Finally, He *et al.*<sup>[69]</sup> demonstrated high sensitivities (81%-84%) and specificities (87%-90%) for noninvasive blood-based testing for initial-phase CRC, using multiplex MethyLight PCR assay to detect concomitantly, aberrant methylation pattern of *ALX4*, *SEPT9*, or transmembrane protein with EGF-like, and two follistatin-like domains 2 (*TMEFF2*) genes.

Apart from the aforementioned *SEPT9*-combined panels, there are recently studies showing even greater CRC detection rates if combined analysis of several genes is used. Alhquist *et al.*<sup>[70]</sup> presented high overall sensitivity (87%) for the CRC detection compared with *SEPT9* (60%), using the combination of methylated genes such as bone morphogenetic protein (BMP3), N-myc downstream regulated family member 4 (NDRG4), vimentin, tissue factor pathway inhibitor-2 (TFPI2), mutant *KRAS* and  $\beta$ -actin. According to Carmona *et al.*<sup>[71]</sup>, there is a 78% sensitivity for CRC early diagnosis when combining angiotensin II receptor type 1 (*AGTR1*), wingless-type MMTV integration site family member 2 (*WNT2*), slit homolog 2 (*Slit2*) (*SLIT2*) genes. Moreover, Cassinotti *et al.*<sup>[72]</sup> exhibited the potential use of gene panel, consisting of D-type cyclin gene (*CYCD2*), hypermethylated in Cancer 1 (*HIC1*), *PAX5*, Ras association domain family 1, isoform A (*RASSF1A*), retinoblastoma tumor suppressor (*RB1*) and sheep red blood cells (SRBC) with sensitivity nearly 84% and specificity 68%. Comparable results revealed by others studies making these panels powerful tools for future large-scale trials<sup>[73-95]</sup> (Table 2).

In parallel, several blood-based methylated genes as potential biomarkers have been studied either alone or within panels as previously demonstrated, and a summary of them exhibited in Table 2, concerning their detection rate<sup>[41,64-67,70,72,74-92]</sup>. Some of them (*SEPT9*, *ALX4*, *SDC2*, *RUNX3*, *TMEFF2*, *NEUROG1*) present high sensitivity and



**Table 2** Abnormally methylated genes as potential circDNA blood-based colorectal cancer detection biomarkers

Potential biomarkers	CRC sensitivity (%)	CRC specificity (%)	Ref.
ALX4	40-83	70-82	[67,76]
TFPI2	76-89	-	[77-80]
SDC2	92	-	[81]
RUNX3	65	100	[82,83]
NEUROG1	52-64	91	[84]
MGMT	39	96	[74]
RARβ2	24	100	[74]
NGFR	51	84	[85]
9-Sep	48-90	86-93	[64-67,70,86]
TMEFF2	65	69	[85]
Vimentin	59	93	[41]
RASSF2A	58	100	[74]
Wif-1	74	98	[74]
APC	6	100	[87]
hMLH1	43	98	[87]
HTLF	21-34	98-100	[87,88]
SFRP2	67	94	[89]
CDKN2A/P16	71	100	[83]
Panel: SEPT9, HTLF and ALX4	74-88	90-96	[68]
Panel: SEPT9 and ALX4	-	-	[67]
Panel: MGMT, RASSF2A, Wif-1 gene	86.5	-	[74]
Panel: BMP3, NDRG4, vimentin, TFPI2, mutant KRAS and β-actin	87	-	[69]
Panel: AGTR1, WNT2, SLIT2	78	-	[71]
Panel: CINP1, FBN1, INA, SNCA, MAL and SPG20	90-99	-	[73]
Panel: CYCD2, HIC1, PAX5, RASSF1A, RB1 and SRBC	84	68	[72]
Panel: THBD and C9orf50	71	80	[75]
RASSF1A, E-cadherin	-	-	[72,90]
CAHM	-	-	[91]
FRP2, TPEF/HPP1	-	-	[83,84,92]

ALX4: Aristaless-like homeobox 4; TFPI2: Tissue factor pathway inhibitor 2; SDC2: Syndecan 2; RUNX3: Runt-related transcription factor 3; NEUROG1: Neurogenin 1; MGMT: O-6-methylguanine-DNA methyltransferase; RARβ2: Retinoic acid receptor β2; NGFR: Nerve growth factor receptor; SEPT9: Septin 9; TMEFF2: Transmembrane protein with EGF-like, and two follistatin-like domains 2; RASSF2A: Ras association domain family 2 (isoform A); Wif-1: Wnt inhibitory factor-1; APC: Adenomatosis polyposis coli; hMLH1: MutL homolog 1; HTLF: Helicase-like transcription factor; SFRP2: Secreted frizzled-related protein 2; CDKN2A/P16: Cyclin-dependent kinase inhibitor 2A; BMP3: Bone morphogenetic protein; NDRG4: N-myc downstream regulated family member 4; KRAS: Kirsten rat sarcoma 2 viral oncogene homolog; AGTR1: Tissue fac angiotensin II receptor type 1; WNT2: Wingless-type MMTV integration site family member 2; SLIT2: Slit homolog 2 (Drosophila); CINP1 FBN1: Fibrillin 1; SNCA: α-synuclein gene; SPG20: Spastic paraplegia-20; CYCD2: D-type cyclin gene; HIC1: Hypermethylated in cancer 1; RASSF1A: Ras association domain family 1 (isoform A); RB1: Retinoblastoma tumor suppressor; SRBC: Sheep red blood cells; THBD: Thrombomodulin; C9orf50: Chromosome 9 open reading frame 50; FRP2: Frizzled related protein 2; TPEF/HPP1: Transmembrane protein containing epidermal growth factor, follistatin domain/hyperplastic polyposis 1.

specificity for CRC detection during initial stages when

analyzing methylation status of circDNA<sup>[67,69]</sup>. Although the evaluation some of these aberrant methylated genes may demonstrate better diagnostic results than the *SEPT9* analysis, their cost effectiveness, further technical improvement and low testing uptake issues impede their use within large-scale clinical trials<sup>[70,93]</sup>. Thus, *SEPT9* as the most common blood-based methylation analysis biomarker holds promising example of sending on real life the laboratory methylation studies upon circDNA, for early CRC diagnosis of average-risk individuals.

### circDNA pre-analysis considerations

As we stated before, analysis of *SEPT9* gene methylation could be subject of further technical advance<sup>[93]</sup>. Therefore, it is reasonable to presume that several factors could play crucial role such as blood sampling and circDNA processing. It is well-established that blood-based circDNA could be extracted from both plasma and serum with the latter one exhibiting higher concentration of DNA<sup>[94,95]</sup>. However there are studies suggesting that this high amount of DNA in serum reflects the *in vitro* lysis of leucocytes when the procedures of coagulation and/or fibrinolysis take place<sup>[95]</sup>. Another theory highlights the significant effect that chemicals differences between serum and plasma have during DNA extraction<sup>[96]</sup>. Other factors that researches should take into account are: The interval time of blood drawn and centrifugation; the sample storage modality; the anticoagulant used; temperature; and the plasma-based DNA isolation protocol<sup>[96]</sup>. All these parameters exhibit enormous significance as concerns the efficiency and quality of circDNA analysis, illustrating the reliability that newer methods of circDNA analysis, should have.

## CONCLUSION AND FUTURE ASPIRATIONS

Colorectal cancer is one of the deadliest malignancies to date even though various techniques are available to prevent and detect its emergence. Although these preventive modalities (sigmoidoscopy, colonoscopy, FOBT, FIT) exhibit high CRC detection sensitivity and specificity, the acceptance rate among population remains low. In parallel, the rapid progression of molecular biology has revealed new translational research fields related to discovery of potential CRC biomarkers in body "liquid fluids". These markers evaluate the fragments of DNA, RNA or proteins in the blood or feces demonstrating an increasingly cost-effective and sensitive way to detect premalignant modification of genome in individuals on average risk for CRC development. Thus, with this review we tried to highlight those circDNA blood-based biomarkers that offer an easy, cost-effective and with minimal invasiveness diagnosis of colonic neoplasia (Table 2). We believe that this research demonstrate in depth the need for further studies to be done which should be large randomized and will try to evaluate or elucidate the clinical value of all these new proposed screening tests which

could be combined the older ones as a critical strategy to improve quality of the existing life expectancy as well as to advance the latter one.

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## Basic Study

**Effect of *Clostridium perfringens* enterotoxin on gastric cancer cells SGC7901 which highly expressed claudin-4 protein**

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**Abstract****AIM**

To investigate the effects of *Clostridium perfringens* enterotoxin (CPE) on gastric cancer cells which highly expressed claudin-4 (CL4) protein.

**METHODS**

In this study, we detected expression of CL4 protein in different gastric cancer cell lines. Then, we investigated the effects of CPE on SGC7901 cells which highly expressed CL4 protein and the effects of CPE on subcutaneous tumor in nude mice models.

**RESULTS**

CL4 are highly expressed in SGC7901 cells. CPE expressed

significant cytotoxicity in SGC7901 cells. Suppression of CL4 expression significantly decreased CPE-mediated cytotoxicity. CPE also inhibited tumor growth in subcutaneous tumor xenograft models.

## CONCLUSION

CPE showed CL4 mediated cytotoxicity on gastric cancer cells SGC7901 and inhibited tumor growth in nude mice models.

**Key words:** Gastric cancer; *Clostridium perfringens* enterotoxin; Claudin-4 protein; Cytotoxicity; Tight junction

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**Core tip:** This study firstly investigated the effects of *Clostridium perfringens* enterotoxin (CPE) on gastric cancer cells SGC7901, and indicated CPE's potential effect in gastric cancer therapy.

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## INTRODUCTION

Gastric cancer is the second leading cause of cancer-related death through the world<sup>[1]</sup>. System therapy including radical surgery, adjuvant chemotherapy, biology therapy and so on. However, 5 year survival rate in advanced gastric cancer patients was still low<sup>[2,3]</sup>.

Tight junction is the important structure between epithelial cells maintaining the cell polarity and membrane integrity<sup>[4]</sup>. Tight junction are formed by some tight junction proteins including occludin, claudins, ZO-1 and so on<sup>[5]</sup>. Recently, some studies show claudin-4 (CL4) protein plays a crucial role in tumor's proliferation, transformation, and metastasis<sup>[6,7]</sup>. CL4 protein are highly expressed in many kinds of malignant tumors, such as ovarian cancer<sup>[8]</sup>. In 2015, Liu *et al*<sup>[9]</sup> reported that overexpression of CL4 protein was associated with progress of gastric cancer and poor prognosis of gastric cancer patients. More and more researches indicated that CL4 may be an emerging target for cancer therapy.

*Clostridium perfringens* enterotoxin (CPE), a 35-kDa single polypeptide comprised of 319 amino acids, could bind with CL4 and formed CPE-CL4 complex. CPE-CL4 complex induced resultant pore formation on cell membranes of epithelial cells, and caused cell apoptosis via the influx of Ca<sup>2+</sup> into the cell<sup>[10]</sup>. In pancreatic cancer cell lines HPAC cells, CPE showed a dose-dependent cytotoxic effect<sup>[11]</sup>. In ovarian tumors, CPE also showed a dose-dependent cytotoxic effect *in vitro*. CPE significantly

inhibited tumor growth and progression in SCID mouse xenografts of human ovarian cancer<sup>[12]</sup>. However, little was known about the effect of CPE on gastric cancer cells.

In this study, we assessed the expression of CL4 protein in different gastric cancer cell lines. Then we investigated the effects of CPE on SGC7901 cells which highly expressed CL4. In addition, we observed CPE effects on subcutaneous tumor growth of gastric cancer cell SGC7901 in nude mice model.

## MATERIALS AND METHODS

### Antibody

Goat polyclonal antibodies against CL4 were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, United States). Rabbit polyclonal antibodies were obtained from Abcam (Cambridge, MA, United States). The secondary antibodies were horseradish peroxidase (HRP)-conjugated anti-rabbit or anti-goat immunoglobulin (Ig) G (ZSGB-BIO, Beijing, China), Alexia Flour 488 (green)-labeled donkey anti-goat IgG (abcam, Cambridge, CA, United States).

### Cell lines and cell culture

The human gastric cancer cell line SGC7901, MKN45, AGS, MGC803, BGC823 and HGC27 were used to assess the expression of CL4 protein. Colon cancer cell line Caco-2 was considered as CL4 positive control. Normal gastric epithelium cell line GES-1 was considered as negative control. All these cell lines were obtained from Cell Bank, Shanghai Institutes for Biological Sciences. All cells were maintained in RPMI-1640 with 10% fetal bovine serum (FBS), 100 U/mL penicillin and 100 mg/mL streptomycin and cultured at 37 °C in a humidified 5% CO<sub>2</sub> atmosphere.

### Western blot

Cells were cultured in 25-mm<sup>2</sup> Tissue Culture Flasks. Total protein was extracted by the Protein extraction kit (KeyGEN BioTECH, Shanghai, China). Twelve percent SDS-PAGE was used for electrophore. After completely separated, the target protein was transferred onto a polyvinylidene difluoride membrane (Immobilon; Millipore). The membrane was saturated with PBS containing 4% skim milk, and then incubated for one night at 4 °C with primary antibodies (diluted 1:1000) in PBS. After rinsing in PBS containing 0.1% Tween 20, the membrane was incubated for 2 h at room temperature with HRP-conjugated anti-rabbit or anti-goat IgG (diluted 1:10000) in PBS. It was then rinsed again, and finally reacted using an immobilon western chemiluminescent HRP substrate (Immobilon; Millipore). Signals in immunoblots were quantified using Quantity One-4.4.0 (Bio-Rad).

### Preparation of CPE

The DNA sequence of CPE was synthesized and amplified by polymerase chain reaction (PCR) and subcloned into

vector pET28a, and the sequence was transfected to *Escherichia coli*. The CPE protein expression was induced by isopropyl- $\beta$ -D-thiogalactoside (IPTG) and purified by Ni-IDA. This process was made by the company of Biogot technology (Nanjing, China).

### Cytotoxicity assay

Cells were grown on 96-well plates to reach confluent density and incubated for 24 h with either the vehicle or CPE. Then 20  $\mu$ L 5 mg/mL methylthiazolyldiphenyl-tetrazolium bromide (MTT) was added to each well and incubated for 4 h. One hundred and fifty microlitre dimethyl sulfoxide was added to every well after the supernatant was wiped off. Then the plates were wobbled for 10 min. The optical density at the wavelength of 490 nm (OD<sub>490</sub>) was detected with a microplate reader ELX800 (BioTek, VT). The inhibition ratio was calculated by formula  $[\text{OD}_{490} (\text{CPE group}) / \text{OD}_{490} (\text{control group})] \times 100\%$ . The final results were the average of 3 times and the image was made by OriginPro 9.2 (OriginLab).

### RNAi and transfection

Stealth siRNA duplex oligonucleotides against human CL4 were synthesized by Invitrogen. The sequences were as follows: Sense (UCUGUUUUGUAAUUUAAGATT) and antisense (UCUUAUUUACAAAACAGAAA). SGC7901 cells were transfected with siRNAs (final concentration was 10 nmol/L) or a Stealth RNAi negative control by using Lipofectamine RNAiMAX Reagent (Invitrogen) according to the manufacturer's protocols.

### Confocal microscope

Cells grown on coverslips were fixed in paraformaldehyde for 15 min at room temperature. After being covered with 10% BSA for 1 h, they were washed three times with PBS and incubated for a night at -4 °C with primary antibodies and rinsed again with PBS, followed by reaction for 2 h at room temperature with appropriate secondary antibodies. All samples were examined using a laser scanning confocal microscope (LSM710, Carl Zeiss, Jena, Germany). Photographs were recorded using a computer (Fujitsu) and ZEN 2009 (Carl Zeiss) and processed with Zeiss LSM Image Browser (Carl Zeiss) and Photoshop CS6 (Adobe).

### Animal studies

SGC7901 cells ( $2 \times 10^6$  cells in 100  $\mu$ L of medium RPMI-1640) were subcutaneously injected into the inguinal region of 6-wk-old nude male mice (BALB/c, nu/nu, SLRC Laboratory Animal Technology Co, Shanghai, China). The mice were killed to get the tumor mass at 2 wk after injection. The tumor mass was cut into pieces with a diameter of 2 mm and planted into the subcutaneous of inguinal region of 6-wk-old nude male mice. After 2 wk the mice were divided into two groups (+CPE and -CPE), and 2  $\mu$ g of CPE in 100  $\mu$ L of saline, or 100  $\mu$ L of PBS was injected around the tumor every day for 10 d. The tumor volume (mm<sup>3</sup>) was calculated

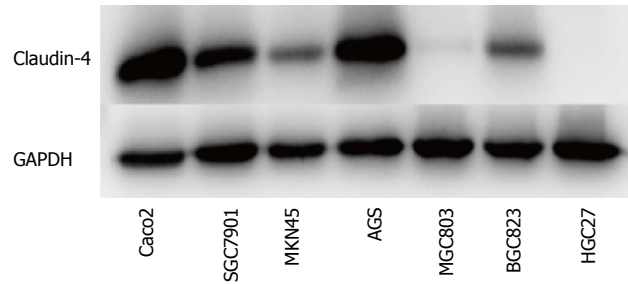


Figure 1 Expression of claudin-4 protein in different gastric cancer cells.

by the formula  $0.5 \times \text{long diameter (mm)}^2 \times \text{short diameter (mm)}$ . On day 10, the tumors were removed and the diameters of tumor were measured. All aspects of the study were approved by the Animal Use and Care Committee of Nanjing Drum Tower hospital (Nanjing, China).

### Statistical analysis

All measured values are presented as the mean  $\pm$  SD. Statistical significance of differences was evaluated using One-Way ANOVA analysis and LSD test. Repeated measures analysis of variance was used for evaluating the animal studies.

## RESULTS

### CL4 protein expression in gastric cancer cells

We firstly evaluated the expression level of CL4 protein in different gastric cancer cells (SGC7901, MKN45, AGS, MGC803, BGC823 and HGC27 cells). Colon cancer cell line Caco-2 which highly expressed CL4 protein was considered as positive control cell. We found CL4 protein was highly expressed in two types of cell lines: SGC7901 and AGS cells (Figure 1). Because AGS cells can not be used in nude mice models, we select SGC7901 cells in further study to investigate CPE effects.

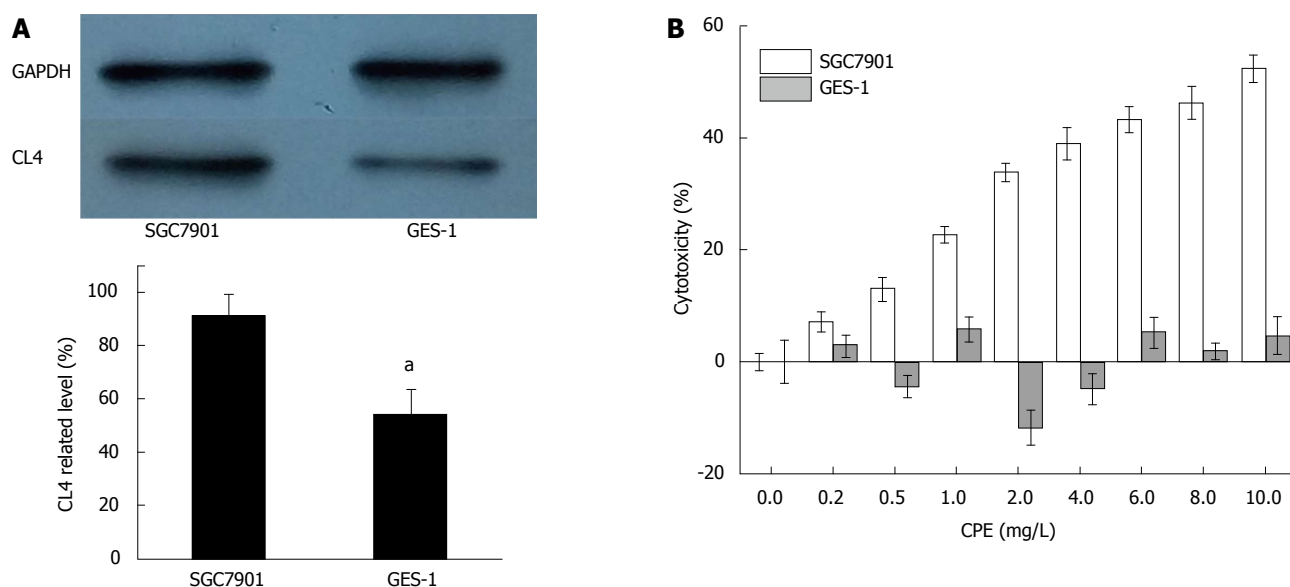
### CPE cytotoxicity in SGC7901 cells

We confirmed the CL4 level of gastric cancer cells SGC7901 and normal gastric epithelium cells GES-1 by western blot. The relative level of CL4 on GES-1 cells was significantly lower than SGC7901 cells (Figure 2A). Cytotoxicity of up to 56% was observed 24 h in SGC7901 cells after CPE treatment and the cytotoxicity of CPE (2, 4, 6, 8, 10 mg/L) showed significant differences ( $P < 0.05$ ) compared with CPE (0.2 mg/L). However, CPE had no significant cytotoxic effects on GES-1 cells under the same conditions (Figure 2B).

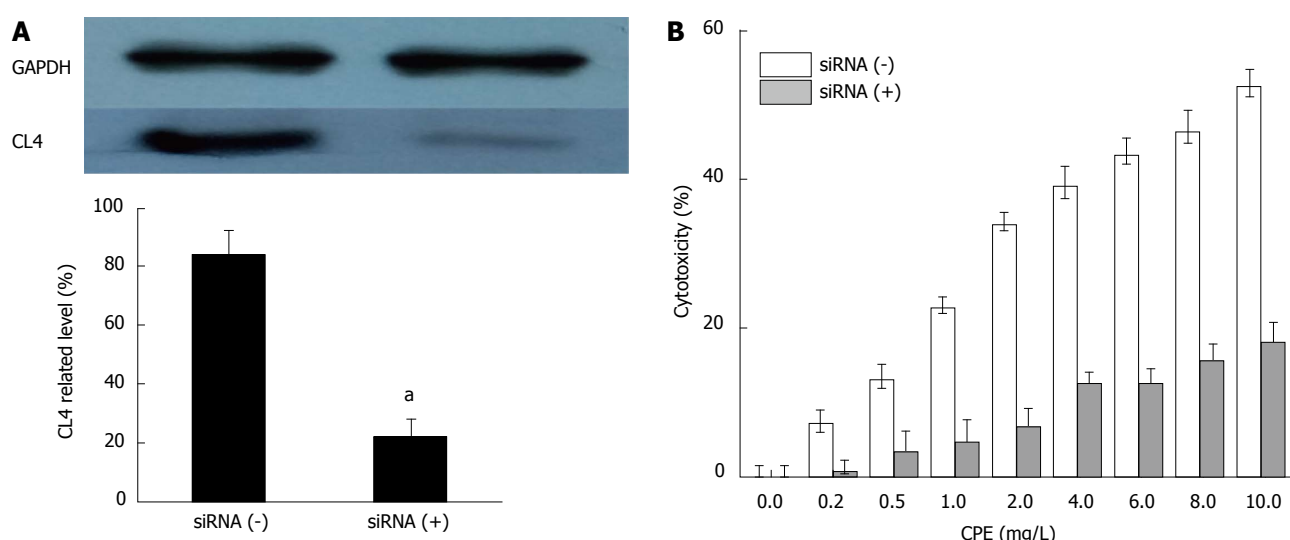
### Suppression of CL4 expression decreased CPE cytotoxicity

We used an RNAi approach to knock down the CL4 protein in SGC7901 cells. Cells were transfected with the siRNA against human CL4 and incubated for 48 h. Western blot analysis showed that the siRNA significantly reduced CL4 protein expression in SGC7901 cells (Figure





**Figure 2** *Clostridium perfringens* enterotoxin effects on SGC7901 cells and GES-1 cells. A: CL4 expression in SGC7901 and GES-1 cells. The intensity levels represent as mean  $\pm$  SD ( $n = 3$ ).  $^aP < 0.05$  vs SGC7901 cells; B: Cells were treated with CPE (0, 0.2, 0.5, 1, 2, 4, 6, 8 and 10 mg/L) for 24 h, and the cytotoxicity of CPE was measured by MTT assay. Values represent as mean  $\pm$  SD ( $n = 3$ ). CPE: *Clostridium perfringens* enterotoxin; CL4: Claudin-4.



**Figure 3** *Clostridium perfringens* enterotoxin effects on SGC7901 cells with siRNA transfection. A: SGC7901 cells were transfected with siRNA, and incubated for 24 h. CL4 expression in siRNA (-) and siRNA (+) cells were shown in A.  $^aP < 0.05$  vs control group; B: The siRNA (-) and siRNA (+) SGC7901 cells were treated with different concentrations of CPE for 24 h. The MTT assay values represent as mean  $\pm$  SD ( $n = 3$ ). CPE: *Clostridium perfringens* enterotoxin; CL4: Claudin-4.

3A). When the expression of CL4 was suppressed, CPE-mediated cytotoxicity was significantly decreased in SGC7901 cells according to MTT assay (Figure 3B).

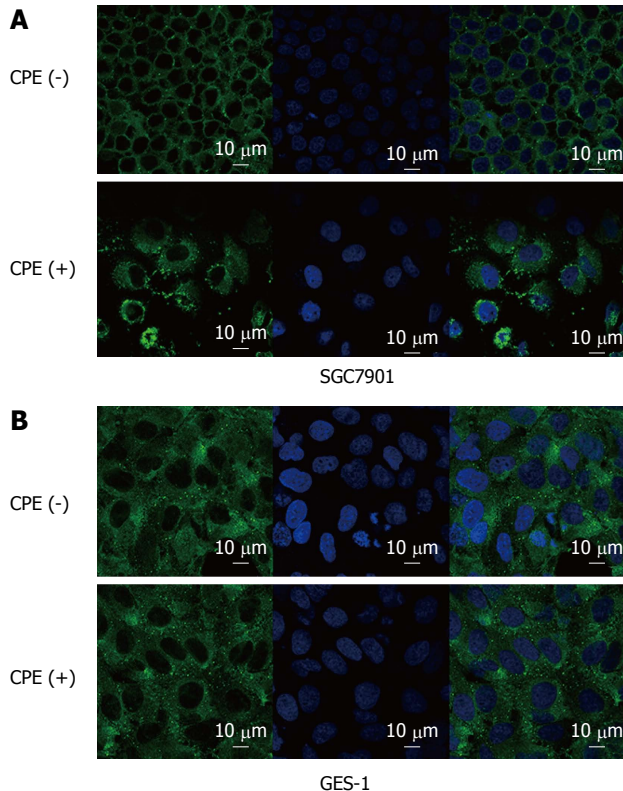
### CPE effect on CL4 expression in membrane

Cells were performed immunostaining and observed under a laser-scanning confocal microscope. The results showed that CL4 mainly expressed on the cell membrane in SGC7901 cells. After treatment with CPE (10 mg/L) for 24 h, CL4 protein expression in cell membrane were suppressed and partly translocated to cytoplasm (Figure 4A). However, CL4 expressed both on cell membrane and in cytoplasm in GES-1 cells. Cell membrane damage was not observed in GES-1 after CPE treatment (10 mg/L)

for 24 h (Figure 4B).

### CPE inhibits tumor growth of SGC7901 xenografts in nude mice

To evaluate the cytotoxic effect of CPE *in vivo*, SGC7901 cells were used to establish the xenograft models. When the subcutaneous tumor diameter reached about 5-7 mm, the nude mice were randomly divided into two groups [CPE (+) group,  $n = 7$ ; CPE (-) group,  $n = 7$ ]. CPE (+) group was injected with CPE (2 mg in 100  $\mu$ L of PBS) around the tumors once a day for 10 d, while CPE (-) group was injected with PBS (100  $\mu$ L) around the tumors once a day for 10 d. Tumor volumes were measured on days 0, 5, and 10 after treatment. Mice were killed and



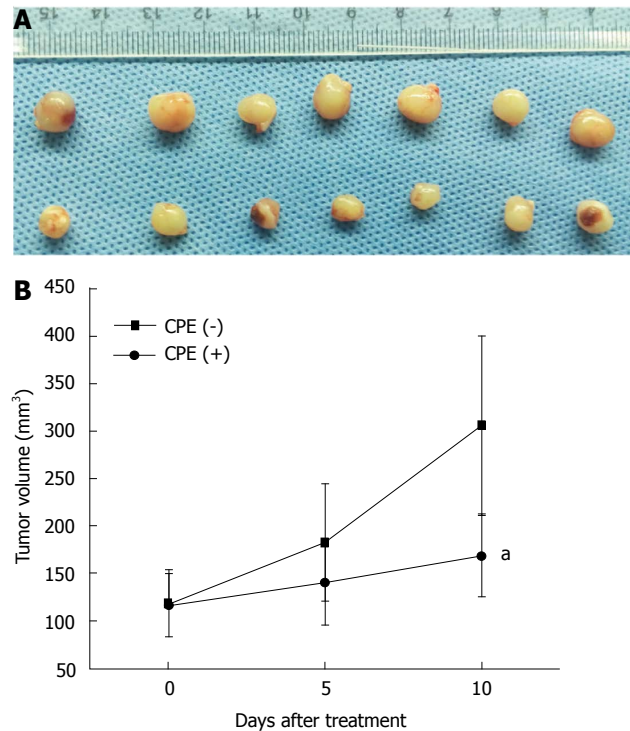
**Figure 4** *Clostridium perfringens* enterotoxin effect on claudin-4 protein in cell membrane. SGC7901 Cells (A) and GES-1 cells (B) were subjected to CL4 immunostaining under a laser-scanning confocal microscope. Green strains illuminated CL4 protein expression. CPE: *Clostridium perfringens* enterotoxin; CL4: Claudin-4.

the tumors were removed after 10 d. The tumor tissues were shown in Figure 5A, CPE significantly suppressed tumor growth, and obvious reduction of tumor volume was observed in CPE (+) group compared with CPE (-) group (Figure 5B). However, in CPE (+) group, injection site skin necrosis and enteritis were also observed in 3 mice.

## DISCUSSION

The receptors of CPE were mainly considered as CL3 and CL4 protein. CL4 has been found highly expressed in some gastric cancer tissues<sup>[13-16]</sup>. Hung Jung found the expression rate of CL4 was 44.4% in gastric cancer tissues, and expressions of CL4 was significantly lower in cases with positive lymphatic invasion<sup>[13]</sup>. Liang *et al.*<sup>[14]</sup> found the expression of CL4 in normal stomach samples was only 15.9%. Maeda's study found that inhibiting the expression of CL4 significantly reduced the CPE toxicity, but inhibiting the expression of CL4 slightly increased the toxicity of CPE in prostate cancer<sup>[17]</sup>. In this study, we also observed inhibiting CL4 expression significantly reduced CPE-mediated toxicity in gastric cancer cells. These results revealed CL4 protein could be a potential target agent in gastric cancer therapy.

Our study found that CPE almost had no significant toxicity on normal gastric epithelium cells GES-1 and



**Figure 5** *Clostridium perfringens* enterotoxin effects on SGC7901 xenograft tumor in nude mice model. A: SGC7901 xenografts were randomly divided into two groups. Mice were killed on day 10 after CPE treatment and the tumors were measured; B: Obvious reduction of tumor volume was observed in CPE (+) group. The data present as mean  $\pm$  SD. <sup>a</sup> $P < 0.05$  vs CPE (-) group. CPE: *Clostridium perfringens* enterotoxin.

the laser confocal microscopy confirmed that CPE had little effects on membrane morphology in GES-1 cells. According to the former study, the toxicity of CPE was associated not only with CL4 expression, but also the subcellular localization of CL4. As the target of CPE, CL4 mainly distributed in the cell membrane in SGC7901 cells, but distributed both in cell membrane and cytoplasm in GES-1 cells. While, the overall expression of CL4 in GES-1 was significantly lower than SGC7901 cells according to the Western blot test. We speculated that little CL4 protein distributed in membranes in GES-1 cells. In addition, recent study found the formation of intact tight junction could alleviate the cytotoxicity of CPE<sup>[17]</sup>. Studies also found solid tight junctions could be formed between GES-1 cells<sup>[18]</sup>. These findings maybe explained the different effects of CPE on GES-1 cells and SGC7901 cells.

Tight junction plays a very important role in the proliferation, differentiation and cell polarity of epithelial cells<sup>[19]</sup>. In our experiment, SGC7901 cell membrane was integrity and the size was substantially uniform. After CPE treatment, part of the cell membrane was not complete. Some nucleus split into smaller pieces after CPE treatment (10 mg/L). This phenomenon agreed with Smedley's study, which found CPE caused apoptosis at low concentrations while oncosis at high concentrations<sup>[10]</sup>.

Although CPE showed potential therapeutic effects

on some malignant tumors, there were still no clinical data or trials available. CPE's side effect limited its clinical application in tumor therapy. In this study, CPE injection site skin necrosis and enteritis were observed in 3/7 mice. Garcia *et al.*<sup>[20]</sup> also found the rabbit's small intestine and colon were damaged after as little as a 1-h treatment with 50 µg/mL of CPE. These studies indicated that serious adverse effects should be considered in CPE-based cancer therapy. To overcome these disadvantages, some researchers cut off the N-terminal region of CPE which mainly cause cell death and then obtain C-terminal CPE (C-CPE) which mainly target to the cells. C-CPE is a smaller molecule without cytotoxicity but also combined with CL4 protein. C-CPE can disrupt the tight junction and increase paracellular permeability, enhance chemotherapy drugs to get into the cells<sup>[21]</sup>. Li *et al.*<sup>[22]</sup> observed the safety of the C-terminal of CPE and confirmed that injection of CL-targeted toxin injured the liver but not the kidney. To alleviate the side effect of C-CPE is highlight in future research.

In summary, this study investigated the effects of CPE on gastric cancer cells SGC7901. CPE showed CL4 mediated cytotoxicity on gastric cancer cells, and inhibited tumor growth in nude mice models. These results provide CPE may be a novel potential tool for gastric cancer's therapy. More studies need to be performed to overcome the limitation of CPE before its clinical application.

## COMMENTS

### Background

*Clostridium perfringens* enterotoxin (CPE) showed therapeutic effects on malignant tumors which highly expressed claudin-4 (CL4) protein. However, little was known about the effects of CPE on gastric cancer cells.

### Innovations and breakthrough

In this study, the authors firstly investigated the effects of CPE on SGC7901 cells which highly expressed CL4 protein. CPE showed CL4 mediated cytotoxicity on gastric cancer cells SGC7901 and inhibited tumor growth in nude mice models.

### Applications

These results provide CPE may be a novel potential tool for gastric cancer's therapy.

### Peer-review

This is an interesting article reporting the therapeutic effect of CPE on gastric cancer cells (SGC7901 cells) and on a subcutaneous tumor in nude mice model.

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## Observational Study

**Bayesian adjustment of gastric cancer mortality rate in the presence of misclassification**

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**Author contributions:** Pourhoseingholi MA was principal investigator and contributing in writing the manuscript; Hajizadeh N contributed to study conception and data analysis; Baghestani AR and Abadi A contributed to study conception and design; Zali MR contributed to interpretation the results; all authors contributed to editing, reviewing and final approval of the article.

**Institutional review board statement:** The study was reviewed and approved by research committee of research institute for gastroenterology and liver diseases (Tehran).

**Informed consent statement:** Hereby it is attested that this manuscript which is submitted for publication in World Journal of Gastrointestinal Oncology has been read and approved by all authors, has not been published, totally or partly, in any other journal.

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**Abstract****AIM**

To correct for misclassification error in registering causes of death in Iran death registry using Bayesian method.

**METHODS**

National death statistic from 2006 to 2010 for gastric cancer which reported annually by the Ministry of Health and Medical Education included in this study. To correct the rate of gastric cancer mortality with reassigning the deaths due to gastric cancer that registered as cancer without detail, a Bayesian method was implemented with Poisson count regression and beta prior for misclassified parameter, assuming 20% misclassification in registering causes of death in Iran.

## RESULTS

Registered mortality due to gastric cancer from 2006 to 2010 was considered in this study. According to the Bayesian re-estimate, about 3%-7% of deaths due to gastric cancer have registered as cancer without mentioning details. It makes an undercount of gastric cancer mortality in Iranian population. The number and age standardized rate of gastric cancer death is estimated to be 5805 (10.17 per 100000 populations), 5862 (10.51 per 100000 populations), 5731 (10.23 per 100000 populations), 5946 (10.44 per 100000 populations), and 6002 (10.35 per 100000 populations), respectively for years 2006 to 2010.

## CONCLUSION

There is an undercount in gastric cancer mortality in Iranian registered data that researchers and authorities should notice that in sequential estimations and policy making.

**Key words:** Misclassification; Bayesian method; Cause of death; Gastric cancer; Iran

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**Core tip:** In some mortality cases, causes of deaths are registered as causes that cannot or should not be considered as the underlying causes of death like cancer without mentioning the type. These cases are not included in the estimations of cause specific mortality rates and leads to under-estimate health risks and burden of disease. The aim of this study is to correct the misclassification of gastric cancer deaths in cancer without label group using a Bayesian method.

Hajizadeh N, Pourhoseingholi MA, Baghestani AR, Abadi A, Zali MR. Bayesian adjustment of gastric cancer mortality rate in the presence of misclassification. *World J Gastrointest Oncol* 2017; 9(4): 160-165 Available from: URL: <http://www.wjgnet.com/1948-5204/full/v9/i4/160.htm> DOI: <http://dx.doi.org/10.4251/wjgo.v9.i4.160>

## INTRODUCTION

Cancer is one of the major health problems in the world and is the third cause of death (after cardiovascular disease and injuries) in Iran<sup>[1]</sup>. Gastric cancer is a disease in which the cells of the inner lining of the stomach start to divide abnormally and uncontrollably, that forming a mass called tumor<sup>[2]</sup>. Gastric cancer is the seventh cause of all deaths in Iran and is the first cause of cancer death in Iranian men and the second cause of cancer death (after breast cancer) in Iranian women<sup>[3]</sup>. The mortality of gastric cancer is high because this cancer does not show symptoms in early stages and diagnosed when the cancer is in its final stages<sup>[4]</sup>.

Burden of disease is used to evaluate the health

status of a country and determining priority of risk factors in order to setup cancer control programs. Cancer registry data are important to estimate the burden of disease, monitoring the screening programs effects, early diagnostics and other prognostic factors, and can be used to guide policy makers to appropriate cancer prevention programs. Among medical indices, mortality is a familiar projection to assess the burden of diseases. But achieving this aim requires a reliable death registry systems that reports death statistics accurately and completely<sup>[5-7]</sup>. In Iran, among four vital events (births, marriages, divorces and mortality) which were registered by the National Organization for Civil Registration (NOCR), mortality was the worst in quality. There was some progress in registering deaths but some problems like delayed registration and inaccurate recording of causes of death remained until 2002, that Ministry of health and medical education Deputy of Research and Technology, started up a system to record the causes of deaths. This system did not allow to delayed deaths registry, but the causes of death were susceptible to information bias due to misclassification<sup>[8]</sup>. Most high-income and many middle-income countries have a complete vital registration system in which the majority of deaths get a death certificate completed by a physician<sup>[9]</sup>. But still, a number of causes of death in the process of completing death certificates and the coding of underlying cause of death based on standardized international rules, remains challenging<sup>[10-13]</sup>. In some cases, especially in developing countries, the cause of death is recorded with error<sup>[14,15]</sup>. For example if a death due to gastric cancer being labeled as a death due to any other cause, the misclassification error in outcome is occurs. Misclassification error makes the registered data inaccurate and often leads to major problems like biased estimates of burden and health risks in epidemiological analysis<sup>[16,17]</sup>.

According to the Iranian death registry, about 15% to 20% of death statistics are recorded in misclassified categories such as cardiopulmonary arrest, old age without dementia, septicemia, unknown, cancer without mention of details, and other ill-defined conditions. Murray and Lopez in 1996, for the first time, introduced the term "garbage coding" for assigning deaths to causes that are not useful for public health analysis of cause-of-death data<sup>[18-21]</sup>.

In developing countries like Iran that registration is not completely accurate, statistical methods can be very helpful to overcome this problem. Two statistical approaches are recommended to deal with misclassification; first is using a small valid sample and extending the results to the population<sup>[22]</sup> and the second is Bayesian analysis which is a flexible method that makes the possibility of combining the prior information regarding the subset of the parameters with the observed data to achieve a posterior distribution which will be the basis of inferences to correct the statistics. Bayesian models also can easily accommodate unobserved variables such as an individual's true information in the presence of Misclassification error<sup>[23]</sup>. The aim of this study is to use

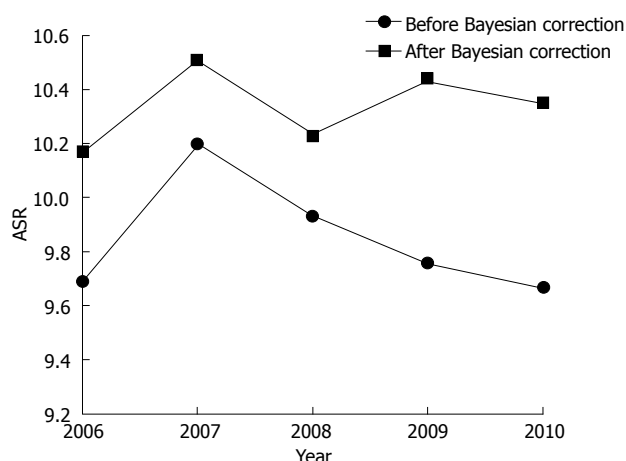


Figure 1 Age standardized rate of gastric cancer mortality in Iran from 2006 to 2010, before and after Bayesian correction of misclassification in causes of death. ASR: Age standardized rate.

Bayesian method to estimate the rate of misclassification that occurs by registering cancer (with no label) as the cause of death instead of deaths that have occurred because of gastric cancer in Iran's cancer registry system.

## MATERIALS AND METHODS

Mortality rates due to gastric cancer and also cancer without label from 2006 to 2010 are extracted from Iranian annual of death statistics which reported annually by Iran's Ministry of Health and Medical Education, in two sex groups (male and female) and four age groups (under 15 years - 15 to 49 years - 50 to 69 years - 70 years and more).

To reassign deaths from garbage codes to valid causes, the approach can be divided into three steps: The first is identifying garbage codes. The second is identifying the target causes where the deaths assigned to a garbage code should in principle be reassigned to; for example if a death cause is registered as cancer and the type of cancer is not mentioned, we face with a garbage code that should be reassigned to a specific cancer. The third step is choosing the fraction of deaths that are assigned to the garbage code that should be reallocated to the target cause<sup>[13]</sup>. In this study we consider cancer without label as garbage code because cancer with no label is most likely to be registered as cause of death instead of a specific cancer like gastric cancer. The data were entered to the Bayesian model by two vectors  $y_1 = [y_{11}, y_{21}, \dots, y_{r1}]$  for gastric cancer and  $y_2 = [y_{12}, y_{22}, \dots, y_{r2}]$  for cancer without label. Both  $y_1$  and  $y_2$  are count data and follow the Poisson distribution. The subscript  $r$  shows the number of covariate patterns that is made by age and sex group combinations.  $\theta$  is considered to be the probability of incorrectly register a mortality from gastric cancer as mortality due to cancer without label group. To perform Bayesian inference, an informative beta prior distribution was assumed for the misclassified parameter, *i.e.*,  $\theta \sim \text{beta}(a, b)$ . The initial value for the parameter of beta distribution

are taken to be  $a = 20$  and  $b = 80$ , based on Iranian annual cancer registration reports. Since  $\theta$  (misclassified parameter) is an unknown parameter, a latent variable approach was employed to simplify the full conditional models; considering  $U_i | \theta, y_1, y_2 \sim \text{Binomial}(y_{i2}, P_i)$  as the number of counts from the first group that are incorrectly labeled as being in the misclassified group that  $P_i = (\lambda_{i1}\theta) / (\lambda_{i1}\theta + \lambda_{i2})$ , finally the posterior distribution appears in the following form;  $\theta | U_i, y_1, y_2 \sim \text{Beta}(\sum U_i + a, \sum y_i + b)$ . The misclassified parameter is estimated using a Gibbs sampling algorithm and averaging of the outcome. Analyses were done using R software version 3.2.0.

## RESULTS

Mortality data consisting of all deaths due to gastric cancer from 2006 to 2010 were considered in this study. Age standardized rate (ASR) of gastric cancer mortality was 9.69 per 100000 populations in 2006, 10.2 per 100000 populations in 2007, 9.93 per 100000 populations in 2008, 9.76 per 100000 populations in 2009 and 9.67 per 100000 populations in 2010 respectively. According to the Bayesian estimation, in year 2006, there was between 3% to 7% misclassification in registering cause of death as cancer without mentioning details while the underlying cause of death has been gastric cancer. The estimated percent of misclassification based on implemented Bayesian method for year 2006 to 2010 is shown in Table 1. This percent were subtracted from deaths that had registered as cancer without mentioning details and added to the number of deaths due to gastric cancer. The age standardized rate per 100000 populations for gastric cancer was estimated to be 10.17 in 2006, 10.51 in 2007, 10.23 in 2008 10.44 in 2009 and 10.35 in 2010, after Bayesian correction respectively. The age standardizes rate of gastric cancer before and after Bayesian correction for 2006 to 2010 is visualized in Figure 1. The number of gastric cancer death before and after Bayesian correction of misclassification for years 2006 to 2010 is shown in Table 1 and its trend is shown in Figure 2.

## DISCUSSION

Iran's death registry is subject to misclassification in reporting the underlying cause of death. About 3%-7% of deaths due to gastric cancer are registered as cancer without mentioning the type of cancer. After correcting misclassification error in death registry data, the number of deaths due to gastric cancer and its age standardized rate were increased. Gastric cancer crude mortality count in Iran had an increasing trend from year 2006 to 2010 except for 2008 that might be because of incompleteness of data; but the age standardized rate of gastric cancer was decreasing from year 2007 onward (except for 2008). About two-thirds of gastric cancer occurs in developing countries<sup>[24-27]</sup> and its rates are generally about twice as high in men as in women<sup>[28]</sup>. The age standardized rate (ASR) of gastric cancer incidence and

**Table 1** Misclassified parameter and the number of gastric cancer death before and after Bayesian correction and percent of increase in number of deaths after Bayesian correction in Iran's death registry 2006-2010

Year	Misclassified parameter	Before Bayesian correction			After Bayesian correction		
		Male	Female	Total	Male	Female	Total
2006	5%	3887	1642	5529	4081	1724	5805
2007	3%	4001	1690	5691	4121	1740	5861
2008	3%	3912	1652	5564	4029	1702	5731
2009	7%	3907	1650	5557	4180	1766	5946
2010	7%	3944	1665	5609	4220	1782	6002

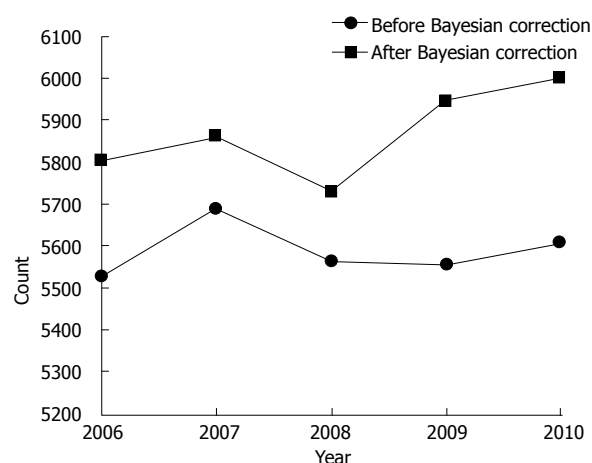
**Table 2** Incidence and mortality age standardized rates per 100000 populations due to gastric cancer for some continents, reported by GLOBOCAN 2012

Continent	Incidence ASR	Mortality ASR
World	12.1	8.9
Asia	15.8	11.7
Europe	9.4	6.9
South America	10.3	8.5
North America	4.0	2.1
Africa	3.8	3.5

ASR: Age standardized rates.

mortality per 1000000 populations based on GLOBOCAN report 2012 is shown in Table 2. The rates show that the ASR of gastric cancer incidence (15.8 per 100000) and also the ASR of gastric cancer mortality (11.7 per 100000) is highest in Asia compared to other continents; It is moderate in Europe and South America and lowest in Northern America and most parts of Africa<sup>[3,28]</sup>.

The age standardized rates of incidence and mortality per 100000 populations in different regions of Asia based on GLOBOCAN report 2012 are shown in Table 3. The incidence and mortality rates are also higher in Eastern Asia in comparison with other Asian regions. This region includes China, Japan and South Korea, that are three countries with the highest gastric cancer incidence and mortality rates<sup>[29]</sup>. Gastric cancer is the most frequently diagnosed form of cancer in Iran<sup>[30]</sup>, with incidence rate 15.3 per 100000 and mortality rate 12.9 per 100000 populations based on GLOBOCAN report 2012<sup>[3]</sup>. A steady decline has been observed in gastric cancer incidence and mortality rates in the most of countries in Northern America and Europe since the middle of the 20<sup>th</sup> century<sup>[31,32]</sup>. In recent years similar decreasing trends have been noted in areas with high rates of gastric cancer history, including some countries in Asia (Japan, China, and South Korea), Latin America (Colombia and Ecuador), and Europe (Ukraine)<sup>[33]</sup>. This reduction maybe due to improved sanitation and antibiotics and consequently reduction in chronic *H. pylori* infection<sup>[34]</sup>. Although the age-adjusted rates have been decreased, it is estimated to have a substantial rise in the crude rates between the years 2000 to 2020 because of the increasing the size and age of the world population, especially in developing countries<sup>[35,36]</sup>.

**Figure 2** Crude number of gastric cancer mortality in Iran from 2006 to 2010, before and after Bayesian correction of misclassification in causes of death.

Gastric cancer is a major health problem in the world, especially in Asia. So it is needed to make appropriate policy making for allocation of resources for gastric cancer control and prevention. To achieve this aim an accurate registry system is needed, while there are some misclassifications in registering causes of death especially in developing countries<sup>[14,15]</sup>. Misclassification of causes of death has been a concern in cancer trends analysis and researches on cancer epidemiology for decades<sup>[14]</sup>. Misclassification error leads to under-estimation of cause specific mortality rates and consequently under-estimation in burden of disease and influences the policy makings and health risk prioritizations<sup>[10-12,37]</sup>. In the study of Khosravi *et al*<sup>[38]</sup>, validated data from hospital death was used to measure the impact of misclassification on rates of cardiovascular disease mortality. But they didn't employ Bayesian method. Bayesian approach has received much attention to correct for misclassification in mortality data. Whittemore and Gong<sup>[39]</sup> used a Bayesian approach to estimate cervical cancer mortality rates and Sposto *et al*<sup>[40]</sup> developed maximum likelihood method for assessing the effect of diagnostic misclassification on non-cancer and cancer mortality in atomic-bomb survivors. Stamey *et al*<sup>[41]</sup> provided a Bayesian approach, which extends the models introduced by Whittemore and Gong<sup>[39]</sup> and Sposto *et al*<sup>[40]</sup>. They assume that the misclassification parameters are unknown. They used the prior information on the misclassification parameters instead of using valid



**Table 3** Incidence and mortality age standardized rates per 100000 populations due to gastric cancer for different regions of Asia, reported by GLOBOCAN 2012

Region	Incidence ASR	Mortality ASR
Eastern Asia	24.2	16.5
Western Asia	9.5	8.1
South-Central Asia	6.7	6.1
South-Eastern Asia	6.0	5.3

ASR: Age standardized rates.

data. They applied their Bayesian approach for estimating the number of deaths due to cancer and non-cancer after correcting for misclassification in registering causes of deaths among survivors of Hiroshima and Nagasaki after atomic bombings<sup>[41]</sup>. Pourhoseingholi *et al*<sup>[42]</sup> extended the models proposed by Stamey *et al*<sup>[41]</sup> to re-estimate the rates of cause specific deaths in cancer registry data after correcting for misclassification<sup>[25,42,43]</sup>. Based on his study on gastric cancer mortality in Iranian population from 1995 to 2004, there were between 30%-40% misclassification in recording deaths due to gastric cancer<sup>[44]</sup>. The current study reveals that the accuracy of death registration in Iran is getting better in recent years.

In conclusion there is an undercount of gastric cancer mortality in Iranian registration system. Because of misclassification error in registering causes of death. Although it seems that the misclassification rate has been reduced, it still exists as a major problem. So, policy makers who use mortality data to determine priorities for disease control and prevention, should notice to this underreported data and registration of causes of deaths should be done more accurately. Increase in data accuracy, requires more expert staffing, refining foundations, and powerful hardware and software resources<sup>[45]</sup>. In the absence of valid data, Bayesian approach is a good and flexible alternative to reduce the effects of Misclassification in registered cancer mortality data.

## COMMENTS

### Background

Mortality data registries are subject to misclassification; because some deaths assigned to causes that cannot be considered as underlying death cause. For example if mortality due to a special cancer be registered as cancer without mentioning the type of cancer, misclassification error occurs. The aim of this study is to estimate the rate of misclassification in registering deaths due to gastric cancer in cancer without label group using a Bayesian method and re-estimate the rate of gastric cancer mortality in Iran.

### Research frontiers

In Iran, death registries data is subject to misclassification. Reviewing the medical records or verbal autopsy as a practical solution for misclassification is time consuming. The hotspot of this study is using the Bayesian method for estimating the rate of misclassification in registering causes of death, which is rapid and cost-effective.

### Innovations and breakthroughs

By using the Bayesian method, it is not needed to valid the data for estimating the rate of misclassification. Data validation is very costly and time consuming

and in many cases it is not possible to obtain valid data. For implementing the Bayesian method only prior information about the misclassification rate is enough.

### Applications

Since registered mortality data is used for health policy making and estimating the burden of disease, after correcting the misclassification in death registry system, more precise estimates of death rates and cause specific burden of disease will be achieved. Consequently there will be a better planning for disease control and prevention.

### Terminology

Misclassification is lack of agreement between the observed value and the true value in categorical data. Bayesian method is one of the statistical approaches that assign a distribution or a probability to events or parameters based on previous experience or an expert's idea and revise those probabilities and distributions after obtaining experimental data with applying Bayes' theorem.

### Peer-review

This is an interesting research.

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## Observational Study

# Macroscopic appearance of Type IV and giant Type III is a high risk for a poor prognosis in pathological stage II / III advanced gastric cancer with postoperative adjuvant chemotherapy

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## Abstract

### AIM

To evaluate whether a high risk macroscopic appearance (Type IV and giant Type III) is associated with a dismal prognosis after curative surgery, because its prognostic relevance remains elusive in pathological stage II / III (pStage II / III) gastric cancer.

### METHODS

One hundred and seventy-two advanced gastric cancer (defined as pT2 or beyond) patients with pStage II / III who underwent curative surgery plus adjuvant S1 chemotherapy were evaluated, and the prognostic relevance of a high-risk macroscopic appearance was examined.

### RESULTS

Advanced gastric cancers with a high-risk macroscopic appearance were retrospectively identified by preoperative recorded images. A high-risk macroscopic appearance showed a significantly worse relapse free survival (RFS) (35.7%) and overall survival (OS) (34%) than an average risk appearance ( $P = 0.0003$  and  $P < 0.0001$ , respectively). A high-risk macroscopic appearance was significantly associated with the 13<sup>th</sup> Japanese Gastric Cancer Association (JGCA) pT ( $P = 0.01$ ), but not with the 13<sup>th</sup> JGCA pN. On univariate analysis for RFS and OS, prognostic factors included 13<sup>th</sup> JGCA pStage ( $P < 0.0001$ )

and other clinicopathological factors including macroscopic appearance. A multivariate Cox proportional hazards model for univariate prognostic factors identified high-risk macroscopic appearance ( $P = 0.036$ , HR = 2.29 for RFS and  $P = 0.021$ , HR = 2.74 for OS) as an independent prognostic indicator.

### CONCLUSION

A high-risk macroscopic appearance was associated with a poor prognosis, and it could be a prognostic factor independent of 13<sup>th</sup> JGCA stage in pStage II/III advanced gastric cancer.

**Key words:** Macroscopic feature; Gastric cancer; Type IV; Giant type III; Stage II/III

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**Core tip:** In this study, we for the first time clarify the clinicopathological relevance of the macroscopic high risk patients with pathological stage II/III gastric cancer who underwent curative surgery with postoperative S1 adjuvant chemotherapy in Japan.

Yamashita K, Ema A, Hosoda K, Mieno H, Moriya H, Katada N, Watanabe M. Macroscopic appearance of Type IV and giant Type III is a high risk for a poor prognosis in pathological stage II/III advanced gastric cancer with postoperative adjuvant chemotherapy. *World J Gastrointest Oncol* 2017; 9(4): 166-175 Available from: URL: <http://www.wjgnet.com/1948-5204/full/v9/i4/166.htm> DOI: <http://dx.doi.org/10.4251/wjgo.v9.i4.166>

### INTRODUCTION

Gastric cancer is the fifth most common malignancy and the third leading cause of cancer-related deaths worldwide<sup>[1]</sup>. Advanced gastric cancer with depth of invasion of T2 or beyond continues to show unsatisfactory survival outcomes despite progress in multidisciplinary therapy, especially for postoperative S1 adjuvant therapy<sup>[2,3]</sup>, while early gastric cancer is largely a curable disease<sup>[4,5]</sup>. Among advanced gastric cancers, macroscopic features and patient age were recently proven to be simple but the most potent independent prognostic factors<sup>[6]</sup>. Type IV and large type III gastric cancer have the most dismal prognosis<sup>[6-8]</sup>.

The gastric cancer section of the Japan Clinical Oncology Group (JCOG) has also classified advanced gastric cancer into macroscopic high risk and average risk to conduct clinical trials to propose novel multimodal treatment strategies. Giant type III (designated as 8 cm in length or greater) and type IV gastric cancer are being proposed as high-risk gastric cancer with dismal prognoses, for which neoadjuvant chemotherapy of cisplatin/S1 (CS) may be promising as a novel therapeutic strategy<sup>[9]</sup>. This strategy may be successful because of the clinical success of neoadjuvant chemo-

therapy for gastric cancer in the Western world, where neoadjuvant chemotherapy with epirubicin/cisplatin/5-fluorouracil (ECF) improved progression free survival (PFS) and overall survival (OS) better than surgery alone in aggressive advanced gastric cancer; gastric cancer in Western countries has shown a more aggressive phenotype than in Eastern countries<sup>[10]</sup>.

Macroscopic features have been repeatedly reported to be a prognostic factor independent of stage as earlier described<sup>[6,7]</sup>, but there have been no investigations of their relevance in advanced gastric cancer patients with pathological stage II/III who underwent curative gastrectomy together with postoperative S1 adjuvant chemotherapy. In this study, the clinicopathological relevance of macroscopic high-risk with pathological stage II/III gastric cancer in patients who underwent curative surgery with postoperative S1 adjuvant chemotherapy was examined for the first time.

### MATERIALS AND METHODS

#### Registration of patients

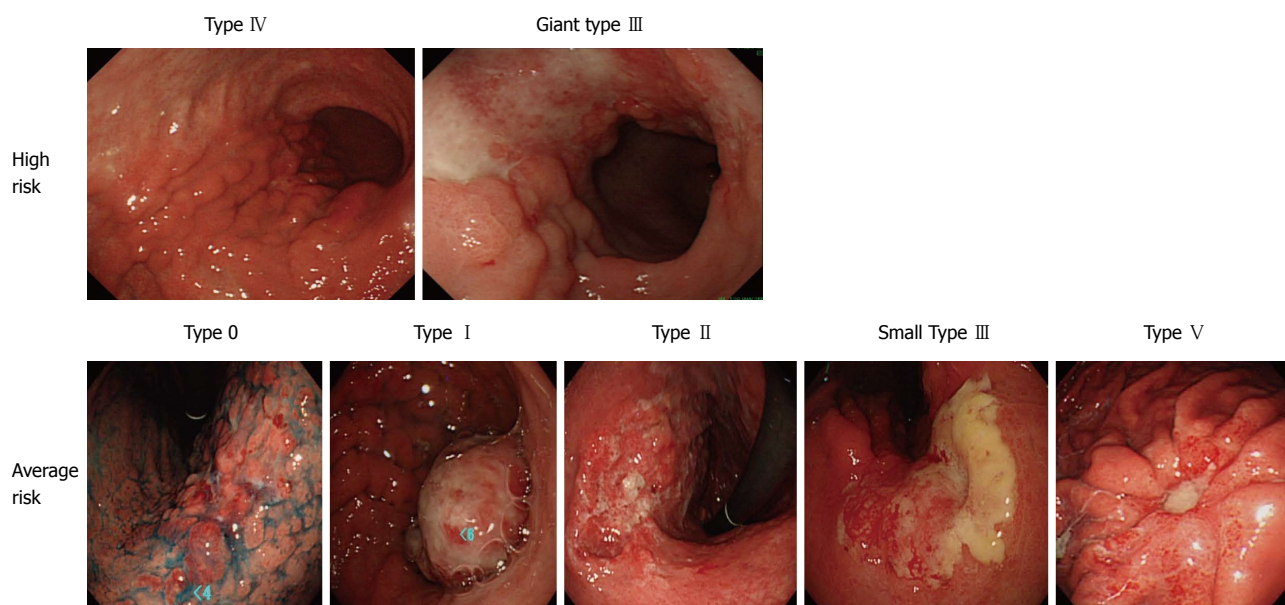
Between January 1, 2000, and December 31, 2010, 1673 patients underwent gastrectomy for gastric adenocarcinoma in the gastrointestinal surgery division, Kitasato University Hospital. A total of 396 patients with 13<sup>th</sup> Japanese Gastric Cancer Association (JGCA) stage II/III advanced gastric cancer underwent curative gastrectomy with D1-D2 lymph node dissection, and 67 underwent neoadjuvant chemotherapy or postoperative chemotherapy other than S-1 as previously reported<sup>[11-13]</sup>. Advanced gastric cancer was defined as pathological T2 (13<sup>th</sup> JGCA stage) or beyond, and pT1 gastric cancers were excluded from this study even when they were pathological stage II. Older age, defined as 67 years of age or older was used from a prognostic point of view from the previous reports<sup>[11]</sup>. Among the 329 patients with pStage II/III, 172 agreed to undergo adjuvant S-1 therapy after curative resection. The 172 patients who underwent adjuvant S-1 chemotherapy after surgery for at least one day were registered in the S-1 group. The clinicopathological features of the 172 patients in this study were investigated.

We participated in the ACTS-GC trial<sup>[2]</sup>, and started postoperative adjuvant use of S-1 for pStage II/III gastric cancer from October, 2001. Since 2007, when the interim analysis of the trial results was disclosed and recommended annual S-1 therapy after curative operation<sup>[2]</sup>, we recommended S-1 postoperative adjuvant therapy to patients with 13<sup>th</sup> JGCA pStage II/III advanced gastric cancer.

Among the 172 patients, D1 lymph node dissection ( $n = 26$ ) was performed for various reasons: preoperative diagnosis of clinical T1 cancer ( $n = 12$ ), omitted D2 dissection in the operative views (surgical T1) during the surgery ( $n = 4$ ), omitted D2 dissection because of systemic complications ( $n = 6$ ), surgery of remnant stomach cancer ( $n = 3$ ); and elderly ( $n = 1$ ).

The dose of S-1 was determined based on body





**Figure 1** Representative gastroendoscopy images of advanced gastric cancer by macroscopic classification. Upper panels include high-risk macroscopic features of type IV (left) and giant type III (right). Lower panels include average risk macroscopic features of type 0, type I, type II, small type III, and type V (in order from left to right).

surface area:  $< 1.25 \text{ m}^2$  (80 mg daily);  $\geq 1.25 \text{ m}^2$  but  $< 1.50 \text{ m}^2$  (100 mg daily);  $\geq 1.50 \text{ m}^2$  (120 mg daily). The adjuvant S-1 chemotherapy regimen was administered for 4 wk followed by 2 wk of rest. This 6-wk cycle was repeated during the first year after surgery. Toxicity of chemotherapy was assessed using Common Toxicity Criteria of the National Cancer Institute, version 4.0 (NCI-CTC)<sup>[14]</sup>. If patients had hematologic toxic effects of grade 3 or 4 or nonhematologic toxic effects of grade 2, 3 or 4, their daily dosage was reduced, or their treatment was postponed or stopped according to each physician's judgment.

### 13<sup>th</sup> JGCA stage

In the present study, the 13<sup>th</sup> JGCA stage classifications were used<sup>[15]</sup>, because ACTS-GC was established based on this staging system. In the 13<sup>th</sup> edition, the T category is classified into four categories: T1, the depth of invasion is mucosal or submucosal; T2, the depth of invasion is muscularis propria or subserosa; T3, the depth of invasion is serosa exposed; and T4, the depth of invasion is infiltrating into other organs. On the other hand, the status of lymph node metastasis is classified into four categories according to the anatomical classification of the involved lymph nodes. The descriptions are as follows: N0, no evidence of lymph node metastasis; N1, metastasis within the first tier of lymph nodes; N2, metastasis within the second tier of lymph nodes (extra-perigastric regional lymph nodes); and N3, metastasis to the third tier of lymph nodes (extra-regional lymph nodes). The latest (7<sup>th</sup>) UICC TNM stage is shown for reference purposes.

### Clinicopathological factors

Macroscopic features were retrospectively determined by

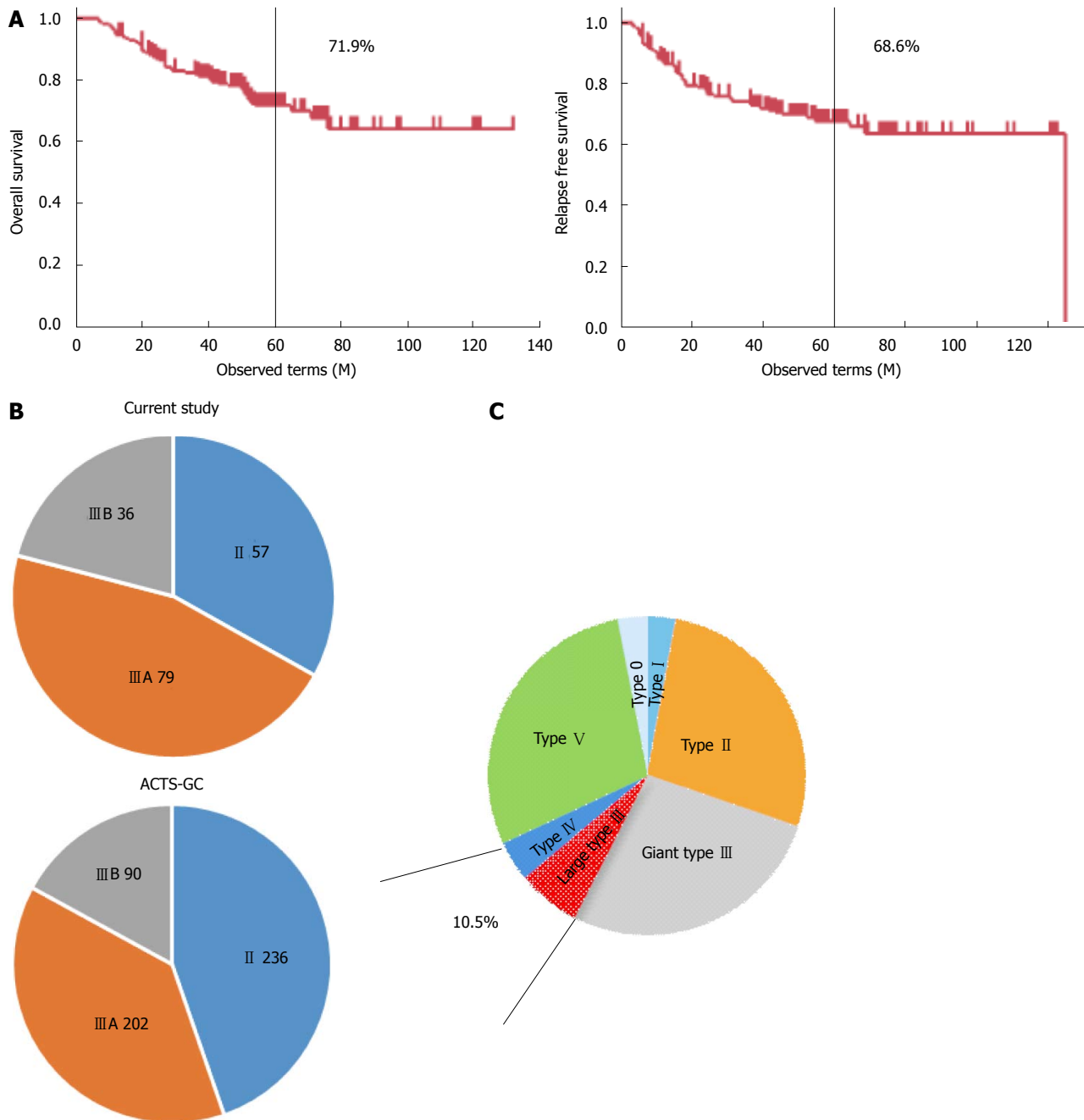
gastro-endoscopy based on the 13<sup>th</sup> JGCA classification<sup>[15]</sup> in combination with computed tomography (CT). Type 0, mucosal or submucosal; Type I, polypoid; Type II, fungating, ulcerated with sharp raised margins; Type III, ulcerated with poorly defined infiltrative margins; Type IV, infiltrative, predominantly intramural lesion, poorly demarcated; Type V, unclassified features. Representative tumors were shown in Figure 1. Giant type III was defined by its maximal diameter (8 cm or greater) assessed by upper gastrointestinal (UGI) barium contrast series as recently described<sup>[8]</sup>.

All histologic and clinicopathological factors were assessed independently and blindly by any of 20 well trained histopathologists. Lymphatic invasion (ly) and vascular invasion (v) were defined as ly0, 1, 2, and 3 and v0, 1, 2, and 3 by infiltrative grade. Histologically, there are two major types of gastric adenocarcinoma (Lauren's classification). In this study, cancers were classified into diffuse type (por1, por2, sig, muc) and intestinal type (pap, tub1, tub2).

### Statistical analysis

Cumulative 5-year OS was estimated by the Kaplan-Meier method, and statistical differences were tested by the log rank test. OS was measured from the date of surgery to the date of death or the last follow-up. Fatal cases in the analysis of OS included those who died from causes other than gastric cancer. Cumulative 5-year relapse free survival (RFS) was estimated by the Kaplan-Meier method, and statistical differences were tested by the log-rank test. RFS was measured from the date of surgery to the date of recurrence or the last follow-up. Deaths from other reasons were not defined as events for RFS.

Blood tests and physical examinations were done every



**Figure 2** Prognosis of pathological stage II/III advanced gastric cancer patients who underwent curative gastrectomy followed by S1 postoperative adjuvant chemotherapy. A: Kaplan-Meier curves for overall survival (OS) (upper panel) and relapse free survival (RFS). Five year survival is shown; B: Stage distribution of pathological stage according to the 13<sup>th</sup> Japanese Gastric Cancer Association stage in Kitasato University in comparison with the ACTS-GC trial; C: Rate of each macroscopic feature in pathological stage II/III advanced gastric cancer. High-risk macroscopic features (type IV and giant type III) are seen in 10.5% as shown in this figure.

3 mo and imaging examinations were performed every 6 mo. Blood tests included a complete blood count and serum biochemistry including tumor markers such as CEA, CA19-9, and CA125. Diagnosis of recurrences was based on clinical reports of radiologists with reference to clinical findings (symptoms and blood test) or histological findings.

The median observation was 56 mo (range, 11 to 122 mo). Variables that had prognostic potential on univariate analysis ( $P < 0.05$ ) were subjected to multivariate analysis with a Cox proportional hazards regression model. A value of  $P < 0.05$  was considered significant. All statistical analyses were done with JMP, version 11 (SAS Institute, Cary, NC).

## RESULTS

### **Prognosis of advanced gastric cancer patients with pathological stage II/III who underwent curative surgery followed by S1 postoperative adjuvant chemotherapy**

The prognosis of gastric cancer patients with pathological stage II/III who underwent curative gastrectomy followed by S1 adjuvant chemotherapy was investigated first. Pathological stage II/III cases did not include those with pathological stage II T1 gastric cancer. Five-year OS and 5-year RFS were 71.9% and 68.6%, respectively (Figure 2A). These survival rates are almost the same as the survival outcomes in the ACTS-GC trial (71.7%

**Table 1** Univariate prognostic analysis in pathological stage II/III advanced gastric cancer

Clinicopathological factors	Classification	Number	Univariate analysis (5-yr RFS)	Univariate analysis ( <i>P</i> value)	5-yr OS	<i>P</i> value
Age	Young	74	77.40%	0.0082	82.30%	0.0024
	Elderly	98	56.90%		58.10%	
Sex	Male	120	62.50%	0.018	63.50%	0.0054
	Female	52	81.90%		83.10%	
Tumor location	Upper	54	59.60%	0.12	81.10%	0.027
	Middle	74	68.40%		76.10%	
	Lower	44	80.50%		59.20%	
Method	Total	100	67.40%	0.51	69.00%	0.18
	Distal	72	70.00%		76.00%	
Lymphadenectomy	D1	10	68.60%	0.95	56.00%	0.53
	D1+	16	64.30%		79.60%	
	D2	146	69.00%		72.20%	
Laparoscopic	Yes	25	77.30%	0.16	77.30%	0.2
	No	147	67.00%		70.90%	
Splenectomy	Yes	51	61.50%	0.2	65.80%	0.31
	No	121	71.50%		74.50%	
Transfusion	Yes	23	63.90%	0.58	68.00%	0.37
	No	149	69.30%		72.70%	
13 <sup>th</sup> JGCA pT	T2	65	80.30%	0.019	83.00%	0.021
	T3	105	61.90%		65.80%	
	T4	2	50.00%		50.00%	
13 <sup>th</sup> JGCA pN	N0	24	90.50%	0.0043	89.40%	0.022
	N1	82	74.00%		77.30%	
	N2	66	54.30%		59.10%	
13 <sup>th</sup> JGCA pStage	II	57	92.10%	< 0.0001	86.80%	< 0.0001
	III A	79	63.90%		76.00%	
	III B	36	43.00%		42.80%	
Lauren histology	Intestinal	60	63.60%	0.23	68.30%	0.27
	Diffuse	112	71.30%		74.20%	
INF	Alpha	13	76.90%	0.83	84.60%	0.53
	Beta	75	69.90%		77.50%	
	Gamma	84	66.40%		67.50%	
Lymphatic invasion	ly0	9	100.00%	0.2	100.00%	0.19
	ly1	45	74.80%		77.10%	
	ly2	62	63.70%		71.20%	
	ly3	56	63.90%		63.60%	
Vascular invasion	v0	16	87.10%	0.055	85.60%	0.043
	v1	55	69.90%		65.20%	
	v2	55	72.80%		83.50%	
	v3	46	55.70%		62.70%	
Macroscopic feature	High risk	18	35.70%	0.0003	34.00%	< 0.0001
	Average risk	154	72.60%		76.60%	

JGCA: Japanese Gastric Cancer Association.

and 65.4%)<sup>[3]</sup>. On the other hand, the stage distribution included a lower rate of stage II gastric cancer and a higher rate of stage III gastric cancer than in the ACTS-GC trial (Figure 2B). These findings indicated that the patient population treated in our institute included more advanced gastric cancer than the ACTS-GC trial.

#### **Classification of macroscopic features in pathological stage II/III advanced gastric cancer**

Retrospective diagnosis with regard to the macroscopic features of gastric cancer was done by review of the recorded gastroscopic images in combination with the CT scan images (if primary tumors were visible on CT scan images, they were considered type I to IV macroscopic features, not type 0 macroscopic features). Among the type III macroscopic features, maximal tumor size was assessed by UGI series, and tumors with size of 8 cm or

beyond were defined as giant type III gastric cancers as previously described<sup>[8]</sup>. As a result, high risk macroscopic features (type IV and giant type III) were identified in 18 cases (10.5%) (Figure 2C).

#### **Multivariate Cox proportional hazards model for RFS identified macroscopic high risk as an independent prognostic factor in pathological stage II/III gastric cancer**

RFS was compared with regard to various clinicopathological factors including macroscopic features (Table 1). There was a significant difference in RFS ( $P = 0.0003$ ) between macroscopic high risk gastric cancer and average risk gastric cancer (Figure 3A). Five-year RFS of macroscopic high-risk gastric cancer was 35.7%, while that of average-risk gastric cancer was 72.6%. Other negative prognostic factors were older age ( $P = 0.0082$ ),

**Table 2** Multivariate Cox proportional hazards model in pathological stage II/III advanced gastric cancer

Clinicopathological factors	Classification	Number	Multivariate analysis for PFS (Hazard ratio)	Multivariate analysis for OS (95%CI)	P value	Hazard ratio	95%CI	P value
Age	Young	74	Reference		0.029	Reference		0.008
	Elderly	98	1.83	1.07-3.20		2.35	1.25-4.58	
Sex	Male	120	Reference	1.06-4.34	0.031	Reference	0.87-4.91	0.11
	Female	52	2.05			1.93		
Tumor location	Upper	54				2.84	1.24-7.19	0.013
	Middle	74				1.73	0.70-4.66	0.24
	Lower	44				Reference		
13 <sup>th</sup> JGCA pStage	II	57	Reference			Reference		
	III A	79	6.17	2.42-20.83	< 0.0001	2.25	0.93-6.27	0.08
	III B	36	8.48	3.11-29.70	< 0.0001	4.81	1.79-14.72	0.002
Vascular invasion	v0	16				Reference		
	v1	55				1.46	0.38-9.55	0.62
	v2	55				0.71	0.17-4.80	0.68
	v3	46				1.34	0.33-9.01	0.71
Macroscopic feature	High risk	18	2.29	1.06-4.63	0.036	2.74	1.17-6.15	0.021
	Average risk	154	Reference			Reference		

JGCA: Japanese Gastric Cancer Association.

male sex ( $P = 0.018$ ), 13<sup>th</sup> JGCA pT ( $P = 0.019$ ), 13<sup>th</sup> JGCA pN ( $P = 0.0043$ ), and 13<sup>th</sup> JGCA stage ( $P < 0.0001$ ). These significant prognostic factors for RFS excluding TNM factor components were applied to a multivariate Cox proportional hazards model, which identified the 13<sup>th</sup> JGCA stage ( $P < 0.0001$ ), macroscopic high risk ( $P = 0.036$ ), sex ( $P = 0.031$ ), and age ( $P = 0.029$ ) as independent prognostic factors as shown in Table 2. Kaplan-Meier survival curves are shown in terms of age (left panel of Figure 3B), sex (left panel of Figure 3C), and 13<sup>th</sup> JGCA stage (left panel of Figure 3D).

#### **Multivariate Cox proportional hazards model for OS identified macroscopic high risk as an independent prognostic factor in pathological stage II/III gastric cancer**

OS was compared with regard to various clinicopathological factors including macroscopic features (Table 1). There was significant difference in OS ( $P < 0.0001$ ) between macroscopic high risk gastric cancer and average risk gastric cancer (Figure 3A). Five-year OS of macroscopic high risk gastric cancer was 34.0%, while that of average-risk gastric cancer was 76.6%. Other negative prognostic factors were older age ( $P = 0.0024$ ), male sex ( $P = 0.0054$ ), tumor location ( $P = 0.027$ ), 13<sup>th</sup> JGCA pT ( $P = 0.021$ ), 13<sup>th</sup> JGCA pN ( $P = 0.022$ ), 13<sup>th</sup> JGCA stage ( $P < 0.0001$ ), and vascular permeation ( $P = 0.043$ ). These significant prognostic factors for OS excluding each TNM factor components were applied to the multivariate Cox proportional hazards model, which identified the 13<sup>th</sup> JGCA stage ( $P = 0.0015$ ), macroscopic high risk ( $P = 0.021$ ), age ( $P = 0.0082$ ), and tumor location ( $P = 0.013$ ) as independent prognostic factors as shown in Table 2. Each TNM factor was excluded, because these 3 factors are confounders for stage definition. Kaplan-Meier survival curves are shown in terms of age (right panel of Figure 3B), sex (right panel of Figure 3C), and 13<sup>th</sup> JGCA stage (right panel of Figure 3D).

#### **Clinicopathological features of macroscopic high risk among pathological stage II/III gastric cancer patients who underwent standard treatment**

Clinicopathological backgrounds with regard to the negative prognostic factors were then compared between the high-risk group and the average-risk group (Table 3). The macroscopic high-risk group included more patients with higher pathological T ( $P = 0.0025$ ), and higher 13<sup>th</sup> JGCA pathological stage ( $P = 0.0004$ ), while there were no significant differences in pN distribution and lymph node dissection level between the macroscopic high-risk group and the average-risk group. In our previous reports, lymph node dissection level was proven not to affect prognosis in these 172 cases<sup>[11]</sup>.

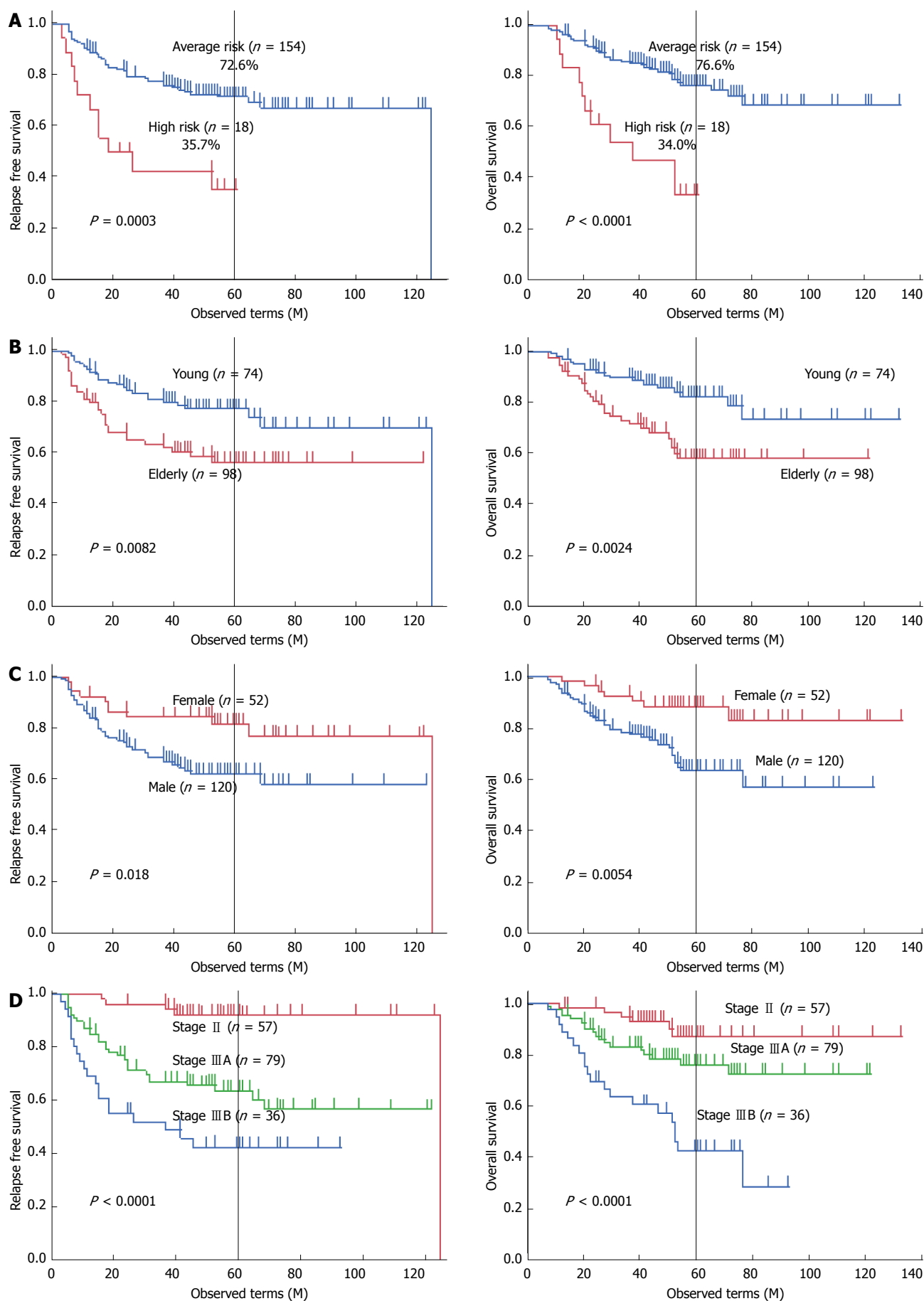
#### **Recurrence patterns of macroscopic high risk gastric cancer**

Recurrent cases were seen in 11 out of 18 cases with macroscopic high risk (Table 4). The 11 cases were composed of 7 giant type III gastric cancers and 4 type IV gastric cancers. Giant type III gastric cancer tended to have extra-regional lymph node recurrences, while type IV gastric cancer had peritoneal dissemination. We recently reported RTKs expression in gastric cancer, and HER3 and EGFR were of prognostic relevance in pathological stage II/III advanced gastric cancer<sup>[12]</sup>. The expression patterns of RTKs such as EGFR, HER2, HER3, IGF1R and EphA2 are also included in Table 4 from the previous studies<sup>[12]</sup>. Among the 11 recurrent cases, 9 showed strong expression (2+/3+) of EGFR, and 10 showed positive immunostaining (1+/2+) for HER3, which were both remnant independent prognostic factors in pathological stage II/III advanced gastric cancer<sup>[12]</sup>.

## **DISCUSSION**

This study reported for the first time the outcomes of macroscopic high-risk gastric cancer (giant type III and





**Figure 3** Survival curve of independent prognostic factors with regard to relapse free survival (left panel) and overall survival (right panel). A: Survival curve according to macroscopic features for the high-risk group and the average-risk group. Five-year survival is shown; B: Survival curve by age; C: Survival curve by sex; D: Survival curve by pathological stage according to the 13<sup>th</sup> JGCA stage. JGCA: Japanese Gastric Cancer Association.

**Table 3** Relations of high risk macroscopic features to prognostic factors in pathological stage II/III advanced gastric cancer

Clinicopathological factors	Classification	Number	High risk gastric cancer <i>n</i> = 18	Average risk gastric cancer <i>n</i> = 154	<i>P</i> value
Age	Young	74	8	66	0.26
	Elderly	98	10	88	
Sex	Male	120	13	107	0.81
	Female	52	5	47	
Lymphadenectomy	D1	26	4	22	0.37
	D2	146	14	132	
Tumor location	Upper	54	3	51	0.32
	Middle	74	9	65	
	Lower	44	6	38	
13 <sup>th</sup> JGCA pT	T2	65	1	64	0.0025
	T3	105	17	88	
	T4	2	0	2	
13 <sup>th</sup> JGCA pN	N0	24	2	22	0.11
	N1	82	5	77	
	N2	66	11	55	
13 <sup>th</sup> JGCA pStage	II	57	3	54	0.0004
	III A	79	4	76	
	III B	36	11	25	
7 <sup>th</sup> UICC pT	T2	29	0	29	0.02
	T3	36	1	35	
	T4a	105	17	88	
7 <sup>th</sup> UICC pN	T4b	2	0	2	0.08
	N0	24	2	22	
	N1	45	1	44	
7 <sup>th</sup> UICC pStage	N2	40	4	36	< 0.0001
	N3	63	11	52	
	II A	13	0	13	
	II B	37	2	35	
	III A	46	2	44	
	III B	38	3	35	
Vascular invasion	III C	38	11	27	0.94
	v0	16	1	15	
	v1	55	6	49	
	v2	55	6	49	
	v3	46	5	41	

JGCA: Japanese Gastric Cancer Association.

type IV) treated by “local” standard therapy in Japan (or partly in some Asian countries) in stage II/III advanced gastric cancer. The ACTS-GC trial demonstrated that postoperative S1 chemotherapy could improve the prognosis of pathological stage II/III advanced gastric cancer<sup>[2,3]</sup>, but there has been no report on the prognosis of macroscopic high risk gastric cancer patients with pathological stage II/III who underwent standard treatment. In this study, 5-year RFS and OS of the macroscopic high-risk group were 35.7% and 34.0%, respectively, and the prognosis of gastric cancer patients with macroscopic high-risk was significantly poorer than that of those with average risk (72.6% and 76.6%, respectively). These results suggest that the present S1 postoperative chemotherapy is not sufficient to control such high risk disease, and novel therapeutic strategies are needed.

In the Western world, perioperative ECF chemotherapy has been shown to improve survival of gastric cancer patients when, ECF chemotherapy was compared to surgery alone<sup>[10]</sup>. Gastric cancer with ECF chemotherapy showed 5-year OS of 36.3%, compared to 23.0% for surgery alone. This outcome is totally different from average-risk advanced gastric cancer in the Eastern

world, with an OS of 60%-70% of OS, whereas it is similar to gastric cancer with macroscopic high-risk. In the present cases, gastric cancer patients who were peritoneal cytology test-positive were excluded, because it represents stage IV in Japan, while the MAGIC trial may have included cytology test positive cases. In any case, the MAGIC trial demonstrated that potent preoperative chemotherapy has a great clinical potential in aggressive gastric cancer. In Japan, preoperative neoadjuvant chemotherapy was evaluated to validate the actual clinical effects including improvement of prognosis in very limited gastric cancer such as macroscopic high risk gastric cancer, namely giant type III and type IV gastric cancer<sup>[9]</sup>; CS (cisplatin/S1) neoadjuvant chemotherapy was proposed as an effective regimens in gastric cancer with macroscopic high risk, and 5-year survival was recently reported to be around 30% in JCOG0210. This is inferior to our standard therapy results, likely because peritoneal cytology test negativity was not mandatory to register in JCOG0210.

Neoadjuvant therapy is a promising therapeutic strategy for giant type III and type IV gastric cancer. We have developed a docetaxel/cisplatin/S1 (DCS) chemotherapeutic regimen in metastatic gastric cancer<sup>[16]</sup>, and

**Table 4** Initial recurrent sites and RTKs expression in high risk gastric cancer with relapse

Case	Age	Sex	13 <sup>th</sup> JGCA pT	13 <sup>th</sup> JGCA pN	13 <sup>th</sup> JGCA pStage	Macroscopic features	EGFR	HER2	HER3	IGF1R	EphA2	Initial recurrences
1	74	M	3	2	III B	Giant type III	2+	1+	2+	2+	1+	#16 LN
2	62	M	3	2	III B	Giant type III	2+	0+	1+	2+	0+	#20 LN
3	79	F	3	2	III B	Giant type III	2+	1+	1+	0+	1+	#13 LN
4	68	M	3	2	III B	Giant type III	3+	1+	1+	1+	0+	#16,13
5	68	M	3	2	III B	Giant type III	3+	0+	1+	1+	1+	#13 LN
6	45	M	3	2	III B	Giant type III	2+	3+	2+	0+	2+	#13 LN
7	69	M	3	2	III B	Giant type III	3+	3+	1+	1+	2+	liver
8	71	M	3	1	III A	Type IV	2+	0+	1+	1+	0+	#13 LN
9	69	F	3	1	III A	Type IV	2+	0+	2+	1+	0+	Peritoneum
10	69	M	3	1	III A	Type IV	1+	2+	0+	1+	1+	Peritoneum
11	59	F	3	2	III B	Type IV	1+	1+	1+	1+	0+	Peritoneum

JGCA: Japanese Gastric Cancer Association; M: Male; F: Female; EGFR: Epidermal growth factor receptor; HER2: Human epidermal growth factor receptor-2; IGF1R: Insulin-like growth factor 1.

KDOG1001 was developed to validate the clinical effect of DCS NAC in aggressive gastric cancer including giant type III and type IV. We are registering patients in this clinical phase II trial for such high-risk patients, and registration has almost been completed. DCS was recently compared to CS in neoadjuvant settings in high-risk gastric cancer with bulky N2 disease in JCOG1002, and detailed results of the clinical outcomes will be available soon. The first report of patients with high-risk advanced gastric cancer who underwent CS neoadjuvant chemotherapy should appear in April, 2017. Such potent chemotherapy would have a promising potential to improve the prognosis of aggressive gastric cancer.

Another therapeutic strategy we can propose in such aggressive gastric cancer is long-term postoperative adjuvant S1 chemotherapy<sup>[17,18]</sup>. Gastric cancer that was cytology test-positive (CY1) or type IV showed a dismal prognosis, but detailed prognostic analysis showed that there were long-term survivors among the patients who underwent long-term postoperative adjuvant S1 chemotherapy. Okuyama *et al.*<sup>[19]</sup> actually showed that 2-year administration of postoperative chemotherapy showed a better prognosis than 1-year administration in gastric cancer. This strategy might be very promising due to its easy feasibility, and should be considered as another therapeutic option. Giant type III and type IV gastric cancers are unique in their recurrence patterns, because minimal residual peritoneal disease is fundamental with regard to disease progression<sup>[8,18]</sup>. This means that minimal residual disease of the peritoneum should be a primary therapeutic target. S1 is more effective against peritoneal disease than against other distant metastases such as liver metastases<sup>[2]</sup> due to unknown mechanisms, thus, long-term S1 administration may be a reasonable rationale in macroscopic high-risk gastric cancer.

We previously identified HER3 immunostaining positive (defined as +1/+2 immunostaining) as an independent prognostic factor, and HER3 could be a promising therapeutic target<sup>[12]</sup>. HER2 immunostaining (defined as +3 immunostaining) is the well-established molecular target in far advanced gastric cancer using trastuzumab<sup>[20]</sup>, but

HER2-positive cases are infrequently found in recurrent gastric cancer<sup>[12]</sup>. Even in high-risk advanced gastric cancer, HER2-positive cases were infrequently seen (Table 4), while HER3-positive cases were frequently found. Moreover, EGFR-positive (defined as +2/+3) together with HER3-positive showed a dismal prognosis in advanced gastric cancer with pathological stage II/III<sup>[12]</sup>, and EGFR-positive together with HER3-positive was found in 9 of 11 recurrent cases among the high-risk advanced gastric cancer patients in this study. We are now investigating in vitro efficacy for tumor reduction by using cetuximab together with HER3 antibody. The combination treatments could have potential in the recurrent cases of high-risk gastric cancer.

The limitations of this study were that it was a single-center study, and the follow-up period was insufficient for definitive conclusions. Moreover, the sample size was small, especially for high-risk advanced gastric cancer. If this result is validated in a larger sample size in the near future, the conclusions would be strengthened. Moreover, this study only collected patients who underwent curative surgery plus adjuvant S1 chemotherapy, we didn't mention if these results can be seen from other patients with advanced gastric cancer.

In conclusion, this study demonstrated for the first time that macroscopic high-risk gastric cancer showed a poorer prognosis than average risk gastric cancer, and a novel therapeutic strategy should be urgently developed in order to improve outcomes in such cases in the near future.

## COMMENTS

### Background

High risk macroscopic appearance (giant type III and type IV) is known to show dismal prognosis in advanced gastric cancer, however it remains elusive whether it is true or not in advanced gastric cancer who underwent curative gastrectomy and the latest evidenced postoperative S1 adjuvant chemotherapy.

### Research frontiers

This study investigated whether the high risk macroscopic appearance could be an independent prognostic factor in advanced gastric cancer who underwent

curative gastrectomy and postoperative adjuvant chemotherapy.

### Innovations and breakthroughs

Macroscopic appearance can be preoperatively diagnosed, and it could be designated as a kind of preoperative surrogate marker for prognosis.

### Applications

Macroscopic appearance is a good candidate for promising therapeutic strategy of neoadjuvant chemotherapy, the novel method in East Asia, if it is true.

### Terminology

The size of the giant type III gastric cancer is defined as 8 cm or beyond in the preoperative imaging such as endoscopy, upper gastrointestinal series, and/or computed tomography.

### Peer-review

Yamashita *et al* presented a study title as "Macroscopic appearance of Type IV and giant Type III is a high risk for a poor prognosis in pathological stage II /III advanced gastric cancer with postoperative adjuvant chemotherapy". The study has some new and interesting findings which authors believe they add some contribution to the literature. Authors were well summarized results, they have novel findings and discussion was pretty good.

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Prospective Study

# Incidence of venous thromboembolism and the role of D-dimer as predictive marker in patients with advanced gastric cancer receiving chemotherapy: A prospective study

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**Author contributions:** Kang MJ designed the research; Park K and Ryu MH wrote the study; Park SR, Kim JH and Kang YK supported clinical data and clinical advice; Han S contributed to the statistical analysis; Ryoo BY designed the research and supervised the report.

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## Abstract

### AIM

To investigate the incidence and risk factors of venous thromboembolism (VTE) in patients with advanced gastric cancer (AGC) receiving chemotherapy.

### METHODS

All consecutive chemotherapy-naïve patients with AGC who would receive palliative chemotherapy between November 2009 and April 2012 in our hospital were recruited. Their pretreatment clinical and laboratory variables, including D-dimer, were recorded. The frequency of VTE development and survival rates during each chemotherapy cycle and regularly thereafter were assessed.

### RESULTS

A total of 241 patients enrolled between November 2009

and April 2012 were analyzed. During a median follow-up duration of 10.8 mo (95%CI: 9.9-11.7), 27 patients developed VTE and the incidence of VTE was 17.5% (95%CI: 10.5-24.0, 12.0 events/100 person-years). The 6-mo and 1-year cumulative incidences were 7.8% (95%CI: 4.2%-11.4%) and 12.4% (95%CI: 7.3-17.2), respectively. Thirteen (48.1%) patients were symptomatic and the other 14 (51.9%) patients were asymptomatic. In multivariate analysis, pretreatment D-dimer level was the only marginally significant risk factor associated with VTE development (hazard ratio = 1.32; 95%CI: 1.00-1.75,  $P = 0.051$ ).

### CONCLUSION

The incidence of VTE is relatively high in patients with AGC receiving chemotherapy, and pretreatment D-dimer level might be a biomarker for risk stratification of VTE.

**Key words:** Advanced gastric cancer; D-dimer; Venous thromboembolism

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**Core tip:** The incidence of venous thromboembolism (VTE) is relatively high in patients with advanced gastric cancer receiving chemotherapy, and pretreatment D-dimer level is a risk factor for VTE. Considering the usefulness of D-dimer as a biomarker given its ease of use and low cost, pretreatment D-dimer might be a risk stratification factor for VTE development and patient selection for thromboprophylaxis.

Park K, Ryoo BY, Ryu MH, Park SR, Kang MJ, Kim JH, Han S, Kang YK. Incidence of venous thromboembolism and the role of D-dimer as predictive marker in patients with advanced gastric cancer receiving chemotherapy: A prospective study. *World J Gastrointest Oncol* 2017; 9(4): 176-183 Available from: URL: <http://www.wjgnet.com/1948-5204/full/v9/i4/176.htm> DOI: <http://dx.doi.org/10.4251/wjgo.v9.i4.176>

### INTRODUCTION

In general, patients with cancer carry a significantly higher risk of venous thromboembolism (VTE) than case-control subjects without cancer<sup>[1,2]</sup>. Gastrointestinal cancers including gastric cancer were ranked third in incidence of VTE, following hematological malignancies and lung cancer<sup>[1]</sup>. Lee *et al*<sup>[3]</sup> reported that patients with advanced gastric cancer (AGC) had a much higher likelihood of developing VTE (24.4%) than patients with lower-stage gastric cancer (0.5%-3.5%), and Blom *et al*<sup>[1]</sup> observed that chemotherapy increased the VTE risk 2.2-fold. These findings suggest that VTE might be more common in patients with AGC receiving chemotherapy, who have several potential risk factors of VTE development including cancer, especially highly vulnerable gastric cancer, advanced stage, and chemotherapy. On the other hand,

patients with cancer who develop VTE also have shorter survival durations than those who do not develop VTE<sup>[4-6]</sup>. Activation of hemostasis, as indicated by development of VTE reflects more aggressive tumor biology<sup>[7]</sup>, additionally, VTE development might hinder the continuation of chemotherapy, resulting in poor outcomes. Considering the relatively high incidence of VTE and its impact on survival, information about the VTE is important in patients with AGC receiving chemotherapy. While, the information about VTE in this cohort is not yet clear. Because most of the previous results were retrospectively analyzed with heterogeneous population and included this cohort as a part of small fraction among heterogeneous various groups<sup>[8-11]</sup>.

It has been challenging for oncologists to conduct a practical use of thromboprophylaxis effectively to prevent VTE. The major problem is that the increased rates of complications such as bleeding outweigh its efficacy<sup>[12]</sup>. Thus, we must more precisely target thromboprophylaxis, especially in gastric cancer, since it has a high risk of bleeding at the endothelial lesion. Therefore, we need to confirm the exact incidence and identify the predictive factors of future VTE in this cohort. To address these issues, we conducted this prospective cohort study to determine the exact incidence and risk factors of VTE in patients with AGC who are undergoing palliative chemotherapy and assess whether VTE development correlates with survival.

### MATERIALS AND METHODS

#### Study design and patients

This is a prospective observational single-center study. The cohort consisted of all consecutive patients with histologically confirmed adenocarcinoma of the stomach or esophagogastric junction that was in an advanced state (*e.g.*, initially metastatic, locally inoperable, or recurrent), and who started palliative chemotherapy between November 2009 and April 2012 at Asan Medical Center, South Korea. All patients were chemotherapy-naïve or had undergone adjuvant chemotherapy 6 mo before being included in the study. All patients had adequate bone marrow, renal, hepatic, and cardiac functions that would allow them to withstand chemotherapy. The following patients were excluded: Those who presented initially with brain metastasis, had been treated with warfarin or low-molecular-weight heparin 4 wk before being screened for inclusion in the study, had undergone major surgery or experienced significant traumatic injuries 4 wk before screening, or were lost to follow up during the first two weeks without evidence of disease progression or VTE. All included patients were observed prospectively for at least 1 year after enrollment of the last patient or until death, loss of follow-up, or withdrawal of consent occurred. The study was approved by the institutional review board of Asan Medical Center and conducted in accordance with the Declaration of Helsinki and Good Clinical Practice guidelines (ClinicalTrials.gov identifier: NCT01047618).

All participants provided written informed consent before enrollment.

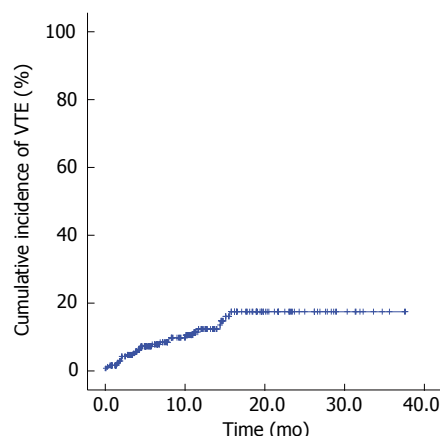
### Treatment and work-up

The chemotherapy regimens mainly included fluoropyrimidine plus platinum-based for the 1<sup>st</sup>-line, taxane-based for the 2<sup>nd</sup>-line, and irinotecan-based for the 3<sup>rd</sup>-line chemotherapy. Each chemotherapy line was continued until disease progression, intolerable toxicity, patient demand, or the attending physician's decision. Routine erythropoiesis-stimulating agent was not applied during the study period.

Within one week before starting palliative chemotherapy, we checked the clinicopathologic factors and laboratory tests including D-dimer. Close history taking, a physical examination, CBC, and chemistry analyses were performed at every chemotherapy cycle and regularly during the follow-up period. The response of each patient to the treatment was assessed every 6 wk. When VTE was suspected due to a constellation of new clinical symptoms or signs, imaging analyses were performed. Doppler sonography and/or CT venography were used to detect DVT, while chest CT and/or pulmonary artery CT angiography were employed to detect pulmonary embolism (PE). Incidentally detected VTE was also counted as an event.

### Statistical analysis

The primary endpoint of this study was the incidence of VTE in patients with AGC receiving chemotherapy, and the secondary endpoints were to identify risk factors of VTE and to evaluate the impact of VTE on survival in this cohort. For risk factor analysis of VTE, we used time to VTE to discriminate early vs delayed development considering the different clinical significance. The baseline characteristics such as baseline biomarkers levels, clinical parameter, cancer diagnosis information, medication and therapy are expressed as the median value with a range (continuous variables) or frequency with proportion (%) (categorical variables). The incidence of VTE was calculated as both cumulative incidence and person-time (events/100 person-years). The statistical significance of the difference in characteristic between symptomatic and incidental VTE was assessed using the  $\chi^2$  test or Fisher's exact test. The time to VTE or overall survival (OS) were measured from the date of first chemotherapy to the date of diagnosis of VTE or death or were censored at the last follow-up date. The time to event data were summarized using the Kaplan-Meier method. Uni- and multivariate Cox regression models were used to detect the association between clinical or pretreatment factors and time to VTE or OS. In the multivariate regression model, all potential factors with a *P* value  $\leq 0.15$  on univariate analyses were employed to detect adjustment factors. The final models were determined by backward elimination. In the regression modeling, log-transformation for severely skewed variables was used to obtain more stable regression coefficients. All statistical analyses were



**Figure 1** Cumulative incidence of venous thromboembolism. VTE: Venous thromboembolism.

performed by using SPSS software, version 21.0 (SPSS Inc., Chicago, IL, United States) and R software 2.10.1. All reported *P* values are two-sided and *P* values  $< 0.05$  are considered to be statistically significant.

## RESULTS

### Patient characteristics and anticancer treatment

Between November 2009 and April 2012, a total of 261 consecutive patients were enrolled in this study. Of these patients, 20 (7.7%) did not receive chemotherapy or were lost to follow-up during the first 2 wk without evidence of disease progression or DVT, so they were excluded from the analysis. The remaining 241 patients had a median age of 56 years (range, 24-83 years) and 169 patients (70.1%) were male. The pretreatment clinicopathological and laboratory characteristics of all patients and those who developed VTE during palliative chemotherapy are summarized in Table 1. All patients received one or more cycles of chemotherapy; the median numbers of 9 cycles (range, 1-42) and 2 lines (range, 1-4).

### Frequency and treatment of VTE

During a median follow-up duration of 10.8 mo (95%CI: 9.9-11.7), 27 patients developed VTE and the VTE incidence was 17.5% (95%CI: 10.5%-24.0%; 12.0 events/100 person-years). The 6-mo and 1-year cumulative incidences of VTE were 7.8% (95%CI: 4.2%-11.4%) and 12.4% (95%CI: 7.3-17.2%), respectively (Figure 1). Regarding VTE type, 19 of 27 patients (70.4%) developed deep vein thrombosis (DVT) only, 4 patients (14.8%) developed PE only, and 4 patients (14.8%) had both DVT and PE. The median time to VTE in these patients who developed VTE was 3.9 mo (95%CI: 2.8-5.1). VTE was detected within 3 mo in 10 patients (37.0%), from 3 to 6 mo in 6 patients (22.2%), from 6 mo to 1 year in 7 patients (25.9%), and after 1 year in the remaining 4 patients (14.8%). Thirteen patients (48.1%) had symptomatic VTE and the other 14 (51.9%) had incidentally detected VTE. A total of 25 patients received treatments for VTE, 22 took warfarin or

**Table 1** Patients' pretreatment characteristics and risk of venous thromboembolism on univariate analysis

Variable	Total patients	Patients with VTE	Risk of time to VTE		
			HR	95%CI	P value
Total, n (%)	241 (100.0)	27 (11.2)			
Age (yr)					
< 65	178 (73.9)	18 (10.1)			
≥ 65	63 (26.1)	9 (14.3)	1.632	0.732-3.641	0.231
Gender					
Male	169 (70.1)	18 (10.7)			
Female	72 (29.9)	9 (12.5)	1.052	0.472-2.345	0.9
ECOG PS					
0-1	229 (95.0%)	27 (11.8)			
2	12 (5.0%)	0	0.046	0.000-99.991	0.432
BMI					
< 25	202 (83.8)	23 (11.4)			
≥ 25	39 (16.2)	4 (10.3)	0.793	0.274-2.296	0.669
Previous CVC					
No	233 (96.7)	25 (10.7)			
Yes	8 (3.3)	2 (25.0)	3.13	0.738-13.266	0.122
Previous gastrectomy					
No	209 (86.7)	26 (12.4)			
Yes	32 (13.3)	1 (3.1)	0.196	0.027-1.448	0.11
Primary tumor site					
Antrum/pylorus	94 (39.0)	10 (10.6)			
Body	32 (13.3)	9 (11.0)	1.131	0.458-2.791	0.79
Cardia/fundus	28 (11.6)	3 (10.7)	1.138	0.313-4.144	0.844
Diffuse	25 (10.4)	5 (20.0)	2.413	0.823-7.073	0.108
Histology					
W/D or M/D	84 (34.9)	6 (7.1)			
P/D or SRC	154 (63.9)	21 (13.6)	2.084	0.840-5.166	0.113
Unclassified	3 (1.2)	0	NA		
Peritoneal seeding					
No	137 (56.8)	11 (8.0)			
Yes	104 (43.2)	16 (15.4)	1.945	0.902-4.191	0.09
Liver metastasis					
No	156 (64.7)	20 (12.8)			
Yes	85 (35.3)	7 (8.2)	0.741	0.313-1.754	0.495
Lung metastasis					
No	220 (91.3)	26 (11.8)			
Yes	21 (8.7)	1 (4.8)	0.509	0.069-3.764	0.509
Bone metastasis					
No	225 (93.4)	24 (10.7)			
Yes	16 (6.6)	3 (18.8)	2.344	0.701-7.835	0.167
Number of metastatic sites					
0-1	109 (45.2)	9 (8.3)			
≥ 2	132 (54.8)	18 (13.6)	1.898	0.851-1.898	0.118
CEA (log) median (range, ng/mL)	2.5 (0.3-8070)		1.133	0.947-1.355	0.173
CA19-9 (log) median (range, U/mL)	19.3 (1.4-30800)		0.882	0.726-1.073	0.209
CA72-4 (log) median (range, U/mL)	5.1 (1.7-6490)		1.099	0.885-1.364	0.395
Hb median (range, g/dL)	12.0 (7.0-17.6)		0.956	0.786-1.163	0.653
WBC median (range, × 10 <sup>9</sup> /L)	7100 (2600-19300)		1	1.000-1.000	0.026
Platelet median (range, × 10 <sup>9</sup> /L)	274 (107-731)		1.001	0.998-1.005	0.508
CRP median (range, mg/dL)	2.01 (0.10-19.22)		1.056	0.952-1.053	0.302
Fibrinogen (log) median (range, × 10 <sup>3</sup> /L)	360 (66-897)		1.132	0.337-3.796	0.841
PAI-1 (log) median (range, × 10 <sup>9</sup> /L)	35.0 (2.0-112.0)		1.197	0.662-2.165	0.551
D-dimer (log) median (range, × 10 <sup>9</sup> /L)	1.02 (0.06-82.3)		1.401	1.069-1.836	0.015

BMI: Body mass index; CI: Confidence interval; CRP: C-reactive protein; CVC: Central venous catheter; log: Log-transformation; M/D: Moderate differentiation; P/D: Poor differentiation; SRC: Signet ring cell; VTE: Venous thromboembolism; W/D: Well-differentiated.

low-molecular-weight heparin, and the other three were treated with an inferior vena cava filter. The remaining two patients were treated conservatively. VTE-associated delay or discontinuation of chemotherapy occurred in 10 patients and was significantly more common in patients with symptomatic VTE than in patients with incidental VTE ( $P =$

0.018). There were no cases of VTE-induced death (Table 2).

#### **Risk factors of VTE**

The pretreatment characteristics and laboratory data were assessed to determine the association with time to VTE development. In univariate analyses, log-transformed



**Table 2 Clinical features of venous thromboembolism in advanced gastric cancer patients receiving chemotherapy *n* (%)**

	Total ( <i>n</i> = 27)	Symptomatic VTE ( <i>n</i> = 13)	Incidental VTE ( <i>n</i> = 14)	<i>P</i> value
Time to VTE duration (median, mo)	6.1	7.5	4.7	0.16
VTE incidence				
< 3	10 (37.0)	4 (30.8)	6 (42.9)	0.68
3-6	6 (22.2)	3 (23.1)	3 (21.5)	
6-12	7 (25.9)	3 (23.1)	4 (28.6)	
> 12	4 (14.8)	3 (23.1)	1 (7.2)	
Types of VTE				
DVT	19 (70.4)	8 (61.5)	11 (78.6)	0.608
PTE	4 (14.8)	3 (23.1)	1 (7.2)	
PTE + DVT	4 (14.8)	2 (15.4)	2 (14.3)	
Treatment of VTE				
Medication (anticoagulation)	22 (81.4)	10 (76.9)	12 (75.7)	0.031
IVC filter	3 (11.1)	3 (23.1)	0	
No treatment	2 (7.5)	0	2 (14.3)	
Delay of chemotherapy				
None	17 (62.9)	5 (38.5)	12 (85.7)	0.018
Yes	10 (37.1)	8 (61.5)	2 (14.3)	

DVT: Deep vein thrombosis; PTE: Pulmonary thrombosis; VTE: Venous thromboembolism; IVC: Inferior vena cava.

**Table 3 Multivariate analysis of risk factors for venous thromboembolism<sup>1</sup>**

Variable	HR	95%CI	<i>P</i> value
Prior gastrectomy (no <i>vs</i> yes)	0.25	0.03-1.89	0.178
History of CVC (no <i>vs</i> yes)	2.21	0.51-9.50	0.286
D-dimer (log)	1.32	1.00-1.75	0.051

<sup>1</sup>All potential prognostic factors with a *P* value ≤ 0.15 on univariate analyses were entered into the multivariate Cox models. The final models were determined by backward elimination.

D-dimer levels and WBC count had a statistically significant association with time to VTE (Table 1). Prior history of central venous catheter, differentiation, prior history of gastrectomy, peritoneal seeding, and number of metastatic sites trended toward a potential risk factor for VTE (*P* ≤ 0.15). In multivariate analysis, any prognostic factors were not statistically significant, but the log transformed D-dimer level was only a marginally significant risk factor in the final model (HR = 1.32, 95%CI: 1.00-1.75, *P* = 0.051) (Table 3).

In case of patients with VTE, the mean levels of D-dimer were 4.19 × 10<sup>9</sup>/L (baseline) and 11.18 × 10<sup>9</sup>/L (at the time of VTE development), respectively, with a significant increase of D-dimer levels at the time of VTE development (*P* = 0.004). Additionally, according to symptom development of VTE, D-dimer levels were increased significantly in patients with symptomatic VTE (*P* = 0.004), on the other hand, those in patients with incidental VTE showed only numerical increase (*P* = 0.198) (Table 4).

There was no significant difference in the time to VTE according to fluoropyrimidine or different platinum use as the 1<sup>st</sup>-line treatment or the addition of targeted agents or angiogenesis inhibitors (Table 5).

### VTE as a prognostic factor

During a median observational duration of 13.8 mo

**Table 4 Comparisons of D-dimer levels between baseline and time of venous thromboembolism development**

	Total ( <i>n</i> = 27)	Symptomatic VTE ( <i>n</i> = 13)	Incidental VTE ( <i>n</i> = 14)	<i>P</i> value
Baseline D-dimer	4.19	3.62	4.72	0.835
D-dimer at the time of VTE development	11.18	14.11	8.45	0.436
	<i>P</i> value	<i>P</i> value	<i>P</i> value	
	0.004	0.01	0.198	

VTE: Venous thromboembolism.

(95%CI: 11.6-14.8), the median OS was 14.2 mo (95%CI: 11.8-16.6). There was no significant difference in OS between patients with and those without VTE (13.8 mo, 95%CI: 9.3-18.3; 14.2 mo, 95%CI: 11.7-16.7, *P* = 0.559) (Figure 2). According to symptom development of VTE, patients with symptomatic VTE was not also noted statistically significant difference in OS compared with patients without VTE (*P* = 0.337). According to VTE type, there was no significant difference in OS between patients with DVT alone and with PE alone or PE/DVT (*P* = 0.597). In the analysis that excluded four patients who developed VTE after 12 mo, there was no significant difference in OS between patients with or without VTE (*P* = 0.609). On the contrary, in the analysis that excluded 10 patients who developed VTE before 3 mo, there was no significant difference in OS between patients with or without VTE (*P* = 0.337).

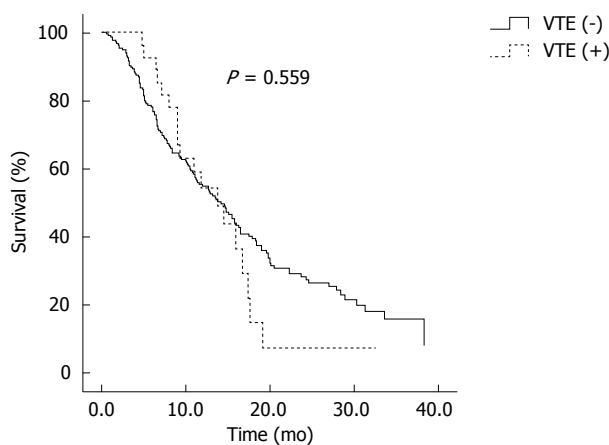
## DISCUSSION

The current study assessed the incidence and risk factors of VTE as well as the influence of VTE on survival in AGC patients undergoing palliative chemotherapy. To our knowledge, this is the first prospective study to evaluate VTE in a homogeneous cohort. This study showed a relatively high cumulative incidence of VTE and the role

**Table 5** Time to venous thromboembolism according to chemotherapeutic agent used for 1<sup>st</sup>-line chemotherapy

Variable	Patients (n)	Patients with VTE n (%)	Risk of time to VTE		
			HR	95%CI	P value
Fluoropyrimidine					
SP	96	11 (11.5)			
XP	22	3 (13.6)	1.67	0.47-6.18	0.422
Platinum					
XP	22	3 (13.6)			
XELOX	27	4 (14.8)	0.94	0.21-4.25	0.94
Addition of targeted agents					
Conventional chemotherapy	158	17 (10.8)			
+ targeted agents	83	10 (12.0)	1.21	0.56-2.65	0.627
Addition of VEGFR inhibitors					
Conventional chemotherapy	217	27 (12.4)			
+ VEGFR inhibitors	24	0	0.04	0.00-12.13	0.227

SP: TS-1 + cisplatin; XP: Capecitabine + cisplatin; XELOX: Capecitabine + oxaliplatin, targeted agents, Vorinostat, sorafenib, Bevacizumab, or trastuzumab +/- pertuzumab; VEGFR: Inhibitors, sorafenib or bevacizumab; VTE: Venous thromboembolism.

**Figure 2** Overall survival according to the development of venous thromboembolism. VTE: Venous thromboembolism.

of pretreatment D-dimer as a risk factor for development of VTE. Although the development of VTE was not correlated with poor survival, patients with symptomatic VTE had more interruptions or delays of chemotherapy than those with incidental VTE.

The 6-mo, 1-year, and 2-year cumulative incidences of VTE were 7.8%, 12.3%, and 17.3%, respectively. Our results showed a relatively high incidence of VTE despite AGC being a representative frequent site in cancer-associated VTE<sup>[13]</sup>. In our previous report of retrospective analysis of a similar patient population, the 1- and 2-year cumulative incidences of venous VTE were 3.5% and 4.9%, respectively<sup>[8]</sup>. Other prior studies also reported VTE incidences of 5.3%-11.4%<sup>[14-16]</sup>, which was somewhat lower than our results. The reason for this discrepancy should be preferentially presumed that this study was conducted for homogeneous patients who were high tumor burden of advanced state and receiving palliative chemotherapy. Regarding tumor burden in cancer-associated VTE, Lee *et al.*<sup>[3]</sup> reported that the VTE incidence increased with stage in gastric cancer patients. Considering that stage is correlated with

tumor burden in cancer patients, we can give careful consideration to our results that a history of previous gastrectomy and multiple metastases were slightly related with the development of VTE, but the correlation was not statistically significant. Regarding chemotherapy of cancer-associated VTE, prior studies reported that chemotherapy is another important risk factor for VTE development in cancer patients<sup>[1,17]</sup>. In the current study, 13 (46.4%) and 20 (71.4%) patients developed VTE within 3 and 6 mo, respectively, which suggests that starting chemotherapy is an important risk factor for VTE development. Blom *et al.*<sup>[1]</sup> also reported that the risk of thrombosis was the highest in the first 3 mo after the diagnosis of cancer and declined thereafter, which supports the finding of the current study.

In multivariate analysis, pretreatment D-dimer level was the only marginally significant risk factor for VTE development. D-dimer, a general biomarker that globally indicates hemostasis and fibrinolysis activation, is a well-studied biomarker in the diagnosis and management of VTE in non-cancer patients<sup>[18]</sup>. Khorana *et al.*<sup>[17]</sup> proposed a predictive model for chemotherapy-associated VTE that included the primary cancer site, pretreatment platelet count, hemoglobin, leukocyte count, and body mass index. However, D-dimer level was not included in this model, which might be because the pretreatment D-dimer level was not routinely checked in the target population. In the subsequent studies, Ay *et al.*<sup>[19]</sup> reported that a high D-dimer level predicted occurrence of VTE in a variety of cancer patients, and Arpaia *et al.*<sup>[20]</sup> demonstrated that pretreatment D-dimer was correlated with chemotherapy-associated VTE. However, these studies only included a small number of gastric cancer patients, 35 of 821 (4.3%) and 10 of 124 (8.1%). Moreover, subcohorts of these patients were heterogeneous since the patients were treated variably with surgery or radiotherapy (or even untreated). Thus, the role of D-dimer as a risk factor of VTE in AGC patients receiving chemotherapy remains to be clarified. We demonstrated here that pretreatment D-dimer was indeed an independent risk factor of VTE in

this homogeneous cohort. This means that pretreatment D-dimer might be used to more precisely target patients for thromboprophylaxis and lead to enhanced use of thromboprophylaxis in AGC patients treated with palliative chemotherapy. On the other hand, prior established risk factors including the aforementioned Khorana score were not associated with VTE in this study (data not shown). A possible explanation might be the small sample size; however, the unique characteristics of stomach cancer or even ethical characteristics might also contribute.

The occurrence of VTE has been reported to have a significant adverse effect on survival in many studies<sup>[3,5,6]</sup>. However, we found no statistical difference in survival according to VTE development. The results did not change after adjustment for the presence of symptoms and signs, VTE type, or detection time. The reason for the negative result in the current study may be too small a sample size to detect a difference. On the other hand, due to the short survival of patients with advanced-stage disease, VTE may have a greatly reduced impact on survival. Previous studies reported that an impact of VTE on survival might be somewhat different from that in our study because they mainly included localized stage of cancer<sup>[3]</sup> or showed a prominent impact of VTE on survival in subgroups of patients with localized cancer<sup>[5]</sup> or in those with breast cancer, which has a characteristic longer survival duration<sup>[6]</sup>. Our previous retrospective study with a larger cohort of similar patients also reported that VTE was not a statistically significant factor for survival<sup>[8]</sup>. As such, whether VTE really has adverse effect on survival in this cohort should be further clarified. Meanwhile, more patients with symptomatic VTE than asymptomatic VTE had chemotherapy interruptions or delays. Although symptomatic VTE did not show poor survival in the subgroup analysis, we should make an effort to detect VTE before symptoms develop.

This study has several limitations. First, we did not evaluate serial measurements of D-dimer; thus, we could not identify the usefulness of the serial changes as a predictive marker for VTE. Considering that the current study showed that D-dimer level at the time of VTE development is significantly increased compared with that at baseline in patients with VTE, serial measurements of D-dimer might detect early changes and predict the development of VTE. Another issue is that the current study did not calculate the proper number of patients, so it might not have been adequately powered to detect statistically significant differences in other characteristics such as risk factors or survival. For these reasons, it is obvious that the present study might not be a confirmative, rather preliminary study for hypothesis generation. Despite these limitations, the current study showed the incidence of VTE and role of pretreatment D-dimer as risk factors in a homogeneous group of AGC patients receiving palliative chemotherapy. Considering the usefulness of D-dimer as a biomarker such as its ease of use and low cost, pretreatment D-dimer might be used as a risk stratification factor for VTE and in patient selection for thromboprophylaxis.

In conclusion, the incidence of VTE is relatively high in patients with AGC receiving chemotherapy, and pretreatment D-dimer level is a risk factor for VTE. Considering the usefulness of D-dimer as a biomarker given its ease of use and low cost, pretreatment D-dimer might be a risk stratification factor for VTE development and patient selection for thromboprophylaxis.

## COMMENTS

### Background

Patients with cancer, especially gastric cancer, are higher risk of venous thromboembolism (VTE). Among cancer, advanced status such as recurrent, metastatic, or locally advanced, is high risk factor and treatment with chemotherapy is also risk factor of VTE. So, information about the VTE in patients with advanced gastric cancer (AGC) receiving chemotherapy is important. However, there has been no report about this homogeneous group, so they planned this study.

### Research frontiers

This study is the first prospective study focused to evaluate VTE and risk factor of VTE in this homogeneous cohort. They reported an incidence of VTE and role of pretreatment D-dimer as risk factor.

### Innovations and breakthroughs

The incidence of VTE is relatively high in patients with AGC receiving chemotherapy 17.5% (95%CI: 10.5%-24.0%; 12.0 events/100 person-years), and pretreatment D-dimer level is a risk factor for VTE.

### Applications

Considering the usefulness of D-dimer as a biomarker given its ease of use and low cost, pretreatment D-dimer might be a risk stratification factor for VTE development and patient selection for thromboprophylaxis.

### Terminology

AGC: Gastric cancer of advanced status such as initially metastatic, locally inoperable, or recurrent.

### Peer-review

Naturally an increased level of circulating fibrin d-dimer may be a good parameter to control the evolution of gastric cancer venous originated metastasis.

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## ***Helicobacter pylori* recurrence after eradication in Latin America: Implications for gastric cancer prevention**

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### **Abstract**

#### **AIM**

To estimate *Helicobacter pylori* (*H. pylori*) recurrence rate in Latin America, a region with a significant *H. pylori* prevalence and gastric cancer burden.

#### **METHODS**

PubMed, LILACS, SciELO, Cochrane databases and abstracts from relevant meetings were reviewed. Information collected included: Participants' characteristics, recruitment strategy, diagnostic modality, treatment arms, follow-up and recurrence rates. Recurrence was calculated using 100-patients-year rates, and data were pooled using a random effects model. The  $I^2$  statistic assessed between study heterogeneity. Meta-regression analyses evaluated for effect modifying variables.

#### **RESULTS**

Literature search yielded 163 articles. Twelve studies involving 4848 patients from 9 countries met inclusion criteria. Four hundred and thirty-two reinfections were recorded in 5487 person-years of follow-up. Pooled analysis showed a recurrence rate of 7.9 cases per 100 person-years (95%CI: 5.3-10.5). Meta-regression revealed that neither the antibiotic schema, a second antibiotic course, nor the diagnostic modality had an impact on the observed risk of recurrence. The recurrence rate in the first year after treatment, predominantly recrudescence,

was 11.2 (6.1-16.4) per 100 patient years. Recurrence in subsequent years, was only 6.2 (3.8-8.7).

## CONCLUSION

*H. pylori* recurrence rates in Latin America are significant, and with geographic variability, yet are acceptable based upon the current literature for consideration of large scale intervention trials. Further research in Latin America is warranted to evaluate the efficacy, cost-effectiveness, and potential adverse outcomes of proposed eradication programs.

**Key words:** Gastric cancer; Reinfection; Hispanic; *Helicobacter pylori*; Latin America

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**Core tip:** Latin America has a high burden of gastric cancer mortality, with significant geographical variability, which offers the opportunity for prevention trials and interventions. Recent trials and meta-analysis show that *Helicobacter pylori* (*H. pylori*) eradication reduces the risk of gastric adenocarcinoma. *H. pylori* reinfection rates in Latin America are similar to those seen in Asian trials. Recurrent cases occur mostly within the first year suggesting treatment failure (re-growth), not reinfection. These findings were not significantly modified by diagnostic modality, the antibiotics selected, retreatment, or the time check for eradication success. Eradication programs are a potentially attractive strategy for gastric cancer prevention in Latin America.

Corral JE, Mera R, Dye CW, Morgan DR. *Helicobacter pylori* recurrence after eradication in Latin America: Implications for gastric cancer prevention. *World J Gastrointest Oncol* 2017; 9(4): 184-193 Available from: URL: <http://www.wjgnet.com/1948-5204/full/v9/i4/184.htm> DOI: <http://dx.doi.org/10.4251/wjgo.v9.i4.184>

## INTRODUCTION

Gastric cancer is the third most common cause of cancer mortality globally, and the leading infection-associated cancer<sup>[1,2]</sup>. Of the 989000 gastric cancer cases in the world in 2008, 78% (770000) were estimated to be attributed to *Helicobacter pylori* (*H. pylori*) chronic infection<sup>[3]</sup>. Gastric cancer has a marked geographic variability<sup>[4,5]</sup>. Latin America has a particularly high burden of prevalent *H. pylori* infection and gastric cancer incidence and mortality<sup>[6-8]</sup>. Estimated age-standardized mortality rates for males per 100000 are elevated in Honduras (22.3), Costa Rica (16.8), Peru (18.2), Chile (15.0), and Ecuador (20.7)<sup>[5,9]</sup>. A concentration of incident gastric cancer is observed in the mountainous regions along the Pacific littoral, including in lower incidence countries (e.g., Mexico), which may offer the opportunity for focused prevention trials and interventions<sup>[5]</sup>.

Recent trials and a meta-analysis suggest that

screening and eradication of *H. pylori* can reduce the risk of gastric cancer<sup>[10,11]</sup>. The Shangdong Intervention Trial, the largest randomized clinical trial to date, had a 53% *H. pylori* cumulative recurrence rate at 7 years, yet demonstrated a significant reduction in gastric cancer at 14.8 years (OR = 0.6, 95%CI: 0.4-0.9)<sup>[10]</sup>. Trial participants were principally middle-aged Asian adults, and the generalizability of results to other populations is uncertain<sup>[12]</sup>. Two subsequent meta-analyses confirmed the findings, while noting that the results were primarily driven by trials conducted in Asia<sup>[11,13]</sup>. The International Agency for Cancer Research (IARC) has recently called for the design and study of large scale interventions for gastric cancer prevention in high incidence regions of the world, including Latin America<sup>[12]</sup>.

The *H. pylori* infection recurrence rate after eradication therapy is the critical determinant of the efficacy of an *H. pylori* eradication program designed to reduce the burden of gastric cancer. This review aims to estimate the reinfection rate of *H. pylori* after completion of antibiotic treatment in Latin America based upon existing literature. We present overall recurrence rates which includes both recrudescence (also called re-growth: Same strain, dominant in the first year after eradication) and reinfection (new strain, dominant in subsequent years), as the majority of studies do not genotype *H. pylori* strains.

## MATERIALS AND METHODS

Review methods and reporting were performed according to the PRISMA guidelines<sup>[14]</sup>. Literature databases PubMed (United States National Library of Medicine), LILACS (Latin America and the Caribbean Literature on Health Sciences), SciELO (Scientific Electronic Library Online) and Cochrane (the Cochrane Collaboration) were included as well as the abstracts from three major gastroenterology and infectious disease meetings [Digestive Disease Week (DDW), American College of Gastroenterology Scientific Meeting (ACG), and ID Week (IDW)]. Studies evaluating *H. pylori* reinfection in the 20 countries comprising Latin America, as defined by the United Nations Educational Scientific and Cultural Organization<sup>[15]</sup>, published in any language up to November 1<sup>st</sup> 2014 were included.

The search was performed in PubMed using the following sequence: *H. pylori* (MeSH term) AND [Recurrence (MeSH) or Recrudescence (MeSH) or Reinfection (not MeSH term)] AND (MeSH terms Latin America or Central America or South America or Argentina or Bolivia or Brazil or Colombia or Costa Rica or Cuba or Chile or Dominican Republic or Ecuador or El Salvador or Guatemala or Honduras or Mexico or Nicaragua or Panama or Paraguay or Peru or Puerto Rico or Uruguay or Venezuela). No other filters or limits were used. Analogous strategies were used to search the other two databases and the meetings' abstracts. Three additional meta-analyses relevant to the study were reviewed for further references<sup>[16-18]</sup>.

## Information coding

Three investigators (Juan E Corral, Corey W Dye and

Douglas R Morgan) independently reviewed titles and abstracts for selection of potentially relevant articles. For journal manuscripts, full text articles were retrieved for further review. Titles that could not be associated with an abstract were excluded from review. A priori, studies with a sample smaller than 50 patient-years (PYs) and studies reporting same populations as other previously registered were excluded from meta-analysis. Citations of retrieved articles were reviewed for studies that may have been missed or were absent from our database queries. Authors were not contacted to provide additional information.

The following information was abstracted from each article: Year of publication, first author, country, information regarding participants (age, recruitment strategy), treatment arms (number of arms, medications used and duration in each arm), follow-up details (duration, intervals of appointment), diagnostic modality and recurrence rates. The interval of possible recurrence started with the last day of antibiotic regimen treatment, and ended with the day of follow-up *H. pylori* diagnostic testing; the last day of treatment was chosen to optimally account for eradication regimens of varying duration. In a given study, if there was more than one follow-up *H. pylori* diagnostic test for recurrence, each testing result was documented independently. The earliest time interval to consider infection recurrence and to be included in the review was 6 mo.

The quality of data (risk of bias) was assessed recording 5 variables, using the same methodology as Camargo *et al.*<sup>[17]</sup> Antibiotic strategy was recorded in detail (medications and length of treatment) and was also scored as an ordinal variable [0 = only one antibiotic without a proton pump inhibitor (PPI), ranitidine or bismuth; 1 = either one antibiotic and a PPI or two antibiotics but no PPI (ranitidine or bismuth allowed); 2 = includes two antibiotics and a PPI (regardless of scheme, for example, triple, quadruple, sequential)].

### Statistical analysis

All treatment arms in each study were reviewed individually. Cases were allocated in two groups: The patients that received antibiotics and those that received either placebo or an antacid medication (PPI, H<sub>2</sub> blocker or bismuth) but without antibiotics. Only antibiotic arms were included in meta-analysis. The number of patients with a negative test immediately after treatment (range 4–8 wk after antibiotic course) were recorded for the intention to eradicate analysis. The patients compliant with subsequent *H. pylori* testing were analyzed for our main analysis, and per our protocol, in this group, the patients lost to follow-up between eradication test (post antibiotics) and subsequent testing were not included. We also documented whether a second antibiotic course was offered for those patients with persistent infection or not.

We used a random-effects model to summarize recurrence rates. Summary reinfection rates and corresponding

95%CIs were calculated using the Poisson distribution. Forest plot graphs were created with 95%CIs. Given the relevance of differentiating between recrudescence (re-growth) and reinfection, subgroup analysis was performed for studies that looked for *H. pylori* recurrence at one year or less (< 53 wk cutoff) and those with longer follow-up<sup>[16]</sup>. Pooled recurrence rates were calculated for different points in time for Latin America, starting at the first six months after completing antibiotics and for all subsequent years where data was available.

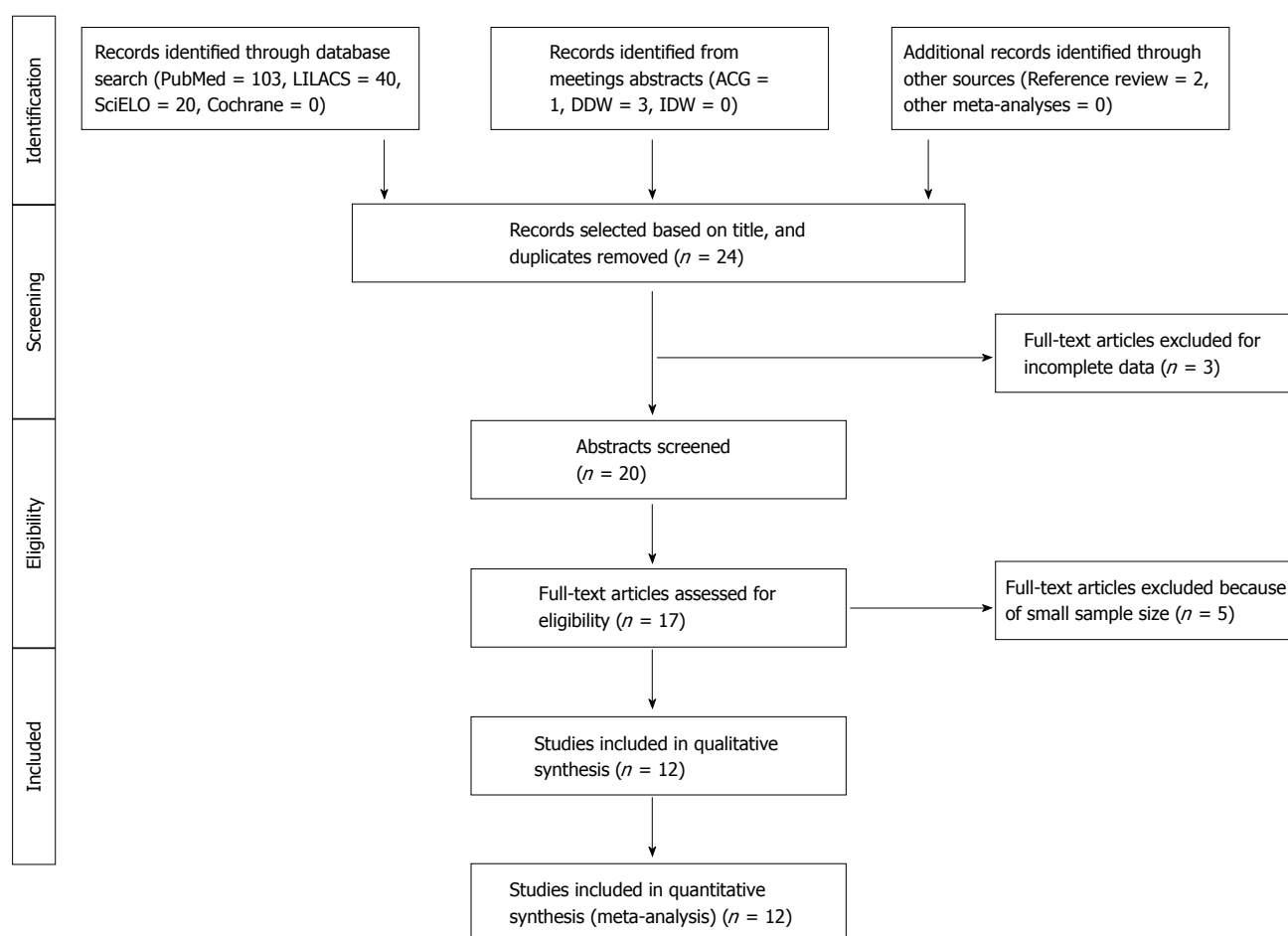
A secondary analysis was conducted with four additional comparisons: Recurrence 3 years after eradication, recurrence in studies enrolling children compared to studies restricted to adults, antibiotics regimens with high (> 75%) or low (≤ 75%) eradication success, and studies that assessed recurrence with endoscopy and biopsy compared to other diagnostic methods.

Meta-regression analyses were performed to evaluate for five effect modifying variables: Study population (community volunteers, patients with duodenal ulcers, dyspepsia, or intestinal metaplasia), *H. pylori* diagnostic modality (with or without urea breath test), quality of antibiotic treatment (0 to 2 points), possibility of a second antibiotic course, and length of follow-up (in years). Between-study heterogeneity was quantified using the *I*<sup>2</sup> statistic. Finally, publication bias was investigated by visual inspection of funnel plots. All statistical analyses were performed with Stata version SE 11.2 (Stata-Corp, College Station, TX, United States). The study plan and results were reviewed by a biomedical statistician (Robertino Mera).

## RESULTS

The literature search resulted in 164 articles from the following sources: PubMed (104), LILACS (40), SciELO (20), and Cochrane (0) (Figure 1). Four abstracts were considered relevant from our review of conference reports (ACG 1, DDW 3, IDW 0). Two additional articles were identified after screening the references of manuscripts found in first review. After excluding 139 irrelevant or duplicate publications, 25 full text articles were retrieved for further evaluation, of which 7 were excluded because of incomplete data or duplicate samples and six additional per protocol for their small sample size (< 50 person-years of follow-up).

In summary, 12 studies from 9 countries met criteria for inclusion, which were published between 1991 and 2014 (Table 1). Ten studies included only adults, and an additional 2 studies included both adults and children. Eleven manuscripts were written in English and one in Spanish. Time to evaluate *H. pylori* eradication success ranged from 4 to 13 wk after last day of antibiotics (3 studies reported percentage of successful treatment “after randomization” without further details). Follow-up ranged from 6 mo to 16 years. These twelve studies encompassed 4848 patients [4685 patients received a treatment regimen that included antibiotics, 163 were



**Figure 1** Latin America *Helicobacter pylori* recurrence: Study selection flow diagram (PRISMA 2009). DDW: Digestive Disease Week; ACG: American College of Gastroenterology Scientific Meeting; IDW: ID Week.

assigned to a placebo group or other treatment arm without antibiotics (only anti-acids)]. In these studies, the mean eradication rate after initial treatment was 72.2%, with a range of 30.2% to 100%. Reinfection rates ranged from 1.8% to 85.4%. In total, there were 432 reinfection events recorded in 5487 PYs follow-up in patients with sufficient data to calculate recurrence rates. Of the 4848 patients, 2172 (44.8%) did not complete follow-up diagnostic testing (Table 2).

Pooled analysis showed an overall recurrence rate of 7.9 cases per 100 PYs (95%CI: 5.3-10.5) (Figure 2). Analysis on an intention to eradicate basis (those with a negative test immediately after treatment) had a recurrence rate of 7.1 (4.7-9.6) per 100 PYs. The recurrence rate in the first year after treatment, postulated to be predominantly recrudescence, was 11.2 (6.1-16.4) per 100 PYs (all 12 studies included), while recurrence in subsequent years, an estimate of reinfection, was 6.2 (3.8-8.7) per 100 PYs. The cumulative reinfection rate at the 5 and 7 year time points were 36.2 and 48.6 per 100 PYs, respectively. Recurrence rates from different countries were combined for the first 6 years, 12 and 16 years (only one study had follow-up beyond 5 years<sup>[19]</sup>). Recurrence rate were

higher in the first six months and decreased afterwards. Data from year 4 and 5 were combined as they had few PY follow-up (132 and 98, respectively). After the first year, reinfection rates ranged from 3.4 per 100 PYs in year 2 to 6.3 per 100 PYs in the combined 4-5 year period (Figure 3).

In a secondary analysis, the reinfection rate was lower when using a 3-year time cutoff; with an estimated rate of 3.8 (95%CI: 1.6-6.1) cases per 100 PYs. Recurrence rates were two times higher in studies that enrolled children compared to those that only enrolled adults 12.3 (95%CI: 9.6-14.9) vs 6.9 (95%CI: 4.2-9.6) cases per 100 PYs. There was no significant difference in recurrence rates among trials with high or low initial eradication success [7.8 (95%CI: 3.4-12.3) vs 8.4 (95%CI: 4.6-12.1), respectively]. Recurrence rates were higher in studies that evaluated eradication by endoscopy, 11.6 (95%CI: 9.9-13.3), compared to those that used non-invasive diagnostic methods, 6.6 (95%CI: 4.0-9.1).

### Meta-regression

In the meta-regression, neither the study population, the method used to detect *H. pylori*, the initial antibiotic



**Table 1** Characteristics of eradication trials included in Latin America

Ref.	Year	Country	Patients enrolled or randomized	Mean age $\pm$ SD (age range)	Patient population	Treatment arm(s)	Antibiotic duration (d)	Second antibiotic treatment	Eradication success rate	Waiting time (wk)	Diagnostic method(s)	Follow-up, yr	Study design and quality <sup>[17]</sup>
Morgan <i>et al</i> <sup>[22]</sup>	2013	6 countries <sup>a</sup>	1463	(21-65)	Community populations	3 options: PPI + A + C PPI + A/PPI + A + M PPI + A + C + M	Variable: 14 5/5 5	PPI + M + Bis + Tetra <sup>b</sup>	Total 77.4% 82.20% 76.50% 73.60%	6-8	13C, CagA IgG	1	5
Silva <i>et al</i> <sup>[34]</sup>	2010	Brazil	150	46.7 (16-85)	Duodenal ulcer	PPI + A + C	7	PPI + Tetra + Furazolidone	92.50%	13	14C, H (RUT, PCR)	5	3
Mesquita <i>et al</i> <sup>[35]</sup>	2005	Brazil	50	49 $\pm$ 14 (> 18)	Duodenal ulcer	H2 + Bis + C	14	NA	100%	13	H (RUT, H and E)	3	2
Coelho <i>et al</i> <sup>[36]</sup>	1991	Brazil	48	40.4 (adults)	Duodenal ulcer	A + M + Furaz	5	NA	60.40%	8.5	14C	1.5	2
Rollan <i>et al</i> <sup>[37]</sup>	2000	Chile	111	38 (16-75)	Duodenal ulcer	2 options: H2 + A + M PPI + A + Tinidazole	14 14	Cross-over	Total 75.7% 79% 73%	4-6	14C, H (RUT, Warthin-S, PCR)	3	3
Figueroa <i>et al</i> <sup>[38]</sup>	1996	Chile	57	49.1 (16-65)	Duodenal ulcer	PPI + A + M + Bis	28	NA	80.70%	4	H (RUT, Gram, Clt)	1	5
Novoa-Reyes <i>et al</i> <sup>[39]</sup>	2014	Peru	140	48.9 $\pm$ 12.3 (18-85)	Non-ulcer dyspepsia	PPI + A + C	10	NA	72.10%	4	14C, H (H and E)	2	3
Soto <i>et al</i> <sup>[40]</sup>	2003	Peru	235	37 $\pm$ 8.7 (18-55)	Non-ulcer dyspepsia	PPI + A + C	14	NA	85.50%	4	14C, H (Warthin-S, Clt)	1.5	5
Leal-Herrera <i>et al</i> <sup>[41]</sup>	2003	Mexico	467	(> 5) <sup>c</sup>	Non-ulcer dyspepsia	PPI + A + C	14	NA	30.20%	4-6	14C, H (Giemsa, Clt, PCR), Serology	2	4
Mohar <i>et al</i> <sup>[42]</sup>	2002	Mexico	131	51.4 $\pm$ 9.3 (> 40)	Healthy volunteers	PPI + A + C	7	NA	76.30%	6	H (H and E, Elisa), CagA IgG	1	4
Sivapalingam <i>et al</i> <sup>[43]</sup>	2014	Bolivia	848	(> 6 mo) <sup>d</sup>	Community populations	PPI + A + C	10	"Triple therapy"	64.00%	6	13C, CagA IgG	1	3
Mera <i>et al</i> <sup>[19,44]</sup>	2005	Colombia	976	50.8 (29-69)	Intestinal metaplasia	Variable (the majority A + M + Bis)	14	NA	51.60%	156	13C, H (H and E, Steiner)	16	5

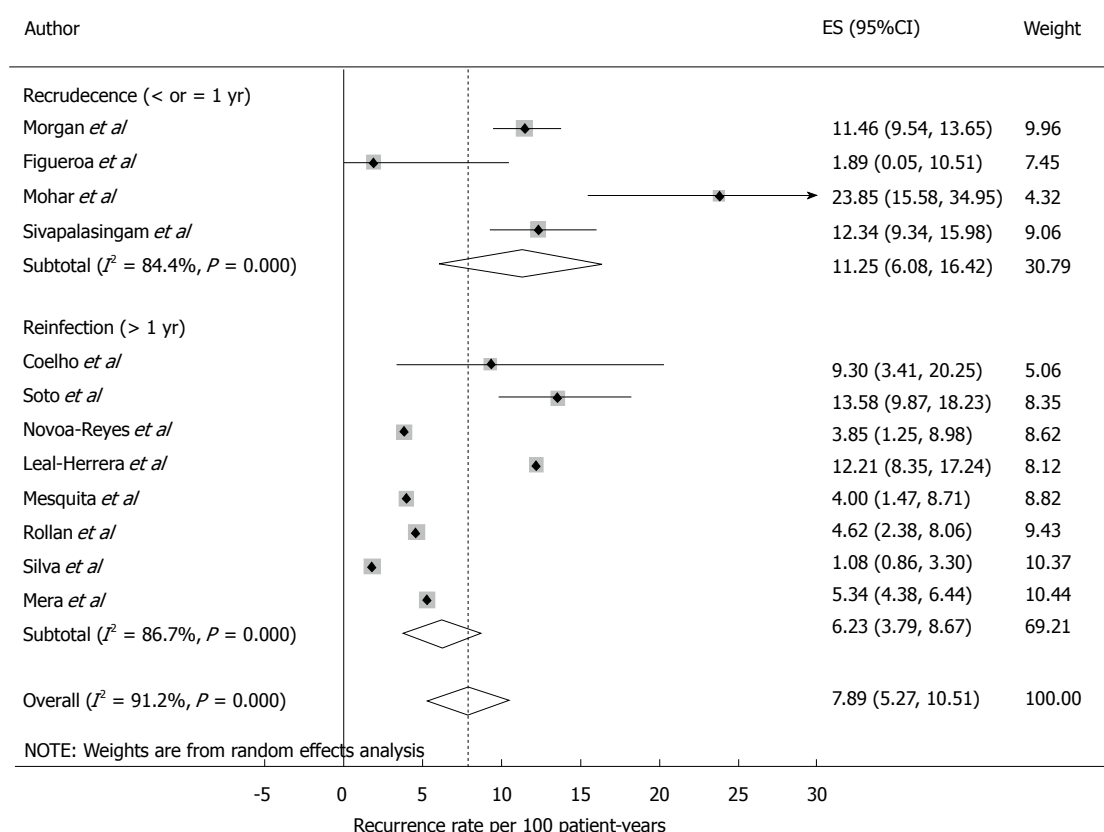
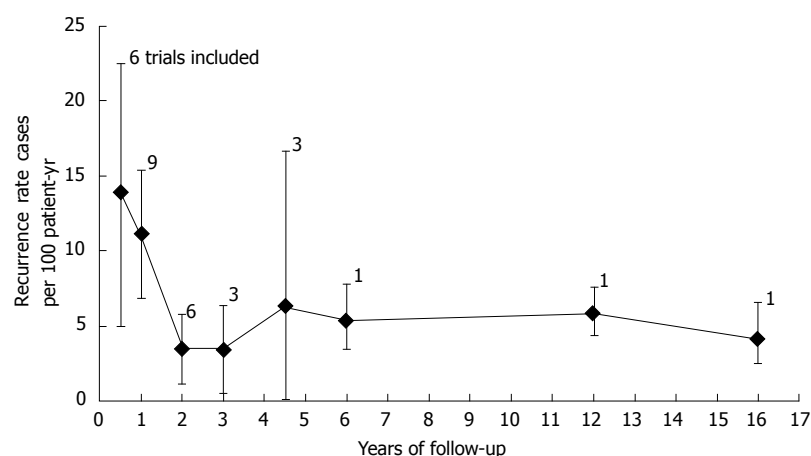
<sup>a</sup>Six countries were Colombia, Costa Rica, Nicaragua, Chile, Honduras, and 2 sites in Mexico (Sonora and Chiapas); <sup>b</sup>Voluntary treatment; <sup>c</sup>66.7% were > 30 years old; <sup>d</sup>41.2% were > 15 years old. PPI: Proton pump inhibitor; A: Amoxicillin; C: Clarithromycin; H2: H2 Blockers; Bis: Bismuth; Furaz: Furazolidone; C: Urea breath test; H: Histology; RUT: Rapid urea test; Clt: Culture.

strategy, the use of a second antibiotic course, nor the length of follow up had a significant impact on the observed risk of reinfection. As anticipated, the recurrence rates decreased after the first year by 40%, but this was not statistically significant after including all five variables ( $P = 0.6$ ).

### Assessment of bias and heterogeneity

Risk of bias according to Camargo scale ranged from 2 to 5 points. Even though reviewed studies used various

methods to assess *H. pylori* recurrence, 10 (83%) used at least two different methods, including 9 (75%) that used urea breath tests. Most studies lost points because of sampling techniques or because they failed to describe salient patient characteristics. The  $I^2$  for the model was 90% and adjusted R2 was -38.8%. Funnel plot showed asymmetry towards higher recurrence rates, with a lack of missing studies with low sample size (high SD) where the rates are lower. The top of the funnel plot demonstrated a low risk for publication bias.

Figure 2 Forest plot of *Helicobacter pylori* recurrence rates in Latin America.Figure 3 Yearly trends of *Helicobacter pylori* recurrence after eradication treatment in Latin America.

## DISCUSSION

*H. pylori* recurrence after eradication is a critical determinant of the efficacy of potential gastric cancer prevention programs utilizing antibiotic treatment. This measure may be more important than the choice of initial antibiotic regimen and bacterial resistance rates, and is likely to differ by global region<sup>[19-21]</sup>. Latin America populations have high colonization rates of *H. pylori*, as well as a significant burden of gastric adenocarcinoma. Our meta-analysis estimates a recurrence rate of 7.9 cases per 100 PYs in Latin America, 11.2 in year-one

and 6.2 in subsequent years. This overall rate is higher than the estimated global recurrence rate of 4.5 (95%CI: 4.2-4.8), but significantly lower than that reported for resource-limited nations 8.7 (7.8-9.6) and 13.0 (6.0-21.0) observed in two independent meta-analysis<sup>[16,18]</sup>.

Is there a maximum *H. pylori* infection recurrence threshold for potential intervention programs? In the Shangdong trial reported by Ma *et al*<sup>[10]</sup> with healthy volunteers in East Asia, *H. pylori* eradication significantly reduced incident gastric cancer compared to placebo after 14.8 years of follow up [OR 0.6 (0.4-0.9),  $P = 0.3$ ]. These results have been supported by recent

**Table 2** Estimated *Helicobacter pylori* recurrence rates in Latin America studies

Ref.	Patients that received antibiotics	Patients present at f/u appointment	Recurrent cases total	Crude reinfection rate <sup>1</sup>	Follow-up (yr)	Year patients (present at f-u appointment)	Recurrence rate per 100 PY (95%CI)
Morgan <i>et al</i> <sup>[22]</sup>	1133	1091	125	11.46	1	1091	11.46 (9.54-13.65)
Silva <i>et al</i> <sup>[34]</sup>	147	112	10	8.98	5	557	1.80 (0.86-3.30)
Mesquita <i>et al</i> <sup>[35]</sup>	50	50	6	12.00	3	150	4.00 (1.47-8.71)
Coelho <i>et al</i> <sup>[36]</sup>	29	43	6	13.95	1.5	64.5	9.30 (3.41-20.25)
Rollan <i>et al</i> <sup>[37]</sup>	84	96	12	12.50	3	260	4.62 (2.39-8.06)
Figueroa <i>et al</i> <sup>[38]</sup>	47	53	1	1.89	1	53	1.89 (0.05-10.52)
Novoa-Reyes <i>et al</i> <sup>[39]</sup>	101	65	5	7.69	2	130	3.85 (1.25-8.98)
Soto <i>et al</i> <sup>[40]</sup>	201	216	44	20.37	1.5	324	13.58 (9.87-18.23)
Leal-Herrera <i>et al</i> <sup>[41]</sup>	141	131	32	24.43	2	262	12.21 (8.35-17.24)
Mohar <i>et al</i> <sup>[42]</sup>	183	109	26	23.85	1	109	23.85 (15.58-34.95)
Sivapalasingam <i>et al</i> <sup>[43]</sup>	543	462	57	12.34	1	462	12.34 (9.34-15.98)
Mera <i>et al</i> <sup>[19,44]</sup>	679	126	108	85.37	16	2024	5.34 (4.38-6.44)
Total	3338	2554	432	16.92		5487	7.89 (5.27-10.51)

<sup>1</sup>Crude reinfection rate: Recurrent cases total/Patients present at follow-up appointment.

**Table 3** Implementation of *Helicobacter pylori* eradication programs for gastric cancer prevention in Latin America

Components	Challenges and considerations	Implementation approaches
Public policy	Lack of awareness among the Ministries of Health, stakeholders, and the public	Large scale education campaigns for cancer and gastric cancer Joint initiatives with international stakeholders: WHO, IARC, PAHO, UICC, NCI, and CDC
Economic investment	Cost of <i>H. pylori</i> eradication program	Conduct CEAs at the country and regional level. The CEAs may differ for HICs and LMICs
Program design	Economics of growing gastric cancer burden Uncertainties and regional variation for target age, screening approach, treatment regimen, and follow-up	Pilot-test eradication campaigns and perform community implementation trials Adapt evidence from cost-effectiveness models and available epidemiologic data. Incorporate screening into existing public health practices ( <i>e.g.</i> , cervical cancer)
Appropriate technologies	Technical difficulties in <i>H. pylori</i> testing Consistent eradication confirmation norms Management of high risk patients	Develop economic, point-of-care <i>H. pylori</i> testing Coordinate endoscopy protocols for high risk patients ( <i>e.g.</i> , premalignant lesions) Implement information networks to coordinate eradication programs, health centers, and endoscopy centers
Adherence measures	Poor compliance with <i>H. pylori</i> eradication regimen, leading to treatment failure and increased infection recrudescence	Consider medication side effect profiles Pre-regimen counseling for common side effects Consider adherence measures, usual ( <i>e.g.</i> , direct observed therapy), or novel ( <i>e.g.</i> , cell phone contact)
<i>H. pylori</i> recurrence	Elevated reinfection rate may affect program efficacy and feasibility	Improve living conditions to reduce potential environmental sources of reinfection Consider the family or the village as the intervention target
Potential overall program risks and unknowns	Alteration of the human microbiome Induction of antibiotic resistance Potential increased risk for certain diseases ( <i>e.g.</i> , allergic diseases, esophageal cancers) Unknown role(s) of <i>H. pylori</i> as a component of the human microbiome: Commensal and pathogen, which may be strain and/or age dependent	<i>H. pylori</i> eradication programs should be considered investigational, with use of rigorous methodology and long term surveillance Monitoring of antibiotic resistance and microbiome profiles Global antibiotic stewardship programs ( <i>e.g.</i> , OTC antibiotic use, veterinary use)
Parallel research agendas	Incorporate evolving approaches and technologies	Develop novel biomarkers for host risk and <i>H. pylori</i> virulence Develop biomarkers for premalignant lesions ( <i>e.g.</i> , intestinal metaplasia) to facilitate endoscopy surveillance Incorporate endoscopy technologies, including advanced imaging and low-cost approaches
<i>H. pylori</i> Vaccination	Unknown long-term effectiveness and side effects Lack of data showing impact in clinical outcomes	Evaluate long-term (> 3 yr) effectiveness in other centers, populations and countries <sup>[23]</sup> Complete regulatory evaluations, collect additional safety data and approval by national agencies. Phase IV studies

WHO: World Health Organization; IARC: International Agency for Research on Cancer; PAHO: Pan American Health Organization; UICC: Union for International Cancer Control; NCI: National Cancer Institute; CDC: Centers for Disease Control and Prevention; HIC: High income country; LMIC: Low/middle income country; OTC: Over the counter; CEAs: Cost-effectiveness analyses; *H. pylori*: *Helicobacter pylori*.

meta-analyses<sup>[11]</sup>. In the Shangdong study, omeprazole and amoxicillin comprised both the treatment and the retreatment regimen, and only 47% of subjects were *H. pylori* negative at the 7-year post-eradication point. In general terms, this may suggest a 50% threshold at the 5 to 7 year time point as a minimum eradication efficacy target. Our estimated 5-year and 7-year reinfection rates in the current meta-analysis are lower or at least similar to the 7-year reinfection rate observed in the Shangdong study: 36.2% and 48.6%, vs 53%, respectively. Thus, *H. pylori* screening and eradication in asymptomatic populations may be an attractive strategy for gastric cancer prevention in Latin America. Further research to evaluate feasibility, cost-effectiveness, acceptance, and adverse consequences of eradication programs in the region is needed. For example, the 1-year recurrence analysis in the large 6-country *H. pylori* eradication trial in Latin America suggested that potential programs may need to be tailored based upon region, gender and age of the participants<sup>[22]</sup>. Uncertainties about *H. pylori* screening and treatment have to be answered and significant challenges are foreseen before such programs can be implemented at a population level (Table 3).

*H. pylori* infection recurrence represents the combination of recrudescence and reinfection, and different strategies may be required to effectively reduce these component rates. Recrudescence or re-growth, usually occurs during the first year after treatment at a rate primarily driven by antibiotic treatment failure, in the setting of a false negative test immediately after treatment. This common scenario may be difficult to distinguish from reinfection with the same strain from a family member in the same household. Reinfection, is the principal component of recurrence after the first year, and persists at a lower but steady state. Molecular analysis comparing pre- and post-treatment strains of patients have shown that 80% of recurrent cases are genetically identical, whereas differing strains were found in only a minority of the cases<sup>[23]</sup>. This suggests that the majority of initial recurrent cases are a product of treatment failure, or reinfection with a strain common to close contacts or family members. In this meta-analysis, recurrence rates significantly decreased after the first year and remained stable in subsequent intervals, ranging from 3.4% to 5.8% per 100 PYs.

Strategies aiming to reduce these two types of recurrence should be different. The first scenario requires a clinical approach where cost-effective antibiotic selection and medication compliance measures are crucial, whereas the second involves a broader public health strategy. Reducing reinfection rate is complex as it involves improving living conditions and reducing potential environmental sources of reinfection, including consideration of interventions at the family or the village levels, and possibly vaccination<sup>[24]</sup>. In this approach, children become a challenging target group with higher therapeutic failure and higher reinfection as seen in most studies (including this meta-analysis)<sup>[25-27]</sup>.

Our results are significantly influenced by two trials:

The study by Morgan *et al*<sup>[22]</sup> as the largest trial with 1463 PYs follow up, and the study by Mera *et al*<sup>[19]</sup> as the cohort with the longest follow-up time. The Mera study was the only cohort followed for more than 5 years; subjects with preneoplastic gastric lesions were enrolled from a geographically circumscribed region of Colombia, and thus, the results may not be generalizable to the remainder of Latin America. Of note, the Caribbean was not represented, where the higher African ancestry, different diets and other environmental exposures may affect generalization. In this review, we observed geographic variability in *H. pylori* recurrence rates, as had been previously described<sup>[22]</sup>. This likely represented both regional *H. pylori* ecology differences, as well as socioeconomic differences in the study populations. Improved socioeconomic status in subsequent birth cohorts may help explain lower acquisition rates. For example, Chile and Peru are countries with divergent development rates, yet similar ethnography and comparable *H. pylori* prevalence rates-lower reinfection rates are observed in Chile<sup>[16,28]</sup>. One likely explanation is that in Chile, the generation  $\geq 40$  years who contracted *H. pylori* in their childhood and remains colonized, coexists with younger generations that have grown in improved living conditions with reduced *H. pylori* prevalence. This paradox of high prevalence but low reinfection rates has been previously described in Japanese patients with peptic ulcer disease<sup>[29]</sup>.

In our meta-regression analysis, the findings were not significantly modified by any of the evaluated factors: Study population, *H. pylori* diagnostic modality, the antibiotic strategy selected, retreatment (a second antibiotic course), or the time interval to check for *H. pylori* eradication success. Antibiotic selection varied among different studies, but half of them used the standard triple therapy regimen. This 14-d regimen has been proven to be superior to sequential and concomitant therapy in Latin America post-eradication time, but not at the 1-year time point<sup>[22,30]</sup>. Diagnostic modalities were appropriate, and 8 out of 12 studies used two methods to diagnose *H. pylori*, wherein one of them was the urea breath test<sup>[31]</sup>. Studies that used endoscopy-based diagnostic methods noted higher recurrence rates, which may be an incidental finding, related to occasional iatrogenic infection, or reflect the improved sensitivity of this approach<sup>[32]</sup>. One limitation of this analysis was the study designs which were not able to differentiate whether cases were secondary to reinfection or recrudescence by molecular fingerprinting. Finally, heterogeneity was significant and there is a possibility of publication bias<sup>[33]</sup>. The Forrest plot suggested missing studies with low sample size (wide standard deviation) wherein the recurrence rates may be lower, with the exception of the Peru study, but this is attributed to the inclusion criteria of at least 50 PYs of follow-up.

## Conclusion

The meta-analysis of studies in Latin America suggests that the *H. pylori* recurrence rate in the first year is



11.2 (95%CI: 6.1-16.4) per 100 person-years, and 6.2 (95%CI: 3.8-8.7) per 100 person-years in subsequent years, or approximately 50% at 7 years. Overall, the reinfection rates are lower than initially reported, making *H. pylori* screening and eradication a reasonable strategy for gastric cancer prevention programs in Latin America, within the context of well-designed clinical trials<sup>[18]</sup>. Further research is needed to evaluate the feasibility, cost-effectiveness, and the potential adverse outcomes (e.g., microbiome effects, antibiotic resistance) of eradication programs, while in parallel, to explore novel biomarkers and eradication strategies.

## ACKNOWLEDGMENTS

Authors would like to thank Leonardo J Tamariz for assisting with statistical analysis.

## COMMENTS

### Background

Recent trials and systematic reviews suggest that *Helicobacter pylori* (*H. pylori*) eradication may reduce the risk of gastric adenocarcinoma if the 5 to 7-year recurrence rate is less than 50%.

### Research frontiers

There is limited evidence of *H. pylori* recurrence after treatment outside of Asia.

### Innovations and breakthroughs

The authors' estimated 5-year and 7-year recurrence rates are similar to the recurrence rates observed in the literature, including the Shangdong study. Recurrent cases occur mostly within the first year suggesting treatment failure.

### Applications

*H. pylori* screening and eradication in asymptomatic populations with chronic gastritis may be an attractive strategy for gastric cancer prevention in Latin America. Further research is needed to evaluate the feasibility, cost-effectiveness, and the potential adverse outcomes (e.g., microbiome effects, antibiotic resistance and stewardship) of eradication programs in the region.

### Peer-review

This manuscript is well written and clinically interesting. Results are presented clearly and conclusions are supported by results.

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*World Journal of Gastrointestinal Oncology* (*World J Gastrointest Oncol*, *WJGO*, online ISSN 1948-5204, DOI: 10.4251) is a peer-reviewed open access academic journal that aims to guide clinical practice and improve diagnostic and therapeutic skills of clinicians.

*WJGO* covers topics concerning carcinogenesis, tumorigenesis, metastasis, diagnosis, prevention, prognosis, clinical manifestations, nutritional support, molecular mechanisms, and therapy of benign and malignant tumors of the digestive tract. The current columns of *WJGO* include editorial, frontier, diagnostic advances, therapeutics advances, field of vision, mini-reviews, review, topic highlight, medical ethics, original articles, case report, clinical case conference (Clinicopathological conference), and autobiography. Priority publication will be given to articles concerning diagnosis and treatment of gastrointestinal oncology diseases. The following aspects are covered: Clinical diagnosis, laboratory diagnosis, differential diagnosis, imaging tests, pathological diagnosis, molecular biological diagnosis, immunological diagnosis, genetic diagnosis, functional diagnostics, and physical diagnosis; and comprehensive therapy, drug therapy, surgical therapy, interventional treatment, minimally invasive therapy, and robot-assisted therapy.

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## Molecular classifications of gastric cancers: Novel insights and possible future applications

Silvio Ken Garattini, Debora Basile, Monica Cattaneo, Valentina Fanotto, Elena Ongaro, Marta Bonotto, Francesca V Negri, Rosa Berenato, Paola Ermacora, Giovanni Gerardo Cardellino, Mariella Giovannoni, Nicoletta Pella, Mario Scartozzi, Lorenzo Antonuzzo, Nicola Silvestris, Gianpiero Fasola, Giuseppe Aprile

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### Abstract

Despite some notable advances in the systemic management of gastric cancer (GC), the prognosis of patients with advanced disease remains overall poor and their chance of cure is anecdotic. In a molecularly selected population, a median overall survival of 13.8 mo has been reached with the use of human epidermal growth factor 2 (HER2) inhibitors in combination with chemotherapy, which has soon after become the standard of care for patients with HER2-overexpressing GC. Moreover, oncologists have recognized the clinical utility of conceiving cancers as a collection of different molecularly-driven entities rather than a single disease. Several molecular drivers have been identified as having crucial roles in other tumors and new molecular classifications have been recently proposed for gastric cancer as well. Not only these classifications allow the identification of different tumor subtypes with unique features, but also they serve as springboard for the development of different therapeutic strategies. Hopefully, the application of standard systemic chemotherapy, specific

targeted agents, immunotherapy or even surgery in specific cancer subgroups will help maximizing treatment outcomes and will avoid treating patients with minimal chance to respond, therefore diluting the average benefit. In this review, we aim at elucidating the aspects of GC molecular subtypes, and the possible future applications of such molecular analyses.

**Key words:** Molecular biology; Immunotherapy; Gastric cancer; Classification; Targeted therapy

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**Core tip:** TCGA individuates four molecular subtypes: Chromosomal instability, microsatellite instability, genomically stable and Epstein-Barr virus positive tumors. Asian Cancer Research Group classification partially overlaps with the previous one. Although not prospectively validated, these novel classifications suggest that different subtypes of gastric cancer might be treated with specific therapeutic strategies in the near future.

Garattini SK, Basile D, Cattaneo M, Fanotto V, Ongaro E, Bonotto M, Negri FV, Berenato R, Ermacora P, Cardellino GG, Giovannoni M, Pella N, Scartozzi M, Antonuzzo L, Silvestris N, Fasola G, Aprile G. Molecular classifications of gastric cancers: Novel insights and possible future applications. *World J Gastrointest Oncol* 2017; 9(5): 194-208 Available from: URL: <http://www.wjgnet.com/1948-5204/full/v9/i5/194.htm> DOI: <http://dx.doi.org/10.4251/wjgo.v9.i5.194>

## INTRODUCTION

Gastric cancer (GC) is among the most common malignancies worldwide and the second leading cause of cancer related deaths<sup>[1]</sup>. In fact, it represents the fifth most commonly diagnosed cancer (6.8% of oncologic diagnoses) resulting in an annual estimated incidence of 18 cases out of 100000 individuals among men and 9 out of 100000 for women<sup>[2]</sup>.

The mainstay of first-line therapy for GC is still represented by a chemotherapy backbone composed by platinum compounds and fluoropyrimidines resulting in a median overall survival (OS) of about 11 mo. Still, the disappointing 5-year survival rate is estimated to be about 25%-30% and slightly higher for some Asian experience. Historically, many attempts have been made in order to re-classify gastric cancer with the aim of clustering some new subgroups that could have different prognostic and predictive value: Anatomical classification (Borrmann classification and Siewert and Stein classification), histological classification (WHO classification and Lauren's classification), and extent of disease (early gastric cancer vs advanced cancer).

The first effective molecular novelty came from the TOGA trial which demonstrated a significant im-

provement in OS with the addition of trastuzumab to chemotherapy when compared to chemotherapy alone in patients with HER2 overexpressing GCs (13.8 mo vs 11 mo, respectively;  $P = 0.046$ )<sup>[3]</sup>. Another clue to the "heterogeneity theory" comes from the observation that Asian patients demonstrate different pattern of disease and outcomes if compared to the Caucasian western population included in the largest trials.

Nowadays, with mounting biological information available, almost every solid cancer type is considered as a "collection" of multiple very molecularly heterogeneous diseases. Very important advances have been made in the molecular classification of breast cancers<sup>[4]</sup>, lung tumors (by the identification of some tyrosine-kinase-inhibitor targetable subtypes), colorectal adenocarcinomas (predictive and prognostic classes sorted by mutations in *RAS* and *BRAF* genes), and malignant melanoma (identification of *BRAF* codon 600 mutation).

Nevertheless, the poor anatomical and molecular selections of GC patients entering clinical trials have potentially limited the effect of many therapeutic agents including chemotherapy, antiangiogenic drugs and the newly tested immune-modulators. In fact, the benefit of those drugs may have been diluted when tested in the overall population. Recently something has changed the way of thinking GC starting from the TCGA group publication appeared in 2014<sup>[5]</sup>.

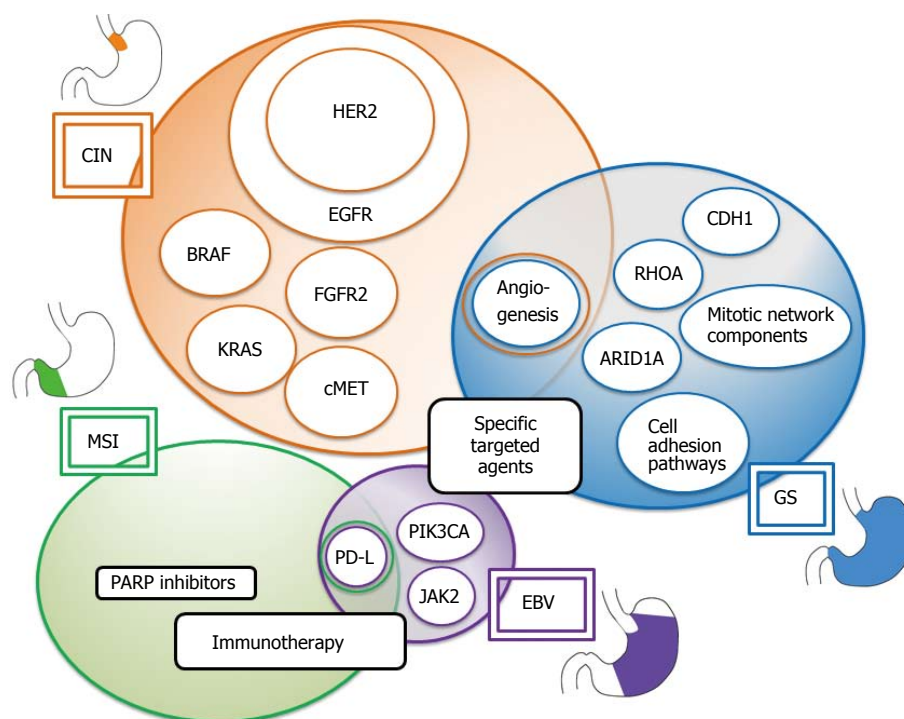
A more profound understanding of the molecular clustering of stomach cancer could give us the chance to obtain new insights into prognostic and predictive categorization of this cancer and could definitely provide the scientific knowledge for developing modernly conceived clinical trials that could maximize the effect of novel agents in the proper patient population, avoiding the use of costly drugs in non-stratified populations.

Finally, the aim of this review is to give a general picture of the current knowledge of the emerging molecular classification of GC and to explore the new possibilities connected to the latest discoveries made on the extreme heterogeneity of this disease.

## THE IMPORTANCE AND LIMITATIONS OF MOLECULAR CLASSIFICATIONS

The first attempt to generate a comprehensive molecular classification for GC was made in 2013 by Singapore Researchers<sup>[6]</sup>. They identified three main types of gastric cancer, namely proliferative (characterized by high genomic instability and *TP53* mutation), metabolic (more sensitive to 5-FU therapy) and mesenchymal (stem cell-like tumors sensitive to PIK3CA-mTOR pathway inhibitors), based on genome expression. Soon after the TCGA research group published a classification dividing GCs into four main subgroups clustered on the basis of six different molecular biology approaches: Copy number variation (CNV) analysis, exome sequencing analysis, DNA methylation profile, mRNA sequencing, micro-RNA (miRNA) sequencing and reverse phase protein array<sup>[5]</sup>. The result





**Figure 1** Four molecular subtypes of gastric cancer (chromosomal instability, genomic stability, microsatellite instability, and Epstein-Barr virus) are represented. Particular anatomic distribution and prospective therapeutic strategies. The areas represent the epidemiologic extent of each of the subtypes. On the side of each subtype the anatomical distribution is displayed. CIN: Chromosomal instability; GS: Genomic stability; MSI: Microsatellite instability; EBV: Epstein-Barr virus.

is the subdivision of GC into four genomic subtypes: Epstein-Barr virus (EBV) positive cancers (9% of all gastric tumors with frequent *PIK3CA* mutation and PD-L1/PD-L2 overexpression), Microsatellite Instability tumors (MSI, representing 22% and hypermutated), chromosomal instability (CIN, 50%, predominantly junctional, *TP53* mutated with RTK-RAS activation, with a high rate of CNV) and Genomically Stable (GS, 20%, presenting mutation in motility and adhesion molecules). Specific TCGA molecular subtypes are represented in Figure 1.

In the meantime, the Asian Cancer Research Group (ACRG) too proposed a novel molecular classification<sup>[7]</sup>, and the resulting taxonomy divided GCs into: Mesenchymal subgroup (MSS/EMT, characterized by hallmarks of epithelial-to-mesenchymal transition), Microsatellite Instability subgroup (MSI), Microsatellite Stable *TP53* positive (MSS/*TP53*<sup>+</sup>, somehow overlapping with EBV type of TCGA classification) and Microsatellite Stable *TP53*-tumors (MSS/*TP53*<sup>-</sup>, overlapping with CIN by TCGA).

These novel classifications create a new paradigm in the definition of cancer biology and allow the identification of relevant genomic subsets by using different techniques such as genomic screenings, functional studies and molecular or epigenetic characterization. However, some limitations should also be openly recognized. First, these classifications are based on a highly complex methodology and currently they should not be replicated in standard laboratories lacking in the uttermost technologies. Attempts towards simplification are ongoing although results may not fully capture the underpinning complexity of the

disease. Second, these classifications lack of a prospective validation on a large scale, including patients with different ethnicity and age. Third, the two proposed classifications have more differences than similarities; in particular, they are different in terms of demographics, baseline molecular mechanisms, driver genes, and association with prognosis. Moreover, there are notable dissimilarities in the distribution of Lauren's diffuse subtype among the different subgroups. Since different molecular subgroups may be identified across a number of independent gene expression profile studies, a collaborative international effort is warranted to aggregate a consensus classification. Fourth, the follow-up of included patients is limited, factor that may decrease their prognostic power, and subgroups were evaluated on resected specimens, with different prevalence of subgroups between localized, locally advanced and advanced settings. Fifth, both classifications insist on epithelial cells, but none of them take into account the active, nonmalignant stromal cells. Actually, not only gene expression profiles deriving from stromal tissues may influence assignment to a specific molecular category, thus creating interpretative troubles<sup>[8]</sup>, but also novel stromal-based distinctive signatures have been proposed and related to the predominant cancer phenotype<sup>[9]</sup>.

## GC WITH CHROMOSOMAL INSTABILITY

CIN subtype represents approximately 50% of GCs<sup>[10]</sup> and it mostly occurs in the esophagogastric junction (EGJ)/cardia. CIN GC is related to intestinal type histology,

to copy number gains of chromosomes 8q, 17q and 20q, while, gains at 12q and 13q are associated with diffuse GC<sup>[11]</sup>. Interestingly, CIN showed elevated frequency in the EGJ/cardia, as demonstrated in TCGA characterization (65%,  $P = 0.012$ ). CIN is characterized by somatic mutations at cytogenetic level, particularly involving loci that control mitotic checkpoints, thus gatekeeper and caretaker genes implicated in carcinogenesis. CIN comprises both altered DNA copy number and structural abnormalities in some chromosomal regions. Those alterations could result in gain or loss of whole chromosomes<sup>[12]</sup> (aneuploidy), non-reciprocal translocations, amplifications, deletion or the loss of one allele with loss of heterozygosity. Altogether, CIN results in the loss or gain of function of some “key genes”, including oncogenes and tumor suppressor genes that may be efficaciously targeted by specific inhibitor molecules<sup>[13]</sup>. Notably, CIN GC is enriched in mutations in *TP53* gene and receptor tyrosine kinases (RTKs), furthermore it shows amplifications of cell cycle genes (Cyclin E1, Cyclin D1, and Cyclin-dependent kinase 6)<sup>[14]</sup>.

Evaluation of the biological characteristics among CIN cancers demonstrated that *TP53* mutations occurs in 71% of GCs<sup>[5]</sup>. Furthermore, CIN also display amplification in oncogene pathways such as RTK/RAS/MAPK signaling, including HER2, BRAF, epidermal growth factor (EGFR), MET, FGFR2, RAS<sup>[5,15]</sup>.

A recent work reviewed the pathogenic and molecular similarities between gastric intestinal-type adenocarcinoma and esophageal adenocarcinoma (EAC)<sup>[16]</sup>, suggesting that treatment of EAC should recall that of gastric adenocarcinoma rather than being similar to the approach used for upper esophageal cancers (mostly squamous). In fact, not only EAC may arise from progenitor cells deriving from the cardia of the stomach but also the majority of EAC express a chromosomal instability that closely resembles the one found in CIN GC. All these findings suggest both the need for better subtyping esophageal cancers and the opportunity of developing specific therapeutics strategies in this disease as well.

## HER2

The proto-oncogene HER2 is a member of the EGF receptor family with tyrosine kinase activity. It is known that HER2 positivity may vary depending on the primary tumour location as well as on the histotype of gastric cancer. Indeed, *HER2* overexpression/amplification is detected in more than 30% of the tumours arising from the gastroesophageal junction whereas less than 20% of tumours in the gastric body are HER2-positive. In addition, intestinal and diffuse histotype display a rate of HER2 positivity of 34% and 6% respectively<sup>[17]</sup>. HER2 plays a key role in a large number of cellular processes, including cell differentiation, proliferation, motility and signal transduction. After the combination of chemotherapy and HER2 targeted therapy with trastuzumab had defined a new standard of care for HER2-positive metastatic GC<sup>[3,18,19]</sup>, other HER2 inhibitors were tested.

Lapatinib, a multi-kinase inhibitor, was evaluated in

two randomized phase III trials enrolling GC patients with advanced disease. The LOGiC trial tested the efficacy of lapatinib in combination with capecitabine plus oxaliplatin given upfront. The addition of lapatinib did not significantly increase OS [12.2 mo vs 10.5 mo, hazard ratio (HR) 0.91,  $P = 0.349$ ], although progression-free survival (PFS) was longer (6.0 mo vs 5.4 mo, HR 0.82,  $P = 0.0381$ ) and objective response rate (ORR) was higher (53% vs 39%,  $P = 0.0031$ ) in the lapatinib arm<sup>[19]</sup>. The TyTAN trial randomized 261 Asian patients to receive lapatinib plus paclitaxel or paclitaxel alone in second-line treatment. Disappointingly, no marked survival differences between treatment groups were noted: Median OS (11.0 mo vs 8.9 mo,  $P = 0.1044$ ) and PFS (5.4 mo vs 4.4 mo,  $P = 0.2441$ ). Overall, 15 patients (6%) had previously received trastuzumab, 8 in the lapatinib/paclitaxel arm and 7 in the paclitaxel alone arm<sup>[20]</sup>.

JACOB, a large randomized phase III trial designed to test the efficacy of pertuzumab in combination with trastuzumab and standard chemotherapy (cisplatin plus fluoropyrimidine) has recently completed the accrual<sup>[21]</sup>. Results of the trial are eagerly awaited. Novel anti-HER2 drugs have been developed to try to overcome secondary trastuzumab resistance, as in the case of trastuzumab-emtansine (T-DM1). Data from phase III GATSBY trial were recently presented concluding that TDM-1 did not improve patients' outcome compared to second-line taxanes at the 2015 clinical cut-off<sup>[22]</sup>.

The majority of gastric cancer patients who achieve an initial response to trastuzumab-based regimens develop resistance within 7 mo<sup>[23]</sup>. These unsatisfactory results may be attributed to primary (*de novo*) or secondary (acquired) resistance to the HER2-targeted therapy. Therefore, as it happened for breast cancer, the onset of trastuzumab resistance has been investigated also in gastric cancer, showing several molecular mechanisms underlying the acquired resistance to HER2 inhibitors<sup>[24]</sup>. Lee *et al*<sup>[25]</sup> identified that *HER2*-amplified GC patients have diverse pattern of various concurrent molecular events. Zuo *et al*<sup>[26]</sup> employed the human gastric carcinoma cell line NCI-N87 with high HER2 expression to create trastuzumab-resistant NCI-N87/TR cells by stepwise exposure to increasing doses of trastuzumab. They showed that activation of the PI3K-AKT signalling pathway downstream of HER2 was one of the major mechanisms leading to resistance of NCI-N87/TR gastric cancer cells to trastuzumab, which was probably associated with *PTEN* gene down-regulation and mutation, as well as with over-activity of the IGF-1R signalling pathway<sup>[26]</sup>. The study conducted by Piro *et al*<sup>[27]</sup> identified the FGFR3/AKT axis as an escape pathway responsible for trastuzumab resistance in gastric cancer, indicating that the inhibition of FGFR3 could be a potential strategy to modulate this resistance. Recently, Arienti *et al*<sup>[28]</sup> explored the role of the IQ-domain GTPase-activating protein 1 (IQGAP1), a multifunctional scaffold protein, which interacts with diverse proteins to regulate cell adhesion and cell migration. IQGAP1 governs HER-2 expression, phosphorylation and signalling in breast cancer cell lines<sup>[29]</sup>, it is overexpressed in aggressive form

of gastric cancer<sup>[30]</sup> and its overexpression is correlated with trastuzumab-induced resistance in breast cancer cell lines<sup>[31]</sup>. The study of Arienti *et al*<sup>[28]</sup> revealed that high IQGAP1 expression leads to resistance to trastuzumab in gastric cancer; in addition, they found two new mutations of the *HER2* gene that may be correlated with acquired resistance to the drug. Moreover, a functional cross-talk between the receptor tyrosine kinase MET and HER family members has been reported in the context of the acquisition of aggressive phenotypes<sup>[32]</sup>. The hepatocyte growth factor (HGF) mediated activation of MET may also cause resistance to lapatinib in *HER2*-amplified GC cell lines by stimulating downstream signalling<sup>[33]</sup>. De Silva *et al*<sup>[34]</sup> confirmed *in vitro* that MET is likely to be a significant mechanism of lapatinib resistance *in vivo*. Finally, we recently showed that *HER2* loss may be associated with acquired resistance to first-line trastuzumab-based treatment in patients with initially *HER2*-positive GC<sup>[35]</sup>. All these evidences enhance the complex cross-talk between *HER2* and its downstream pathway and stress the importance of further elucidating the strategies to overcome resistance to *HER2*-targeted therapy. Indeed, identifying the mechanisms underlying treatment resistance would increase the benefit from *HER2*-targeted therapy in patients with *HER2*-positive gastric cancer. Certainly, development of inhibitors targeting multiple receptors or common downstream signalling proteins deserves further investigation.

### EGFR

The EGFR (or ERBB1) belongs to RTKs and it is the second most frequent RTK playing a key role in GC initiation and progression. Despite the wide use of anti-EGFR monoclonal antibodies in colorectal cancer, demonstration of efficacy in GC has not yet been provided. EGFR overexpression has been reported in 24%-27% of all gastric adenocarcinomas<sup>[36]</sup>. Several studies have evaluated the efficacy and safety of different anti-EGFR therapy, based on preclinical data<sup>[37]</sup>. The phase III EXPAND trial evaluated the addition of cetuximab to first-line capecitabine and cisplatin in a non-selected cohort of GC patients. This trial showed no significant advantage in median PFS (4.4 mo vs 5.6 mo in favor of control arm,  $P = 0.32$ )<sup>[38]</sup>. The REAL-3 phase III trial evaluated the addition of panitumumab to epirubicin, oxaliplatin, and capecitabine (EOC). It demonstrated that the addition of panitumumab is detrimental as to OS (11.3 mo for EOC and 8.8 mo for EOC plus panitumumab, HR 1.37, 95%CI:1.07-1.76,  $P = 0.013$ )<sup>[39]</sup>. These disappointing results have been confirmed with another anti-EGFR drug, nimotuzumab<sup>[40]</sup>. The failure of anti-EGFR monoclonal antibodies in advanced GC may lie in the lack of a proper selection, as happened to the patients treated in the aforementioned trials. A recent publication from Birkman *et al*<sup>[41]</sup> studied the prevalence of EGFR overexpression/genomic amplification in gastric intestinal-type adenocarcinoma. In this work, 220 paraffin-embedded samples of GC were collected with the aim of elucidating the prevalence of EGFR over-

expression/amplification, the *HER2* overexpression/amplification and the combination of the previous two. Interestingly, EGFR overexpression was more frequent in intestinal-type GCs (32.7% of the specimens) and its genomic amplification was demonstrated in 14.1% of the patients. It has also been shown that EGFR amplification was associated to a deeper tumor invasion (pT3-4 vs pT1-2, OR 2.15,  $P = 0.029$ ). This unfavourable clinical feature correlated also to a shortened time to cancer recurrence ( $P = 0.026$ ) and cancer specific survival ( $P = 0.033$ ). Furthermore, *HER2* overexpression/amplification has been shown to be less frequent when compared to EGFR overexpression/amplification and EGFR/*HER2* co-amplification (3.6% of the cases), indicating that these two different populations may bear specific genomic alterations potentially approachable with different treatments. All these data strongly suggest that modern trials should be designed with a careful stratification according to EGFR amplification to properly assess the clinical effectiveness of anti-EGFR drugs in GC patients.

### RAS and BRAF

*KRAS* mutation occurs in less than 5% of GC and may have a negative prognostic value in GC patients. *KRAS* activates critical pathways involved in carcinogenesis and tumor progression, such as PI3K-Akt, RAF, MEK-extra-cellular signal regulated kinase and NF- $\kappa$ B. However, no target therapies are currently approved for this molecular aberration<sup>[42]</sup>. Other drugs, such as MEK inhibitors were tested in *KRAS* mutated cancer cell lines with promising results. Since preclinical study suggested that the combination of MEK-inhibitors and PI3K or BCL-XL inhibitors may be efficacious in *KRAS* mutant lung cancer patients<sup>[43]</sup>, it would be intriguing to evaluate MEK inhibitors in monotherapy or in combination with PI3K inhibitors or BCL-XL in GC patients who carry this mutation. In GC patients, *BRAF* mutations are rare (2.2% in TCGA database) and are mostly represented by *BRAF* V599M<sup>[42]</sup>. The role of this mutation in GC is yet to be assessed.

### FGFR2

*FGFR2* amplification is associated with tumor cell proliferation and survival of GC cell lines and indicates poor prognosis. In the TCGA classification, approximately 9% of CIN GC patients had *FGFR2* gene amplification. Several drugs and studies targeting this mutation are ongoing<sup>[5]</sup>. A phase II randomized trial is evaluating the activity of AZD4547 (a FGFR 1-2 and 3 inhibitor) compared to paclitaxel in second-line treatment. Other ongoing trials are testing dovitinib in *FGFR2* amplified GC patients or in combination with docetaxel<sup>[18]</sup>.

### C-MET

Mesenchymal epithelial transition factor (MET) alteration was rarely observed in GC (8%)<sup>[44]</sup>. MET is an RTK that interacts with its native ligand HGF. Deregulated expression of C-MET in GC has been related to worse



prognosis. In fact, the HGF/c-MET signal is involved in cancer growth, invasion, angiogenesis, anti-apoptosis and epithelial to mesenchymal transition<sup>[45]</sup>. Two monoclonal antibodies, rilotumumab (an anti-HGF antibody) and onartuzumab (an anti c-MET antibody) were tested. In a phase I b/II study, rilotumumab was effective and it improved PFS<sup>[46]</sup>. Based on these data, the phase III RILOMET-1 trial, conducted on selected *c-MET* amplified patients, evaluated OS and ORR in the experimental arm with rilotumumab plus ECX compared to control arm with placebo plus ECX. The trial results were negative, and demonstrated that rilotumumab does not improve survival<sup>[47]</sup>. A similar phase III study called RILOMET-2 is ongoing for Asian patients in the same setting<sup>[48]</sup>.

Onartuzumab, a monoclonal antibody directed to c-MET, was tested in MET-Gastric study, in which patients were randomized to receive FOLFOX alone or in combination with onartuzumab. Once again, results were negative (OS: 11.0 mo in the experimental arm vs 11.3 mo in the control arm, HR = 0.82,  $P = 0.24$ )<sup>[49]</sup>. Recently results on a specific MET kinase inhibitor have been presented at ASCO 2016<sup>[50]</sup>. For the first time AMG337 was tested, in a phase I study, in humans with solid tumors: 51 patients were treated and among them 10 had *MET*-amplified gastrointestinal cancers: 4 partial responses and 1 complete response were observed. At the end of the study a maximum tolerated dose of 300 mg was reached. Although an expansion phase on *MET*-amplified patients was on the way, it was early interrupted for excess of toxicity. Despite these negative results, the interest on c-MET as a potential molecular target for novel therapies has not vanished, since better molecular selection of the patients and optimal combination/drugs may finally achieve the expected results.

### VEGF and VEGFR-2

Another frequently amplified gene in CIN subtype is *VEGF*, a mediator of angiogenesis that is essential for cancer growth and metastasis as it ensures oxygen and nutrients supply to proliferating cancer cells<sup>[51]</sup>. Bevacizumab, a monoclonal antibody that targets VEGF, was tested in the AVAGAST trial. This study did not meet its primary endpoint of improved OS (median OS 12.1 mo vs 10.1 mo, HR 0.87 95%CI: 0.73-1.03,  $P = 0.1$ ), but improvements in median PFS and tumor response rate were reported<sup>[52]</sup>. Similarly, the AVATAR trial showed no survival benefit with antiangiogenic therapy added to cisplatin and capecitabine-based regimens (HR 1.1)<sup>[53]</sup>. Although the addition of bevacizumab to standard therapy showed disappointing results, antiangiogenic strategy was further investigated beyond first line treatment. Ramucirumab, a fully human monoclonal IgG directed against VEGFR-2, was evaluated both as single agent and in combination with chemotherapy<sup>[54-56]</sup>. In the REGARD trial, ramucirumab demonstrated a statistically significant improvement when compared to the best supportive care in pretreated GC patients with advanced disease (OS: 5.2 mo vs 3.8 mo respectively, HR = 0.776;  $P = 0.047$ )<sup>[54]</sup>. In the RAINBOW trial,

patients were randomized to receive paclitaxel with or without ramucirumab. Median OS was 9.63 mo for the combination therapy and 7.36 mo for paclitaxel alone (HR = 0.807, 95%CI: 0.678-0.962;  $P = 0.017$ )<sup>[55]</sup>. Recently, a novel VEGFR-2 tyrosine kinase inhibitor, apatinib, was evaluated in Asian patients who had previously received 2 or 3 lines of chemotherapy<sup>[57]</sup>. Patients exposed to apatinib had an improved median OS (6.5 mo vs 4.7 mo; HR = 0.709; 95%CI: 0.537-0.937;  $P = 0.156$ ) and median PFS (2.6 mo vs 1.8 mo; HR = 0.444; 95%CI: 0.331-0.595;  $P < 0.001$ ) compared to patients who received placebo. Therefore, multitarget TKIs represent another potential approach to block angiogenesis by simultaneously targeting VEGFR and other signaling pathways. Notably, the role of antiangiogenic strategy seems to gain importance in subsequent lines of treatment, but its role in first-line therapy is still unclear. An ongoing randomized phase III trial is assessing the potential survival benefit of ramucirumab in combination with cisplatin and capecitabine given upfront<sup>[56]</sup>.

### GC WITH MICROSATELLITE INSTABILITY

According to the TCGA's molecular classification, the enrichment for microsatellite instability (MSI) characterizes a distinct molecular subgroup of GC. MSI occurs in about 15%-30% of GCs, and more frequently correlates with intestinal histotype, location in the distal part of the stomach, female gender and older age at diagnosis<sup>[5,58,59]</sup>.

MSI is a genetic alteration consisting of the expansion or contraction of regions of repetitive nucleotide sequences, called microsatellites. The alteration is triggered by a dysfunction of DNA mismatch repair (MMR) enzymes, caused by mutations in one of several different DNA mismatch repair genes (*i.e.*, *MLH1* or *MSH2*). In a single cell, bi-allelic inactivation of *MMR* genes causes an increased mutation rate (genomic instability) due to the failure of DNA mismatch repair that usually occurs during normal DNA synthesis<sup>[60]</sup>.

Defective DNA mismatch repair is the hallmark of Lynch syndrome. Moreover, approximately 15% of sporadic colorectal cancers also displays MSI since both alleles of a *MMR* gene are inactivated<sup>[61]</sup>. Different *MMR* genes are probably involved in MSI-high (MSI-H) sporadic gastric cancer without *MLH1* hypermethylation, which represents the main mechanism leading to MMR deficiency in MSI GC<sup>[62,63]</sup>.

MSI-H colorectal cancer have better prognosis compared to MSI low, and should not receive adjuvant chemotherapy with fluoropyrimidine after resection for stage II disease<sup>[64]</sup>. In gastric cancer, 5-FU is frequently used and information about sensitivity to this agent may be very useful. A meta-analysis of Zhu *et al.*<sup>[65]</sup> showed a 37% mortality risk reduction and improved median OS in patients with MSI-H compared to MSI-L(low) or microsatellite stable (MSS) GC patients. The relationship between MMRd, MSI and survival has been examined in patients with resectable GC randomized to surgery alone or perioperative chemotherapy within the MRC MAGIC



trial. MSI and *MLH1* deficiency was associated with a better outcome in patients treated with surgery alone while it had a negative prognostic effect in those treated with chemotherapy<sup>[62]</sup>.

Despite MSI cases generally lack of targetable amplifications, mutation in *PIK3CA*, *ERBB3*, *ERB22* and *EGFR* are noted<sup>[5,59]</sup>; *BRAF* V600E mutations, commonly seen in MSI colorectal cancer, are absent in MSI GC<sup>[5]</sup>. However, the predictive role of these mutations in MSI GC population is uncertain. The combination of olaparib with paclitaxel as second-line therapy was found to be more active compared with paclitaxel alone in patients with metastatic or recurrent GC. Although the trial did not meet its primary endpoint (namely PFS), olaparib prolonged survival in patients with low levels of ataxia telangiectasia mutated, a key activator of DNA damage response<sup>[66]</sup>. A phase III trial in this setting is under way and detailed analysis in MSI GC could be attractive.

The hypothesis of an increased activity of immunotherapy in MSI non-colorectal cancer has recently generated interest. In fact, the increased number of somatic mutations may amplify the number of neoantigens, thus stimulating the immune system and conferring higher sensitivity to PD-1 blockade to tumor<sup>[67,68]</sup>. Interestingly, the tendency to have a lymphocytic infiltrate, observed in MSI tumors, likely reflects immune activation of T-cells directed against tumor-specific carboxy-terminal frameshift peptides that are associated with MSI<sup>[69]</sup>. In addition to that, genomic aberrations in tumor cells lead to aberrant PD-L1 expression, suggesting a predictive role for MSI.

MSI has already been reported as a strong predictive factor for the use of immune check-point inhibitors in the treatment of patients with colorectal cancer<sup>[70]</sup>. The immune-related objective response rate and immune-related 6-mo PFS rate were 40% and 78%, respectively, for patients with dMMR and 0% and 11% for those with MMR-proficient cancer, with a higher median PFS and survival in the cohort with dMMR colorectal cancers vs 2.2 and 5.0 mo, respectively, in the cohort with MMR-proficient tumors. Le *et al*<sup>[68]</sup> enrolled 41 consecutive patients (9 patients with MMR deficient solid tumors other than colorectal cancer, only 1 patient with GC) to explore the activity of PD-1 blockade according to MMR status in non colorectal cancer too. Although data are not ready for clinical application, 30% of GC have been shown to present with a burden of nonsynonymous mutations that may define who are the optimal candidates for immune checkpoint inhibitors treatment<sup>[71]</sup>. Of note, a phase 2 study of pembrolizumab in subjects with advanced gastric or gastroesophageal junction adenocarcinoma who progressed after first-line therapy with platinum and fluoropyrimidine is currently recruiting participants<sup>[72]</sup>. Muro *et al*<sup>[73]</sup> have recently reported the activity of pembrolizumab in GC in a phase I trial. The authors showed a decrease in tumor burden in 41% of the study patients. The ORR was 32% in Asian patients and 30% in non-Asian patients<sup>[73]</sup>. A phase 2 trial of nivolumab or nivolumab plus ipilimumab is recruiting patients to evaluate the response to checkpoint inhibitors in MSI-H gastrointestinal

cancers<sup>[74]</sup>. Interestingly, a preventive vaccine, set-up using neopeptides frequently affecting MSI tumorigenesis, has been shown to delay the onset of dMMR tumors. It remains to be proven if vaccination against these neopeptides might be a promising approach for novel adjuvant treatment strategies in patients with MSI-H tumors<sup>[75]</sup>.

## GC WITH GENOMIC STABILITY

GS GCs account for around 20% of all the tumors analyzed by the TCGA project. This subtype occurs with equal frequency in males and females. GS gastric tumors are enriched for the diffuse histological variant [58% according to Lauren's classification) and for the poor cohesive variant (58% according to World Health Organization (WHO) classification]. One quarter of GS GCs arise in the antrum, about 20% in the gastroesophageal junction/cardia, and approximately 15% in the gastric body/fundus. The principal somatic genomic alterations observed in GS gastric tumors involve *CDH1*, *ARID1A* and *RHOA*. In addition, a recurrent interchromosomal translocation (between *CLDN18* and *ARHGAP26*) implicated in cell motility was found in GS gastric tumors<sup>[5]</sup>.

### CDH1

The *CDH1* gene is located on chromosome 16q22.1 and encodes E-cadherin, which belongs to the cadherin superfamily of calcium-dependent cell adhesion molecules. E-cadherin plays a well-documented role in the progression of epithelial cancers. Inactivating mutations in the *CDH1* gene are frequently found in gastric cancer, especially in hereditary diffuse gastric cancer<sup>[76]</sup>. *CDH1* promoter methylation is also frequently found in sporadic gastric cancer<sup>[77]</sup>. During epithelial tumorigenesis, the protein is downregulated and E-cadherin has been categorized as a tumor suppressor gene<sup>[78]</sup>. Li *et al*<sup>[79]</sup> reported that in diffuse-type GC, *CDH1* mutation is associated with shortened patients survival, independently from disease stage. In the analysis of the TCGA Research Network *CDH1* somatic mutations were enriched in the GS subtype (37% of cases). Therefore, the prognostic value of *CDH1* as well as its potential as therapeutic target in gastric cancer has yet to be fully understood and explored.

### ARID1A

Inactivating mutations of *ARID1A* were found in GS gastric cancer, as in the EBV-subtype<sup>[5]</sup>. The *ARID1A* gene, located in chromosome 1p35.3, encodes adenine-thymine-rich interactive domain-containing protein 1A, which participates in chromatin remodeling, therefore is involved in regulating cellular processes including DNA repair, differentiation, and development<sup>[80]</sup>. As shown by Wang *et al*<sup>[81]</sup>, loss of *ARID1A* expression was significantly correlated with tumor stage and grade; moreover, it was also significantly correlated with poor survival in GC patients. Restoring *ARID1A* expression in gastric cancer cells significantly inhibited cell proliferation and colony formation, whereas silencing *ARID1A* expression

in gastric epithelial cell lines significantly enhanced cell growth rate<sup>[81]</sup>.

### RHOA

Rho belongs to the Ras-related family of small molecular weight GTP-binding proteins, and it works as a molecular switch between the GDP-bound inactive form and the GTP-bound active form<sup>[82]</sup>. It regulates cytoskeletal organization, cell adhesion, intracellular membrane trafficking, gene transcription, apoptosis, and cell cycle progression<sup>[83]</sup>; moreover, it activates STAT3 to promote tumorigenesis<sup>[84]</sup>. RhoA plays a role in these processes through a variety of effectors including ROCK1, mDia and protein kinase N<sup>[85]</sup>. mDia is involved in nucleation and polymerization of actin filaments, while ROCK intervenes in induction of actinomyosin bundles and contractility. The balance between mDia and ROCK regulates cell morphogenesis, adhesion, and motility activities. In addition, the Rho-ROCK pathway is involved in Ras-mediated transformation, the amoeboid movement of tumor cells in the three-dimensional matrix, and transmigration of tumor cells through the mesothelial monolayer<sup>[86]</sup>. According to the TCGA, RHOA mutations were clustered in two adjacent amino-terminal regions that are predicted to be at the interface of RHOA with ROCK1 and other effectors, leading to a modulation of signaling downstream of RHOA<sup>[5]</sup>. Interestingly, diffuse-type GCs, characterized by malignant phenotype and stromal differentiation, frequently have gain-of-function mutations of RHOA<sup>[87]</sup>.

The TCGA network discovered a recurrent inter-chromosomal translocation between claudin 18 (*CLDN18*) and Rho GTPase-activating protein 6 (*ARHGAP26*), resulting in the *CLDN18-ARHGAP26* fusion gene, which primarily occurs in GS GC<sup>[5]</sup>. *ARHGAP26* (also known as GTPase Regulator Associated with Focal Adhesion Kinase, GRAF) is a GTPase-activating protein that facilitates conversion of RHO GTPases to the GDP state and has been implicated in enhancing cellular motility<sup>[88]</sup>. *CLDN18* is a component of the tight junction adhesion structures<sup>[89]</sup>. Yao *et al.*<sup>[90]</sup> showed that expression of *CLDN18-ARHGAP26* fusion gene in gastric epithelial cells resulted in epithelial-mesenchymal transition, which is indicative of cell transformation in cancer development. A recent trial tested IMAB362, a chimeric IgG1 antibody against *CLDN18.2* showing clinical activity in patients with 2 + /3 + immunostaining<sup>[91]</sup>.

The *CLDN18-ARHGAP* fusions were mutually exclusive with *RHOA* mutations; within the GS subtype, 30% of cases had either *RHOA* or *CLDN18-ARHGAP* alterations<sup>[5]</sup>.

Given the role of *RHOA* in cell motility, modulation of *RHOA* may contribute to the disparate growth patterns and lack of cellular cohesion that are hallmarks of diffuse tumors.

Rho/Rho-kinase inhibitors have been explored as putative therapeutic targets in various diseases, including cancers<sup>[92]</sup>. The development of drugs that inhibit Rho GTPase signaling would be of great potential in this

setting.

### Other notable patterns

The GS subtype exhibited elevated expression of cell adhesion pathways, including the B1/B3 integrins, syndecan-1-mediated signaling, and angiogenesis-related pathways. Also in the GS subtype, hierarchical clustering of samples and pathways revealed several notable patterns, including elevated expression of mitotic network components such as AURKA/B and E2F, targets of MYC activation, FOXM1 and PLK1 signaling and DNA damage response pathways<sup>[5]</sup>. Specific inhibitors of AURKA are currently under investigation in phase I / II clinical trials in advanced GC<sup>[93]</sup>. PLKs, mitotic kinases of the polo family, play a pivotal role in the normal cell cycle, and their overexpression is involved in the pathogenesis of multiple human cancers<sup>[94]</sup>. PLK1 is overexpressed in approximately 80% of human tumors, including gastric cancer, and it is associated with poor prognosis<sup>[94]</sup>. Currently, inhibitors of PLK1 are being developed<sup>[95]</sup>. In a phase I trials enrolling patients with advanced solid cancers, including gastric cancer, volasertib, a potent and selective PLK inhibitor that induces mitotic arrest and apoptosis, demonstrated anti-cancer activity with a manageable safety profile<sup>[96]</sup>.

### EBV ASSOCIATED GC

Latent EBV infection is associated with about 10% of GCs, as demonstrated by *in situ* hybridization EBV encoded miRNA detection, by whole genome sequencing or by PCR EBV genome detection<sup>[5]</sup>.

EBV associated GC has been related to different epidemiological and clinico-pathological features. In a meta-analysis of 39 case-control studies, Bae *et al.*<sup>[97]</sup> investigated the strength of association between EBV infection and GC risk, and showed a 10 fold increase (95%CI: 5.89-17.29). It was also reported that there is a higher risk of EBV associated GC in Far East Asia if compared to Europe<sup>[98]</sup>.

In a meta-analysis of 70 studies the pooled prevalence of EBV-positive GC resulted 8.7% (95%CI: 7.5%-10.0%) with similar distributions across the three analyzed geographic regions (America, Asia and Europe). Moreover, a two-fold difference in male/female ratio favored men as to prevalence of EBV positive GC. The antral location was less frequently associated with EBV infection when compared to other types. In contrast, there was no statistically significant difference in the proportion of EBV-positive disease between intestinal (9.5%; 95%CI: 7.2%-12.5%) and diffuse (7.6%; 95%CI: 5.7%-10.3%) histology<sup>[98]</sup>.

In addition, EBV-positive GC was more prevalent in younger patients compared to older subjects<sup>[99]</sup>.

As to possible therapeutic approaches, Kim *et al.*<sup>[100]</sup> observed that EBV infected GC patients had a higher rate of alteration in pathways related to immune response which may also be related to a more favorable prognosis

in these patients. According to TCGA, *PD-L1* gene was frequently amplified in EBV-positive GC, adding proofs to the hypothesis of higher immunogenicity of this class of GC. Based on the evidence that 15% of EBV positive GC harbor amplification of chromosomal region 9p24.1, the locus of *PD-L1* and *PD-L2*, potential role of *PD-L1* expression in EBV-positive GC was investigated in a study<sup>[101]</sup>. In EBV-associated GC, *PD-L1* expression was present in 50% (16/32) and 94% (30/32) of tumor and immune cells, respectively. In contrast, EBV-negative GC showed a lower *PD-L1* expression (10% and 39% of tumor and immune cells, respectively,  $P < 0.001$ ), thus providing a further rationale for testing *PD-1* expression in this GC subtype to potentially identify a predictive response factor for immunomodulatory therapeutic strategies.

Besides *PD-L1* and *PD-L2* expression, *PIK3CA* mutations, DNA hypermethylation, and *JAK2* mutations are also present<sup>[5]</sup>. In a large retrospective study, 855 GC specimens were analyzed to verify protein expression levels and prognostic values of *PIK3CA*, *JAK2*, *PD-L1* and *PD-L2*. Only 59 samples were found to be EBV positive. *PIK3CA* and *PD-L2* were more highly expressed in EBV positive GC than in negative ones, but no prognostic value of *PIK3CA*, *JAK2*, *PD-L1* or *PD-L2* was found. No differences in *JAK2*, *PD-L1* or *PD-L2* expression were seen between EBV positive and negative cases. Moreover, the expression of *PIK3CA*, *JAK2*, *PD-L1* or *PD-L2* was not significantly associated with any clinico-pathological feature, maybe due to the small number of EBV-associated GC cases, and the prognostic value of these mutations remains uncertain<sup>[102]</sup>.

## THE ACRG CLASSIFICATION

The ACRG proposed a different molecular classification for gastric cancer in 2015<sup>[7]</sup>. This classification has some overlapping features with the one proposed by TCGA even though some differences can be highlighted. The clustering process included a first subdivision into MSI (22.7%, better prognosis, mainly intestinal type) and EMT tumours (15.3%, worse prognosis mainly diffused type) with two exclusive gene expression profiles, the first characterized by the loss of function of genes involved in the MMR and the second by alterations in cell adhesion, angiogenesis, and motility. Notably, the MSI subtype was associated with a hypermutation in genes such as: *KRAS* (23.3%), *PI3K*-*PTEN*-*mTOR* pathway (42%), *ALK* (16.3%) *ARID1A* (44.2%), *ERBB2* (16.3%) and *ERBB3* (14%). The remaining tumours were further divided into *MSS/TP53*<sup>+</sup> (26.3%, *P53* function intact) and *MSS/TP53*<sup>-</sup> (35.7%, loss of oncosuppressor function). In terms of survival, the MSI subtype showed the best overall prognosis, followed by *MSS/TP53*<sup>+</sup>, *MSS/TP53*<sup>-</sup> and *MSS/EMT*. The *MSI/TP53*<sup>+</sup> subtype was more frequently associated with EBV infection if compared to the other groups and showed an active *TP53* pathway and a higher prevalence (compared to *MSI/TP53*<sup>-</sup>) of *APC*, *ARID1A*,

*KRAS*, *PI3KCA*, and *SMAD4* mutations. Finally, the *MSI/TP53*<sup>-</sup> subtype showed the highest prevalence of *TP53* mutations, relevant copy number variations (CNVs), a greater aneuploidy and recurrent focal amplifications in *MDM2*, *ROBO2*, *GATA6*, *MYC*, *ERBB2*, *EGFR*, *CCNE1* and *CCND1*. These latter two amplifications were mutually exclusive, so they could be considered driver alterations.

A comparison of the ACRG categories with the TCGA subtypes showed similarities in the tumors with *MSI*, while *GS* was approximated to *MSS/EMT*, *EBV* to *MSS/TP53*<sup>+</sup>, and *CIN* to *MSS/TP53*<sup>-</sup>. Nevertheless, in the TCGA cohort the EBV positive cancers represented a separated subgroup (with a favourable phenotype), whereas in the ACRG classification EBV infection occurred more frequently in the *MSS/TP53*<sup>+</sup> subtype, without CNVs, hypermethylation or hypermutation. Moreover, *PI3KCA* and *ARID1A* mutations were more prevalent in *EBV*<sup>+</sup> gastric cancers compared to *MSS* subtypes.

Although both the *MSS/EMT* and the *GS* molecular subgroups included tumors with a prevalent diffuse histology, the TCGA classification showed a lower percentage of Lauren's diffuse subtype compared to the ACRG database (24% vs 45% respectively); additionally, *CDH1* and *RHOA* mutations did not appear prevalent in the *MSS/EMT* subgroup, unlike the *GS* subtype. Finally, *GS* tumours were also present in the ACRG *MSS/EMT*, *MSS/TP53*<sup>+</sup> and *MSS/TP53*<sup>-</sup> molecular subgroups. All these findings showed that the *GS* and the *MSS/EMT* subgroups were not equivalent.

The comparison of the *CIN* TCGA subtype to ACRG *MSS/TP53*<sup>-</sup> subtype showed that the first is quite homogeneously distributed in the subtypes classified by ACRG.

Overall survival associations were weaker when using the TCGA genomic scheme in the ACRG cohort compared to the original prognosis trends: While the *MSI* subtype showed a better prognosis in both classifications, there were no differences in prognosis in *CIN* and *GS* subtypes when they were identified based on application of the TCGA classification on the ACRG patient population.

## CONCLUSION

While the advent of novel molecular classifications has faded the "one size fit-all" era, a more profound understanding of the underpinning tumour biology has set the dawn of a more contemporary clinical approach called precision medicine. At present, the two aforementioned genomic classifications of GC represent the state-of-the-art achieved so far. Somehow it is possible to find an overlap between the TCGA and ACRG subtypes even though some difference can still be found. Emerging data clearly individuate a category of GC characterized by *MSI* that may benefit from immunotherapeutic approaches. For this subgroup, with good prognosis, the development of anti *PD-1/PD-L1* drugs could be the leading research avenue. High mutational burden is also a driving feature of *EBV* positive GC that could be targeted with immunotherapy as

**Table 1 Clinical outcomes of recent trials in gastric and esophagogastric adenocarcinomas**

Trial name	Phase of study	Line of treatment	Selected biomarker	Treatment arms	n	Primary endpoint	Outcomes
<b>CIN</b>							
TOGA <sup>[3]</sup>	III	First	HER2 expression/ amplification	CF/CX CF/CX + trastuzumab	296 298	OS	OS: 13.8 mo <i>vs</i> 11.1 mo (HR = 0.74, <i>P</i> = 0.005) PFS: 6.7 mo <i>vs</i> 5.5 mo (HR = 0.71, <i>P</i> = 0.0002) ORR: 47% <i>vs</i> 35% ( <i>P</i> = 0.001)
LOGIC <sup>[19]</sup>	III	First	HER2 expression/ amplification	CapeOX CapeOx + lapatinib	273 272	OS	OS: 12.2 mo <i>vs</i> 10.5 mo (HR = 0.91, <i>P</i> = 0.34) PFS: 6.0 mo <i>vs</i> 5.4 mo (HR = 0.82, <i>P</i> = 0.038) ORR: 53% <i>vs</i> 39% ( <i>P</i> = 0.003)
TyTAN <sup>[20]</sup>	III	Second	HER2 amplification by FISH	Paclitaxel Paclitaxel + lapatinib	129 132	OS	OS: 11.0 mo <i>vs</i> 8.9 mo (HR = 0.84, <i>P</i> = 0.104) PFS: 5.4 mo <i>vs</i> 4.4 mo (HR = 0.85, <i>P</i> = 0.244) ORR: 27% <i>vs</i> 9% ( <i>P</i> < 0.001)
JACOB <sup>[21]</sup>	III	First	HER2 expression/ amplification	Pertuzumab + tFP Placebo + tFP		OS	Ongoing
GATSBY <sup>[22]</sup>	II / III	Second	HER2 expression/ amplification	TAX T-DM1	117 228	OS	OS: 8.6 mo <i>vs</i> 7.9 mo (HR = 1.15, <i>P</i> = 0.86) PFS: 2.9 mo <i>vs</i> 2.7 mo (HR = 1.13, <i>P</i> = 0.31) ORR: 19.6% <i>vs</i> 20.6%
EXPAND <sup>[38]</sup>	III	First	Unselected	CX CX + cetuximab	449 445	PFS	OS: 10.7 mo <i>vs</i> 9.4 mo (HR = 1.0, <i>P</i> = 0.95) PFS: 5.6 mo <i>vs</i> 4.4 mo (HR = 1.09, <i>P</i> = 0.32)
REAL-3 <sup>[39]</sup>	III	First	Unselected	EOC EOC + panitumumab	275 278	OS	OS: 11.3 mo <i>vs</i> 8.8 mo (HR = 1.37, <i>P</i> = 0.013) PFS: 7.4 mo <i>vs</i> 6.0 mo (HR = 1.22, <i>P</i> = 0.068) ORR: 42% <i>vs</i> 46% ( <i>P</i> = 0.42)
RILOMET-1 <sup>[47]</sup>	III	First	MET positive by IHC HER2 negative	ECX ECX + rilotumumab	305 304	OS	OS: 11.5 mo <i>vs</i> 9.6 mo (HR = 1.37, <i>P</i> = 0.016) PFS: 5.7 mo <i>vs</i> 5.7 mo (HR = 1.30, <i>P</i> = 0.016) ORR: 39.2% <i>vs</i> 30% (OR = 0.67, <i>P</i> = 0.027)
METGastric <sup>[49]</sup>	III	First	MET positive by IHC HER2 negative	mFOLFOX mFOLFOX + ornatuzumab	562	OS	OS: 11.3 mo <i>vs</i> 11.0 mo (HR = 0.82, <i>P</i> = 0.244) PFS: 6.8 mo <i>vs</i> 6.3 mo (HR = 0.90, <i>P</i> = 0.429) ORR: 41% <i>vs</i> 46% ( <i>P</i> = 0.253)
AVAGAST <sup>[52]</sup>	III	First	Unselected	CX CX + bevacizumab	387 387	OS	OS: 10.1 mo <i>vs</i> 12.1 mo (HR = 0.87, <i>P</i> = 0.1) PFS: 5.3 mo <i>vs</i> 6.7 mo (HR = 0.80, <i>P</i> = 0.037) ORR: 37.4% <i>vs</i> 46.0% ( <i>P</i> = 0.03)
AVATAR <sup>[53]</sup>	III	First	Unselected	CX CX + bevacizumab	102 100	OS	OS: 11.4 mo <i>vs</i> 10.5 mo (HR = 1.11, <i>P</i> = 0.55) PFS: 6.0 mo <i>vs</i> 6.3 mo (HR = 0.89, <i>P</i> = 0.47) ORR: 34% <i>vs</i> 41% ( <i>P</i> = 0.35)
REGARD <sup>[54]</sup>	III	Progression after TP	Unselected	BSC BSC + ramucirumab	117 238	OS	OS: 3.8 mo <i>vs</i> 5.2 mo (HR = 0.77, <i>P</i> = 0.047) PFS: 1.3 mo <i>vs</i> 2.1 mo (HR = 0.48, <i>P</i> < 0.001)
RAINBOW <sup>[55]</sup>	III	Second	Unselected	Paclitaxel Paclitaxel + ramucirumab	335 330	OS	OS: 7.4 mo <i>vs</i> 9.6 mo (HR = 0.80, <i>P</i> = 0.017) PFS: 2.9 mo <i>vs</i> 4.4 mo (HR = 0.63, <i>P</i> < 0.0001)
Apatinib <sup>[57]</sup>	III	Third or more	Unselected	Placebo Apatinib	91 176	OS	OS: 4.7 mo <i>vs</i> 6.5 mo (HR = 0.70, <i>P</i> = 0.015) PFS: 1.8 mo <i>vs</i> 2.6 mo (HR = 0.44, <i>P</i> < 0.001) ORR: 0% <i>vs</i> 2.84% ( <i>P</i> = 0.16)
<b>MSI</b>							
NCT01063517 <sup>[66]</sup>	II	Second	ATM expression	Paclitaxel Paclitaxel + olaparib	62 61	PFS	OS: 8.3 mo <i>vs</i> 13.1 mo (HR = 0.56, <i>P</i> = 0.01) PFS: 3.55 mo <i>vs</i> 3.91 mo (HR = 0.80, <i>P</i> = 0.13)
NCT02589496	II	Second	Unselected	Pembrolizumab		RR	Ongoing
<b>GS</b>							
FAST <sup>[91]</sup>	II	First	CLDN18.2	EOX EOX + IMAB362	161	PFS	OS: 8.7 mo <i>vs</i> 12.5 mo (HR = 0.5) PFS: 5.7 mo <i>vs</i> 7.9 mo (HR = 0.5, <i>P</i> = 0.001)

Most significant target-oriented phase II and phase III trials are presented. In the table are shown in order: name of the trial, phase of the study, line of treatment, biomarker selection, treatment arms, number of enrolled patients, primary endpoint and key outcome results. tFP: Trastuzumab + Platinum + fluorouracil; PF: Platinum + fluoropyrimidine; TAX: Taxane, CF: Cisplatin + fluorouracil; CX: Cisplatin + capecitabine; EOC (or ECX): Epirubicin + oxaliplatin + capecitabine; BSC: Best supportive care; CIN: Chromosomal instability; GS: Genomical stability; MSI: Microsatellite instability.

efficaciously as in MSI tumours.

It is also possible to clearly segregate another class of GC classified either as GS or MMS/EMT, in which the prevalent deregulation is represented by EMT pathway alterations. Development of inhibitors of HGF/c-Met pathway, Rho/Rho-kinase, AURKA/AURKB, PLK1 could be a strategy adopted in the near future.

The category corresponding to CIN, and partially to MSS/TP53<sup>-</sup>, represents a cluster of GC with high CNV variation leading to deregulation of specific biological

targets such as receptors and kinases. Since these driver alterations are mostly mutually exclusive, they could be easily targeted using specific monoclonal antibodies or TKIs. On the other side, tumour heterogeneity may limit the efficacy of targeted strategies through alternative mechanisms of primary and acquired resistance<sup>[103]</sup>.

The overall landscape is complex and our knowledge on this topic is still just at the starting point and novel trials should be designed accordingly (Table 1)<sup>[3,19-22,38,39,47,49,52-55,57,66,91]</sup>. Doubtlessly, dissecting and genotyping different



tumour subtypes and setting apart patients with different diseases will represent the future of gastrointestinal oncology. The key landmark comprehensive efforts made by TCGA and ACRG have just paved the way for precision oncology.

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Basic Study

# Extramural vascular invasion and response to neoadjuvant chemoradiotherapy in rectal cancer: Influence of the CpG island methylator phenotype

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## Abstract

### AIM

To identify whether CpG island methylator phenotype (CIMP) is predictive of response to neoadjuvant chemoradiotherapy (NACRT) and outcomes in rectal cancer.

### METHODS

Patients undergoing NACRT and surgical resection for rectal cancer in a tertiary referral centre between 2002-2011 were identified. Pre-treatment tumour biopsies were analysed for CIMP status (high, intermediate or low) using methylation specific PCR. *KRAS* and *BRAF* status were also determined using pyrosequencing analysis. Clinical information was extracted from case records and cancer services databases. Response to radiotherapy was measured by tumour regression scores determined upon

histological examination of the resected specimen. The relationship between these molecular features, response to NACRT and oncological outcomes were analysed.

## RESULTS

There were 160 patients analysed with a median follow-up time of 46.4 mo. Twenty-one (13%) patients demonstrated high levels of CIMP methylation (CIMP-H) and this was significantly associated with increased risk of extramural vascular invasion (EMVI) compared with CIMP-L [8/21 (38%) *vs* 15/99 (15%),  $P = 0.028$ ]. CIMP status was not related to tumour regression after radiotherapy or survival, however EMVI was significantly associated with adverse survival ( $P < 0.001$ ). Intermediate CIMP status was significantly associated with *KRAS* mutation ( $P = 0.01$ ). There were 14 (9%) patients with a pathological complete response (pCR) compared to 116 (73%) patients having no or minimal regression after neoadjuvant chemoradiotherapy. Those patients with pCR had median survival of 106 mo compared to 65.8 mo with minimal regression, although this was not statistically significant ( $P = 0.26$ ). Binary logistic regression analysis of the relationship between EMVI and other prognostic features revealed, EMVI positivity was associated with poor overall survival, advanced "T" stage and CIMP-H but not nodal status, age, sex, *KRAS* mutation status and presence of local or systemic recurrence.

## CONCLUSION

We report a novel association of pre-treatment characterisation of CIMP-H with EMVI status which has prognostic implications and is not readily detectable on pre-treatment histological examination.

**Key words:** Rectal cancer; CpG islands; Methylation

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**Core tip:** There is wide and unpredictable response of rectal cancer to neoadjuvant therapy which carries significant side effects and relies on limited pre-treatment risk stratification. Methylation specific PCR was used to determine CpG island Methylator phenotype (CIMP) status in 160 rectal cancers and compared with response to therapy, clinical and pathological outcomes. CIMP status was not directly related to tumour regression but was related to extramural vascular invasion which confers an adverse survival risk.

Williamson JS, Jones HG, Williams N, Griffiths AP, Jenkins G, Beynon J, Harris DA. Extramural vascular invasion and response to neoadjuvant chemoradiotherapy in rectal cancer: Influence of the CpG island methylator phenotype. *World J Gastrointest Oncol* 2017; 9(5): 209-217 Available from: URL: <http://www.wjgnet.com/1948-5204/full/v9/i5/209.htm> DOI: <http://dx.doi.org/10.4251/wjgo.v9.i5.209>

## INTRODUCTION

Locally advanced rectal cancer is usually treated with neoadjuvant chemoradiotherapy to downstage and/or downsize the tumour prior to surgery<sup>[1,2]</sup>. The response of rectal cancer to neoadjuvant therapy varies significantly between patients. The most successful outcome is a pathological complete response (pCR) in which no viable tumour cells are seen upon subsequent histological examination of the resected bowel. In this scenario patients have a significantly improved 5-year survival of up to 85%-100%, although any residual lymph-nodal involvement is associated with a significantly worse survival despite complete local tumour regression<sup>[3]</sup>. This compares favourably with those showing minimal response to radiotherapy who may expect 5-year survival of between 55%-66%<sup>[4]</sup>.

pCR occurs in between 10%-20% of patients undergoing neoadjuvant chemoradiation therapy<sup>[4-6]</sup>, however up to 30% of patients do not show any response<sup>[7]</sup>. Furthermore, those patients not responding to neoadjuvant treatment risk progression of their disease with either local progression or distant metastases during preoperative treatment. The use of imaging technology including magnetic resonance imaging (MRI) and endo-rectal ultrasound are not sufficiently reliable<sup>[8,9]</sup> to be implemented as a sole means of discriminating between those with pCR and those without.

The adverse prognostic value of extramural vascular invasion (EMVI) is well established and is known to be associated with poor survival<sup>[10]</sup>, increased risk of local recurrence<sup>[11]</sup> and death<sup>[11-14]</sup>. Furthermore, the presence of EMVI has a relative risk of 3.7 for the development of systemic recurrence when detectable on preoperative MRI scanning<sup>[15]</sup>. The role of EMVI in directing treatment is relatively new and not well established. In particular, National Institute of Health and Care Excellence recommend that EMVI may confer a higher risk of recurrence in stage II rectal cancers and suggest adjuvant chemotherapy may be considered in those patients with EMVI where the relative benefits of this treatment are not otherwise clear<sup>[16]</sup>. EMVI status may also influence the decision to offer neoadjuvant radiotherapy, as it has been demonstrated that chemoradiation (CRT) can cause vessel fibrosis in EMVI-positive tumours, which may influence survival outcomes<sup>[17]</sup>.

EMVI is detectable in rectal cancer patients on MRI, however, sensitivity and specificity are relatively low at 62% and 88% respectively<sup>[17]</sup>. It is therefore important that not only is EMVI accurately characterised but should be available early to inform decisions regarding neoadjuvant chemoradiotherapy and influence overall treatment outcomes.

Developments in genetics and epigenetics lend support to the notion that tumours display characteristic clinicopathological and morphological features depending on the nature of specific combinations of molecular pat-

terms<sup>[18]</sup>. In particular, the CpG island methylator phenotype (CIMP), which may account for up to 20% of all colorectal cancers<sup>[19,20]</sup>, is associated with differences in tumour location, patient gender and association with characteristic gene mutations including *KRAS*, *BRAF* and *p53*<sup>[18]</sup>, although this relationship has not been explored in EMVI. CpG islands are typically short (300-3000 base pairs) Cytosine-Guanine phosphodiester bonded sequences found in or around the promoter region of a gene where they are usually unmethylated if the genes are expressed. The CIMP phenotype is characterised by epigenetic DNA hyper-methylation and consequent suppression of key genes important in controlling cell growth and survival, which is associated with poor survival in rectal cancer<sup>[21,22]</sup>. It is becoming increasingly clear that epigenetic factors affecting specific gene promoter regions (CpG islands) can be equally as important as genetic alterations in all disease processes, as these can affect every component of gene regulation. Previous work has demonstrated that genetic factors such as *KRAS* mutation has an inverse relationship with EMVI<sup>[23]</sup> but little is known of the influence of epigenetic factors in the development of EMVI. The purpose of this study was to explore the relationship between CIMP and response to chemoradiotherapy and EMVI in rectal cancer.

## MATERIALS AND METHODS

Patients undergoing neoadjuvant chemoradiotherapy and subsequent surgical resection for rectal adenocarcinoma with curative intent were identified from a prospectively maintained pathology database of all colorectal cancers between the years 2002 and 2011. All patients underwent endoscopic diagnostic biopsy in order to confirm histological evidence of rectal adenocarcinoma prior to treatment. After pre-treatment staging with thoracic-abdominal-pelvic computed tomography (CT), pelvic MRI, clinical examination under anaesthesia (EUA) and in some cases endorectal ultrasound (ERUS), patients were discussed by the multidisciplinary team and offered neoadjuvant chemoradiotherapy according to the local protocol. Local indications for neoadjuvant CRT were extensive mesorectal or pelvic sidewall nodal disease, predicted mesorectal fascia involvement by tumour and/or lymph nodes based on MRI imaging, or clinical fixity of tumour to surrounding structures. After a 6 to 8 wk period following completion of chemoradiotherapy patients underwent restaging investigations (MRI, CT, ERUS and/or EUA) to assess response to treatment and to plan surgical resection. Standardised surgical techniques to maximise complete excision were used including total mesorectal excision and extralevator pelvic floor excision. In some cases multivisceral resection was required for tumours beyond conventional planes. Neoadjuvant radiotherapy in all cases was administered at South West Wales Oncology Centre (Singleton Hospital, Swansea, United Kingdom) and delivered with concurrent 5-fluorouracil (Capecitabine) according to local protocol.

Pre-treatment biopsy specimens stained with Hae-

matoxylin and Eosin were examined by a consultant histopathologist to ensure they contained at least 60% adenocarcinoma tissue. Post treatment resection specimens were examined by two consultant histopathologists who were blinded to patient details and recorded their reports conforming to the Royal College of Pathologists colorectal cancer data set (2<sup>nd</sup> edition 2007) on separate sheets which were stored in a locked cabinet and not seen by other investigators until the data analysis stage. If the reports given by pathologists differed, a third pathologist would be asked to give an opinion and the final report reflected the consensus. When examining tumour regression scores, to ensure there was agreement between the two pathologists scoring the regression, Cohen's kappa statistic was utilised to measure agreement between both raters. For the Royal College of Pathologists tumour regression score there was almost perfect agreement ( $k = 0.856$   $P < 0.001$ ). Patients not completing a full course of neoadjuvant CRT or those not proceeding to surgery were excluded from this study. Patients with rectosigmoid junction tumours, history of inflammatory bowel disease or known high risk genetic predisposition to colorectal cancer (familial adenomatous polyposis or Lynch syndrome) and those undergoing treatment for recurrent cancer were also excluded.

Demographic and clinical outcome data for patients in this study were gathered from patients' case notes, clinic letters and computerised patient hospital records. Patients with local and systemic recurrence were also identified in this way. To identify patients who had died following their treatment, the NHS Wales Informatics Service (Myrddin) database was utilised which records the date of death for each patient if this has occurred. Overall survival, local and systemic recurrence free survival were calculated from the date of surgical resection until either the date of death or the date that recurrence was confirmed clinically, radiologically or histopathologically. If no death or recurrence had occurred, the reference date of last known follow-up was used to calculate survival. These data were also cross referenced against the Cancer Information Network System Cymru database which records data for all patients undergoing cancer treatment in South Wales to ensure its accuracy. Ethical approval for this study was granted by South West Wales REC (Project Ref No.:11/WA/0256). Consent was not required in accordance with the Human Tissue Act 2004 (chapter 30).

## DNA extraction

Formalin fixed paraffin embedded pre-treatment biopsy specimens were utilised for this study. Several representative 5  $\mu$ m sections of the biopsy were cut and mounted unstained onto glass slides and DNA from these tissues was obtained using the MasterPure Complete DNA and RNA purification kit (Epicentre, Illumina, WI, United States).

The quantity and quality of DNA was measured at absorbance between 230 nm and 320 nm using spectrophotometry (Nanodrop ND-1000, Software v3.1.2, ThermoScientific, DE, United States). DNA quantity was



calculated by multiplying the measured concentration following spectrophotometry at 260 nm with the dilution factor. DNA was diluted to a working concentration of 20 ng/ $\mu$ L. Purity was further analysed by calculating the absorbance at 260 nm to absorbance at 280 nm ratio.

### **Bisulfite conversion and methylation specific PCR**

Methylation specific PCR is accomplished by performing bisulfite conversion of genomic DNA (Imprint DNA Modification Kit, Sigma Aldrich, United States). The PCR products were resolved using gel electrophoresis on a 30% polyacrylamide gel. Depending on the methylation status of each CpG island, each patient could be classified as one of three epigenotypes; CIMP-High, Intermediate or Low using a two panel approach<sup>[24,25]</sup>. The first panel consists of SOCS1, MINT-1 and hMLH, which are associated strongly with CIMP-H. The second panel consist of NEUROG1, THBD, HAND1, ADAMTS1, IGFBP3. CIMP status could then be determined using the following system: (1) CIMP-High if  $\geq 2/3$  group 1 markers methylated; (2) CIMP-Intermediate if  $< 2/3$  group 1 but  $\geq 3/5$  group 2 methylated; and (3) CIMP-Low if  $< 2/3$  group 1 and  $< 3/5$  group 2 methylated.

### **KRAS and BRAF mutational analysis**

Pyrosequencing analysis was performed in collaboration with the Leeds Cancer Research United Kingdom Centre, (Leeds Institute of Cancer Studies and Pathology, Clinical Sciences Building, level 6, St. James's University Hospital, Leeds, LS9 7TF). Pyrosequencing conditions used were as previously published by this group<sup>[26]</sup>. Substitution and insertion/deletion mutations in *KRAS* codon 12, 13 and 61 and *BRAF*-600 were examined for all specimens using this method.

### **Definitions**

Tumours were defined as low (0-5 cm from anal verge), mid (5-10 cm) or high (10-15 cm) rectal based on pre-operative rigid sigmoidoscopy and according to where the majority of the tumour was located. Predicted circumferential resection margin (CRM) involvement was defined by the presence of tumour foci (primary, nodal or extranodal deposit) within 1 mm of the mesorectal fascia or cylindrical resection margin for low tumours. An involved CRM was defined pathologically as tumour within 1 mm of the CRM. The original definition of EMVI describes "a rounded mass of tumour in an endothelium-lined space either surrounded by a rim of smooth muscle or containing red blood cells<sup>[27]</sup>". More recent definitions suggest venous invasion may also be suspected when a rounded or elongated tumour profile is identified adjacent to an artery, especially when no separate accompanying vein can be identified (the "orphan" artery sign), or where smooth tongues of tumour extend into pericolic/perirectal fat ("protruding tongue" sign)<sup>[28]</sup>.

### **Statistical analysis**

Statistical analysis was performed using SPSS v.18

Chicago: SPSS Inc. Data was tested for normality using a Kolmogorov-Smirnov test, and a Student's *t*-test was for analysis of normally distributed continuous data. Categorical variables were compared using  $\chi^2$  or Fishers exact test where expected frequencies were less than 10. Relationship between independent variables and time to event was compared using Kaplan-Meier methodology using the Log Rank test to determine significance. Multivariable analysis was performed using bivariate logistical regression and Cox Proportional Hazards modelling. Statistical significance was assumed at the 5% level.

## **RESULTS**

### **Patient and tumour characteristics**

There were 160 patients included in this study. There were 113 (71%) males and 47 (29%) females and the average age by the time of surgery was 65.4 years. By the time of this analysis, 53 (33%) patients had died and the median time from surgery to death was 26.2 mo (IQR 11.9-48.5).

Of the surviving patients, the median follow-up time from surgery was 46.4 mo (IQR 33.8-56.0). Local recurrence data were available for 152 patients and of these, 8 (5%) had evidence of local recurrence a median of 19.7 mo after surgery. Systemic recurrence data were available for 151 patients and of these, 37 (25%) had evidence of systemic recurrence at median 16.3 mo after surgery. Overall survival for all patients was estimated using Kaplan-Meier analysis at 73.3 mo (95%CI: 63.3-83.2). 4 (3%) patients had an involved CRM which was related to worse overall survival (74.1 mo vs 37.2 mo,  $P = 0.047$ ).

There were 14 (9%) patients with a pCR compared to 116 (73%) patients having no or minimal regression after neoadjuvant chemoradiotherapy. Of those undergoing pCR, 8 were male, 6 were female and had a mean age of 66 years. None of the pCR patients demonstrated CIMP-H, whereas 2 were CIMP-I and 12 were CIMP-L. Those patients with pCR had median survival of 106 mo compared to 65.8 mo with minimal regression, although this was not statistically significant ( $P = 0.26$ ). There were 52 patients (33%) with demonstrable *KRAS* mutation, but only a single *BRAF* mutation was detected in the study sample.

### **CIMP status analysis**

CIMP status was determined in all patients, 21 (13%) were CIMP-H, 40 (25%) were CIMP-I and 99 (62%) were CIMP-L. Comparison of patient characteristics by CIMP status revealed no differences in mean age, gender, "T" or "N" stage, presence of systemic or local recurrence, CRM involvement, survival or tumour regression scores. Sub-analysis of individual CIMP markers with tumour regression scores revealed no significant differences. However, CIMP-H was significantly related to EMVI positivity with 8/21 (38%) CIMP-H patients demonstrating

**Table 1** Comparison of pathological features by CpG island methylator phenotype status

	CIMP-H	CIMP-I	CIMP-L	P value
Mean age	66	69.2	63.9	
Sex				
Female	5	14	28	
Male	16	26	71	
ypT stage				
0 or pCR	2	2	16	
1	3	1	7	
2	2	10	20	
3	11	24	48	
4	3	3	8	
ypN stage				
0	11	27	65	
1	6	8	21	
2	4	5	13	
Systemic recurrence				
Absent	14	30	66	
Present	5	2	22	
Local recurrence				
Absent	20	37	87	
Present	0	2	6	
EMVI				
Negative	13	33	84	
Positive	8	7	15	CIMP-L vs CIMP-H $P = 0.028$
KRAS status				
Wildtype	15	20	73	
Mutant	6	20	26	KRAS Mut + CIMP-I $P = 0.01$
CRM				
Not involved	21	39	95	
Involved	0	0	4	
RC path score				
1 (pCR)	0	2	12	
2	6	9	14	
3	15	29	73	
Total	21	40	99	

CRM: Circumferential resection margin; CIMP: CpG island methylator phenotype; EMVI: Extramural vascular invasion; pCR: Pathological complete response.

EMVI compared with 15/99 (15%) who were CIMP-L. (CIMP-H/ EMVI<sup>+</sup> 38% vs CIMP-L/EMVI<sup>+</sup> 15%, Fishers exact,  $P = 0.028$ ). Furthermore, a higher proportion of CIMP-I patients demonstrated *KRAS* mutation than other CIMP groups [CIMP-I + *KRAS* mutation 20/40 (50%) vs CIMP-H/L + *KRAS* mutation 32/120 (27%), Fishers exact,  $P = 0.01$ ] (Table 1).

None of 21 (0%) patients with CIMP-H tumours experienced a pCR compared with 12/99 (12%) CIMP L patients, however this was not statistically significant (Fishers exact = 0.12). There were 30 (19%) patients with EMVI-positivity on histopathological examination of the specimen. This was associated with a significant reduction in median overall survival (83.8 mo vs 43.9 mo,  $P < 0.001$ , Figure 1).

No patient with pCR displayed EMVI, whereas 29 (25%) with RC Path score of 3 (minimal regression) displayed EMVI ( $P = 0.039$ , Table 2).

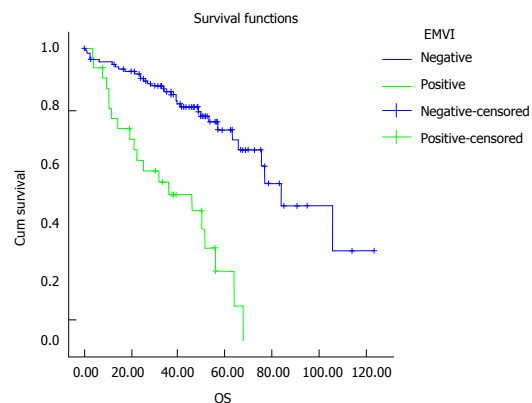
### Multivariable analysis

Cox hazard regression analysis revealed that EMVI-

**Table 2** Tumour regression scores (Royal College Pathologists data set) by extramural vascular invasion status

	EMVI +	EMVI -	P value
RC Path 1 (pCR)	0	14	0.039
RC Path 2	1	28	
RC Path 3	29	88	

EMVI: Extramural vascular invasion; pCR: Pathological complete response.



No at risk (mo)	0	0-20	20-40	40-60	60-80	80-100	100-120
EMVI negative	130	114	77	23	8	3	1
EMVI positive	30	19	9	2	1	0	0

**Figure 1** Overall survival by extramural vascular invasion positivity. Positive vs negative,  $P < 0.001$ . EMVI: Extramural vascular invasion; OS: Overall survival.

positivity was the only factor that was significantly related to adverse survival (Table 3).

Binary logistic regression analysis of the relationship between EMVI and other prognostic features revealed, EMVI positivity was associated with poor overall survival, advanced "T" stage and CIMP-H but not nodal status, age, sex, *KRAS* mutation status and presence of local or systemic recurrence (Table 4).

## DISCUSSION

### CIMP as a prognostic marker

CIMP-positivity has been implicated as an adverse survival predictor in patients with colorectal cancer<sup>[29-31]</sup>, however, the majority of studies investigating survival outcomes in relation to methylation status regard colon and rectal cancers as one entity. Most investigators identify CIMP as an adverse prognostic feature, particularly in colorectal cancer taken as a whole and this was also corroborated by a recent meta-analysis including all colorectal sub sites, which found shorter survival in CIMP positive patients<sup>[32,33]</sup>.

The current understanding of the role of CIMP in colorectal cancer is that tumours with a greater level of CpG island methylation (CIMP-High or CIMP +) have distinct molecular and clinical characteristics compared to low levels of CpG methylation (CIMP-Low or CIMP -)<sup>[34]</sup>. There is some evidence that CIMP-Positivity is related to shorter overall survival<sup>[35]</sup> and disease free survival<sup>[36]</sup>, however the populations in these studies generally lack

**Table 3** Multivariable analysis of pathological and molecular variables against overall survival

	Wald statistic	OR	95%CI (lower)	95%CI (upper)	P value
T stage	1.735	1.392	0.851	2.279	0.188
N stage	0.268	0.857	0.479	1.535	0.605
EMVI	9.422	4.041	1.657	9.857	0.002
CIMP status	0.982	0.791	0.498	1.257	0.322
KRAS status	2.162	1.740	0.832	3.640	0.141
Sex	0.439	0.764	0.344	1.695	0.508
Local recurrence	0.861	1.763	0.532	5.839	0.353
Systemic recurrence	2.165	1.729	0.834	3.584	0.141
Tumour regression (pCR)	0.052	0.793	0.109	5.785	0.819
Involved CRM	0.146	1.339	0.299	6.002	0.703

CRM: Circumferential resection margin; CIMP: CpG island methylator phenotype; EMVI: Extramural vascular invasion; pCR: Pathological complete response.

**Table 4** Binary logistic regression analysis; extramural vascular invasion positivity against overall survival and other pathological, demographic and molecular features

	OR	95%CI (lower)	95%CI (upper)	P value
Overall survival	0.936	0.893	0.981	0.006
T stage	7.764	1.749	34.463	0.007
N stage	2.552	0.851	7.651	0.095
Age	1.024	0.969	1.081	0.405
Systemic recurrence	0.865	0.200	3.749	0.846
Sex	0.564	0.119	2.668	0.470
Local recurrence	1.841	0.193	17.562	0.596
Involved CRM	0.276	0.009	8.376	0.459
KRAS mutation	1.577	0.389	6.391	0.524
CIMP-H	6.368	1.091	37.162	0.040

CRM: Circumferential resection margin; CIMP: CpG island methylator phenotype.

homogeneity of factors such as *KRAS* and *BRAF* mutation status, MSI status and tumour stage<sup>[34]</sup>.

The present study did not demonstrate any relationship between CIMP status and survival. CIMP status was however significantly associated with EMVI positivity which itself was associated with worse survival. Therefore it is likely that the relative contribution of these phenomena to prognosis is more complex than previously understood and should be studied in more detail and with particular distinction of rectal cancers from colon cancers.

### Predicting response to chemoradiotherapy

Relatively few studies have studied the role of CIMP as a predictive marker of rectal cancer response to neoadjuvant chemoradiotherapy. A factor that complicates the evidence is that there is no agreed definition on CIMP classification, and therefore widely ranging and contradicting results are found in the literature. Our research did not find that CIMP status was a predictor of response to chemoradiotherapy, although others have found that detecting the methylation status of individual gene promoter-regions affected the response to neoadjuvant treatment.

Ebert *et al*<sup>[37]</sup> examined a total of 294 patients with colorectal cancer undergoing neoadjuvant chemotherapy (5-fluorouracil, oxaliplatin and irinotecan), and analysed the expression, methylation and function of the *TFAP2E*

gene. They demonstrated that hypermethylation of the promoter regions of *TFAP2E* was associated with down-regulation of the gene, and the subsequent up-regulation of a down-stream target. Furthermore, *TFAP2E* hypermethylation was a marker of 5-fluorouracil resistance in CRC in this study, but there was no effect on response to treatment with oxaliplatin or irinotecan.

### CIMP and KRAS mutation

Ogino *et al*<sup>[38]</sup> examined methylation in 840 colorectal cancers led to the proposal that a further subset of intermediate methylation associated tumours exist but which do not fulfil the criteria for CIMP-High. These tumours (termed CIMP-intermediate) were independently associated with male gender and *KRAS* mutation. The three epigenotype model was further supported by Yagi *et al*<sup>[24]</sup>, who used a large scale mass spectrometry analysis and hierarchical clustering to identify two panels of markers, the first to identify CIMP-High tumours and then a second panel to distinguish between CIMP-intermediate and low tumours. In our research, CIMP-I had a significant association with *KRAS*-mutation compared to CIMP-H or CIMP-L tumours ( $P = 0.01$ ), confirming this association in our patients, although no difference with regards to survival was demonstrated.

### CIMP classification and EMVI status

The adverse prognostic value of EMVI is well established

and is known to be associated with poor survival<sup>[10]</sup> and has a relative risk of 3.7 for the development of systemic recurrence when detectable on preoperative MRI scanning<sup>[15]</sup>. This is supported by data from the present study, which revealed significantly decreased survival with EMVI.

EMVI was also associated with a lack of response to neoadjuvant chemoradiotherapy. If EMVI is present before treatment and is absent after treatment, then this would indicate a response, whereas failure of EMVI to regress would indicate a lack of response. However, the presence of EMVI is not currently detectable on histological analysis of pre-treatment biopsy specimens. In the present study, a novel association between EMVI and CIMP-H status was identified. This finding does provide a novel insight into potential mechanisms for the association of poor survival with CIMP-H seen in other studies.

There are several mechanisms which may explain the link between CpG island hypermethylation and EMVI. For example, angiogenesis and subsequent local invasion of colorectal tumours has previously been linked to hypermethylation and silencing of micro-RNA-126 (miRNA-126), which is associated with up-regulation of vascular endothelial growth factor and subsequent increased likelihood tumour invasion<sup>[39]</sup>. Other research has suggested that silencing the gene that codes for E-Cadherin (a molecule that forms the adherens junctions between normal cells, preventing spread of tumour cells across the epithelial basement membrane)<sup>[40]</sup> is associated with increased risk of EMVI and reduced response to neoadjuvant chemoradiotherapy and worse survival in rectal cancers<sup>[41]</sup>. Finally, the invasion of cancer cells into the surrounding extracellular matrix depends on the function of matrix metalloproteinases (MMPs), which are themselves regulated by tissue inhibitors of matrix metalloproteinases (TIMPs). *In vitro* and animal studies have demonstrated that aberrant epigenotypes affecting the MMP/TIMPs axis can lead to increased tumour invasion and migration *in vitro* and increased tumorigenesis and therapeutic reversal of this aberrant methylation can suppress these tumorigenic phenomenon<sup>[42,43]</sup>.

Given that CIMP is deemed to represent a phenotypic hypermethylated state, it is likely that the presence of the CIMP-H state explains the association of EMVI-positivity and poor survival seen in rectal cancer patients. The detection of a hypermethylated state in individual gene promoter regions may well further our understanding of the response to chemoradiotherapy in the future.

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Singleton Hospital for her assistance with outcome data collection as well as consultant colorectal surgeons Mr. Umesh Khot, Mr. TV Chandrasekaran, Mr. Mark Davies and Mr. Martyn Evans for their collaboration with patient data collection.

## COMMENTS

### Background

There is wide variation in response to neoadjuvant chemoradiotherapy (NACRT) in rectal cancer, which has a significant impact on survival. There is currently no reliable means to predict response to NACRT, which carries significant side effects. The CpG island methylator phenotype (CIMP) is characterised by epigenetic DNA hyper-methylation and suppression of key genes controlling cell growth and survival and occurs in approximately 20% of colorectal cancers. The role of CIMP status in the prognosis and response of rectal cancer to neoadjuvant therapy is not well understood but evidence is emerging that it may be an adverse prognostic indicator.

### Research frontiers

Previous studies have demonstrated an association of high levels of CIMP associated methylation with adverse survival and differential responses to neoadjuvant treatment where methylation is seen in specific genes in rectal cancer, however, the mechanism and exact nature of this association is not clear.

### Innovations and breakthroughs

This study reports a novel association of CIMP related methylation with extramural vascular invasion which represents an adverse prognostic indicator and provides a novel insight into potential mechanisms for the association of poor survival with CIMP H which may be related to epigenetic silencing of the normal inhibitory mechanisms which prevent cell migration, proliferation and vascular invasion.

### Applications

Extramural vascular invasion (EMVI) has recently been associated with adverse survival and risk of metastasis and although it features in the National Institute of Health and Care Excellence United Kingdom guidelines for the treatment of rectal cancer, suggesting that short course neoadjuvant therapy should be considered in these patients on this basis, the current guidelines concede that the risks and benefits in this group are unclear and further research is needed. Indeed the prediction of EMVI on preoperative imaging is notoriously difficult and non-reproducible. EMVI is detectable in rectal cancer patients on magnetic resonance imaging, however, sensitivity and specificity are relatively low at 62% and 88% respectively and it is possible that in future, CIMP status could be used to enhance preoperative EMVI detection and subsequent risk stratification.

### Terminology

CpG islands are typically short (300-3000 base pairs) Cytosine-Guanine phosphodiester bonded sequences found in or around the promoter region of a gene where they are usually unmethylated if the genes are expressed. The CIMP phenotype is characterised by epigenetic DNA hyper-methylation and consequent suppression of key genes important in controlling cell growth and survival. High levels of CIMP associated methylation (deemed CIMP-High), are associated with poor survival in rectal cancer. Extramural vascular invasion of a tumour is defined as "a rounded mass of tumour in an endothelium-lined space either surrounded by a rim of smooth muscle or containing red blood cells". Venous invasion may also be suspected when a rounded or elongated tumour profile is identified adjacent to an artery, especially when no separate accompanying vein can be identified or where smooth tongues of tumour extend into pericolic/perirectal fat.

### Peer-review

The authors aimed to identify whether CIMP status is predictive of response to neoadjuvant chemoradiotherapy and outcomes in rectal cancer. They found



that a novel association of CIMP status with extramural vascular invasion which represents an adverse prognostic indicator and provides a novel insight into potential mechanisms for the association of poor survival with CIMP-H rectal cancers. The study is well-designed and presented. The results are all clear and understandable, the descriptions of methods and materials are also clear.

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## Observational Study

# Critical evaluation of contemporary management in a new Pelvic Exenteration Unit: The first 25 consecutive cases

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## Abstract

### AIM

To critically appraise short-term outcomes in patients

treated in a new Pelvic Exenteration (PE) Unit.

## METHODS

This retrospective observational study was conducted by analysing prospectively collected data for the first 25 patients (16 males, 9 females) who underwent PE for advanced pelvic tumours in our PE Unit between January 2012 and October 2016. Data evaluated included age, co-morbidities, American Society of Anesthesiologists (ASA) score, Eastern Cooperative Oncology Group (ECOG) status, preoperative adjuvant treatment, intra-operative blood loss, procedural duration, perioperative adverse event, lengths of intensive care unit (ICU) stay and hospital stay, and oncological outcome. Quantitative data were summarized as percentage or median and range, and statistically assessed by the  $\chi^2$  test or Fisher's exact test, as applicable.

## RESULTS

All 25 patients received comprehensive preoperative assessment *via* our dedicated multidisciplinary team approach. Long-course neoadjuvant chemoradiotherapy was provided, if indicated. The median age of the patients was 61.9-year-old. The median ASA and ECOG scores were 2 and 0, respectively. The indications for PE were locally invasive rectal adenocarcinoma ( $n = 13$ ), advanced colonic adenocarcinoma ( $n = 5$ ), recurrent cervical carcinoma ( $n = 3$ ) and malignant sacral chordoma ( $n = 3$ ). The procedures comprised 10 total PEs, 4 anterior PEs, 7 posterior PEs and 4 isolated lateral PEs. The median follow-up period was 17.6 mo. The median operative time was 11.5 h. The median volume of blood loss was 3306 mL, and the median volume of red cell transfusion was 1475 mL. The median lengths of ICU stay and of hospital stay were 1 d and 21 d, respectively. There was no case of mortality related to surgery. There were a total of 20 surgical morbidities, which occurred in 12 patients. The majority of the complications were grade 2 Clavien-Dindo. Only 2 patients experienced grade 3 Clavien-Dindo complications, and both required procedural interventions. One patient experienced grade 4a Clavien-Dindo complication, requiring temporary renal dialysis without long-term disability. The R0 resection rate was 64%. There were 7 post-exenteration recurrences during the follow-up period. No statistically significant relationship was found among histological origin of tumour, microscopic resection margin status and post-operative recurrence ( $P = 0.67$ ). Four patients died from sequelae of recurrent disease during follow-up.

## CONCLUSION

By utilizing modern assessment and surgical techniques, our PE Unit can manage complex pelvic cancers with acceptable morbidities, zero-rate mortality and equivalent oncologic outcomes.

**Key words:** Colorectal cancer; Advanced pelvic tumour; Sacrectomy; Oncological outcome; Pelvic exenteration; Chordoma

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**Core tip:** Pelvic exenteration surgery was introduced by Brunswick in 1948 as a palliative treatment for advanced pelvic tumour, which carries high morbidity and mortality rates. However, decades of medical evolution in preoperative imaging, adjuvant therapy, better anatomical knowledge of the pelvis and modernized surgical techniques has made this procedure safe and effective for treating complex pelvic tumours. This study describes and demonstrates how our new Pelvic Exenteration Unit utilises the advantage of modern assessment and contemporary surgical techniques to achieve excellent outcomes.

Chew MH, Yeh YT, Toh EL, Sumarli SA, Chew GK, Lee LS, Tan MH, Henedige TP, Ng SY, Lee SK, Chong TT, Abdullah HR, Goh TLH, Rasheed MZ, Tan KC, Tang CL. Critical evaluation of contemporary management in a new Pelvic Exenteration Unit: The first 25 consecutive cases. *World J Gastrointest Oncol* 2017; 9(5): 218-227 Available from: URL: <http://www.wjgnet.com/1948-5204/full/v9/i5/218.htm> DOI: <http://dx.doi.org/10.4251/wjgo.v9.i5.218>

## INTRODUCTION

Surgeries for advanced pelvic tumours constitute technical challenges. Despite better understanding of the pelvic anatomy due to superior imaging modalities, the resection of tumours and extirpation of any contiguous organs continue to be associated with considerable morbidity and risks. In addition, tumours originating from the rectum, gynaecological organs or urological organs behave differently and indications of surgery for each require multidisciplinary coordination and evaluation.

Pelvic exenteration (PE) surgery was first introduced by Brunswick<sup>[1]</sup> in 1948 but was associated with a high morbidity rate, a perioperative mortality rate of 23%, and a poor postoperative quality of life. As such, a non-surgical approach with chemotherapy and radiotherapy has traditionally been offered to the majority of the patients with pelvic tumours. These approaches may provide transient relief of symptoms but as the disease progress, many of the patients suffer from refractory pain, obstruction, bleeding, malodorous fistulating or erosive malignant cutaneous lesions, and pelvic sepsis. Survival may be increased up to 12-14 mo but remains poor, with < 4% of patients surviving beyond 4 years<sup>[2-5]</sup>.

As a result of better patient selection, perioperative adjuvant chemotherapy and irradiation, careful planning and multidisciplinary involvement as well as advances in surgical techniques in the modern era, PE has become accepted as a procedure that can maintain adequate local disease control, prolong survival and achieve potential cure for advanced pelvic tumours. The most significant advances in surgical techniques have allowed for achievement of an R0 resection, as demonstrated by large-scale reviews which predominantly investigated for the locally-advanced and recurrent types of rectal cancers<sup>[6-8]</sup>.



Accomplishing an R0 resection requires complete or partial removal of the pelvic vessels, muscles, ligaments and bony structures-including the ileum, ischium, pubic rami, sacrum or coccyx-as well as pelvic viscera. Experience gained over the years has led to acceptable morbidity risks and a low mortality rate. In a systematic review, Heriot *et al*<sup>[6]</sup> reported exenteration-related morbidity and mortality rates of 27% and 0.6% respectively. Similar trends were found in an Australian study of 148 patients who underwent PE, which reported a 0% 30-d mortality rate and good postoperative quality of life<sup>[9]</sup>.

The development of a dedicated PE Surgical Unit in our institution was borne from recognition of the advantages afforded by an aggressive approach to tackling these advanced pelvic tumours. Nonetheless, the initial phase of conceptualization necessitated discussion on the understanding of pelvic cancer biology and pathophysiology among the various subspecialties, as well as of the appropriate surgical indications. The core members of this PE Unit included: A colorectal surgeon, who had received comprehensive training in PE; a gynaecologist, who specialized in gynaecological malignancies; an urologist, who specialized in urological cancers; and a team of experienced anaesthesiologists. Other subspecialty surgeons-including plastic, vascular and orthopaedic surgeons-were referred on an *ad hoc* basis. While the concept of PE surgery was not new to this Unit at its inception, the latest surgical techniques for achieving R0 margins had only recently been introduced into its practice.

This article reports our systematic evaluation of the short-term oncological outcomes achieved by the newly established PE group using modern techniques.

## MATERIALS AND METHODS

### Definitions

Definitions of the PE surgeries described herein correspond to those published in a 2013 systematic review from Yang *et al*<sup>[10]</sup>, and include.

### Total (T)PE

Whereby rectum, distal colon, genitourinary viscera, internal reproductive organs, draining lymph nodes and pelvic peritoneum are removed. If a sacrectomy is performed, it is specified as TPE with sacrectomy.

### Anterior PE

Whereby upper rectum, reproductive organs and bladder are removed. The lower rectum may be spared or a perineal excision may be performed.

### Posterior PE

Whereby the rectum and reproductive organs are removed. The bladder may be spared. If a sacrectomy or coccygectomy was performed, it is specified.

### Lateral PE

Whereby a lateral pelvic node dissection is performed,

with *en bloc* resection of all involved structures, including viscera and vascular structures. If the sciatic nerve can be preserved, its perineural sheath is excised.

### Study design

After approval was obtained by the Institutional Review Board of our hospital, a retrospective review of patient records was conducted to identify the first consecutive 25 patients who underwent PE through our new PE Unit. No exclusion criteria were applied. These patients had been treated between January 2012 and October 2016, and all had received or were undergoing follow-up consisting of 3-mo outpatient clinic visits for at least 2 years following the surgery. The follow-up routine included monitoring of carcinoembryonic antigen level (each clinic visit) and computed tomography (CT) chest, abdomen and pelvis scans (once annually for the first 2 years). No patient was lost to follow-up.

Data were expressed as median, maximum range and minimum range due to smaller sample size. Statistical analysis was performed by the Microsoft Excel 2010 software, with Fisher's exact test used to determine significance, indicated by *P* value.

### Patient selection

All 25 patients had been evaluated by the multidisciplinary team of the PE Unit, which included medical and radiation oncologists as well as surgeons. For each case, all findings from imaging modalities had been retrieved and carefully re-evaluated by a dedicated radiologist. The extent of local regional disease, as well as the potential for distant metastatic disease, had been determined, with the plan for multi-visceral resection and its approach being formulated accordingly.

Patients considered for surgical resection were those who had no evidence of metastatic disease, had good performance status, and represented those who the multidisciplinary team deemed that the ability to achieve a R0 resection was possible. Patients who did not meet operative criteria were those with either unresectable metastatic disease (for who surgery was performed with palliative intent) or unresectable large volume disease, or who were deemed physically or psychosocially unfit for extensive surgery.

Typically, in our institute, patients with primary advanced colorectal cancer undergo long-course neoadjuvant chemoradiotherapy. Upfront surgery is planned only in cases with prior chemoradiotherapy treatment for other cancers (e.g., prostate) or with cancers unlikely to benefit from neoadjuvant therapy (e.g., chordomas). A delay of 8-12 wk after neoadjuvant chemoradiation treatment is routinely advocated to achieve maximum down-staging. Repeat imaging is usually performed at 4 wk after completion of the neoadjuvant treatment, in order to determine response. The organs and planes involved before commencing neoadjuvant treatment are resected, as well, in order to ensure negative margin.

The entire team of specialty surgeons and anaesthetists assigned to the case would perform preoperative

counselling in their respective area of resection or reconstruction. The counselling process involved appropriate patient-level explanations on the probability of achieving an R0 resection, the survival benefit post-PE, the morbidity and mortality risks associated with organ-specific resection or reconstruction, the anaesthetic risks and the financial implications. Stoma care and potential need of postoperative rehabilitation were also discussed with both the patient and any caregivers. The surgical candidate was also advised of the potential need for 2-4 wk postoperative inpatient hospital stay, including 1-2 d in the intensive care unit (ICU). The usual consultation process takes 4-6 wk. The majority of that time is allotted to allow patients to decide whether they are keen on the procedure and to come to accept the need for stoma; only after these issues are resolved can the patient provide final consent.

### **Surgical approach**

PE cases are highly heterogeneous, and the surgery types vary considerably; however, our PE Unit adheres to certain principles for all cases. All patients undergo oral bowel preparation, as well as mechanical thromboprophylaxis, prior to surgery. Chemical thromboprophylaxis is not routinely administered, with respect to the potential high-risk of bleeding related to the extra-fascial plane dissection requirement.

All of the 25 cases assessed in this study had dedicated anaesthetists and underwent the PE in the Lloyd-Davis position. For those patients requiring a high sacrectomy (S2 and above), a combined anterior and posterior jack-knife approach was used. After laparotomy and adhesiolysis, any suspicious peritoneal nodules were biopsied and sent for frozen section. Positivity for peritoneal disease would have precluded curative resection, triggering abandonment of the procedure; however, none of the cases in our series showed positivity or peritoneal recurrence during the surgical exploration.

In all of the 25 cases, *en bloc* resection was the surgical aim. The surgical planes had been determined preoperatively by consensus among all involved surgeons. If an organ was abutting the tumour, *en bloc* resection was performed. There was no attempt in any case of a trial of dissection for organ preservation to prevent tumour spillage. Ureteric stents were not routinely inserted if bladder or ureteric resection was planned.

The standard approach of anterior or posterior PE, in our PE Unit, is to mobilise the central pelvic compartment (*i.e.*, the rectum) immediately after ligating the inferior mesenteric vessels and performing transection of the distal sigmoid colon. The dissection continues along the total mesorectal excision (TME) plane, if feasible, and down to the pelvic floor. The dissection stops at the level of the organ involving the tumour. In pelvises restrained by adhesions or tumour, extra-fascial plane dissections are performed, but only after vascular control is obtained. Many of the 25 cases described herein necessitated cranial-to-caudal anterior compartment mobilization (*i.e.*,

urogenital and gynecological organs) and transection, specifically at the urethra or vagina, before the final transection of the rectum.

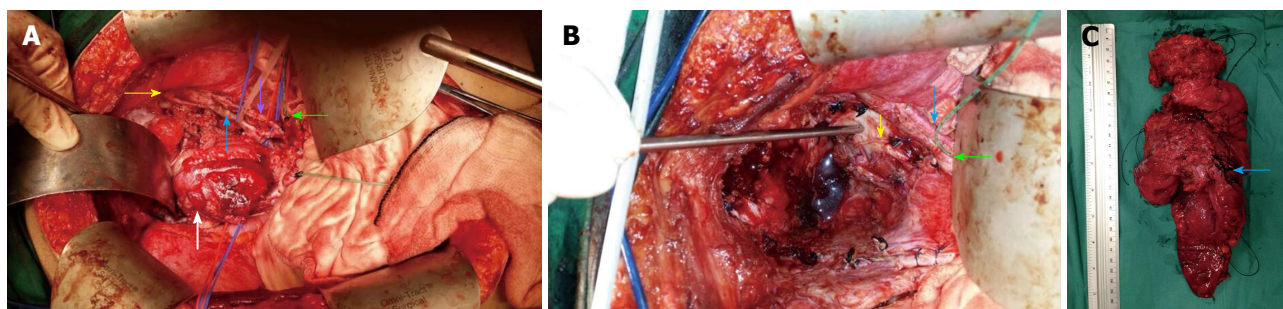
In our PE Unit, frozen section is utilized to confirm clear histopathology margins in areas associated with perioperative doubt. Advanced energy medical devices are commonly employed for TME mobilization and pelvic wall dissection, in order to reduce blood loss. The appropriate laparoscopic lengths of these devices are determined according to the narrow-width and depth of the pelvis.

The technique for lateral PE utilized in our case series to achieve clear margins was that described by Höckel *et al.*<sup>[11]</sup> and Austin *et al.*<sup>[12]</sup>. The anatomic approach of this technique reaches the plane lateral to the internal iliac vessels. Vascular control of common iliac vessels and external iliac vessels is first achieved with vessel loops, and the external iliac vessels are mobilized to allow easy access to the obturator canal. The internal iliac artery is usually ligated first, before the internal iliac vein is accessed and ligated. All subsequent distal branches are suture ligated. These internal iliac vessels are then resected *en bloc* with the tumour specimen.

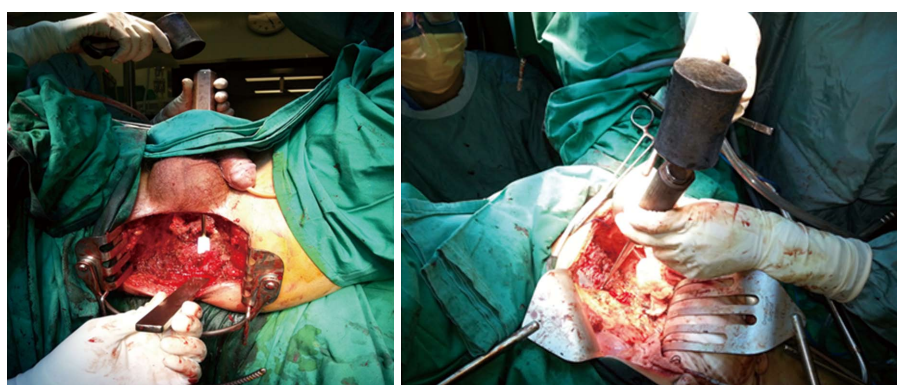
In our case series, the external iliac artery resection was performed only after a graft from the common iliac to the femoral artery was created. In addition, all sciatic nerves were preserved, but the perineural sheath was resected *en bloc*, if required. Lateral node dissection was also performed if there were suspicious nodes noted preoperatively, or if the tumour extended to the area of the lateral pelvic sidewall. This dissection would commence from the aortoiliac bifurcation, proceed down to the nodes around the common and external iliac vessels, and down to the origin of the internal iliac vessels and the obturator canal (Figure 1).

In our PE Unit, for sacrectomies, the abdominal approach is used for low sacrectomy (S3/S4), as described by Solomon *et al.*<sup>[13]</sup>. For the combined abdominal-perineal approach, the abdominal phase incorporates complete mobilization of the posterior plane, up to 1 cm from the level of the sacrectomy. Ligation of the various internal iliac vessel branches, particularly the sacral, visceral and gluteal veins, is performed. Preservation of the upper sacral nerves is paramount, and all presacral fascia and piriformis muscles are dissected free. The perineal phase begins with an elliptical skin incision, which is followed by dissection below the coccyx and up to the level of the S2/S3 junction posteriorly, with the gluteal muscles and sacrococcygeous ligaments being dissected free. The sacrectomy is then performed by 20-mm osteotome, applied transabdominally, in a medial to lateral manner; this is carried out with a surgical assistant located at the perineum and placing an osteotome below the sacrum to prevent damage or button-holing of the perineal skin (Figure 2). For our cases, the perineal defect was reconstructed by the plastic surgeon using either primary closure and biological mesh reinforcement or myocutaneous pedicle flap.

For high sacrectomy (S1/S2), the orthopaedic team



**Figure 1 Total pelvic and lateral exenteration.** A: A Deaver retractor was placed caudally (White arrow: Pelvic tumour; Yellow arrow: Right obturator nerve; Blue and purple arrows: Right internal iliac vein and artery respectively; Green arrow: Transected right distal ureter at pelvic brim with infant feeding tube inserted for intraoperative urinary diversion); B: Post-exenteration view showing the right internal iliac vessels, obturator nerve and pelvic lymph nodes excised and the metal vacuum tube pointed at exposed pelvic bone (Yellow arrow: Sciatic nerve; Blue arrow: Right external iliac vessels; Green arrow: Transected right distal ureter); C: Cicatrizing tumour specimen showing invasion into bladder and right pelvic sidewall (Blue arrow: Right internal iliac vessels).



**Figure 2 Demonstration of abdominal perineal approach for level S3 sacrectomy.** The left panel (perineal view) demonstrates placement of the osteotomes posterior to the sacral bone in order to protect perineal skin while the surgeon transects the sacrum at S3 level in the right panel (abdominal view).

conducts the surgery with the patient in a prone jack-knife position. This procedure is performed only after complete mobilization of all vascular structures and organs off of the sacrum, down to the coccyx. Following ligation of all posterior internal iliac branches and completion of mobilization as described above, a penny towel pack is able to be placed between the sacrum and iliac vessels. The osteotomy site is marked anteriorly, using a drill. A myocutaneous flap is mobilized and tucked deep in the pelvis, a stoma is “matured” if necessary, and finally the abdomen is closed. After turning the patient to prone position, an incision is made down to the level of the sacrectomy and then transected with *en bloc* resection of the tumour. Reconstruction of the defect is then completed using the flap.

In our PE unit, an ileal conduit is commonly performed as the means of permanent urinary diversion. To avoid urinary complications, it is essential to have technical collaboration between the colorectal surgeons and the urologists. The most important technical step in ileal conduit formation is to ensure delicate handling of the ureters and ileum; the former must be meticulously mobilised with care to preserve ureteric vascularity. Transection of the ureters is performed as distal as possible, without compromising the oncological outcome. Ureteroenteric anastomosis is methodically performed, in

order to achieve good tissue vascularity, in a tension-free manner and without malrotation of the ureters. These concepts are crucial to prevent urinary anastomotic leaks, conduit ischaemia and late ureteric strictures, while balancing the need for an adequate resection margin.

## RESULTS

### Patient demographics

Twenty-five consecutive cases were evaluated. The patient demographics and indications for surgeries are summarized in Table 1. The median length of follow-up period was 17.6 mo (range: 6.3–39.0 mo). The most common indications for PE were locally invasive rectal adenocarcinomas (13 cases, including 9 primary and 4 recurrent), followed by advanced colonic adenocarcinomas (5 cases, including 3 primary and 2 recurrent), recurrent cervical carcinomas (3 cases) and malignant sacral chordomas (3 cases). There were 10 TPEs performed, and the majority of these cases were combined with lateral PEs. Three out of those 10 TPE cases also had sacrectomy. Except for 4 isolated lateral PEs, anterior (1 of 4) and posterior PEs (5 of 7) were commonly performed in conjunction with lateral PEs. R0 resection was achieved in 16 cases (64%). These results are summarised in Table 2.



**Table 1** Characteristics of patients who underwent pelvic exenteration

Variable	
Sex, <i>n</i> (%)	
Male	16 (64)
Female	9 (36)
Age, <i>n</i> (%)	Median, 61.9 yr (range, 30-72)
ASA score, <i>n</i> (%)	I : 11 (44)
	II : 13 (52)
	III: 1 (4)
ECOG status	Median, 2
	0: 22 (88)
	1: 2 (8)
	3: 1 (4)
	Median, 0
Co-morbidities, <i>n</i> (%)	
Hypertension	6 (24)
Diabetes mellitus	5 (20)
Hyperlipidaemia	8 (32)
Ischaemic heart disease	1 (4)
Primary cancer type ( <i>n</i> = 16)	
Colorectal	12
Chordoma	3
Gynaecological	1
Recurrent cancer type ( <i>n</i> = 9)	
Colorectal	6
Gynaecological	3

ASA: American Society of Anesthesiologists; ECOG: Eastern Cooperative Oncology Group.

### Morbidity and mortality

The median operative time was 11.5 h (range: 6.3-16.8 h). The median volume of blood loss was 3306 mL (range: 650-11000 mL), and the median volume of red cell transfusion was 1475 mL (range: 222-5565 mL). Of note, procedures combined with lateral PEs had higher blood loss (median: 2500 mL, range: 650-11000 mL). The highest blood loss in our series was 11 L, which occurred in a rectal cancer patient with 2<sup>nd</sup> occurrence of left pelvic wall nodal recurrence, and on who an isolated lateral PE was performed. This surgery was the 3<sup>rd</sup> procedure after initial ultra-low anterior resection and followed a prior attempt at lateral node dissection. After extensive adhesiolysis, the left pelvic nodal recurrence was resected *en bloc* with left distal ureter and internal iliac artery and vein. A segment of left external iliac vein was resected for margin, and a prosthetic graft reconstruction was made from common iliac to left femoral vein. The left ureter was reconstructed and re-implanted with a Boari flap.

The median length of ICU stay was 1 d (range: 0-8 d), and the median length of hospital stay was 21 d (range: 8-136 d). There was no perioperative mortality. The postoperative complications are summarized in Table 3. A total of 20 complications occurred in 12 patients. Three patients (12%) experienced major complications, including 2 patients (8%) with grade 3 Clavien-Dindo postoperative complications, which required further invasive interventions. The first patient with high body mass index (BMI) underwent redo-

**Table 2** Pre-operative and operative treatment details

Incidence of neoadjuvant chemoradiation	
Primary cancer	
Colorectal	75%
Chordoma	0%
Gynaecological	0%
Recurrent cancer	
Colorectal	67%
Gynaecological	0%
Operative procedure, <i>n</i> (%)	
Total PE	1 (4)
Total PE with lateral exenteration	9 (36)
Anterior PE	3 (12)
Anterior and Lateral PE	1 (4)
Posterior PE	2 (8)
Posterior and Lateral PE	5 (20)
Lateral PE	4 (16)
Sacrectomy combined with any above PE procedures	9 (36)

PE: Pelvic exenteration.

**Table 3** Clavien-Dindo classification of surgical complications, *n* = 12

Grade	Feature	<i>n</i>
2	Wound infection	6
	Urinary tract infection	4
	Venous access infection	4
	Prolonged ileus	1
	Deep vein thrombosis	1
	Acute myocardial infarction	1
3	Postoperative bleeding: Re-laparotomy	1
	Donor site-infected seroma percutaneous drainage	1
4	Temporary renal dialysis	1
Total adverse events		20

laparotomy for a torn ileal conduit mesentery bleed on postoperative day 1. The second patient, also with high BMI, underwent vertical rectus abdominis myocutaneous flap reconstruction and developed a postoperative large infective seroma in the abdominal wound site, which required percutaneous drainage on postoperative day 24. There was only one patient who required temporary renal dialysis (grade 4A Clavien-Dindo) following TPE with ileal conduit reconstruction, but no revision surgery was needed; the causes of acute renal failure were multifactorial, but did not include the newly-constructed ileal conduit. This patient's renal function gradually recovered, without long-term disability. The remaining 9 patients had grade 2 complications, which required pharmacological interventions.

### Short-term oncological outcome

During the study period, 18 out of 25 patients were in remission. There were 7 (30.4%) post-PE recurrences that presented during follow-up, and these included 2 with local regional recurrence, 2 with distant metastasis, and 3 with both regional and distant recurrences. The histopathological origin of cancer and postoperative microscopic margin status for each of these cases are



**Table 4** Characteristics of post-pelvic exenteration recurrent diseases

Pre-PE status	Histology origin	Regional recurrence	Distant metastasis	Regional and distant	R0	R1
Primary	Colonic	0	0	1	1	0
Primary	Rectal	0	1	1	0	2
Primary	Sacral chordoma	1	0	0	1	0
Recurrent	Rectal	1	0	0	0	1
Recurrent	Cervical	0	1	1	2	0
Total		2	2	3	4	3

Both microscopic resection margin status and pre-exenteration primary or recurrent tumours do not show any statistically significant influence on post-exenteration recurrence ( $P = 0.67$ ). PE: Pelvic Exenteration.

summarized in Table 4. There were no statistically significant relationships among microscopic resection margin status, histopathological origin of tumour and postoperative recurrence ( $P = 0.67$ ); these results may, however, simply reflect the small size cohort of this study. Among these 7 cases, 4 of the patients died during follow-up. Two of the patients' deaths were attributed to cardiopulmonary failure from systemic disease burden. The remaining 2 patients' deaths were related to sepsis secondary to locoregional recurrences, with 1 having developed urosepsis from ileal conduit malignant stricture and the other having developed pelvic sepsis from malignant pelvic floor fistula. The overall median survival from surgery to death was 12 mo (range: 6.1-17.0 mo).

## DISCUSSION

PE surgery has evolved over the decades. Brunswig<sup>[1]</sup> originally developed PE as a palliative intervention, but-as detailed in the Introduction-the procedure had high morbidity and mortality rates and poor long-term outcome. These drawbacks precluded its widespread application by surgeons and acceptance by patients; and, despite its potentially life-saving benefits, this psychological and physical taxing operative procedure was considered with even more caution. However, constant evolution in chemoradiation interventions and surgical techniques, as well as better patient selection, have increased the safety of this procedure when performed by an experienced multidisciplinary team. Now, besides the survival benefits, there are also marked improvements to patients' quality of life.

Studies have shown that the oncological benefit of PE is best when a negative pathological margin can be achieved<sup>[2,10,14-16]</sup>. To assess our short experience using a multidisciplinary team approach for PE surgery, the outcomes of a series of 25 consecutive patients were evaluated based on morbidity, mortality and recurrence. A systematic review performed by Young *et al.*<sup>[9]</sup>, which incorporated 23 studies and 1049 patients as a benchmark, noted a 73% R0 resection rate (range: 42%-100%). In that same review, the median perioperative mortality rate was low, at 2.2%, with the majority ranging from 0% to 25%. Our case series demonstrated comparable outcomes, namely 64% R0 resection rate and 0% in-hospital or 30-d perioperative

mortality rates. The postoperative complication rate in our case series was 48% but the actual serious morbidity (grades 3 and 4 Clavien-Dindo) was 12%, and two-third of the adverse events in our case series were grade 2 Clavien-Dindo that necessitated pharmacological treatment alone. This finding is comparable to the median rate of 57% that was reported from the systematic review<sup>[9]</sup>. Short-term follow-up in our case series found a recurrence rate of 28%. There was, however, no statistically significant relationship among pathological resection margin status and post-exenteration recurrence in our study; since this is likely due to a small sample size, we must await our series to expand further before survival benefit can be commented on.

While our case series was large enough to generally assess the learning curve of our PE Unit, our procedures were highly heterogeneous and included complex lateral and posterior PEs that are not commonly performed. The results provide validation that these techniques applied for PE surgery allow for good short-term outcomes; yet, the authors acknowledge that achieving better outcomes would rely also on better decision-making and patient selection. One of the first criteria of patient selection for such extensive surgery is physical fitness and minimal co-morbidities. In our study cohort, the median age of patients was 62-year-old, and the oldest patient was 72-year-old (who underwent surgery for sacral chordoma). In general, our patients were fit; the median ASA score was 2 and the median ECOG score was 0. The one exception was a 42-year-old woman, who underwent the surgery despite being ECOG grade 3 status due to a symptomatic pelvic recurrence that caused significant disability.

The post-surgery social aspects are other important issues that must be considered in the decision-making process. Many patients are reluctant to accept the physical, psychological and financial sacrifices required for the surgery. It is not uncommon that a patient ends up with two permanent ostomies and are then unable to overcome the perceived lack of independence and social stigma. In our case series, multiple consultations were required in order to obtain the appropriate informed consent from the patient and caregiver, with the time frame often being 4-6 wk.

It was crucial in our preoperative planning that attempts were made to obtain histological proof of the tumour

before PE, especially for cases of recurrent disease. This was achieved *via* endoscopic or percutaneous biopsy for accessible tumours. We also had to perform an open biopsy for 1 patient. Yet, this approach was considered especially important to aid in planning of the extra-fascial planes and because dissection is meant to avoid opening up of tumour planes and subsequent spillage of tumour cells. Obtainment of intraoperative biopsies of the tumours and subsequent frozen section histology can take time before proceeding to a PE, creating anxiety and uncertainty in both the patient and relatives, ultimately making the logistic planning of a multidisciplinary surgery difficult and inefficient. A confirmed preoperative diagnosis allows the patient to be convinced of the necessity of such extensive surgery and may avoid any potential medico-legal pitfalls.

Proper preoperative planning is necessary, with adequate time set aside for preanaesthetic assessment, a dedicated operative theatre list, invasive intraoperative haemodynamic monitoring and Level 1 rapid transfuser device set-up, if necessary. Adequate blood and ICU resources must be ensured before the operation commences. This operative planning incurs costs as well. Therefore, success of the programme long-term would also require cost-conscious practices or may negate support from the administrative side for these highly expensive and complex procedures.

For R0 resections, magnetic resonance imaging (MRI) has been shown to be a valuable tool to identify the anatomy of involved organs and to guide the extent of resection and reconstruction options, especially when reviewed by an experienced radiologist. In an expert's hand, the radiological accuracy of rectal cancer staging improves in sensitivity (from 77% to 96%) and specificity (from 40% to 74%)<sup>[17]</sup>. We have had the benefit in our team of a dedicated radiologist who specializes in evaluating all images after initial reporting. The key questions asked include the likelihood of involvement of contiguous organs, the presence of undiagnosed peritoneal disease, and, often, the difference between post-radiation fibrosis vs tumour. This is especially pertinent to determine if a low sacrectomy will be required to treat advanced or recurrent rectal cancers. On MRI of a previously irradiated rectal cancer, it can be difficult-even for an expert-to differentiate between viable residual tumour and post-treatment fibrosis<sup>[17]</sup>. In these instances, as well as when indeterminate loco-regional or systemic organ or nodal disease is encountered on anatomical imaging, the fluorodeoxyglucose positron emission tomography (PET) with CT scan can be utilized. PET CT scan has reported sensitivity of 91% and specificity of 76% for colorectal metastatic lesions, and sensitivity of 91% and specificity of 91% for colorectal recurrence<sup>[18]</sup>. In addition, PET CT scan can guide the surgical decision for pelvic lymph node dissection to avoid pelvic autonomic nerve injury or late lower limb lymphedema.

MRI PET scan has been introduced for rectal cancer,

and shown improved accuracy of T-staging for cases in which standalone MRI and PET CT failed to define the nature of an avid lesion. A small case series study has shown promising results regarding the use of MRI PET as compared to PET CT, with a true positive rate of 86% for the former vs 71% for the latter in overall TNM staging<sup>[19]</sup>. MRI PET is not readily available in our practice; however, it may represent the next-generation of preoperative imaging for PE planning. In the case of isolated pelvic sidewall or nodal recurrence, where the tumour is not accessible for biopsy and the disease is not apparent, serial imaging and tumour marker surveillance should be conducted after endoscopic re-assessment (if accessible) for anastomosis or luminal recurrence.

For the future of PE surgery, there are proposals to adopt laparoscopic or robotic techniques, especially for colorectal and gynaecological malignancies, due to the potential benefit of the minimally invasive nature of these surgeries. There are some published reports of laparoscopic-assisted anterior PE or TPE in highly selected patients with rectal or gynaecological cancers<sup>[20-23]</sup>. The preliminary data have shown minimal blood loss, short hospital stays, low morbidity rates, and non-comprising short-term oncological outcome. The first report of robotic PE in advanced rectal cancer patients was published by Shin *et al*<sup>[24]</sup> in 2014. The authors reported on 3 consecutive male patients with locally advanced rectal cancer involving prostate and seminal vesicles. The robotic approach was performed with reduced operative time and blood loss. Except for one minor vesical-urethral anastomosis leak requiring temporary suprapubic cystostomy, there were no other major surgical complications. Oncologic outcomes were also favourable in that study. These reports have highlighted the possibilities of minimally invasive surgery in the setting of complex PE. However, the small cohorts on which they are based consist of highly selected patients who have participated in short-term follow-up, and wide adaptation of this novel approach will require larger clinical trials.

Our PE Unit has demonstrated a safe and effective approach to manage complex pelvic cancers, with acceptable morbidity rates, zero-rate mortality and equivalent oncologic outcomes. The success of managing this group of patients was made possible by careful patient selection, detailed preoperative planning, multidisciplinary teamwork and an adaptation of modern operative techniques and technologies.

## COMMENTS

### Background

Advanced pelvic tumour is a debilitating illness, which poses a formidable surgical challenge. Chemotherapy and radiotherapy often improve the symptoms, but the results are transient. As the disease progress to the terminal stage, many patients suffer from refractory pain, bleeding, malodorous fistula or pelvic sepsis. Pelvic exenteration (PE) is a combination of numerous extensive surgical procedures that aims to remove all the diseased organs in order to achieve a negative resection margin. This complex intervention is

currently the only curative option for advanced pelvic tumour.

### Research frontiers

PE has long been associated with high morbidity and mortality rates. However, adaptation of contemporary perioperative medical care approaches and innovative surgical techniques has allowed PE to emerge as the mainstream intervention, offering a good curative rate with low morbidity and mortality rates in selected patients with locally advanced pelvic tumours. Due to the substantial postoperative physiological disturbances associated with PE and the need to attain a negative margin, the focus of recent research has been to identify the suitable patient through comprehensive preoperative screening, detailed radiological staging, and adjuvant downstaging chemoradiotherapy. In addition, the development of methodological lateral PE and abdominal-approach sacrectomy has helped to improve the oncological outcome.

### Innovations and breakthroughs

In this study, the authors describe their initial experience and treatment strategy in a newly-established PE Unit that achieves low morbidity, zero-rate mortality and acceptable R0 resection rate. The short-term result is equivalent to other reports in the recent literature. The authors attribute this success to a dedicated multidisciplinary team, state-of-the art perioperative care and modern operative techniques.

### Applications

This study provides a descriptive patient selection criteria, perioperative non-surgical treatment strategy, and operative techniques that will help to reduce postoperative PE complications and achieve good oncological outcomes.

### Terminology

PE is a generic description of combined surgical procedures that were developed to remove the advanced pelvic tumour. Often, the advanced pelvic tumour has invaded into contiguous organs adjacent to the tumour origin, and therefore multiple surgical procedures are utilised in order to resect all diseased organs and achieve negative pathological resection margin. PE can be subgrouped into four types according to pelvic organs that are resected. Anterior PE involves removal of the upper rectum and genitourinary organs. Posterior PE involves removal of the rectum and reproductive organs, but spares the bladder. Total PE is defined as removal of the rectum, distal colon, genitourinary viscera, internal reproductive organs, draining lymph nodes and pelvic peritoneum. Lateral PE involves removal of the lateral pelvic lymph nodes along with diseased vascular and neural structures. After extensive resection, it is common to combine further procedures, such as permanent faecal or urinary diversion and perineal reconstruction, in order to maintain the physiology and to close the muscular defect.

### Peer-review

The newly-established PE Unit reported by the authors offers state-of-the-art exenteration service in Singapore. This study confirms that the modern perioperative treatment strategy and multidisciplinary approach produced excellent short-term outcomes in the first 25 consecutive cases.

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Prospective Study

# Introduction of laparoscopic gastrectomy for gastric cancer in a Western tertiary referral centre: A prospective cost analysis during the learning curve

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## Abstract

### AIM

To evaluate the costs of the introduction of a laparoscopic surgery program for gastric cancer in a Western community training hospital and tertiary referral centre for gastric cancer surgery.

### METHODS

All patients who underwent surgery for gastric cancer with curative intent in 2013 and 2014 were prospectively included. Primary outcomes were costs regarding surgery and hospital stay.

### RESULTS

Laparoscopic gastrectomy was used in 52 patients [mean age 68 years ( $\pm 9$ , range 50 to 87)] and open gastrectomy was used in 25 patients [mean age 70 years ( $\pm 10$ , range 46 to 85)]. Mean costs (in euro's) of surgical instrumentation were significantly higher for laparo-

scopic surgery:  $2270 \pm 670$  vs  $1181 \pm 680$  in the open approach ( $P < 0.001$ ). Costs of theatre use were higher in the laparoscopic group: mean  $3819 \pm 865$  vs  $2545 \pm 1268$  in the open surgery ( $P < 0.001$ ). Total costs of hospitalization (*i.e.*, costs of surgery and admission) were not different between laparoscopic and open surgery,  $8187 \pm 4864$  and  $7673 \pm 8064$  respectively ( $P = 0.729$ ). Mean length of hospital stay was  $9 \pm 12$  d in the laparoscopic group vs  $14 \pm 14$  d in the open group ( $P = 0.044$ ).

### CONCLUSION

The introduction of laparoscopic gastrectomy for gastric cancer coincided with higher costs for theatre use and surgical instrumentation compared to the open technique. Total costs were not significantly different due to shorter length of stay and less intensive care unit (ICU) admissions and shorter ICU stay in the laparoscopic group.

**Key words:** Laparoscopic surgery; Healthcare costs; Gastric cancer

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**Core tip:** The introduction of laparoscopic surgery for gastric cancer did not seem to result in increased costs as compared to open gastrectomy for gastric cancer. Despite higher operating room costs (longer operating time and more costly operating room materials) costs were similar between the open and laparoscopic group due to reduced length of stay and complication rate in laparoscopic gastrectomy patients.

Tegels JJ, Silvius CE, Spauwen FE, Hulsewé KW, Hoofwijk AG, Stoot JH. Introduction of laparoscopic gastrectomy for gastric cancer in a Western tertiary referral centre: A prospective cost analysis during the learning curve. *World J Gastrointest Oncol* 2017; 9(5): 228-234 Available from: URL: <http://www.wjgnet.com/1948-5204/full/v9/i5/228.htm> DOI: <http://dx.doi.org/10.4251/wjgo.v9.i5.228>

### INTRODUCTION

In patients with gastric cancer, surgical resection is the only treatment that can offer cure or increase long-term survival<sup>[1]</sup>. Laparoscopic surgery for gastric cancer has gained popularity despite initial concerns regarding safety and oncological adequacy<sup>[2]</sup>. Studies conducted in South Korea and Japan reported that laparoscopic gastrectomy is comparable to open gastrectomy with regard to surgical and oncological outcomes<sup>[2-4]</sup>. A meta-analysis by Memon *et al*<sup>[4]</sup> showed that laparoscopic procedures are associated with less blood loss but longer operation time. Many studies have reported outcomes of laparoscopic surgery for early gastric cancer (EGC), but

several authors have shown that a laparoscopic approach can also be used in cases of advanced gastric cancer<sup>[5-7]</sup>. This makes it a potentially important strategy in Europe where the vast majority of patients present at stage II or higher as opposed to Asian countries where EGC is far more common<sup>[8]</sup>.

In the current economic climate, governmental organizations and health insurance companies have a major influence on the regulation of costs in healthcare. Moreover, surgeons often have to prove that new techniques are cost-effective for hospital organizations. To the best of our knowledge, no cost-analysis studies have yet been performed concerning the introduction of the laparoscopic procedure for gastric resections. The aim of this study was to evaluate the costs of the introduction of a laparoscopic surgery program for gastric cancer in a Western community training hospital and tertiary referral centre for gastric cancer surgery.

### MATERIALS AND METHODS

#### Patients

Introduction of laparoscopic gastrectomy was started in January 2013. All consecutive patients with gastric adenocarcinoma eligible for curative surgery from January 2013 to December 2014 were included. Whether the patient would undergo laparoscopic or open surgery depended on the surgeon's experience in laparoscopic gastric surgery and surgeon preferences. Patients who underwent multivisceral resections were not included in this study. All data were collected in a prospective database. If non-equal groups were obtained, a consecutive number of patients who underwent open gastrectomy with curative intent would be retrospectively included to create two groups of equal size. All data, including intraoperatively used materials (*e.g.*, electrosurgical devices, staplers, suture materials and reusable instruments) were all available through the hospitals fully digitized patient information system, also for retrospectively included patients. This observational study collected data concerning direct hospital-related costs and complication rates and admission length. The Charlson comorbidity index (CCI) was used to classify comorbidities in patients<sup>[9]</sup>. Patients received care as usual. Approval for this study was obtained from the medical ethics committee.

#### Preoperative stage

Preoperatively all patients underwent gastroesophageal endoscopy and biopsies were taken to confirm the diagnosis. Further preoperative staging was done with computed tomography (CT) of the abdomen and chest. Magnetic resonance imaging and/or positron emission tomography/CT imaging was selectively performed when liver lesions were visible on CT-imaging. Multidisciplinary consensus regarding the treatment was obtained in all cases. Neo-adjuvant chemotherapy was administered whenever patient condition and comorbidities would

**Table 1** Costs of disposable instruments for laparoscopic surgery and open surgery

Item	Price
Laparoscopic surgery	
Ligasure Impact 5 mm, Medtronic, Ireland <sup>1</sup>	€ 448.22
Ligasure Impact 10 mm, Medtronic, Ireland <sup>1</sup>	€ 376.00
Autosuture endobag, endocatch, Medtronic, Ireland <sup>1</sup>	€ 63.56
Autosuture EndoGIA 12 mm, Medtronic, Ireland <sup>1</sup>	€ 203.00
Reload EndoGIA purple 45, Medtronic, Ireland <sup>1</sup>	€ 173.00
Reload EndoGIA purple 60, Medtronic, Ireland <sup>1</sup>	€ 176.00
Reload EndoGIA Gold 45, Medtronic, Ireland <sup>1</sup>	€ 181.00
Endopaddle 12 mm, Medtronic, Ireland <sup>1</sup>	€ 81.84
Alexis small/medium, applied medical	€ 31.00
Endoshear 5 mm, Medtronic, Ireland <sup>1</sup>	€ 73.50
EEA XL, Covidien United States	€ 439.62
EEA Orvil, Covidien United States	€ 94.20
Bladeless trocar 5 mm, Medtronic, Ireland <sup>1</sup>	€ 48.88
Bladeless trocar 5-12 mm, Medtronic, Ireland <sup>1</sup>	€ 48.88
Blunt trocar 5-12 mm, Medtronic, Ireland <sup>1</sup>	€ 47.81
Pyramidal bladed trocar 10-15 mm, Medtronic, Ireland <sup>1</sup>	€ 80.43
Hem-o-lok L filling, Weck United States	€ 23.00
Hem-o-lok XL filling, Weck United States	€ 23.00
Open surgery	
Ligasure Impact, Medtronic, Ireland <sup>1</sup>	€ 343.80
Purssting stapler, Medtronic, Ireland <sup>1</sup>	€ 57.34
TA Green 30, Medtronic, Ireland <sup>1</sup>	€ 106.30
Reload TA Green 30, Medtronic, Ireland <sup>1</sup>	€ 60.50
TA Green 60, Medtronic, Ireland <sup>1</sup>	€ 110.09
Reload TA Green 60, Medtronic, Ireland <sup>1</sup>	€ 65.00
GIA Blue 60, Medtronic, Ireland <sup>1</sup>	€ 119.68
Reload GIA Blue 60, Medtronic, Ireland <sup>1</sup>	€ 75.51
GIA Blue 80, Medtronic, Ireland <sup>1</sup>	€ 147.48
Reload GIA Blue 80, Medtronic, Ireland <sup>1</sup>	€ 81.74
GIA Green 80, Medtronic, Ireland <sup>1</sup>	€ 148.96
Reload GIA Green 80, Medtronic, Ireland <sup>1</sup>	€ 81.74
CEEA 21, Medtronic, Ireland <sup>1</sup>	€ 408.97
CEEA 25, Medtronic, Ireland <sup>1</sup>	€ 384.48
CEEA 28, Medtronic, Ireland <sup>1</sup>	€ 388.33
CEEA 25 XL, Medtronic, Ireland <sup>1</sup>	€ 439.62

<sup>1</sup>Formerly Covidien United States.

allow.

### Outcome measurement

Primary outcomes included costs regarding surgery and hospital stay. Costs were obtained from the Financial Controllers of the involved departments. Data (*e.g.*, duration of operation, use of disposables) were collected prospectively. For patients who were retrospectively included all data were available through the electronic patient record system. This system also recorded which disposable and reusable operating theatre materials were used during surgery. Costs of ward stay were 180 euro's per day; ICU admission was 665 euro's per day. Costs of operating theatre use were hourly rates for surgery and anesthesiology combined at 800 euro's per hour. Sterilization costs of reusable instruments were also accounted for and varied for different types of surgical sets that were used. All used instruments, disposable and reusable, were noted during the procedure by one of the operating room nurses on a prepared list that was provided to prospectively collect data. Costs of the

disposable instruments for the laparoscopic and open surgery are shown in Table 1. Postoperatively all patients were admitted to the recovery ward before they were transferred to the general ward. For laparoscopic and open gastrectomy, costs of both disposable and reusable instruments, operating theatre use, ICU stay and hospital stay were calculated separately.

Secondary outcomes were estimated blood loss, duration of operation, length of ICU stay, length of hospital stay, anastomotic leakage rate and complications. Complications were graded according to Clavien-Dindo classification: Grade 3a (*i.e.*, complication requiring reintervention) or greater was considered a major complication<sup>[10]</sup>. Tumour stage was classified in line with the American Joint Committee on Cancer tumor node metastasis 5<sup>th</sup> edition.

### Surgical technique and postoperative care

Open surgery was performed by three surgeons prior to introduction of laparoscopic surgery for gastric cancer. All three had extensive experience in open surgery for gastric cancer.

Laparoscopic procedures were performed by two of the abovementioned three surgeons. Both surgeons also had extensive prior expertise in laparoscopic surgery for other gastrointestinal malignancies mainly colorectal surgery and gastric GIST tumours. Prior to introducing laparoscopic surgery for gastric cancer, specific expertise and proficiency was obtained by the laparoscopic surgeons by taking expert courses in the Netherlands and Singapore.

Surgical resections for gastric malignancy were defined as either distal or total gastrectomies. The type of resection performed depended on the localization and depth of invasion of the tumour. In both open and laparoscopic surgery, a standard D2 or D1+ lymph node dissection (dissection of group 1 and number 8a and 9 lymph nodes) was performed in accordance with Dutch guidelines. Continuity of the gastrointestinal tract in subtotal gastrectomies was restored either by a Billroth-II or a Roux-en-Y reconstruction. In the case of a total gastrectomy a Roux-en-Y reconstruction was always performed. Patients were not routinely admitted to the ICU postoperatively. ICU admission was always for complication management (*e.g.*, sepsis, pulmonary complications).

Postoperative care of both open and laparoscopic patients included several aspects of a multimodal perioperative Enhanced Recovery After Surgery (ERAS) program for gastrointestinal cancer<sup>[11]</sup>. These include early enteral feeding (*i.e.*, resumption of liquids on postoperative day one) and early mobilization. Follow-up of the patients after discharge was performed periodically. Follow-up consisted of physical examination, blood tests, and CT-imaging if indicated.

### Statistical analysis

Data were analyzed using SPSS 20 (IBM Corp. Armonk,

**Table 2** Baseline characteristics *n* (%)

	Open gastrectomy ( <i>n</i> = 25, 32%)	Laparoscopic gastrectomy ( <i>n</i> = 52, 68%)	<i>P</i> value
Age	70.0 (± 10, 46-85)	68 ± 9, 50-87)	0.470
Sex (male/female)	17/8	32/20	0.623
BMI <sup>1</sup>	25 ± 4, 18-36	25 ± 5, 15-38	0.824
CCI <sup>2</sup>			0.158
0-2	16 (64)	27 (52)	
3-4	7(28)	11 (21)	
> 4	2 (8)	14 (27)	
Tumour stage <sup>3</sup>			0.681
0	0 (0.0)	2 (4)	
1 <sup>1</sup>	4 (16)	11 (21)	
1 <sup>2</sup>	6 (24)	15 (29)	
2	5 (20)	4 (8)	
3 <sup>1</sup>	4 (16)	10 (19)	
3 <sup>2</sup>	3 (12)	4 (8)	
4	3 (12)	6 (11)	
Subtotal gastrectomy	16 (64)	38 (73)	0.436
Total gastrectomy	9 (36)	14 (27)	
Neoadjuvant chemotherapy	11 (44)	36 (69)	0.046

<sup>1</sup>BMI: Body mass index in kg/cm<sup>2</sup>; <sup>2</sup>CCI: Charlson comorbidity index; <sup>3</sup>In accordance with tumor node metastasis 5<sup>th</sup> edition.

NY). Continuous variables were expressed as mean ± SD or mean (range) if appropriate.  $\chi^2$  tests were used to compare the difference in frequencies of categorical variables. To compare the means of two independent samples, *t*-tests and non-parametric tests were used. The threshold for statistical significance was set at a *P*-value of < 0.05.

## RESULTS

### Baseline characteristics

A total of 77 patients underwent gastrectomy with curative intent from January 2013 to December 2014. The laparoscopic approach was used in 52 (68%) patients. The open approach was used in 25 (32%) patients. There were no statistically significant differences in sex, age, CCI (*i.e.*, comorbidities) or tumour stage. Patients undergoing laparoscopic gastrectomy had significantly more frequently received neoadjuvant chemotherapy (69% vs 44%, *P* = 0.046) (Table 2).

A consecutive series of 30 patients who underwent open surgery were included retrospectively, these patients underwent surgery between May 2012 and January 2013. These patients did not differ from patients who underwent open surgery in the prospective series with regards to the baseline characteristics mentioned in the prospective group.

### Primary outcome

The costs (in euro's) of surgical instrumentation were significantly higher for laparoscopic surgery compared to open gastrectomy, 2270 ± 670 and 1181 ± 680 respectively, *P* < 0.001 (Table 3). Also, the costs of theatre use were significantly higher in the laparoscopic group compared to open gastrectomy, 3819 (± 865) and

**Table 3** Primary outcome, costs of surgery, hospital admission and intensive care unit stay

Costs (in euro's)	Open gastrectomy ( <i>n</i> = 25, 32.5%)	Laparoscopic gastrectomy ( <i>n</i> = 52, 67.5%)	<i>P</i> value
Surgical instrumentation	1181 ± 680	2270 ± 670	< 0.001
Operating theatre use	2545 ± 1268	3819 ± 865	< 0.001
Ward stay	2218 ± 1810	1381 ± 1298	0.023
ICU stay	1729 ± 6499	716 ± 3299	0.366
Admission	3947 ± 6719	2097 ± 4419	0.153
Total costs	7673 ± 8064	8187 ± 4864	0.729

ICU: Intensive care unit.

2545 ± 1268 respectively, *P* < 0.001. Costs of general ward stay were significantly lower in the laparoscopic group compared to open gastrectomy, 1381 ± 1298 and 2218 ± 1810 respectively, *P* = 0.023. ICU stay and total admission costs (*i.e.*, ward stay and ICU stay combined) were not significantly different. The total costs of admission and surgery did not significantly differ between open and laparoscopic gastrectomy, 7672 ± 8064 and 8187 ± 4863 respectively, *P* = 0.729.

When a retrospective consecutive series of open gastrectomies was included to obtain equal sized groups (*i.e.*, 55 open vs 52 laparoscopic gastrectomies), total admission costs were significantly lower in the laparoscopy group, 2097 ± 4420 vs 4611 ± 7991, *P* = 0.048. Costs difference of total hospitalisation (*i.e.*, operating theatre and ward stay) between open and laparoscopic gastrectomy was smaller at 8187 ± 4868 for laparoscopic patients vs 7915 ± 8653 for patients who underwent open surgery, *P* = 0.843.

### Secondary outcomes

Comparison between the two techniques showed that total theatre time utilized was 191 min ± 95 for the open procedure and 286 min ± 65 for the laparoscopic gastric resection (*P* < 0.001) (Table 4). Results for secondary outcome parameters are listed in Table 4. Mean intraoperative blood loss was significantly less in the laparoscopic gastrectomy group (267 mL vs 592 mL, *P* = 0.002). In three cases, the laparoscopic approach was converted to an open procedure. In one case this was due to a splenic rupture, which was caused during laparoscopic surgery. In the other two patients the reason for conversion to an open procedure was a limited view of suspected ingrowth of tumour in the pancreas.

Laparoscopic gastrectomy was associated with a lower rate of overall complications and major complications, 16 (31%) vs 15 (60%), *P* = 0.025 and 6 (12%) vs 7 (28%), *P* = 0.104 respectively. Anastomotic leakage rates were higher in patients undergoing open gastrectomy than laparoscopic gastrectomy 2 (12%) and 2 (4%) respectively, *P* = 0.322. The differences in major complications and anastomotic leakage rates were not statistically significant in the prospective series. Also, patients who underwent laparoscopic resection had a shorter length of hospital stay and ICU stay (Table 4).



**Table 4 Secondary outcome parameters**

	Open gastrectomy ( <i>n</i> = 25, 32.5%)	Laparoscopic gastrectomy ( <i>n</i> = 52, 67.5%)	<i>P</i> value
Intraoperative blood loss (mL)	592 (± 529, 100-2500)	267 (± 316, 20-2000) <sup>1</sup>	0.002
OR time (min)	191 (± 95, range 95-554)	286 (± 65, range 207-597)	< 0.001
Lymph node yield ( <i>n</i> )	25 (± 10, 751)	26 (± 8, 10-47)	0.651
Any complication	15 (60%)	16 (31%)	0.025
Grade Clavien-Dindo ≥ 3a	7 (28%)	6 (12%)	0.104
Anastomotic leakage	3 (12%)	2 (4%)	0.322
Mean length of stay (d)	15 (± 14, 5-59)	9 (± 12, 2-84)	0.044
Mean ICU stay (d)	3 (± 10, 0-49)	1 (± 5, 0-35)	0.366
Readmission	4 (16%)	6 (12%)	0.720

<sup>1</sup>Five missing values for intraoperative blood loss in laparoscopic group. ICU: Intensive care unit.

When comparing equal sized groups (*i.e.*, 55 open and 52 laparoscopic gastrectomies), significantly more major complications occurred in the open surgery group, 17 (31%), compared to the laparoscopic group, 6 (12%), *P* = 0.019. Also, the anastomotic leakage rate was significantly higher in the open surgery group at 10 (18.2%) compared to 2 (4%) in the laparoscopic group, *P* = 0.029.

In the prospective series two patients died after surgery 1 (4%) after open gastrectomy and 1 (2%) after laparoscopic gastrectomy. In the total series (*i.e.*, including the retrospective series of open gastrectomies) four patients (7.3%) died after open gastrectomy, three died after septicemia from anastomotic leakage with one patient who also had a concurrent pancreatic leakage. One patient died of a severe aspiration pneumonia. One patient (1.9%) died after a laparoscopic gastrectomy from intestinal ischemia of the right and transverse colon.

Both techniques had a similar lymph node yield: mean 29 ± 10 and 26 ± 8.5 for open and laparoscopic gastrectomy respectively (*P* = 0.103). There were three cases of microscopically irradical resection: One in the open group and two in laparoscopic gastrectomy group (*P* = 0.614). Analysis of equal sized groups (*i.e.*, 55 open vs 52 laparoscopic gastrectomies) resulted in similar results for the abovementioned secondary outcome parameters.

## DISCUSSION

The aim of this study was to evaluate the costs of laparoscopic surgery for gastric cancer during the introduction of this new technique in a tertiary referral centre. The results show a significant increase in costs of surgery associated with the laparoscopic procedure. These costs are mainly due to increased use of (non-) disposable instrumentation and theatre time. The secondary outcomes suggest that laparoscopic gastrectomy is safe. This is represented by less blood loss, and less (major) post-operative complications in laparoscopic surgery. With regards to oncological safety the number of harvested lymph nodes and microscopically irradical resections were equal in laparoscopic and open surgery. Only two patients died in this study, one following open and one following laparoscopic gastrectomy.

This study was conducted at the time when laparoscopic approach was introduced in our tertiary referral

hospital for gastric cancer. The complexity of the laparoscopic approach is one of the reasons for a more time-consuming procedure. As surgeons gain experience, operative time is expected to decrease and theatre costs (at an hourly rate) will decline. Moreover, knowledge of the postoperative care on the clinical wards and safety of earlier discharge (ERAS) for patients who underwent laparoscopic as well as open surgery may help reduce hospital stay. This study shows positive results with regards to financial aspects of laparoscopic surgery even during the introduction and learning curve phase of its introduction.

Even though the duration of operation is expected to decline, the longer operative time compared to open surgery will probably remain. This has been shown in larger meta-analyses with weighted mean differences ranging from + 48 to + 82 min longer operative time for laparoscopy<sup>[12-14]</sup>.

These meta-analyses also show several other advantages of laparoscopic surgery compared to open surgery such as significantly shorter hospital stay (2.5-3.6 d) and significantly lower complication rates<sup>[13,14]</sup>. These differences can be expected to be associated with lower costs. Moreover, laparoscopic gastrectomy has been shown to be associated with improved quality of life<sup>[15]</sup>. Studies in liver surgery, pancreatectomy and wedge resections for gastrointestinal tumours, have shown that laparoscopic surgery has the same advantages discussed above compared to open surgery (*e.g.*, shorter hospital stay, less intraoperative blood loss, decreased medical complications and no differences in operative mortality)<sup>[16-18]</sup>. For pancreatic and wedge resections this was performed at the cost of a longer operative time and a more expensive procedure due to costly surgical instruments<sup>[16,17]</sup>. In these studies increased costs associated with the procedure and instrumentation are offset by a reduction in other costs (*e.g.*, shorter hospital stay). This possibly makes laparoscopy a viable and cost effective option.

Another potential cost benefit of laparoscopic surgery could be found in long term complications of open abdominal surgery. Incidence of incisional hernia can be expected to be much lower in laparoscopic surgery compared to patients who underwent midline laparotomy. Therefore costs of treating incisional hernia might be

lower in laparoscopic compared to open surgery for gastric cancer.

Multimodal fast-track programs such as ERAS could further decrease hospital stay and complication rates and therefore costs. A fast-track program in laparoscopic gastrectomy for gastric cancer has been shown to be associated with decreased hospital stay and costs<sup>[19]</sup>.

One of the main limitations of this study is its non-randomized design. Therefore a selection bias cannot be excluded. Also the non-equal sized groups is a consequence of this fact. By partially retrospectively studying prospectively maintained digital registration data of used materials an effort could be made to compare equal sized groups. Most data and all costs-related data regarding laparoscopic procedures however were collected prospectively. Despite this, statistically significant differences were shown for the primary and secondary outcomes. No definitive conclusions can be drawn with regard to aspects such as postoperative complications and long term oncological safety. However, secondary outcomes show differences in favor of laparoscopic surgery. These are in line with other studies and show a shorter length-of hospital stay and fewer complications. Another limitation is that only patients who underwent surgery with curative intent for gastric adenocarcinoma were included. No conclusions can be drawn with regard to costs of palliative resections.

In conclusion, during the introduction of a laparoscopic gastrectomy programme for gastric cancer costs for theatre use and surgical instrumentation were higher compared to the open technique but overall costs were similar due to reduced length of stay and lower complication rates (and therefore lower ICU admission rates and costs). Similar results regarding surgical safety, feasibility and post-operative complications between laparoscopic and open gastrectomy were found. Larger prospective studies will be needed to determine cost effectiveness of laparoscopic surgery for gastric cancer.

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## COMMENTS

### Background

Laparoscopic surgery for gastric cancer has gained popularity despite initial concerns regarding safety and oncological adequacy. Studies conducted in Korea and Japan reported that laparoscopic gastrectomy is comparable to open gastrectomy with regard to surgical and oncological outcomes.

### Research frontiers

A meta-analysis by Memon *et al* showed that laparoscopic procedures are associated with less blood loss but longer operation time. Many studies have reported outcomes of laparoscopic surgery for early gastric cancer, but several authors have shown that a laparoscopic approach can also be used in cases of advanced gastric cancer.

## Innovations and breakthroughs

The authors aimed to evaluate the costs of the introduction of a laparoscopic surgery program for gastric cancer in a Western community training hospital and tertiary referral centre for gastric cancer surgery.

## Applications

Larger prospective studies will be needed to determine cost effectiveness of laparoscopic surgery for gastric cancer.

## Peer-review

An interesting manuscript describing cost analysis for laparoscopic gastric cancer surgery. It contains an important message for gastric cancer surgeons, to provide proof in favor of laparoscopic surgery for fellow surgeons, hospital directory boards and insurance companies. Nice short and concise manuscript.

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# World Journal of *Gastrointestinal Oncology*

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## Detecting circulating tumor material and digital pathology imaging during pancreatic cancer progression

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### Abstract

Pancreatic cancer (PC) is a leading cause of cancer-related death worldwide. Clinical symptoms typically present late when treatment options are limited and survival expectancy is very short. Metastatic mutations are heterogeneous and can accumulate up to twenty years before PC diagnosis. Given such genetic diversity, detecting and managing the complex states of disease progression may be limited to imaging modalities and markers present in circulation. Recent developments in digital pathology imaging show potential for early PC detection, making a differential diagnosis, and predicting treatment sensitivity leading to long-term survival in advanced stage patients. Despite large research efforts, the only serum marker currently approved for clinical use is CA 19-9. Utility of CA 19-9 has been shown to improve when it is used in combination with PC-specific markers. Efforts are being made to develop early-screening assays that can detect tumor-derived material, present in circulation, before metastasis takes a significant course. Detection of markers that identify circulating tumor cells and tumor-derived extracellular vesicles (EVs) in biofluid samples offers a promising non-invasive method for this purpose. Circulating tumor cells exhibit varying expression of epithelial and mesenchymal markers depending on the state of tumor differentiation. This offers a possibility for monitoring disease progression using minimally invasive procedures. EVs also offer the benefit of detecting molecular cargo of tumor origin and add the potential to detect circulating vesicle markers from tumors that lack invasive properties. This review integrates recent genetic insights of PC progression with developments in digital



pathology and early detection of tumor-derived circulating material.

**Key words:** Circulating tumor cells; Digital pathology; Early detection; Exosomes; Pancreatic cancer

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**Core tip:** Pancreatic cancer (PC) is a leading cause of cancer-related death. PC mutations accumulate 20 years before patient death with metastatic mutations occurring late in the process. Metastatic risk increases dramatically when tumor diameter is greater than 1 cm. Most PC cases are diagnosed at late metastatic stages when survival is short. Outcomes could be improved if non-invasive methods could detect early stages of the disease and guide treatment decisions. Recent studies indicate this may be possible with application of digital pathology imaging, screening of CA 19-9 with additional markers, and detecting circulating tumor material in early-stage PC patients.

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## INTRODUCTION

Pancreatic cancer (PC) is the third leading cause of cancer-related death in men and women in the United States surpassing breast cancer<sup>[1,2]</sup>. Projections indicate PC will outpace colorectal cancer and become the second leading cause of cancer-related death in the United States by 2020<sup>[2]</sup>. The majority of pancreatic tumors (90%) are classified as adenocarcinomas arising from the ductal epithelium with an annual incidence of 45220 patients diagnosed with pancreatic ductal adenocarcinoma (PDAC) in the United States<sup>[1,3]</sup>. Estimates suggest that only 1.3%-10% of patients diagnosed with PC have familial basis for the disease where a genetic component is inherited from a relative<sup>[4]</sup>. The remaining majority of PDAC cases display large genomic heterogeneity<sup>[5]</sup>. Five-year survival is about 25% for localized stages but only 2% for advanced disease<sup>[1]</sup>. The best curative treatment is surgical resection, if performed early it presents a 5-year survival in 25%-30% lymph node negative patients but only 10% for those with positive lymph nodes<sup>[6-8]</sup>. Less than 20% of PC cases are diagnosed early enough for surgical intervention<sup>[2]</sup>. Relapse rate after surgery is typically high (80%) for this type of cancer and surgery is often followed by adjuvant chemotherapy or chemoradiation<sup>[2,9]</sup>. Approximately 80% of PDAC patients are diagnosed late when the disease becomes locally advanced or metastatic, where palliative chemotherapy

is the only treatment option<sup>[10]</sup>. Since 1997, Gemcitabine has been commonly used over 5-fluorouracil (5-FU) albeit with only a modest median overall survival (OS) advantage of 5.6 mo (Gemcitabine) vs 4.4 mo (5-FU) in patients presented with advanced stage<sup>[11]</sup>. Extensive efforts have been made over the past decade, including numerous randomized phase III clinical trials, to evaluate combinatorial drug treatments for patients with advanced disease<sup>[12]</sup>. To date erlotinib, an epidermal growth factor receptor (EGFR) inhibitor, plus gemcitabine is the only course with a targeted therapy agent approved by the United States Food and Drug Administration (FDA) for first-line use in advanced PC<sup>[13-15]</sup>. FOLFIRINOX, Fluorouracil, IRINotecan, and OXaliplatin (FOLFIRINOX) and nab-paclitaxel/gemcitabine have emerged as combinatorial treatments with results that may reach the one-year survival barrier<sup>[16,17]</sup>. Adjuvant combination chemotherapy comprising gemcitabine with capecitabine has also shown statistically improved survival over gemcitabine monotherapy in PDAC subjects (ESPAC-4, Phase 3)<sup>[18]</sup>. Great focus has been extended into developing methods for improving early detection of the disease and exploring alternate treatment options that can extend survival in patients with late stage presentation<sup>[14]</sup>. This review provides description of the genetic fingerprints that drive disease progression and discusses selected features relevant to detection and treatment in this biological context. We further highlight recent advances in digital pathology, improvements in CA 19-9 testing, and detection of circulating tumor cells and tumor-derived extracellular vesicles (EVs) in biofluids of PC subjects (Table 1). Particular attention is made to literature that provides examples of material isolated from human PC subjects along with cell culture or animal model systems that explore mechanistic underpinnings.

## PC PROGRESSION AND GENETICS

Computational modeling of primary pancreatic tumors supports the observations that metastatic probability increases exponentially with tumor size<sup>[19,20]</sup>. A patient with a primary tumor size of 1 cm in diameter is predicted to have a 28% probability of harboring metastasis at the time of diagnosis. This dramatically increases to 73% probability with a tumor size of 2 cm and elevates to 94% chance for a tumor size of 3 cm<sup>[20]</sup>. This clearly suggests that systemic treatments that target rapidly growing cells need to be administered early before log-phase growth is reached. For conventional therapies to improve survival, it will become paramount to detect early lesions before significant invasion takes course. The term pancreatic intraepithelial neoplasia (PanIN) was first coined in 1999 to describe ductal lesions which form as precursors to invasive cancer<sup>[21]</sup>. A progression model was soon after proposed where *HER-2/neu* overexpression and *KRAS* mutations are observed early, *p16 (CDKN2A/INK4a)* gene inactivation occurs at intermediate stages, with inactivation of *p53*, *DPC4*, and *BRCA2* occurring late<sup>[21]</sup>.

**Table 1** Summary of demonstrated clinical uses for digital pathology, circulating tumor cells and extracellular vesicles for pancreatic cancer

	Digital pathology	CTCs	EVs
Screening in population	Relies on invasive biopsies	Detection of KRAS mutations <sup>[92]</sup>	Early detection possibility (GPC1+ EVs) <sup>[117]</sup>
Diagnosis	Differential diagnosis of mucinous cancers <sup>[62]</sup>	Pancreatic CTC detected by ISET <sup>[82]</sup> and CellSearch <sup>[81]</sup>	GPC1+ EVs detected in IPMNs <sup>[117]</sup> EVs express mutated KRAS and p53 in PDAC serum <sup>[123]</sup> EVs detected in pancreaticobiliary cancers <sup>[124]</sup>
Staging	Early stage detection in mice <sup>[60]</sup> Distinguish Grade I / II in humans <sup>[61]</sup>	(C-MET, CK20, CEA) + CTCs elevated in late stages <sup>[96]</sup>	miR-17-5p in serum exosomes correlates with stage <sup>[128]</sup>
Prognosis	Potential	CTC positivity has prognostic value in locally advanced pancreatic cancer <sup>[81]</sup> CK20 expression in CTC indicates shorter overall survival <sup>[94]</sup>	Potential
Monitor treatment	Potential	CTC levels decrease during 5-FU therapy <sup>[91]</sup>	Potential
Drug sensitivity/ pharmacokinetics	CT scans can predict drug transport <sup>[35]</sup>	CTC apoptosis can be detected after 5-FU therapy <sup>[91]</sup>	Demonstrated for breast cancer <sup>[111]</sup>
Monitor recurrence	Potential	CTC positivity correlates with postoperative staging <sup>[94-97]</sup>	potential

EVs: Extracellular vesicles; CTCs: Circulating tumor cells; 5-FU: 5-Fluorouracil; PDAC: Pancreatic ductal adenocarcinoma; CT: Computed tomography; PDAC: Pancreatic ductal adenocarcinoma; CEA: Carcino-embryonic antigen.

This model predicts that PC evolves slowly with defined mutational characteristics and presents clinically at late stage. This progression paradigm of gradual pace has recently been challenged by Notta *et al.*<sup>[22]</sup> who propose a punctuated equilibrium hypothesis where tumorigenic mutations arise from a cataclysmic event that rapidly leads to invasive cancer and metastasis. Data from this model suggests PC development is neither gradual nor follows the accepted mutation order which may be supported by observations showing that not all clonally expanded precursor lesions lead to a tumor lineage<sup>[5,22,23]</sup>.

Recent evidence suggests that the development of metastatic cancers from primary tumors can take up to two decades, based on genomic sequence comparisons and mathematical analysis. The development of parental clones from an initiated tumor cell is estimated to take an average of 11.7 years, with an additional 6.8 years for expansion of metastatic subclones, and another 2-3 years before tumors disseminate to distant organs leading to patient death<sup>[24]</sup>. The founder mutations present only in the parental clones accumulate in a large number of driver genes involved in tumorigenesis such as *KRAS*, *TP53* and *SMAD4*. The resulting subclones, giving rise to metastatic lesions, contain additional progressor mutations which vary highly among subclones<sup>[24]</sup>. This suggests that distant metastasis occurs late during the genetic evolution of PC also supporting the punctuated equilibrium model of progression. These observations are consistent with findings that show more than 50% of the genomic rearrangements occur early during tumor progression being present in both primary and metastatic clones in the patient<sup>[25]</sup>. If these rearrangements could be narrowed to distinct genes or protein signaling pathways, they could serve as powerful targets for therapeutics made highly effective by reaching both primary and

metastatic sites. In addition to identifying mutation hot-spots in metastatic clones, it will be important to compare founder mutations in primary tumors between patients with different survival outcomes to discover early factors that commit patients to a high risk course<sup>[26]</sup>.

PCs were shown to have gene expression alterations in 69 gene sets, half of which cover at least twelve core signaling pathways with functional relevance in 67%-100% of observed neoplasias<sup>[27]</sup>. Even though these 12 overlapping cascades appear to be genetically altered in majority of the tumors, alterations of the pathway components themselves vary greatly between individual tumors<sup>[27,28]</sup>. This implies that therapies directed against these actionable targets may need to implement multi-targeted approaches based on selected patient subgroups, or consist of cocktails that effectively abolish entire signaling cascades<sup>[14,29]</sup>.

A recent study performing whole-genome sequencing and copy number variation (CNV) analysis found a total of 857971 point mutations, insertions and deletions in 100 samples of PDAC<sup>[30]</sup>. The four most commonly mutated genes observed in PDAC patients are the oncogene *KRAS* (75%-90%), tumor suppressor genes *TP53* (74%), *CDKN2A/p16* (35%), and *SMAD4* (31%), along with inactivating mutations in the Rac exchange factor *PREX2*, the tumor suppressor *RNF43*, and the histone demethylase *KDM6A* observed in 10%-18% of subjects<sup>[30]</sup>. Focal amplification of druggable oncogenes such as *ERBB2*, *MET*, *FGFR1*, *CDK6*, *PIK3R3* and *PIK3CA* is observed at very low prevalence among only 1%-2% of patients<sup>[30]</sup>. Levels of protein expression or activity were not determined in these studies, however, to understand the functional significance of the focal amplifications. Integrated genomic analysis of PDAC identified 32 mutated genes that comprise 10 signaling

pathways: KRAS, transforming growth factor (TGF)-beta, WNT, NOTCH, ROBO/SLIT signaling, G1/S transition, SWI-SNF, chromatin modification, DNA repair and RNA processing<sup>[31]</sup>. Four tumor subtypes were identified based on differential expression of transcription factors and downstream targets: Squamous, pancreatic progenitor, immunogenic, and aberrantly differentiated endocrine exocrine (ADEX) tumors. These tumor subtypes were sorted by gene programs to identify genetic factors that impact OS in PDAC subjects<sup>[31]</sup>.

PDAC primary tumors can also be sorted into three distinct subtypes based on gene expression patterns and drug sensitivity: Classic, quasimesenchymal and exocrine-like<sup>[32]</sup>. The classic subtype, more sensitive to the EGFR inhibitor erlotinib, expresses high levels of adhesion-associated epithelial genes such as *AGR2*, *S100BP* and *GATA6*. The quasi-mesenchymal subtype is more sensitive to gemcitabine and expresses high levels of mesenchymal genes such as *TWIST1* and *S100A2*. The exocrine-like subtype has high expression of tumor cell derived digestive enzyme genes such as *REG3A* and *PRSS1*<sup>[32]</sup>. These findings open the possibility for stratifying patients based on tumor gene expression patterns as a means for predicting drug sensitivity.

Taken together, these observations demonstrate that primary and metastatic tumors of the pancreas are highly heterogeneous and contain several distinct clonal populations with unique molecular signatures which develop over a long period of time. This makes targeted therapy difficult, unless common pathways are found that can be effectively blocked by personalized drug regimens<sup>[5]</sup>.

## PANCREATIC STROMA

Another source of genetic diversity can be found within the pancreatic stroma. PDAC cells are surrounded by a rich stroma that is typically far more abundant in cell types other than the tumor. Pancreatic stroma contains a variety of cells including stellate cells, immune cells, fibroblasts, vascular endothelial cells and the extracellular matrix which make up the tumor micro-environment (TME)<sup>[33]</sup>. TME plays a pivotal role in tumor behavior including proliferation, drug resistance, invasion and localized immune response<sup>[33,34]</sup>. A clinical study investigating intraoperative gemcitabine infusions during PDAC resection showed that high stromal density inhibits hENT1-mediated drug incorporation into the tumor<sup>[35,36]</sup>. Investigators in this study derived mass transport parameter (MTP) cutoff values based on expression of the nucleoside transporter hENT1 in the tumor, and pancreatic stromal density scores calculated from CT scans<sup>[35]</sup>. Applying MTP cutoffs to a cohort of 110 patients, who received gemcitabine therapy, revealed a 5-year survival rate of 40% in subjects with favorable transport parameters compared to a 15% survival rate in subjects who did not reach the parameter cutoff point<sup>[35]</sup>. This study demonstrates that stromal density and drug transport properties can be measured during surgery, using routine contrast-enhanced CT scans

and immunohistochemistry, as a highly effective means for predicting significant response to cancer therapy. hENT1 expression in tumor cells permits bidirectional transport of pyrimidine nucleosides such as gemcitabine, capecitabine and 5-FU<sup>[37]</sup>. High expression of hENT1 in PC patients treated with gemcitabine is predictive of improved survival<sup>[36,38,39]</sup>. These studies open the possibility for determining drug sensitivity in resected patients through screening morphological features of the stroma combined with assessment of pharmacogenomic profiles<sup>[40]</sup>.

The pancreatic stroma is enriched with large diversity of constituents, making it difficult to score clinically. A recent study applied a blind source separation technique called non-negative matrix factorization (NMF) to analyze gene expression from a microarray dataset that included 145 primary and 61 metastatic PDAC tumors in comparison to 134 normal tissue samples<sup>[41]</sup>. This technique effectively generated gene expression signatures sorted by tumor, stromal and normal cellularity. Patients that were identified with a "classical" tumor subtype had a median survival of 19 mo compared to patients with a "basal-like" tumor subtype that demonstrated a significantly worse survival of 11 mo. Additionally, two stromal subtypes were identified in patients: A "normal" subtype with 24 mo-median survival and an "activated" stromal subtype with significantly worse median survival of 15 mo. These techniques lead the way for identifying genetic markers that may otherwise be obscured by confounding material from normal and stromal tissue<sup>[41]</sup>.

Mounting evidence supports the hypothesis that pancreatic TME s play a significant role in pathological outcome and treatment response and should therefore be clinically evaluated as a standard practice. The use of digital imaging combined with pharmacogenomic analysis could extend the application of existing treatments for personalized medicine. Best clinical outcomes come from early diagnosis of the disease. Leveraging the biological properties of pancreatic adenocarcinomas and their surrounding micro-environment for early detection and diagnosis would provide maximum benefit for patient survival.

## CURRENT DIAGNOSTIC METHODS USING SERUM

Presently, there are no suitable PC screening strategies effective for early detection of PC in the general population. Diagnosis of PDAC is made by pathological assessment of a tissue biopsy. The current gold standard is *via* an endoscopic ultrasound technique coupled with fine needle aspirations (EUS-FNA) which has a sensitivity of 75%-94% and specificity of 78%-95%<sup>[8,42]</sup>. For patients who have non-diagnostic FNAs or cannot undergo endoscopy, treatment decisions are based on imaging or determining CA 19-9 serum levels<sup>[8]</sup>. The only serum biomarker approved by the FDA for PC is the sialylated Lewis (a) blood group antigen CA 19-9 which is not tumor specific and is frequently elevated during many malignancies,

**Table 2 Clinical uses for biomarker panels that increase predictive value of CA 19-9 for pancreatic cancer**

	CA 19-9	Sensitivity	Specificity	Ref.
Screening in population	EUS-FNA	75%-94%	78%-95%	[42]
	CA 19-9 <sup>1</sup>	60%-70%	70%-85%	[45,46]
Differential diagnosis	CA 19-9	60%	83%	[44]
	CA 19-9 + CA 125	87%	77%	[44]
	CA 19-9 + ICAM-1 + OPG	78%	94%	[49]
	CA 19-9 + CEA + TIMP-1	71%	89%	[49]
	PAM4-reactive mucins	76%	85%	[51]
Staging	CA 19-9 + PAM4-reactive mucins	84%	82%	[51]
Monitor treatment	Response to chemotherapy			[47]
Monitor recurrence	Low levels post-surgery			
	correlate with survival			[45]

<sup>1</sup>Values reflect subjects presented with pancreatobiliary disease. EUS-FNA: Endoscopic ultrasound and fine needle aspiration; OPG: Osteoprotegerin; ICAM-1: Intercellular adhesion molecule 1; CEA: Carcinoembryonic antigen; TIMP-1: Tissue inhibitor of metalloproteinases 1; clivatuzumab monoclonal antibody (PAM4) to MUC5AC.

pancreatitis, cholangitis, obstructive jaundice, hepatobiliary cancer, and benign biliary obstruction<sup>[43,44]</sup>. CA 19-9 alone has not been shown to be an effective screening marker for PDAC among the general population based on most studies<sup>[45]</sup>. However, sensitivity (60%-70%) and specificity (70%-85%) of CA 19-9 improve significantly in patient cohorts presented with pancreatobiliary disease<sup>[45,46]</sup>. Low serum CA 19-9 levels following surgery correlate with improved survival<sup>[45]</sup>. Oncologists occasionally use CA 19-9 to track response to chemotherapy but the predictive significance of CA 19-9 for this purpose has reported some variability<sup>[43,45,47]</sup>.

Measuring CA 19-9 in combination with other markers such as CEA, CA242, and TIMP1, however, was shown to improve its predictive value (Table 2)<sup>[45,48,49]</sup>. Barnett *et al.*<sup>[49]</sup> could identify PDAC patients using two independent panels: CA 19-9, CEA, and TIMP-1; and a second panel containing CA 19-9, ICAM-1, and OPG. Both panels demonstrated increased sensitivity and specificity over CA 19-9 alone (Table 2)<sup>[49]</sup>. Recently, O'Brien *et al.*<sup>[44]</sup> discovered CA 19-9 (> 37 U/mL) and CA 125 (> 30 U/mL) serum levels can be elevated up to two years before PDAC diagnosis based on a nested case control study. CA 125 has been reported to distinguish malignant from benign PC tumors with 60.8% sensitivity and 83.3% specificity which improved to 87.8% and 77.8% respectively when combined with CA 19-9<sup>[50]</sup>. PAM4, an antibody which binds mucin MUC1 and MUC5AC epitopes expressed in PC, was capable of identifying 64% of stage I PDAC patients with high discriminatory power compared to those with benign pancreatic disease<sup>[51]</sup>. PAM4 is capable of distinguishing normal pancreas from PanIN-1A, PanIN-1B, PanIN-2, and PanIN-3 lesions, intraductal papillary mucinous neoplasia (IPMN) lesions, as well pancreatic adenocarcinomas of various grades<sup>[52]</sup>. Combining CA 19-9 with a PAM4-reactive marker improved sensitivity (84%) without a loss in specificity (82%) in a serum-based enzyme immunoassay (EIS)<sup>[51]</sup>. Despite some propensities for false positivity, CA 19-9 continues to be a benchmark serum marker for evaluating PC in the clinical setting. It will be important

to test combinations of other markers in addition to CA 19-9 to improve its diagnostic utility in larger populations.

## TUMOR IMAGING AND DIGITAL PATHOLOGY

In addition to biopsies and serum marker tests, lesions and primary tumors can be characterized by clinical imaging. Computed tomography (CT) and magnetic resonance imaging (MRI) are the most frequently used imaging method for diagnosis and clinical staging<sup>[33,43]</sup>. Additional screening approaches using imaging multi-modalities include endoscopic ultrasonography (EUS), magnetic resonance cholangiopancreatography (MRCP), and endoscopic retrograde cholangiopancreatography (ERCP)<sup>[4]</sup>. However, these approaches are limited to surveillance centers with robust PC programs and are typically only performed on high-risk patients<sup>[53,54]</sup>. MRI and EUS have been proposed for use as first line modalities but often fail to distinguish benign from malignant lesions<sup>[55]</sup>. Emerging imaging modalities and molecularly targeted imaging agents are of great interest as early detection strategies but may be cost prohibitive and inaccessible to many patients<sup>[56]</sup>.

Upon diagnosis, patients are staged based on the AJCC 7<sup>th</sup> Edition Staging Manual criteria before proceeding to surgery<sup>[8,57]</sup>. This is typically accomplished through cross-sectional imaging (CT or MRI) along with tissue biopsy<sup>[8]</sup>. Among those staged with resectable disease by biopsy, only 70%-85% actually present with resectable tumors, intraoperatively<sup>[8]</sup>. This indicates a need for improvements in staging methodology which may be enhanced by digital pathology<sup>[8]</sup>. The field of digital pathology has recently grown to complement histological diagnosis performed by pathologists<sup>[58]</sup>. These methods extract and quantify histological features from whole slide images thus improving on the subjective nature of the work<sup>[59]</sup>.

Langer *et al.*<sup>[60]</sup> developed a method that can accurately predict early pancreatic lesions with a 93% success rate



in an independent test set using tissue obtained from mouse models of early-stage PDAC. The program uses a top-down object learning paradigm similar to the methodology used by human pathologists. Initially, ducts, nuclei and tumor stroma are identified and segmented. From those, secondary morphological features such as duct deformation and nuclei malformations are measured. These data sets are then used to train a predictive model that distinguishes normal tissue from premalignant cancer lesions<sup>[60]</sup>. Similar techniques can be extended to accomplish classification of PDAC by grade using human tissue samples<sup>[61]</sup>. Diagnosis of PDAC was made based on three parts: Segmentation and feature extraction; model learning and validation; and diagnosis. Training data measuring ducts, consisting of the lumen and epithelial nuclei, can distinguish normal human subjects and those with grade I and grade II PDAC with an accuracy of 94%<sup>[61]</sup>. Automated systems have been developed for making a differential diagnosis of rare lesions such as cystic neoplasms of the pancreas using human biopsy tissue<sup>[62]</sup>. Song *et al.*<sup>[62]</sup> were able to distinguish benign serous from malignant mucinous cystadenomas using a computer-aided design technique. Cystic regions were identified and epithelial cells surrounding the lumen were discerned. Three classes of features were analyzed by the program to achieve a differential diagnosis: The number and size of cysts, characteristics of the surrounding epithelium, and indication of mucus production<sup>[62]</sup>.

Current applications of digital pathology for PC do not offer much more beyond histological diagnosis performed by a pathologist but indicate potential for detecting early lesions. Improvements could be made, for example, by developing digital pathology methods for images annotated with clinical data from population-based repositories. This could potentially aid the discovery of morphological features associated with treatment and survival outcomes.

The intended goal beyond research is to incorporate digital tools into clinical practice as a way to standardize histological diagnosis in patients at high risk of developing the disease. This could improve staging and determination of resectability. Some concerns raised include public health consequences if misdiagnosis is caused by improper use or analysis of poor quality images<sup>[63]</sup>. The Food and Drug Administration recently released a guidance for technical performance assessment of digital pathology whole slide imaging (WSI) devices<sup>[64]</sup>. Currently, WSI devices are classified as Class II for methods that provide adjunct analysis after a primary diagnosis is made using glass slides. WSI devices which make a primary diagnosis alone are classified as high-risk Class III devices if their intended use is new and lacks a Class II predicate. A *de novo* process provides a less resource-intensive approval path to Class I / II classification if special controls are presented that provide reasonable assurance of safety and effectiveness. A more clearly-defined approval process for manufactures would enhance innovation and commercialization potential of digital pathology instruments and software<sup>[65]</sup>.

## DETECTING TUMOR CELLS IN CIRCULATION

Performing invasive biopsies for routine screening of the general population is not reasonably a feasible option. Detecting tumor material in the blood or other biofluids would be ideal for many reasons. A test assessing a panel of markers in biofluids could be ordered by physicians in most clinical centers, and collected by non-invasive or minimally invasive procedures. Performing additional tests using the same starting material could easily lead to diagnostic refinement. Diagnostic tests can be expanded to cover non-tumor biomaterial such as components of the immune system, blood/serum, pancreatic juice, stool, oral and gut microbiota, and markers of metabolic activity. Given patient variability, measuring systemic profiles of markers not directly derived from tumors may not yield the specificity and sensitivity necessary to accurately determine risk for developing advanced PC. A search for "pancreatic cancer" in the published literature can easily generate over 50000 returns which documented more than 2500 individual genes as potential PC biomarkers due to their overexpression patterns<sup>[66]</sup>. A compendium of PC biomarkers identified at least 1000 molecules with evidence of upregulation in precursor lesions<sup>[66]</sup>. Early detection of these precursor lesions particularly before invasive cells establish colonization would be ideal.

A critical study, using genetically engineered mouse models of PanIN, showed that cells from preneoplastic lesions can breach the basement membrane and spread into the stroma<sup>[67]</sup>. Contrary to conventional wisdom, these cells undergo epithelial-to-mesenchymal transition (EMT) and enter the blood into circulation with no evidence of carcinoma. These findings suggest that EMT transition can occur as an early phenomenon even before histologic emergence of cancer. These cells acquire a mesenchymal phenotype, exhibit stem cell properties, have tumor-initiating capacity, and are most abundantly observed at inflammatory foci<sup>[67]</sup>. Induction of pancreatitis and immunosuppressive treatment with dexamethasone have strong effects on dissemination supporting a link between early precursor cell invasion and localized inflammation<sup>[67]</sup>. Typical circulating tumor cell (CTC) markers such as EpCAM are expressed in less than 20% of the PanINs in this model system<sup>[67]</sup>. This has implications for commercially available methods which may overlook these circulating precursors, because they rely on such epithelial markers for CTC detection.

EMT in primary cells was shown to be associated with acquisition of stem cell-like characteristics<sup>[68]</sup>. Both normal and cancer stem cells possess the ability to self-renew and produce differentiated progeny<sup>[29]</sup>. Cancer stem cells are further functionally defined by having enhanced tumor initiating capacity when transplanted to a permissive host<sup>[67]</sup>. *In vivo*, CTCs detach from the primary tumor and enter the blood where they can be

transported to distant sites with only 0.01% surviving to form metastases<sup>[69]</sup>. CTC detection has been extensively used for prognosis (progression free survival and OS) and predicting response to treatment in breast, prostate, colorectal and lung cancers<sup>[70-74]</sup>. CTCs have also been detected in PC patient samples but their prognostic potential remains to be optimized outside the limitations of a small sample of subjects<sup>[69,75-77]</sup>.

## CTC DETECTION METHODS

Circulating tumor cells are present at very low concentrations in the blood, typically one CTC per billion blood cells. For this reason, CTCs need to be enriched to differentiate them from the vast hematopoietic cell background, and characterized to verify their tumor origin<sup>[69]</sup>. Several enrichment media for density-gradient centrifugation are commercially available including LymphoPrep™ (Axis-Shield), Ficoll-HyPaque™ (Sigma-Aldrich), Oncoquick® (Greiner Bio-One), and RosetteSep™ Human Circulating Epithelial Tumor Cell Cocktail with SepMate™ (StemCell Technologies). Enrichment is typically followed by targeted isolation. Four strategies are available to isolate and capture CTCs: Positive selection using antibodies attached to solid-support, negative selection, cell size-based methods such as filtration, and physical property-based methods<sup>[77]</sup>. Most CTC detection methods rely on either positive immunoselection of cells expressing the epithelial cell adhesion molecule (EpCAM) or negative selection by depleting leukocytes from the blood using CD45-binding antibodies<sup>[78]</sup>. Commercial immunomagnetic bead separation systems are available including EasySep cell separation (StemCell Technologies), Dynabeads (Invitrogen), CellSearch CTC system (Janssen Diagnostics) and MACS (Miltenyi). CellSearch CTC is the only system approved by the FDA for capturing and enumerating CTCs of epithelial origin by CD45<sup>-</sup>, EpCAM<sup>+</sup> and cytokeratin<sup>+</sup> selection<sup>[79]</sup>. CellSearch has been cleared by the FDA for management of breast, colorectal and prostate cancers and has also been tested in PDAC patients with detection rates varying from 11%-45%<sup>[44,78,80-85]</sup>.

Once isolated, circulating tumor cells are typically characterized by immunocytochemical (ICC) staining or nested real-time polymerase chain reaction (RT-PCR). Detection strategies typically assess epithelial mRNA profiles which include *EpCAM*, epithelial carcinoembryonic antigen (*CEA*), *CEACAM5*, *CK19*, *BIRC5* and *MUC1*<sup>[76]</sup>. There are currently more than 40 assay platforms for CTC detection and enrichment that have been widely publicized<sup>[86]</sup>. Among these, the utilization of microfluidics and microarray technology in CTC detection is expanding.

CTC detection was investigated as a prognostic tool in a LAP07 international multicenter randomized study to assess if patients with locally advanced pancreatic carcinoma (LAPC) would benefit from chemoradiotherapy over continuation of chemotherapy<sup>[81]</sup>. Bidard *et al.*<sup>[81]</sup> were able to achieve a CTC detection rate of 11% using a low cut-off of one or more CTCs/7.5 mL of blood using the

CellSearch system. This is lower than the 50% detection rate typically reported for metastatic PC patients<sup>[84]</sup>. CTC positivity nonetheless was a prognostic factor for OS which was lower in CTC positive LAPC patients<sup>[81]</sup>. More CTCs can be detected in the blood of PC patients using ISET (Isolation by Size of Tumor Cell) based on a comparative study which found detection of 26 CTCs/7.5 mL blood using ISET compared to 2 CTCs/7.5 mL blood by CellSearch<sup>[82]</sup>. ISET also detected CTCs in a much higher proportion of patients (93%) vs CellSearch (40%)<sup>[82]</sup>. ISET is a filtration-based, marker-independent method that sorts by cell size and morphology using filter modules offered by a company started by the inventor of the technology (Rarecells Diagnostics)<sup>[87,88]</sup>. Thus ISET may offer a significant advantage over CellSearch which relies on expression of EpCAM for CTC identification. PDAC cells, among carcinomas, are more prone to epithelial-mesenchymal transition (EMT) which reduces the expression of EpCAM<sup>[78,89,90]</sup>. This presents a problem for PC detection using CTCs as most of the current CTC detection methods rely on EpCAM or other epithelial molecules for CTC detection<sup>[69,86]</sup>.

## CTC CHARACTERIZATION

There exists a critical need for the development of assays that can additionally identify CTCs which undergo EMT and lose expression of typical epithelial surface antigens. Ren *et al.*<sup>[91]</sup> detected CTCs in peripheral blood of advanced stage PC patients before (in 80% of patients) and after treatment (in 29% of patients) with 5-FU by immunostaining for CA19-9 and CK8/18 expression. The mean concentration of blood CTC decreased from 16.8 cells/7.5 mL of blood before chemotherapy to 3.8 cells/7.5 mL blood after a seven-day cycle of 5-FU chemotherapy<sup>[91]</sup>. Evidence of apoptosis induced by 5-FU was observed in CTCs obtained from patients and in pancreatic cell line models (PL45 and PANC-1 cells)<sup>[91]</sup>. These studies open the possibility for using CTC assays to monitor chemotherapy efficacy and extent of remission although they fail to selectively identify mesenchymal antigens expressed by CTCs. Other potential mesenchymal protein marker candidates include Cadherin 2, Vimentin, Snail/Slug, zinc finger E-box binding homeobox1 (ZEB1), and Twist family basic helix-loop-helix transcription factor 1 (TWIST1)<sup>[79]</sup>. These mesenchymal markers could be combined with PC-specific markers to increase specificity.

To verify tumor origin, isolated CTCs can also be screened for genes expressed or mutated predominantly in PC such as *KRAS*. Court *et al.*<sup>[92]</sup> detected *KRAS* mutations in 92% of PC patients using a NanoVelcro/laser capture microdissection (LCM) platform. This technique captures CTCs on a microfluidic chip using biotinylated anti-EpCAM antibodies and is followed by identification through ICC staining of CD45, CEA, and staining of pancytokeratin for nuclear morphology. Mutations in *KRAS* were not observed in white blood cells and overall reliability of the assay required isolation of only 10-100 circulating tumor cells<sup>[92]</sup>. Chausovsky *et al.*<sup>[93]</sup> detected the

expression of Cytokeratin 20 (CK20) in 22/28 PC patients using RT-PCR analysis of peripheral blood CTCs. Soeth *et al*<sup>[94]</sup> found that CK20 was expressed in CTCs of 33% of patients in a larger cohort ( $n = 154$ ) who had significantly shorter OS. Cytokeratin 7 (CK7) and cytokeratin 20 (CK20) are expressed in a variety of epithelial neoplasms including majority of pancreatic carcinomas (62%)<sup>[95]</sup>. A variety of commercial platforms are now available for detection of amplified CTC DNA such as TruSeq Amplicon (Illumina) and Ion Torrent AmpliSeq™ (Life Technologies).

Levels of RNA expression can also be measured by RT-PCR or directly imaged by *in situ* RNA hybridization using platforms such as ViewRNA™ CTC Platform (Affymetrix). Zhou *et al*<sup>[96]</sup> measured mRNA expression of *h-TERT*, *C-MET*, *CK20*, and *CEA* by RT-PCR in CTCs isolated by immuno-magnetic enrichment using EpCAM. This method can distinguish PC patients from benign control subjects with high degree of specificity. Further, when pancreatic patients were in later stages, the expression rate for C-MET (67%), CK20 (75%) and CEA (75%) were statistically higher than during earlier stages<sup>[96]</sup>. These findings open the utility of CTC detection for monitoring disease progression. Two independent studies have also found that preoperative CTC positivity correlated with postoperative staging<sup>[94,97]</sup>. This indicates that in addition to diagnostic value, CTC detection has prospects for PC staging<sup>[8]</sup>.

The genetic content of CTCs can also be sequenced for molecular discovery<sup>[92]</sup>. Yu *et al*<sup>[98]</sup> adapted a microfluidic device to capture CTCs which were subjected to single-molecule RNA sequencing. Using a mouse PC model, *Wnt2a* gene was identified to be enriched in CTCs isolated from mice and in 5/11 human PC cases<sup>[98]</sup>. Ting *et al*<sup>[99]</sup> used focusing-enhanced microfluidic capture of CTCs (CTC-iChip) from primary PC tumors followed by deep-RNA sequencing. RNA-seq profiles identified enrichment of stem-cell-associated genes such as *Aldh1a2* and the extracellular growth factor binding protein *Igfbp5* which localized focally at the tumor epithelial-stromal interface<sup>[99]</sup>. CTCs of mouse and human origin also expressed elevated levels of gene expression of the stromal-derived extracellular matrix protein (SPARC), which increases invasive and migratory potential of PDAC cell lines<sup>[99]</sup>. Whole exome sequencing of CTCs has been successfully accomplished in metastatic prostate cancer cells, PDACs, and pancreatic carcinoma neoplasms with acinar differentiation<sup>[100-102]</sup>.

To improve prognostic value, CTC analysis can be combined with other methods such as direct detection of circulating free DNA (cfDNA) in the blood. Mutated *KRAS* cfDNA, isolated from plasma, was observed in 26% of patients with resectable and advanced stage disease and correlated strongly with decreased OS compared to mutant *KRAS* free subjects (60 d vs 772 d)<sup>[85]</sup>. Patients with panreatobiliary carcinomas were accurately diagnosed using cfDNA sequencing with a 92% sensitivity and 100% specificity<sup>[103]</sup>. CTCs were detected in peripheral blood of 20% of metastatic disease patients using the CellSearch system by CD45 positive cell depletion. CTC

positive PDAC patients had decreased OS of 88 d (95%CI: 27-206) compared to 393 d (95%CI: 284-501) in CTC negative subjects<sup>[85]</sup>. Circulating tumor DNA (ctDNA) can also serve as a detection strategy on its own or in combination and can be found in other articles that focus on this topic<sup>[78,104,105]</sup>. For example, Berger *et al*<sup>[104]</sup> were able to distinguish patients with Intraductal Papillary Mucinous Neoplasm (IPMN) lesions from controls by detecting mutation hot-spots in circulating cell-free DNA from patient blood samples.

Collectively, the utility of circulating tumor cells as a diagnostic marker in PC is gaining more ground. CTC detection offers the benefit of a low-risk safety profile which may be a cheaper alternative to FNA biopsies<sup>[8]</sup>. The cost of obtaining a diagnosis by EUS-FNA in the United States can be approximately \$16000 compared to \$370 Medicare reimbursement for the CellSearch CTC-based Assay<sup>[106,107]</sup>. A broader range of epithelial and mesenchymal markers are needed to create techniques that adequately capture a wide range of PC-specific circulating tumor cells. Finally, selected CTC techniques need to be tested in larger patient cohorts to pass the same FDA clinical guidelines that made the CellSearch CTC-based assay a successful clinical tool for breast, prostate and colon cancer.

## EVS

The study of EVs has gained significant momentum in recent years, because their cargo represents material of tumor which can shed light on the state of disease progression. EVs are membrane-bound organelles secreted by a variety of cells including cancer cells. The cytosol-derived lumen of EVs is enclosed by a lipid-bilayer forming a delivery vehicle for a variety of nucleic acids, proteins and lipids which can be horizontally transferred into recipient cells altering their biological properties. This allows cancer cells to continually modify their local microenvironment as well as distant sites when EVs enter circulation<sup>[108]</sup>. Because the molecular composition and function of these organelles represents their tumor origin, insight into EV biology provides great potential for tumor screening, diagnosis and prognosis. However, not all EVs are alike. The subcellular origin determines the type of cargo and mechanism of release from the cell. Large microvesicles (100-1000 nm) that bud outward from the plasma membrane are called ectosomes or ARRDC1-mediated microvesicles (ARMMs)<sup>[109]</sup>. Small EVs (30-150 nm) are called exosomes, which originate as intraluminal vesicles found in endosomal membranes and are secreted through fusion of multivesicular bodies (MVB) with the plasma membrane<sup>[110]</sup>. Several studies have identified exosomal subtypes based on molecular content that may hold diagnostic and prognostic value for diseases such as PDAC<sup>[110,111]</sup>.

Exosomes play an active role in disease progression by promoting tumorigenesis, metastasis, tissue remodeling, immune evasion, and chemoresistance<sup>[111,112]</sup>. This is reported to be achieved by the delivery of microRNA,

mutated genomic DNA fragments, proteins and lipids which alter the biology of tissues that take up cancer-derived exosomes<sup>[112]</sup>. Exosomes offer several detection advantages over other biomarkers. Because exosomes travel across the endothelium into circulation they can be detected in serum and/or urine which can be collected over time when monitoring a patient<sup>[112]</sup>. Exosomal content can be dispersed within the lipid membrane bi-layer but can also be found in the lumen where it is protected from degradation by external nucleases and proteases<sup>[113]</sup>. Once exosomes are isolated, their content can be much easier to detect by sensitive techniques such as RT-PCR, next generation sequencing, gene expression microarrays, and mass spectrometry<sup>[111,114]</sup>. The first challenge in establishing exosome biomarkers as clinical tools depends on the ability to isolate them in sufficient quantity at high purity.

Initial isolation depends on crude physicochemical properties such as particle size, density and solubility. Isolation by differential centrifugation is the most classical method used by the biomedical research community. However, differential centrifugation typically results in low yield and always presents with some degree of contamination<sup>[108]</sup>. Recent developments have improved yield and purity through precipitation, affinity-based sorting by magnetic beads, and particle size-based isolation such as ultrafiltration and size exclusion chromatography<sup>[108,113]</sup>. Exosome isolation kits are now readily commercially available<sup>[108,115]</sup>. The identity and enrichment of exosomes in a biochemical fraction can be further defined by detection of endosome-specific tetraspanins (CD9, CD63, CD81), membrane transport and fusion proteins (flotillin, GTPases), MVB biogenesis-related proteins (Syntenin, Alix, ESCRT, TSG101), and heat-shock proteins (Hsp60, Hsp70, Hsp90)<sup>[110,113,116]</sup>.

## EXOSOMAL CARGO

Given that most cells secrete exosomes, it can be a difficult task to distinguish cancer-specific material to that of healthy cells. When evaluating pathological relevance of exosome studies, purification methodology, exosome identification and presence of cancer-specific markers are essential components that should be taken into consideration. One of the most widely acclaimed PDAC exosome studies was recently presented by Melo *et al.*<sup>[117]</sup>. The authors identified the presence of heparin proteoglycan GPC1, in exosomes isolated from breast and PC patients by ultracentrifugation and sucrose density gradient separation (followed by CD9, CD81 and flotillin 1 detection). Baseline GPC1 positivity was found in only 2.3% of healthy donors while elevated GPC1 expression was found in 75% of breast cancer subjects and among 100% of pancreas cancer patients ( $n = 190$ ). Relative concentrations of exosomes were much higher in the sera of cancer patients compared to healthy subjects. GPC1<sup>+</sup> exosomes were also detected prior to formation of PanIN lesions in 16-d-old mouse models of PDAC (Ptf1a<sup>cre/+</sup>; LSL-Kras<sup>G12D/+</sup>; Tgfr2<sup>L/L</sup>)

with increased proportionality over time. Serum GPC1<sup>+</sup> exosomes in these models were present in circulation early before the onset of histological signs or MRI-detectable lesions. Further, the authors were able to use GPC1 positivity to distinguish healthy donors and those with benign pancreatic disease (BPD) from patients with histologically validated PC precursor lesions (intraductal papillary mucinous neoplasm-IPMN) with a high degree of specificity (75%) and sensitivity (82%). Taken together, these findings suggest that PC cells secrete elevated levels of GPC1 positive exosomes which may be useful for early detection of tumors even prior to histologic manifestation<sup>[117]</sup>.

EGFR is a receptor tyrosine kinase (RTK) activated in a subset of PC cells<sup>[118]</sup>. Adamczyk *et al.*<sup>[119]</sup> found pancreatic cell lines (BxPC3, MiaPaca2, Panc1, Paca44 and A818-4 cells) secrete a 110 kDa soluble form of the EGFR ligand-binding extracellular domain (sEGFR) directly into conditioned media. A 170 kDa intact receptor and a 65 kDa processed form, including the intracellular kinase domain, are secreted as constituents of exosomes. Exosomes were separated from the secretome by ultracentrifugation and confirmed by exosome markers Alix, CD9, CD63 and Syntenin. The full-length EGFR was enriched 20-fold in exosomes along with 1600 other proteins found in the fraction by mass spectrometry<sup>[119]</sup>. The reason for compartmentalized release of these processed EGFR forms is currently not known. Soluble EGFR may provide a method for distant receptor transactivation or may confer EGFR positivity in cancer cells lacking EGFR expression. EGFR<sup>+</sup> exosomes may also enhance drug resistance by serving as a decoy for therapeutic antibodies. This has been observed in HER2<sup>+</sup> exosomes secreted by breast cancer cells that were shown to inhibit cell proliferation effects of Trastuzumab but not Lapatinib<sup>[120]</sup>. The next important step will be detecting EGFR<sup>+</sup> exosome in healthy and PC patients. Whether these isoforms possess any oncogenic mutations also needs to be explored before clinical use of these exosomes as cancer-specific biomarkers is considered.

KRAS is an oncogene that is mutated in 90% of PC cases<sup>[121,122]</sup>. Kahlert *et al.*<sup>[123]</sup> isolated large (> 10 kb) double stranded genomic DNA fragments from EVs originating from PC cell lines and from serum of PDAC patients. Exosomes were purified from cell lines (Panc-1, T3M-4) and serum isolated from patients prior to surgical resection using ultracentrifugation after filtration. Exosomes were further verified by expression of CD9, TSG101, and CD63 by FACS analysis. By using whole genome sequencing, the authors demonstrated that PDAC serum exosomes contain not just mutated KRAS and p53 oncogenes but also genomic DNA fragments spanning all chromosomes<sup>[123]</sup>. This suggests that genomic fragments can be isolated from purified PC exosomes and sequenced for analysis. Exosomes isolated from peripheral blood and pleural effusions can be sequenced to profile the genomes and transcriptomes of patients with pancreaticobiliary cancers<sup>[124]</sup>. Traditional tissue biopsies for these deeply located visceral cancers are difficult to safely acquire in



**Table 3** Challenges and potential solutions for pancreatic cancer diagnosis and treatment

Challenges	Potential solutions
Metastatic probability increases dramatically with larger tumor size	Promote development of early detection methods (circulating tumor cells, extracellular vesicles, molecular cargo in CTCs and EVs, cfDNA, ctDNA)
Tumor mutations develop up to two decades with metastatic mutations occurring late in the process	Identify founder mutations that correlate with unusual survival outcomes
Pancreatic stroma influences treatment sensitivity	Promote research on stromal characterization
Transporter expression in the tumor impacts drug delivery	Identify expression features that correlate with treatment sensitivity to a variety of drugs
CA 19-9 is not pancreatic cancer specific	Promote development of assays for biomarker panels that increase CA 19-9 utility that will be eligible for FDA approval
Prediction of resectability is only 70%-85% accurate	Improve staging based on biopsies by implementing clinical use of digital pathology methods
No FDA-approved digital pathology methods exist for pancreatic cancer	Combine digital pathology with accepted primary diagnostic methods and test special controls for digital imaging that will permit FDA application through a more streamlined <i>de novo</i> pathway

CTC: Circulating tumor cells; EVs: Extracellular vesicles; cfDNA: Circulating free DNA; ctDNA: Circulating tumor DNA; FDA: Food and Drug Administration.

less specialized clinical centers. These studies create the possibility of performing genomic panel tests to identify oncogenic material from exosomes isolated from patients suspected of having elevated risk of PC or those where traditional biopsies are not feasible to obtain.

In addition to carrying genomic DNA, exosomes can also directly inhibit translation or target mRNA for degradation through the delivery of microRNA<sup>[125]</sup>. Exosomal miR-21, miR-212-3p and miR-203 and have been shown to enhance chronic pancreatitis, modulate immune response, and induce drug resistance<sup>[125-127]</sup>. Que *et al.*<sup>[128]</sup> found elevated levels of miR-17-5p in serum exosomes of PC patients which correlate with metastatic stage, compared to healthy controls. Levels of exosomal miR-21 were also higher in PC patients vs healthy and chronic pancreatitis subjects but did not correlate with PC differentiation or stage<sup>[128]</sup>. The concentration of EVs in serum or plasma is almost one thousand times higher than in urine, a less invasive biofluid where exosomes remain stable at room temperatures for up to a week<sup>[115]</sup>. Ymir Genomics has developed a novel precipitation reagent, Ymirite, which isolates extracellular nucleic acids and vesicles from urine samples. Exiqon offers two exosome enrichment kits (miRCURY) for serum/plasma and urine isolation and a qPCR detection system (LNA<sup>TM</sup>) for miRNA detection which enables profiling of biofluids where microRNA levels are extremely low. Further improvements in exosome and oncosome cargo characterization will significantly improve the clinical prospects of EVs.

## EXOSOMAL MARKERS THAT INDICATE PATHOGENIC EFFECTS OF PDAC

Once released into circulation, the destination of tumor-secreted exosomes can be directed through expression of membrane proteins that guide cellular targeting such as integrins, tetraspanins, phosphatidylserine receptors and heparin sulfate proteoglycans<sup>[117,129-131]</sup>. These features

enable exosomes to reach distant sites where they can exert pathogenic effects secondary to the primary cancer. For example, PDAC derived exosomes have been shown to promote liver metastasis through expression of macrophage migration inhibitory factor (MIF) which induces a fibrotic microenvironment when taken up by liver resident Kupffer cells<sup>[112,132]</sup>. PDAC derived exosomes can also secrete TGF-beta which activates hepatic stellate cells to secrete fibronectin which in turn arrests bone-marrow derived macrophages and neutrophils to produce pro-tumorigenic cytokines in the liver<sup>[127,132]</sup>.

Diabetes is a risk factor for PC but the association is complex<sup>[133]</sup>. Studies by Javeed *et al.*<sup>[134]</sup> suggest that adrenomedullin (AM), secreted into circulation by pancreatic exosomes, reaches remote pancreatic beta cells to induce beta-cell dysfunction by inhibiting insulin secretion. The authors showed AM<sup>+</sup> exosomes, isolated by differential centrifugation, are secreted into cultured media by PC patient-derived primary cell lines as well as into portal/peripheral venous blood of PC patients. Additionally, these AM<sup>+</sup> exosomes also contain CA 19-9 making them an attractive PC biomarker<sup>[134]</sup>. Another PDAC-exosomal protein Bip, also impairs insulin secretion through interactions with pro-insulin<sup>[127]</sup>. These studies hold promise for potential diagnostic methods which may predict secondary complications to PC.

Detection of exosomes and their cargo presents some attractive qualities as a liquid biopsy technique. Advantages include the ability to capture tumor-derived material circulating before and during metastatic colonization, enable monitoring of treatment effectiveness and recurrence, enhance prognostic capability based on classifying molecular signatures, and serving to indicate secondary complications. Vesicle enrichment methods have been streamlined and standardized through the availability of commercial kits. The discovery of highly cancer-specific exosomal markers such as GPC1 will provide a foundation that could serve as the basis for

accurate and non-invasive diagnostic tests.

## CONCLUSION

The genetic evolution of PC is complex and may take up to two decades with metastatic mutations occurring relatively late in the process. Diagnosis is made at late stage where large genetic heterogeneity is observed within the tumor. With genotyping costs decreasing, it may be possible to predict drug sensitivity following resection through a combination of genomic profiling of the tumor, stromal density image processing and transporter expression determination. Digital analysis of the stroma from CT images has demonstrated the ability to predict a significant survival benefit for patients who undergo gemcitabine treatment. These methods pave the way for future applications in digital pathology as a means to increase prognostic potential and augment treatment decisions for personalized medicine.

While there is some room for refining existing treatment options to extend survival of late-stage PC patients, overcoming challenges for early detection of the disease will be paramount to significantly decrease the burden on the population (Table 3). Detecting physiologically relevant markers in exosomes and circulating tumor cells offers an advantage of testing with little or no discomfort to the patient. This creates the possibility to obtain serial samples of body fluids over time to allow monitoring of disease progression while eliminating risks associated with invasive biopsies. Combining information gained from these two types of tests could potentially increase diagnostic potential during early stages of PC development.

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This article is dedicated to Sgt. Mark Diehl.

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Retrospective Cohort Study

# Value of macrobiopsies and transanal endoscopic microsurgery in the histological work-up of rectal neoplasms: A retrospective study

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**Author contributions:** Bökkerink GMJ designed the study, collected the data, performed analysis and wrote the manuscript; de Jong D provided the endoscopy-reports and contributed to the discussion; van der Wilt GJ assisted in the statistical analysis and calculations to compare various work-up schemes; van Krieken HHJM provided the histopathological reports and contributed to the discussion; de Wilt JHW supervised the first author; Bleichrodt RPB and Bremers AJA designed the study, performed the definitive surgery and supervised the first author.

**Institutional review board statement:** This study was performed in accordance to the ethical standards of the institutional and national research committee and with the 1964 Helsinki Declaration and its later amendments; this study was exempted from informed consent according to Dutch regulations.

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## Abstract

### AIM

To evaluate a step up approach: Taking macrobiopsies and performing excision biopsies in patients with suspected rectal cancer in which biopsies taken through the flexible endoscope showed benign histology.

### METHODS

Patients with a rectal neoplasm who underwent flexible endoscopy and biopsies were included. In case of benign biopsies rigid rectoscopy and macrobiopsies were employed. If this failed to prove malignancy, transanal



endoscopic microsurgery (TEM) was used in a final effort to establish a certain preoperative diagnosis. The preoperative results were compared with the findings after surgical excision and follow up to calculate the reliability of this algorithm.

## RESULTS

One hundred and thirty-two patients were included. One hundred and ten patients with a carcinoma and 22 with an adenoma. Seventy-five of 110 carcinomas were proven malignant after flexible endoscopy. With the addition of rigid endoscopy and taking of macrobiopsies, this number increased to 89. Performing TEM excision biopsies further enlarged the number of proven malignancies to 100.

## CONCLUSION

The step-up approach includes taking macrobiopsies through the rigid rectoscope and performing excision biopsies using transanal endoscopic microsurgery in addition to flexible endoscopy. This approach, reduced the number of missed preoperative malignant diagnoses from 32% to 9%.

**Key words:** Rectal cancer; Histology; Biopsy; Macrobiopsy; Transanal endoscopic microsurgery; Sampling error

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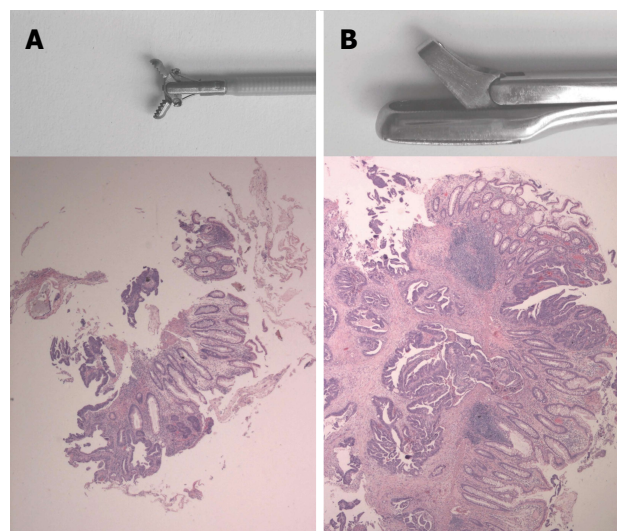
**Core tip:** Increasing the number of biopsies taken through a flexible endoscope, taking macrobiopsies and performing excision biopsies with transanal endoscopic microsurgery can reduce the number of missed preoperative malignant diagnoses in patients with rectal cancer.

Bökkerink GMJ, van der Wilt GJ, de Jong D, van Krieken HHJM, Bleichrodt RP, de Wilt JHW, Bremers AJA. Value of macrobiopsies and transanal endoscopic microsurgery in the histological work-up of rectal neoplasms: A retrospective study. *World J Gastrointest Oncol* 2017; 9(6): 251-256 Available from: URL: <http://www.wjgnet.com/1948-5204/full/v9/i6/251.htm> DOI: <http://dx.doi.org/10.4251/wjgo.v9.i6.251>

## INTRODUCTION

Adequate pre-treatment histological sampling is of paramount importance for the optimal treatment of rectal neoplasms. A wide spectrum of surgical and neoadjuvant treatments is available. In case of benign disease, surgical excision alone, will suffice. For a majority of the malignant tumors however, a combination of neoadjuvant therapy and total mesorectal excision is indicated to optimize local control<sup>[1-5]</sup>. High complete response rates after chemoradiation therapy have led to the development of organpreserving strategies<sup>[6-8]</sup>.

Although the oncological benefits of neoadjuvant treatments are evident, the acute toxicity and long term side effects of chemoradiation therapy are considerable.



**Figure 1** Biopsy forcepses and histological slides. A: Biopsy forceps used with the flexible endoscope: Representative slide of a malignant tumor, HE × 20; B: Biopsy forceps used with the rigid endoscope, representative slide of the same tumor, HE × 20.

Therefore, administration of neoadjuvant chemoradiation therapy requires definite proof of malignancy. As the diagnosis of malignancy based on imaging alone may be erroneous because of the risk of overstaging MRI based imaging, these neoadjuvant treatments require histological evidence of malignancy before treatment can commence.

A preoperative histological diagnosis is usually obtained by taking biopsies through a flexible endoscope. Flexible endoscopy offers a high tumor detection rate<sup>[9]</sup> and the possibility to take biopsies. However, from limited evidence available, sensitivity for malignancy on these biopsies is suboptimal at best<sup>[10-12]</sup>. The most important reason for this is that biopsies taken through flexible endoscopes are small and sometimes too superficial to demonstrate high grade neoplasia<sup>[13]</sup>. In case of superficial biopsies, the diagnosis of malignancy relies solely on tissue structure and atypical appearance of cells (Figure 1). One way to overcome this problem is to take more biopsies. Indeed, several authors demonstrated a correlation between sensitivity and the number of biopsies taken from a suspected lesion. When 3 or 4 biopsies were taken, the sensitivity for invasive growth varied between 50% and 86%<sup>[10-12]</sup>. By taking up to 10 biopsies, the sensitivity increased to 78% to 100% (Table 1).

Another way to increase the sensitivity of pre-treatment histological sampling for the detection of malignancy is to increase the volume and depth of the biopsy. Although considered old-fashioned by many clinicians, rigid rectoscopy is an easy, cost effective, fast and well-tolerated tool for examination of the rectum<sup>[14]</sup>, that enables the endoscopist to take so-called "macrobiopsies". Macrobiopsies are 2-10 times larger in three dimensions and approximately 50 times larger in volume than those obtained with flexible rectoscopy. The

**Table 1 Flexible endoscopy: Sensitivity for invasive growth; correlation with the number of biopsies**

Number of biopsies		≤ 2	3	4	5	6	7	8	≥ 9
Marshall (1993)	Sens <i>n</i> = 70 <sup>1</sup>			68.3 70		78.3 70		78.3 70	78.3 70
Colleypriest (2009)	Sens <i>n</i> = 217	80%	86%	86%	88%	98%	100%	98%	100%
Dabos (2011)	Sens <i>n</i> = 149	Not specified	50%	72%	70%	76%	88%	91%	100%
Current study	Sens <i>n</i> = 113	40% 7	30% 12	76% 21	75% 17	83% 14	50% 16	91% 13	72% 13

<sup>1</sup>Authors studied the value of reviewing 2, 4 and 6 additional biopsies, taken in every patient.

rigidity of the biopsy forceps also enables the endoscopist to push the forceps against the tumor so that deeper layers of the rectal wall can be included in the biopsy, and to “palpate” the lesion and take the biopsies from the firmer parts of the lesion selectively. For these reasons, rigid rectoscopy may perform better with respect to sampling error than flexible endoscopy.

Sometimes, even macrobiopsies may fail to demonstrate invasive growth. In an ultimate effort to obtain sufficient histological confirmation of malignancy without interfering with the optimal treatment strategy, transanal endoscopic microsurgery (TEM) may be used in these cases to perform an excision biopsy. TEM is an invasive way to obtain a histological diagnosis. However, it does have the advantage that it can sometimes be used as a definitive treatment for low risk T1 carcinomas.

Although there are sound theoretical grounds to expect that rigid rectoscopy and TEM can boost the sensitivity of the pre-treatment histological work-up for suspected rectal cancer, this has never been empirically investigated. The aim of this article, therefore, is to assess the accuracy, therapeutic value and tolerability of taking additional macrobiopsies and performing excision biopsies with TEM in patients with suspected rectal cancer: a step-up approach.

## MATERIALS AND METHODS

### Patients

All patients who underwent biopsy through a flexible endoscope, as part of the work-up for surgery of a rectal neoplasm, between January 2005 and January 2011 in the Radboud University Nijmegen Medical Center, Nijmegen, The Netherlands were analyzed. Patient selection was based on the database of surgical procedure in our hospital. All patients who underwent surgical excision of a rectal neoplasm [local excision; transanal endoscopic microsurgery or total or partial mesorectal excision: Abdomino perineal resection or (low) anterior resection] were selected. The medical records of all patients were reviewed for demographic characteristics and for endoscopy, pathology and surgical reports.

### Diagnostic and therapeutic algorithm

This is a retrospective analysis of the diagnostic and

therapeutic step-up algorithm, which was followed during the study period. This algorithm is shown in Figure 2. Macrobiopsies were taken through the rigid sigmoidoscope in case of benign histology after flexible endoscopy and persisting clinical or radiological suspicion for malignancy, macrobiopsies were taken through rigid rectoscopy. TEM was performed in case of a benign or cT1 tumor on endorectal ultrasound (ERUS).

### Equipment

Flexible endoscopes were the CF140S 70 cm sigmoidoscope and CF 140 I colonoscope (Olympus, Tokyo, Japan). For flexible endoscopy, a 2.2 mm radial jaw biopsy forceps was used (Boston Scientific, Natick, United States) (Figure 1). For colonoscopy complete bowel preparation was used. Sedation and analgesia given upon request. During colonoscopy multiple biopsies were taken from any suspicious lesions. A 250 mm × 18 mm disposable rectoscopy tube, Heine, Herrsching, Germany was used for rigid rectoscopy. Biopsies were taken with a Franital biopsy forceps with a 5 mm × 10 mm bite (Figure 1). Bowel preparation before rigid and flexible sigmoidoscopy consisted of a single soap enema. All procedures were performed by, or under direct supervision of, consultant level surgeons or gastroenterologists.

TEM-surgery was performed by one of the authors (AB) as first described by Buess<sup>[15]</sup> using the stereo-optic Wolf rectoscope (Wolf, Knittlingen, Germany).

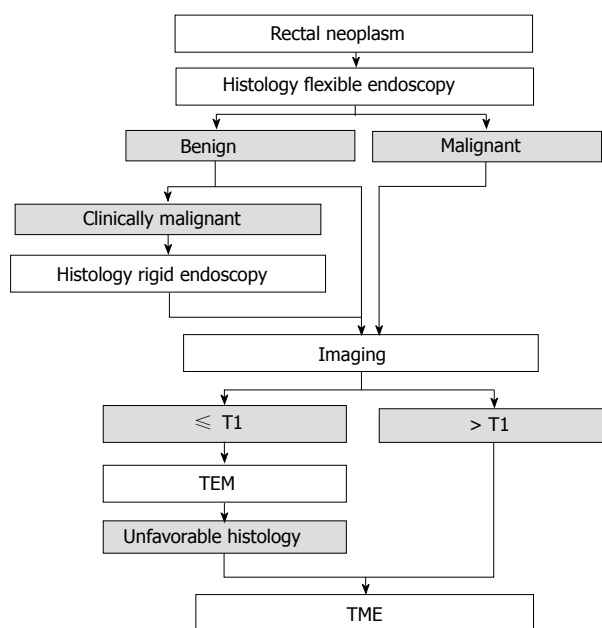
### Statistical analysis

The additional yield of taking macrobiopsies and performing excision biopsies was analyzed by comparing all biopsies with the definitive excision specimen. The differences in sensitivity between the number of samples taken through the flexible endoscope was tested with the chi square test for trends.

## RESULTS

### Patients

One hundred and thirty-two patients (82 males and 50 females) underwent flexible endoscopy with biopsies as part of the work-up for a rectal neoplasm (tumor located below 15 cm from the anal verge). Median age was 63 years (range: 27-92).



**Figure 2 Diagnostic and therapeutic algorithm.** TEM: Transanal endoscopic microsurgery.

### Flexible endoscopy

The histological work-up of all 132 patients is shown in Figure 3. At final pathology 110 patients had an adenocarcinoma, of which 75 (68%) were detected with flexible endoscopy only. The other 22 patients had a villous adenoma. One of the tumors, classified as malignant based on biopsies taken through the flexible endoscope (snare polypectomy), showed benign histology after (transanal) resection.

The number of biopsies was documented for 113 patients and varies from 1 to 14, with a median of 4 biopsies (Table 1). There was a significant correlation between the number of biopsies and a correct histological diagnosis ( $P = 0.020$ ; 2-sided  $\chi^2$  test for trends). Taking 4 or more biopsies resulted in a significant higher sensitivity than taking 3 or less ( $P = 0.004$ ; 2-sided  $\chi^2$  test for trends). Prior probability of malignancy was 83.3% in this group. Sensitivity and specificity were 68% and 95% respectively. A malignant result is useful with a posterior probability of malignancy of 99% (95%CI: 92%-100%). Benign histology after flexible endoscopy is clearly inconclusive, leaving a posterior probability of malignancy of 62% (95%CI: 55%-69%).

### Rigid rectoscopy and macrobiopsies

In 29 of the 56 patients who were diagnosed with a benign tumor after flexible endoscopy, additional rigid endoscopy was performed. With this addition, 14 previously undetected carcinomas were diagnosed. In this selected group of 29 patients who underwent rigid endoscopy, prior probability of malignancy was 75.9%. Sensitivity and specificity were 64% and 100% respectively, which makes a malignant histology after rigid endoscopy useful with a posterior probability of malignancy of 100% (95%CI: 68%-100%). Benign histology after rigid endoscopy leaves a posterior probability of malignancy of 53%

Inclusion	Malignant	Benign	132
Histology flexible endoscopy		56	76
Histology rigid endoscopy		15	14 <sup>1</sup>
TEM	7	4	7
TME	4	2	4

**Figure 3 Yield of macrobiopsies on excision biopsies.** <sup>1</sup>Fourteen not earlier detected carcinomas; <sup>2</sup>Eleven not earlier detected carcinomas.

(95%CI: 41%-68%). The remaining 27 patients did not undergo additional macrobiopsies taken through a rigid endoscope because there was no clinical suspicion of malignancy and endorectal ultrasound did not show invasion deeper than the submucosa (clinical benign or clinical T1). Further management was not dependent on histology analysis, since these lesions were regarded as indication for TEM for complete removal.

### TEM

A total of 44 patients underwent TEM (Figure 3), 32 patients after benign biopsies (combined flexible and rigid), 12 after malignant biopsies (clinical and radiological T1). With this addition, another 11 invasive carcinomas were detected. The number of detected carcinomas increased from 89 out of 110 (81%) to 100 out of 110 (91%).

Histology after TEM showed 18 adenomas, 4 *in situ* carcinomas, and 22 carcinomas. After TEM, 10 patients underwent a completion TME because of unfavorable histological findings. The excision specimen of one of these 10 patients was perforated at the former local excision site. One patient with an ypT3 tumor was unfit to undergo a total mesorectal excision and was treated with short course radiotherapy and TEM after a 6 wk interval. No major complications were observed nor preoperative perforations or conversions to laparotomy after TEM in this group. One patient with postoperative rectal blood loss needed transfusion.

### Neoadjuvant treatment

A total of 79 patients received neoadjuvant treatment in 4 different schemes according to tumor stage and general condition. Thirty-eight patients received 5 Gy × 5 Gy in the week prior to surgery according to protocol for T2 and T3 tumors. Thirty Patients with a radiologically involved circumferential resection margin received neoadjuvant chemoradiation therapy (25 Gy × 2 Gy with concomitant capecitabine) and delayed surgery after 8 wk. Eleven patients whose general condition did not allow chemoradiation therapy (CRT) and who required tumor regression received 5 Gy × 5 Gy ( $n = 9$ ) or long course radiotherapy (24 Gy × 2 Gy) ( $n = 2$ ) and delayed surgery as decided by a multidisciplinary team.

## Surgery

Forty-four patients underwent TEM, 53 underwent a LAR and a further 34 underwent APR, 1 patient with MSH6 mutation underwent a subtotal colectomy with LAR. After TEM 10 patients underwent a completion TME. Definitive histology after resection showed 18 adenomas, 4 *in situ* carcinomas, 101 carcinomas and 9 complete responses after neoadjuvant treatment.

## DISCUSSION

In the present study we demonstrated that macrobiopsies obtained through a rigid endoscope and excision biopsies by TEM are valuable additional tools to obtain a correct preoperative histological diagnosis in a significant number of patients with suspected rectal cancer.

Over time, flexible endoscopy has replaced rigid rectoscopy because of its superior (videoscopic) visualization of the entire colon, better mobility and deeper intubation<sup>[16-20]</sup> and subsequently a good tumor detection rate<sup>[9]</sup>. However, when it comes to the diagnostic sensitivity to detect malignancy in rectal tumors, our results are in accordance with the literature and confirm the disappointing overall performance of flexible endoscopy. The proportion of false negative biopsies after flexible endoscopy alone was 32%. This can be explained by the number of biopsies taken in our study. With a median number of biopsies of 4, a sensitivity of 70% can be expected.

Increasing the number of biopsies with flexible endoscopy can increase the number of detected malignancies in the group of suspicious rectal neoplasms (Table 1). However, increasing the number of biopsies through flexible endoscopy, as suggested by some authors<sup>[10-12]</sup>, was not our main strategy to increase diagnostic sensitivity, because these biopsies are often too superficial to show high grade neoplasia<sup>[13]</sup>. Our algorithm included rigid endoscopy and TEM as additional steps.

In terms of accuracy, the selected group of patients with false negative biopsies after flexible endoscopy, 14 additional patients with a malignancy were identified with rigid endoscopy, and with TEM, another 11 patients. In total, 100 of 110 malignancies could be diagnosed preoperatively. This means that the proportion of carcinomas of which the malignant nature would have been proven in time was 32% with flexible endoscopy alone and was reduced to 9% in the evaluated algorithm. This is a significant reduction with high therapeutic value.

Regarding procedure-related morbidity, both rigid endoscopy and TEM were well-tolerated. In our experience, TEM did not cause an increase in positive circumferential resection margins (CRM) in TME as determined by standardized pathological evaluation according to Quircke<sup>[21]</sup>.

## Conclusion

With the current treatment options for patients with rectal cancer, optimal preoperative histological diagnosis is essential. Besides the combinations with radical surgery, multimodality organ sparing treatments are becoming more and more accepted. Short-term results show high

percentages of pathologic complete response<sup>[6,22]</sup> and acceptable oncological outcome<sup>[6,7]</sup>, adequate histological sampling seems of paramount importance for these new treatment strategies, not only before but also after (chemo)radiation therapy.

In the present study we demonstrated that macrobiopsies obtained through a rigid endoscope and excision biopsies by TEM are valuable additional tools in obtaining a correct preoperative histological diagnosis in a significant number of patients with suspected rectal cancer. Prospective trials are needed to compare the yield of these strategies to increasing the amount of biopsies through flexible endoscopy. Evidence-based recommendations for guidelines regarding the histological work-up of rectal neoplasms can be based on those trials.

## COMMENTS

### Background

Histological sampling is one of the key components of the work-up for rectal neoplasms. For neoadjuvant and radical surgical treatments histological proof of invasive growth is mandatory. It can be difficult to obtain this proof with flexible endoscopy only. There are only a few publications available in which the sensitivity for malignancy of biopsies taken through a flexible endoscope is discussed. The aim of this study was to evaluate a step up approach: Taking macrobiopsies and performing excision biopsies in patients with suspected rectal cancer in which biopsies taken through the flexible endoscope showed benign histology.

### Research frontiers

An important subject in current rectal cancer research is the evaluation of organ sparing treatment techniques. Adequate pre-treatment histological sampling is of paramount importance for this treatment technique.

### Innovations and breakthroughs

Other studies evaluating the value of macrobiopsies and excision biopsies are not available in literature. More studies with larger populations need to be done to confirm the results from this study.

### Applications

This study can motivate the reader to take macrobiopsies and perform excision biopsies in daily practice.

### Terminology

Excision biopsy: Transanal local excision of (a part of) a rectal malignancy with the intention to assess its histology; Macrobiopsy: Large biopsy taken through a rigid recto- or sigmoidoscope.

### Peer-review

Bökkerink *et al* describe the use of macrobiopsies in the diagnosis of rectal cancer.

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## Retrospective Study

# Effects of age on survival and morbidity in gastric cancer patients undergoing gastrectomy

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**Institutional review board statement:** This study was reviewed and approved by the Ethics Committee of the Nara Hospital, Kindai University.

**Informed consent statement:** Comprehensive agreement of clinical study was obtained in all patients at admission of our hospital. The analysis used anonymous and the detail of this study are published on the homepage of Nara Hospital, Kindai University.

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## Abstract

### AIM

To evaluate clinicopathological features and surgical outcomes of gastric cancer in elderly and non-elderly patients after inverse probability of treatment weighting (IPTW) method using propensity score.

### METHODS

We enrolled a total of 448 patients with histologically confirmed primary gastric carcinoma who received gastrectomies. Of these, 115 patients were aged > 80 years old (Group A), and 333 patients were aged < 79 years old (Group B). We compared the surgical outcomes and survival of the two groups after IPTW.

### RESULTS

Postoperative complications, especially respiratory complications and hospital deaths, were significantly more common in Group A than in Group B ( $P < 0.05$ ). Overall survival (OS) was significantly lower in Group A patients than in Group B patients. Among the subset of patients who had pathological Stage I disease, OS was significantly lower in Group A ( $P < 0.05$ ) than Group B, whereas cause-specific survival was almost equal in the two groups. In multivariate analysis, pathological stage, histology, and extent of lymph node dissection were

independent prognostic values for OS.

## CONCLUSION

When the gastrectomy was performed in gastric cancer patients, we should recognized high mortality and comorbidities in that of elderly. More extensive lymph node dissection might improve prognoses of elderly gastric cancer patients.

**Key words:** Gastric cancer; Mortality; Morbidity; Elderly; Lymphadenectomy; Propensity score matching; Prognosis; Survival

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**Core tip:** Inverse probability of treatment weighting (IPTW) attempts to reduce the bias due to confounding variables in estimates of treatment effects. In the present study, we compared the surgical outcomes and survival of elderly gastric cancer patients with that of general population after IPTW. The overall survival of pStage I gastric cancer patients in elderly was lower survival due to death of other diseases. We found that extent of lymph nodes dissection were independent prognostic factors. When the gastrectomy was performed in gastric cancer patients, we should recognized high mortality and comorbidities in that of elderly. This study was reviewed and approved by Nara Hospital, Kindai University review board on human research.

Fujiwara Y, Fukuda S, Tsujie M, Ishikawa H, Kitani K, Inoue K, Yukawa M, Inoue M. Effects of age on survival and morbidity in gastric cancer patients undergoing gastrectomy. *World J Gastrointest Oncol* 2017; 9(6): 257-262 Available from: URL: <http://www.wjgnet.com/1948-5204/full/v9/i6/257.htm> DOI: <http://dx.doi.org/10.4251/wjgo.v9.i6.257>

## INTRODUCTION

Gastric cancer is the fifth most common malignancy after cancers of the lung, breast, colorectal area and prostate; patients in Eastern Asia account for about half of the world's incidence<sup>[1]</sup>. In the past decade, incidence of gastric cancer in elderly patients has increased in Japan because of longer life spans of the general population<sup>[2]</sup>; decisions regarding gastric cancer surgeries in elderly patients have therefore also increased. Many surgeons are reluctant to have elderly patients undergo gastrectomies because of the considerably higher risk of complications from gastrectomies. There were some retrospective studies compared the outcomes of elderly gastric cancer patients to that of general populations, but the effects of age on morbidity, mortality from gastrectomy and/or prognosis are controversial, as no randomized studies have been conducted to our knowledge<sup>[3-18]</sup>. Also, no standard definition of "elderly" exists; thresholds vary from 65 to 80 years. Therefore,

no standard guidelines for the treatment of elderly gastric cancer patients are available.

Recently, the concept of propensity score matching (PSM) and inverse probability of treatment weighting (IPTW) has garnered some attention. PSM and IPTW attempts to reduce bias due to confounding variables in estimates of treatment effects<sup>[19]</sup>. In the present study, we first evaluated the clinicopathological features and surgical outcomes of gastric cancer treated in our department among patients aged 80 years and older, and compared them with those of patients aged 79 years and younger, after IPTW. We then analyzed these data to find optimal cut-off ages for elderly patients with gastric cancer.

## MATERIALS AND METHODS

A total of 448 patients with histologically confirmed primary gastric carcinoma had gastrectomies in our department between 2005 and 2013. Of these, 115 patients were aged  $\geq 80$  years old (Group A), and 333 patients were aged  $\leq 79$  years old (Group B). All patients were American Society of Anesthesiologists risk less than three and there was no selection bias in each groups. Clinicopathological data for these patients were obtained from hospital records. Characteristics of two groups are shown and compared in Table 1. Postoperative complications were evaluated according to CTCAE Version 3.0; complications of grade  $\geq 2$  were regarded as significant<sup>[20]</sup>. Tumor location, clinical or pathological stage, degree of lymph node dissection (D0, D1 or D2), and curability (R0, R1 or R2) were assessed according to the Japanese Classification of Gastric Carcinoma, 13<sup>th</sup>, and then 14<sup>th</sup> editions<sup>[21,22]</sup>. Surgical mortality, morbidity, and hospital mortality were compared between two groups. Mean follow-up time for all patients was 34.57 mo (range: 0.16-113.13 mo). Recurrences were confirmed by computed tomography, tumor markers, and endoscopic examinations. Overall survival (OS) was defined as the time from the date of surgery to patient death (including surgery-associated death or hospital death), or the date of last available information concerning vital status. Cause-specific survival (CSS) is cancer survival in the absence of other cause of death or death from other cancers. CSS and OS were evaluated after IPTW method. This study was approved by our institute's committee on human research (Approval No.399): Comprehensive informed consent was obtained from all patients when they admitted our hospital prior to surgery.

### Statistical analysis

Clinicopathological variables between two groups were compared using the Mann-Whitney test or  $\chi^2$  test. Survival analysis was carried out using Kaplan-Meier methods, and log-rank test was used to assess survival differences.  $P < 0.05$  was considered significant. The propensity score (PS) was calculated using a multivariable logistic regression model with the two age groups as the dependent variables, and sex, cancer site, cT (14<sup>th</sup>

**Table 1** Characteristics of patients in this study

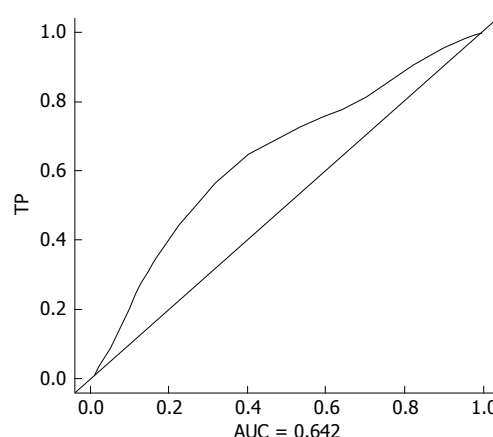
	Group A	Group B	P value
Patients number	115	333	
Sex (Male: female)	73/42	135/198	
Mean age (yr)	83.44	65.87	< 0.05
Occupied lesion			0.693
U	24	81	
M	39	114	
L	52	138	
Clinical stage (13 <sup>th</sup> edition)			0.446
I A	40	137	
I B	30	65	
II	20	48	
III A	9	39	
III B	8	26	
IV	8	18	
Lymph nodes metastasis			0.639
Negative	76	212	
Positive	39	121	
Histological type			0.1224
Intestinal	70	175	
Diffuse	45	158	
Operative procedures			0.074
Distal gastrectomy	68	218	
Total gastrectomy	34	95	
Proximal gastrectomy	5	4	
PPG	3	11	
Partial gastrectomy	5	4	
PD		1	
Lymph nodes dissection			< 0.05
D0	18	8	
D1	60	61	
D2	36	264	
Curability			< 0.05
Curative	97	310	
Non-curative	18	23	

PPG: Pylorus preserving gastrectomy; PD: Pancreaticoduodenectomy.

edition), cN, clinical stage, operative procedures, and histological type (Lauren classification) as independent variables. Inverse probability of treatment weight (IPTW) was then calculated using PS. To evaluate the sensitivity and specificity of age in predicting 3-year OS, a time-dependent receiver operating characteristic (ROC) curve was calculated, and Youden's index was estimated to determine the optimal cutoff age. Univariate and multivariate analyses used the Cox proportional hazard model for OS after IPTW method. A stepwise method was used to estimate predictive variables for OS in multivariate analysis. Statistical analysis was performed using STATA version 14 (Stata Corp LP, College Station, TX, United States), R version 3.1.0 (R Project for Statistical Computing, Vienna, Austria), and SPSS Statistics version 22 (IBM, Tokyo, Japan).

## RESULTS

Patients' characteristics are shown in Table 1. Degree of lymph node dissection was significantly more extensive in Group B ( $P < 0.05$ ), and non-curative dissection was more frequency in Group A ( $P < 0.05$ ). Optimal cutoff age for gastrectomy in terms of OS was 79.2 years



**Figure 1** Receiver operating characteristic curve for three years survival (AUC = 0.642, TP = 0.536, FP = 0.248).

old (AUC = 0.642, TP = 0.536, FP = 0.248, Figure 1). Therefore, we set the cut-off age at 80 years old.

Postoperative complications are shown in Table 2. Respiratory complications and hospital death (including surgery-associated death) were more common in Group A ( $P < 0.05$ ). After IPTW method, we found OS was significantly lower in Group A patients ( $P < 0.05$ ; Figure 2A). The OS rates for Group A were 3-year: 46.6%, 5-year: 36.8%; those for Group B were 3-year: 74.8%, 5-year: 68.8%. Also, estimated CSS rates were significantly lower in Group A patients at 3-year, 5-year: 59.7% for Group A; and 3-year: 74.9%, 5-year: 69.1% in Group B ( $P < 0.05$ , Figure 2B). Among patients with pStage I disease, OS was significantly lower in Group A ( $P < 0.05$ , Figure 3A), whereas CSS was almost equal in both groups (Figure 3B); their estimated 5-year CSS and OS rates were CSS: 92.07%, OS: 62.18% in Group A and CSS, OS: 94.7% in Group B. OS was lower in Group A because of death by other cancers and other diseases, included pneumonia.

Among patients with pStage II-III disease, CSS and OS rates were almost equal in the two groups. The 5-year estimated CSS/OS rates (same rates) for patients with pStage II disease were 67.5% in Group A and 67.96% in Group B. Estimated 5-year CSS and OS rates for patients with pStage III disease were CSS: 42.4%, OS: 22.16% in Group A and CSS, OS: 23.23% in Group B. However, among patients with pStage IV disease, estimated OS/CSS (same rates) were significantly lower in Group A than in Group B; estimated 5-year CSS/OS were 27, 1% in Group B and 0% in Group A, respectively (Figure 4).

Univariate analysis of prognostic factors for OS in Group A is shown in Table 3. We found pStage, radicality, lymph node metastasis and extent of LN dissection significantly affected prognoses ( $P < 0.05$ ). In multivariate analysis, pStage, histology, and extent of lymph node dissection were independent prognostic values for OS (Table 4).

## DISCUSSION

In the present study, we evaluated clinicopathological

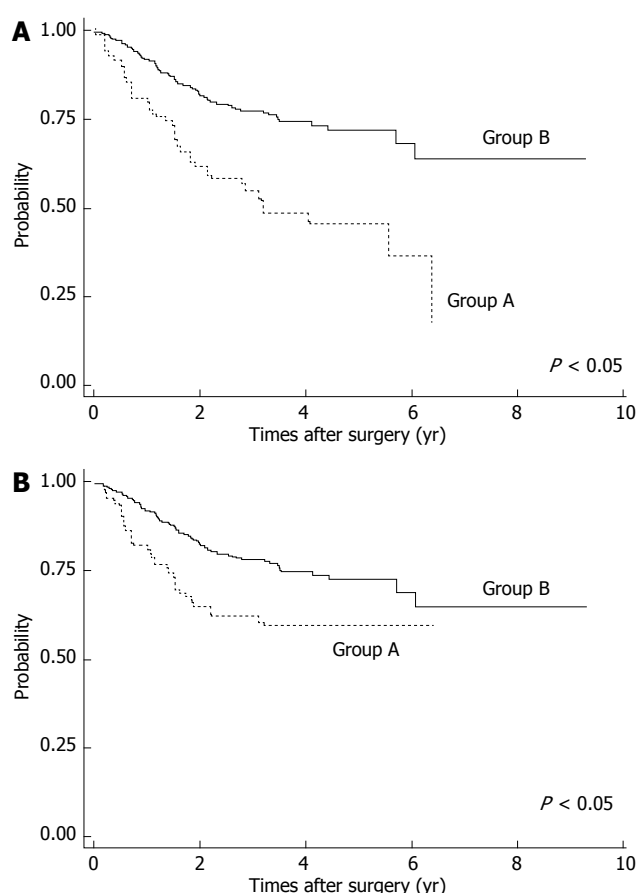
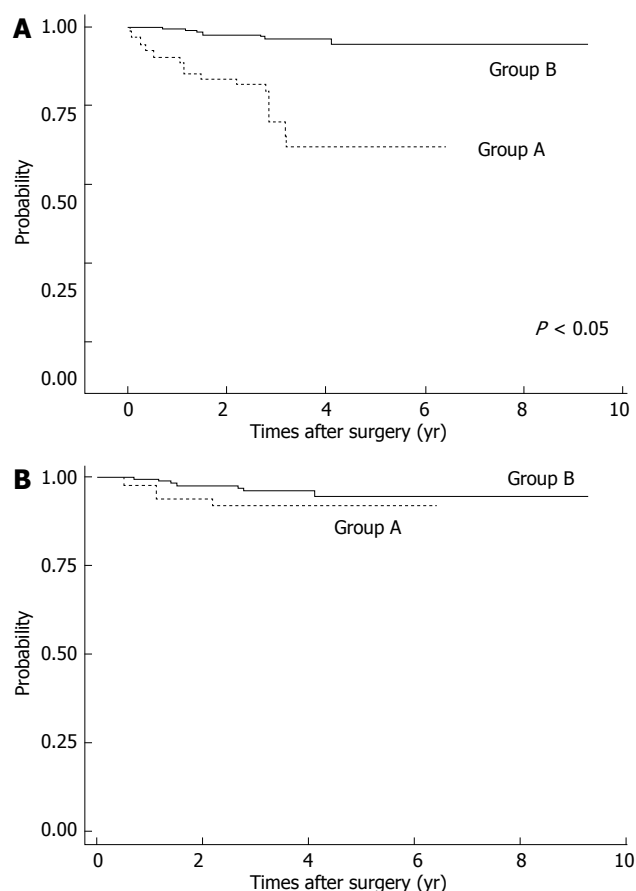


**Table 2** Postoperative complications compared between two aged group

	Group A (n = 115)	Group B (n = 333)	P value
Anastomotic leakage	5 (4.3)	8 (2.4)	NS
Respiratory complications	7 (6.0)	7 (2.1)	< 0.05
Other complications			
Pancreatitis	3 (2.6)	7 (2.1)	NS
Intraabdominal abscess	0 (0)	5 (1.5)	NS
Ileus	1 (0.87)	1 (0.3)	NS
Duodenal stump perforation	1 (0.87)	1 (0.3)	NS
Hepatic failure	1 (0.87)	1 (0.3)	NS
Cholecystitis	0 (0)	1 (0.3)	NS
Hospital death	5 (4.3)	3 (0.9)	< 0.05

**Table 3** Univariate analysis of overall survival in Group A patients after IPTW method

Variants	HR	95%CI	P value
Sex (male:female)	0.941	0.515-1.720	0.845
Tumor location (U:M:L)	0.967	0.779-1.202	0.768
Operative procedures (total:others)	1.005	0.813-1.242	0.961
Extent of LN dissection (D0:D1:D2)	0.661	0.4233-1.032	0.009
pStage (13 <sup>th</sup> edition) ( I : II : III : IV)	2.12	1.616-2.782	0.001
Radicality (curative:non-curative)	1.529	0.083-0.280	0.001
pLN metastasis (negative:positive)	2.332	1.274-4.272	0.006
Postoperative complications (negative: positive)	1.432	0.642-3.195	0.379
Histology (Lowren) (intestinal:diffuse)	2.637	1.470-4.729	0.01

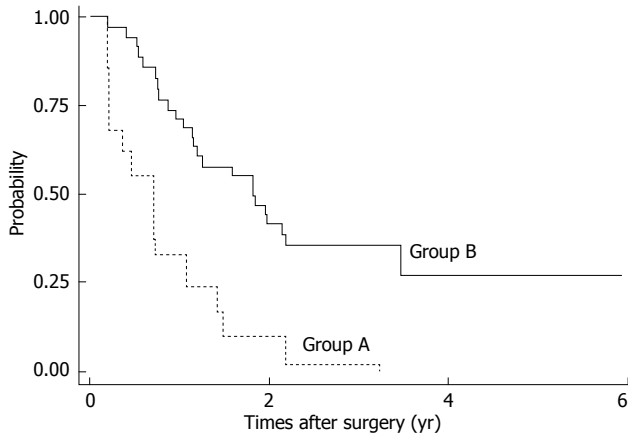
**Figure 2** Overall survival (A) and cause-specific survival (B) in two aged group after IPTW method. OS and CSS were significantly lower in Group A than Group B ( $P < 0.05$ ). OS: Overall survival; CSS: Cause-specific survival; IPTW: Inverse probability of treatment weighting.**Figure 3** Overall survival (A) and cause-specific survival (B) by age group among patients with pStage I gastric cancer who underwent gastrectomy. OS was significantly lower significantly lower in Group A than Group B after IPTW method ( $P < 0.05$ ). OS: Overall survival; CSS: Cause-specific survival; IPTW: Inverse probability of treatment weighting.

features and survival of patients aged 80 years and older, compared with patients aged 79 years and younger after IPTW.

The optimal cut-off age for gastrectomies in elderly patients is controversial. The WHO classification defines “elderly” as older than 65 years old, “young-old” as 65-75 years old and “old-old” as older than 75 years<sup>[23]</sup>. In previously published studies of gastric cancer surgery in older patients, age thresholds ranged from 65 to 80 years old, so “elderly person” was not defined with

regard to stomach cancer<sup>[4,5,7,8,11-17,24]</sup>. In the present study, we therefore used a survival ROC curve in patients with gastric cancer in terms of OS to determine the borderline age for gastrectomies, and concluded the optimal cut-off age is 79.2 years old, regardless of low AUC. Therefore, we divided the gastric cancer patients into two groups: 80 years and older (Group A, elderly group) and 79 years and younger (Group B, general population) in this study.

In general, morbidity and mortality of gastric cancer



**Figure 4** Cause-specific survival and overall survival by age group among patients with pStage IV gastric cancer who underwent gastrectomy; after IPTW method. CCS and OS were significantly lower in Group A than Group B ( $P < 0.05$ ). OS: Overall survival; CCS: Cause-specific survival; IPTW: Inverse probability of treatment weighting.

patients after gastrectomy is controversial; mortality rates for elderly patients with gastric cancer who undergo gastrectomies range from 2% to 8.3% in the published data, which is compatible with our results<sup>[3-9,11,15]</sup>. Most reports did not find significant differences between the age groups, despite varying definitions of “elderly”. In the present study, surgical mortality was significantly higher in Group A (4.8%) than in Group B (0.9%), possibly because the mortality rate of Group B was less than 1% in our institution. Among postoperative complications, respiratory complications were more frequent in Group A in the present study. Although postoperative respiratory complications in elderly patients have been reported, only two reports noted a high complication rate specifically in elderly patients with gastric cancer<sup>[4,6,8,11,15]</sup>. Postoperative respiratory complications of elderly gastric cancer patients might be associated with surgical mortality; they therefore warrant more careful postoperative attention.

In analyzing survival of patients with gastric cancer, we matched the two age groups using propensity scores; IPTW is considered to be a reliable statistical method for evaluating propensity scores<sup>[25]</sup>. Among patients with pStage I disease, OS was significantly lower in Group A, but CSS was not significantly different. Lower OS for elderly pStage I patients was due to surgical mortality, other causes of death, and death from other cancers. Therefore, careful observation after gastrectomy might improve survival of elderly patients with gastric cancer.

In multivariate analysis, we found that extent of lymph node dissection was independent prognostic factors in elderly patients with gastric cancer. Also, postoperative complications, especially respiratory complication and hospital death were more common in elderly group. However, relationships between extent of lymph node dissection and postoperative morbidity, mortality and prognosis in elderly gastric cancer patients are controversial in the literature<sup>[3,4,7,11]</sup>.

Most of these reports showed that more extended lymphadenectomy in elderly patients did not affect

**Table 4** Multivariate analysis of overall survival in Group A

	Stepwise method ( $P < 0.1$ )		
	HR	95%CI	P value
pStage	2.014	1.516-2.675	0.01
Histology (Lauren)	2.039	1.117-3.720	0.02
Extent of LN dissection	0.528	0.343-0.813	0.004

postoperative complication rates or prognosis.

Only Eguchi *et al.*<sup>[4]</sup> reported the extent of lymph node dissection in elderly gastric cancer patients to have influenced postoperative complications. Our findings indicate that more extended lymphadenectomy might improve survival in these patients if postoperative complications could be avoided.

In conclusion, our retrospective study indicated that optimal cut-off ages for elderly patients with gastric cancer was eighty years old, and suggests that even if curative surgery is performed for pStage I disease in elderly gastric cancer patients, careful follow up is needed to stay abreast of other diseases, other cancers as outpatients. Additionally, more extensive lymph node dissection might improve prognosis of elderly patients with gastric cancer if postoperative complications could be minimized. However, postoperative complications lead to hospital death should be noted.

## COMMENTS

### Background

In the past decade, incidence of gastric cancer in elderly patients has increased in Japan. There was no randomized study compare the prognosis, morbidity and mortality of elderly gastric cancer patients and that of younger populations. Propensity score matching (PSM) and inversed probability of treatment weighting (IPTW) attempts to reduce bias due to confounding variables in estimates of treatment effects. They evaluated the clinicopathological features and surgical outcomes of gastric cancer treated in our department among patients aged 80 years and older, and compared them with those of patients aged 79 years and younger, after IPTW.

### Research frontiers

There were some retrospective studies compared the outcomes of elderly gastric cancer patients to that of general populations, but the effects of age on morbidity, mortality from gastrectomy and/or prognosis are controversial, as no randomized studies have been conducted to our knowledge.

### Innovations and breakthrough

PSM and IPTW attempt to reduce bias due to confounding variables in estimates of treatment effects. Quasi randomization is possible when they compared elderly group and younger group, statistically.

### Applications

The clinical significance of elderly gastric cancer patients received gastrectomy were evaluated and revealed the higher postoperative complications and mortality in elderly patients, and more extensive lymph node dissection might improve prognosis of elderly patients with gastric cancer.

### Peer-review

This is interesting to report the effects of age on survival and morbidity in gastric cancer patients undergoing gastrectomy. The author of this manuscript evaluated the gastric cancer patients received gastrectomy in elderly compared to that in younger population. Notably, this manuscript was compared the

results of these patients used propensity score.

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## Gastric plexiform fibromyxoma resected by endoscopic submucosal dissection after observation of chronological changes: A case report

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**Author contributions:** Kawara F, Tanaka S and Morita Y performed endoscopic resection and wrote the manuscript; Kawara F, Ohara Y and Okabe Y performed follow-up endoscopy and endoscopic ultrasound; Yamasaki T, Yokozaki H and Hirose T performed histopathological examinations; Hoshi N, Toyonaga T, Umegaki E and Azuma T contributed to the literature review and manuscript editing.

**Institutional review board statement:** This case report was exempt from approval by the Ethics Committee of Kobe University Hospital.

**Informed consent statement:** Informed consent was obtained from the patient.

**Conflict-of-interest statement:** The authors declare that they have no conflicts of interest.

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### Abstract

A 66-year-old man was diagnosed with a gastric submucosal tumor. Endoscopic ultrasound (EUS) revealed an iso/hypoechoic mass in the third layer. No malignant cells were detected in a histological examination. Yearly follow-up endoscopy and EUS showed the slow growth of the tumor. Endoscopic submucosal dissection (ESD) was performed and a glistening tumor was resected. The lesion showed a multinodular plexiform growth pattern consisting of spindle cells with an abundant fibromyxoid stroma that was rich in small vessels. The tumor was diagnosed as plexiform fibromyxoma (PF) by immunohistochemistry. Although difficulties are associated with reaching a diagnosis preoperatively, chronological changes on EUS may contribute to the diagnosis of PF. ESD may also be useful in the diagnosis and treatment of PF.

**Key words:** Plexiform fibromyxoma; Plexiform angiomyxoid myofibroblastic tumor; Endoscopic ultrasound; Endoscopic submucosal dissection; Gastrointestinal stromal tumor



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**Core tip:** Plexiform fibromyxoma (PF) is a very rare gastric submucosal tumor. Therefore, difficulties are associated with diagnosing PF preoperatively, particularly in a differential diagnosis of gastrointestinal stromal tumors with cystic changes. We suggest that the chronological changes observed by endoscopic ultrasound contribute to the preoperative diagnosis of PF. Furthermore, endoscopic submucosal dissection needs to be considered for the diagnostic treatment of PF without muscle invasion.

Kawara F, Tanaka S, Yamasaki T, Morita Y, Ohara Y, Okabe Y, Hoshi N, Toyonaga T, Umegaki E, Yokozaki H, Hirose T, Azuma T. Gastric plexiform fibromyxoma resected by endoscopic submucosal dissection after observation of chronological changes: A case report. *World J Gastrointest Oncol* 2017; 9(6): 263-267 Available from: URL: <http://www.wjgnet.com/1948-5204/full/v9/i6/263.htm> DOI: <http://dx.doi.org/10.4251/wjgo.v9.i6.263>

## INTRODUCTION

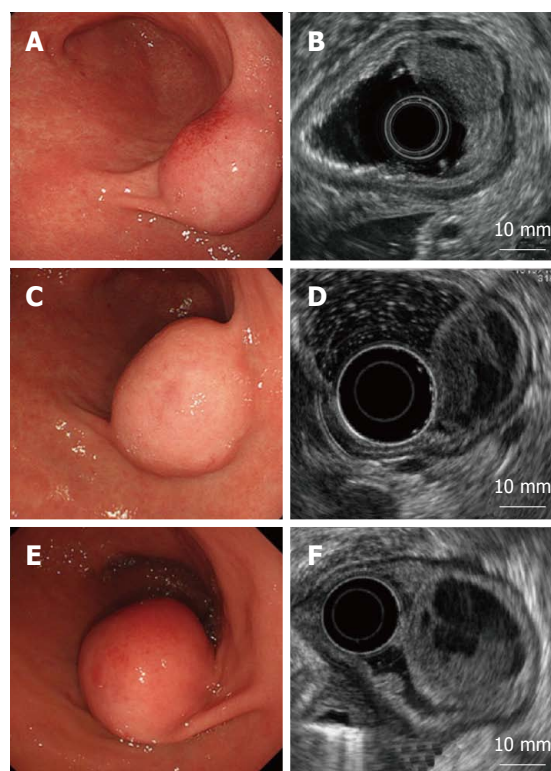
Plexiform fibromyxoma (PF), also known as a plexiform angiomyxoid myofibroblastic tumor (PAMT), is a very rare gastric submucosal tumor (SMT) with a unique plexiform growth pattern of bland spindle cells<sup>[1-3]</sup>. Few studies have described the endoscopic ultrasound (EUS) characteristics of PF, and its chronological changes also remain unclear. We herein report a case of PF resected by endoscopic submucosal dissection (ESD) after a 4-year follow-up period.

## CASE REPORT

A 66-year-old man was referred to our institute for the management of a gastric tumor. An endoscopic examination revealed a SMT, approximately 20 mm in diameter, located in the antrum (Figure 1A). EUS showed an iso/hypoechoic mass in the third layer (Figure 1B). Computed tomography (CT) displayed a poorly enhanced lesion (Figure 2). Endoscopic biopsy and endoscopic ultrasound-guided fine-needle aspiration (EUS-FNA) were performed. Histological findings showed no malignant cells, and no further diagnosis was made.

Yearly follow-up endoscopy revealed the slow growth of the tumor, which became pedunculated and showed transpyloric prolapse (Figures 1C-F). EUS revealed gradual increases in the solid and multicystic components without muscle invasion. Based on these findings, our preoperative diagnosis was a hamartomatous inverted polyp<sup>[4-6]</sup>. In order to avoid outlet obstruction and reach a histological diagnosis, ESD was performed (Figure 3).

On dissection, a glistening, 40 mm × 30 mm tumor covered with normal gastric mucosa was identified. Microscopically, the lesion showed a multinodular plexiform growth pattern, and consisted of bland spindle cells

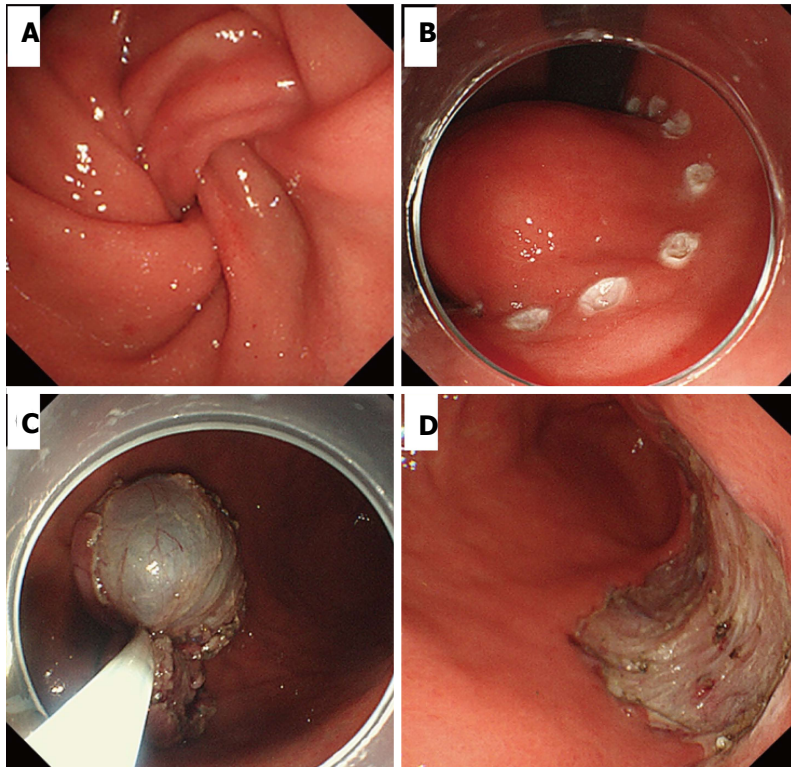


**Figure 1** Endoscopic and endoscopic ultrasound findings. A: A submucosal tumor covered with a normal mucosa; B: An iso/hypoechoic mass with cystic components in the third layer; C, D: One year later; E, F: Four years later. The tumor increased in size and became pedunculated. Solid and multicystic parts both grew larger without muscle invasion.

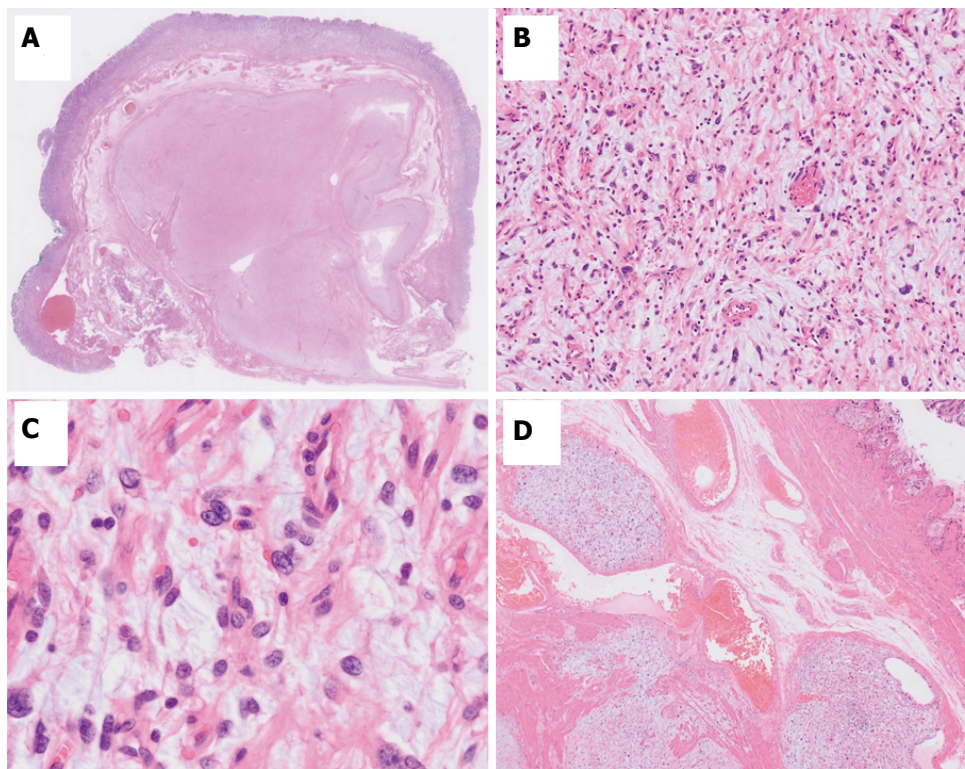


**Figure 2** Computed tomography of the patient. A computed tomography scan revealed a poorly enhanced tumor in the antrum.

separated by abundant intercellular myxoid or fibromyxoid matrix. The stroma was rich in small vessels (Figure 4). Immunohistochemical tests revealed that tumor cells were focally positive for smooth muscle actin (SMA), muscle-specific actin (HHF35), and calponin, but were negative for c-kit, CD34, DOG-1, desmin, the S-100 protein, CD10, and h-caldesmon. The Ki-67 labeling index was approximately 2% (Figure 5). The pathological assessment led to a diagnosis of PF. Resected margins were histologically tumor-free. Although vascular invasion was positive, the patient did not undergo surgery due to the reportedly good prognosis of PF<sup>[1,7]</sup>, and remained under careful observation



**Figure 3 Endoscopic submucosal dissection.** A: Tumor prolapse into the duodenum from the pylorus; B: Circumferential marking around the mass; C: Resected tumor retrieved using a snare; D: The ulcer bed after endoscopic submucosal dissection.



**Figure 4 Histological appearance of the tumor. The margins were histologically tumor-free.** A: The tumor showed a plexiform growth pattern; B, C: The tumor consisted of spindle-shaped cells with an abundant myxoid or fibromyxoid stroma; D: Some tumor cells intruded into the vessel space.

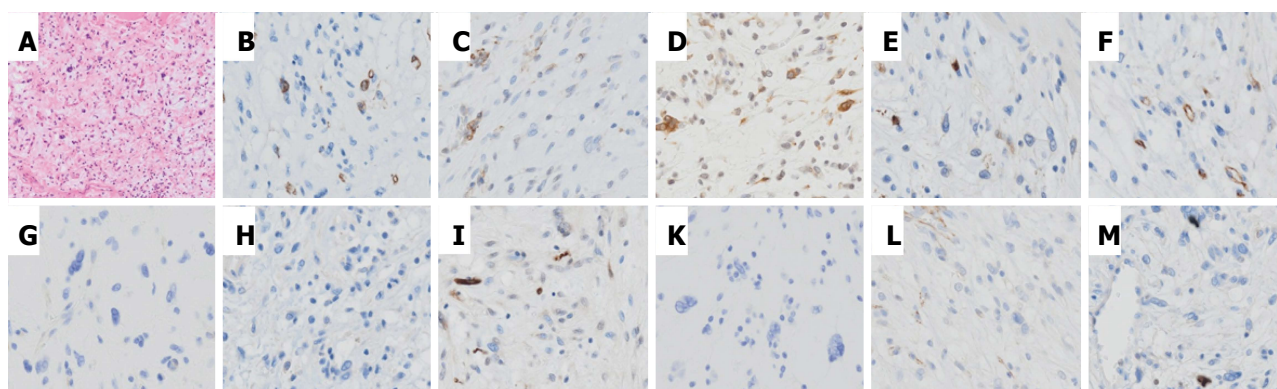
by endoscopy and CT follow-up. There was no recurrence or metastasis in the 12-mo follow-up.

## DISCUSSION

Gastric PF is a new benign mesenchymal tumor that has

been adopted by the 2010 WHO classification of tumors of digestive system<sup>[8]</sup>. The term PAMT is also used for this type of tumor. The distinction between these terms has been controversial<sup>[7,9]</sup>. Previous studies reported that most cases of this tumor are found in the antrum, with approximately half extending into the extragastric





**Figure 5 Hematoxylin and eosin.** A: Histological appearance with hematoxylin and eosin (HE) staining; B-L: Immunohistochemically, tumor cells were focally positive for SMA (B), HHF35 (C), and calponin (D), but negative for c-kit (E), CD34 (F), DOG-1 (G), desmin (H), the S-100 protein (I), CD10 (K), and h-caldesmon (L); M: The Ki-67 labeling index was 2% at most.

soft tissues or proximal duodenum<sup>[2,7]</sup>. The diagnosis of PF is based on its histological features, including immunohistochemical findings<sup>[1]</sup>. Its histology indicates a plexiform growth pattern composed of spindle cells, fine small vessels, and a myxoid matrix. Tumor cells are typically immunoreactive for SMA and HHF35, whereas c-kit, CD34, DOG-1, and the S-100 protein are nearly completely negative. Focal immunoreactivity for CD10, caldesmon, or desmin has occasionally been detected<sup>[1,3,7]</sup>.

In the present case, endoscopy and EUS showed that the tumor grew gradually, with increases in the solid and multicystic components. Spindle cells, with a rich vascular myxoid stroma, were considered to be detected as an isoechoic lesion and fluid leakage was observed as a hypoechoic lesion.

Previous studies reported the lack of recurrence or metastasis of PF after excision<sup>[1,7]</sup>; however, Miettinen *et al*<sup>[1]</sup> demonstrated that some plexiform elements showed intravascular involvement, suggesting that PF occasionally spreads through vessels. Since our case also exhibited vascular invasion, follow-up examinations were carefully performed. Since no patients have developed recurrence, annual endoscopy and CT are considered to be sufficient to monitor patients.

Although PF is considered to be benign, distal or partial gastrectomy is generally performed under the assumption of the presence of GIST<sup>[7]</sup>. Although GIST typically appears as a solid mass, few studies have described myxoid GIST that also shows a plexiform growth pattern<sup>[10]</sup>, and some cases of GIST have shown cystic changes as a result of degeneration or necrosis<sup>[11-13]</sup>. Thus, it may be difficult to distinguish PF from these GIST by performing EUS only once. The chronological changes observed in the present case may contribute to a preoperative diagnosis of PF and the elucidation of its growth process. In this case, even though contrast-enhanced EUS was not performed, it may also be useful for reaching a differential diagnosis<sup>[14,15]</sup>. The distinction of PF from a hamartomatous inverted polyp is also important. EUS-FNA is the first choice for a definite diagnosis of SMT<sup>[16]</sup>. Nevertheless, ESD remains an im-

portant option for diagnostic treatment, including that for cases of gastric SMT of the submucosal layer<sup>[6,17]</sup>. Since EUS-FNA revealed no abnormalities in the present case, ESD was selected as a second choice. We performed *en bloc* ESD, which allowed for the diagnosis of PF. To the best of our knowledge, this is the first case report to describe the successful resection of PF by ESD. Further studies are needed in order to establish the appropriateness of ESD for PF.

## COMMENTS

### Case characteristics

A 66-year-old man presented with a gastric tumor located in the antrum.

### Clinical diagnosis

Gastric submucosal tumor.

### Differential diagnosis

A hamartomatous inverted polyp, myxoid gastrointestinal stromal tumor (GIST), and GIST with cystic degeneration.

### Laboratory diagnosis

Laboratory test results were within normal limits.

### Imaging diagnosis

Endoscopic ultrasound revealed an iso/hypoechoic mass of 20 mm in diameter in the third layer, and it showed gradual increases in the solid and multicystic components without muscle invasion.

### Pathological diagnosis

Plexiform fibromyxoma.

### Treatment

Endoscopic submucosal dissection was performed as a diagnostic treatment.

### Related reports

Few studies have described plexiform fibromyxoma, also known as a plexiform angiomyxoid myofibroblastic tumor. Patients with plexiform fibromyxoma have generally undergone distal or partial gastrectomy.

### Term explanation

Plexiform fibromyxoma is a new mesenchymal tumor entity that shows a unique

plexiform growth pattern of bland spindle cells.

### Experiences and lessons

Plexiform fibromyxoma needs to be considered in a differential diagnosis of gastric submucosal tumors, and follow-up endoscopic ultrasound (EUS) may be able to distinguish plexiform fibromyxoma from other gastric submucosal tumors.

### Peer-review

The rarity of the case could be enriched with a brief review of the literature, due to the scarce number of papers reporting similar tumors. Moreover it could be interesting to expand data about EUS, for example explaining the characteristics of elastometry and eventual contrast enhancement. The quality of the article is augmented by the images, which are impressive and clear. Overall it is a good paper.

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*WJGO* covers topics concerning carcinogenesis, tumorigenesis, metastasis, diagnosis, prevention, prognosis, clinical manifestations, nutritional support, molecular mechanisms, and therapy of benign and malignant tumors of the digestive tract. The current columns of *WJGO* include editorial, frontier, diagnostic advances, therapeutics advances, field of vision, mini-reviews, review, topic highlight, medical ethics, original articles, case report, clinical case conference (Clinicopathological conference), and autobiography. Priority publication will be given to articles concerning diagnosis and treatment of gastrointestinal oncology diseases. The following aspects are covered: Clinical diagnosis, laboratory diagnosis, differential diagnosis, imaging tests, pathological diagnosis, molecular biological diagnosis, immunological diagnosis, genetic diagnosis, functional diagnostics, and physical diagnosis; and comprehensive therapy, drug therapy, surgical therapy, interventional treatment, minimally invasive therapy, and robot-assisted therapy.

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## Emerging molecular targets and therapy for cholangiocarcinoma

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### Abstract

Cholangiocarcinoma (CCA) is a rare cancer arising

from the biliary tree with a poor prognosis and limited therapeutic options. Recent large scale molecular characterisation studies have identified recurrent genetic alterations in CCA which may be amenable to therapeutic targeting. In this review we explore the genomic landscape of CCA and examine results from trials of molecularly targeted agents and immunotherapy in this disease. Challenges in CCA diagnosis, treatment and trial design are discussed and we reflect on future directions which may lead to improved outcomes for CCA patients.

**Key words:** Cholangiocarcinoma; Biliary tract cancer; Targeted therapy; Immunotherapy; Mutation; Molecular; Microenvironment; Stroma; MiRNA

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**Core tip:** Cholangiocarcinoma (CCA) is a clinically challenging malignancy; it is rare, molecularly heterogeneous and associated with a poor prognosis. Here we review recent data on the genomic landscape of CCA and highlight the results of clinical trials using targeted agents and immunotherapy. We find a number of promising therapeutic agents in development and discuss strategies to improve diagnosis and outcomes in this patient group.

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### INTRODUCTION

Cholangiocarcinoma (CCA) is a relatively infrequent malignancy arising from epithelial cells lining the biliary tree. It is associated with poor prognosis and limited

standard therapeutic options. Globally incidence varies considerably according to geographical location with significantly higher rates in South-East Asia compared to Western countries. In North-East Thailand the incidence is high at 85 per 100000<sup>[1]</sup>, whilst in the United States and United Kingdom the incidence is much lower at around 1-3 cases per 100000 population<sup>[2,3]</sup>. The typical age at diagnosis of CCA is around 70 years, with slightly higher incidence in men than women<sup>[4]</sup>. Survival depends on the stage of disease at presentation, but even in patients with localised disease, five-year survival is poor at 15% and 30% for intrahepatic (ICC) and extrahepatic (ECC) CCA respectively<sup>[5]</sup>. For unclear reasons the incidence of ICC is increasing in western countries whilst rates of ECC are falling internationally<sup>[6]</sup>.

## CLASSIFICATION

CCA is now classified according to anatomical location into ICC, perihilar and distal subtypes; the latter two are ECC tumours. Prior to this novel classification the terms intra- and extra-hepatic CCA predominated, and we will use this nomenclature for the purposes of this review. This anatomical classification is useful as in addition to guiding surgical management, it is increasingly recognised that ICC and ECC have differing molecular profiles and may arise from differing cells of origin. There is evidence to suggest that some ICCs arise from the hepatic stem cell lineage whilst other ICCs and most ECCs arise from the biliary tree stem cell lineage<sup>[7]</sup>. Understanding the differences in tumour aetiology and biology between CCA subtypes will help guide stratification of targeted therapies.

## AETIOLOGY

Parasitic infection with the liver flukes *Clonorchis sinensis* and *Opisthorchis viverrini*, which are endemic in parts of South-East Asia, are strongly associated with development of CCA. In non-Asian countries most cases occur sporadically, however conditions causing bile stasis and chronic biliary inflammation are associated with CCA development. Specific risk factors include primary sclerosing cholangitis, hepatolithiasis, choledocal cysts and Caroli's syndrome (congenital cystic dilation of intrahepatic bile ducts). All causes of liver cirrhosis potentially predispose to CCA and studies have identified viral hepatitis and alcoholic liver disease as specific risk factors. The now banned radiocontrast, thorotrast has also been associated with CCA development.

## MANAGEMENT OF CCA

### Localised disease

**Surgery and locoregional therapy:** Less than 40% of patients present with resectable disease<sup>[8]</sup> and 5-year survival rate for patients with completely resected bile duct and GBC is in the range of 20%-50%. Loco-

regional failure occurs in more than half of patients, even in absence of residual disease (R0) and provides the justification for the study of adjuvant therapy.

Previously, the role of adjuvant chemotherapy for resected patients is not clearly defined. Phase III trials in this setting had not demonstrated a survival advantage in CCA, but these studies have included a range of tumour types (including pancreatobiliary, gallbladder and ampullary carcinomas) and have lacked sufficient power to identify a survival difference specifically in CCA<sup>[9,10]</sup>. However, recently the results of the UK BILCAP study have been presented in abstract form. This large phase III randomised trial recruited patients with resected biliary cancer including 368 (plus 79 gallbladder carcinomas) cholangiocarcinoma patients and randomised between no adjuvant therapy or 6 mo of oral capecitabine. Patients treated with capecitabine had improved overall survival [53 mo vs 36 mo HR = 0.75 (95%CI: 0.58-0.97; *P* = 0.028)]. The results will lead to adjuvant capecitabine being adopted as a standard of care in resected biliary cancers.

Liver transplantation is not a standard treatment for CCA due to historically high recurrence rates and donor shortage. More modern series, have reported more encouraging results<sup>[11]</sup>. Potential candidates, such as patients with poor hepatic reserve for extended hepatectomy or those with localised but unresectable perihilar CCA should be enrolled on to suitable clinical trials.

Locoregional therapies, including radiotherapy, photodynamic therapy, chemo/radio-embolisation and radiofrequency ablation may have a role in locally advanced disease or patients who are surgically unfit. There is a lack of comparative clinical trial evidence to support any of these modalities improving survival compared to standard of care chemotherapy<sup>[12]</sup>. However retrospective and phase II data suggest a promising rate of local control by adding radiotherapy in the management of ICC, and warrants further investigation<sup>[13,14]</sup>.

### Unresectable/metastatic disease

The United Kingdom phase 3 ABC-02 trial established cisplatin-gemcitabine combination therapy as standard of care for the first line treatment of advanced CCA, providing a clinically significant survival advantage compared to gemcitabine alone (median OS 11.7 mo vs 8.1 mo, HR = 0.64, 95%CI: 0.52-0.80, *P* < 0.001)<sup>[15]</sup>. There is currently no established second line treatment for advanced CCA and although a number of small phase II studies have shown disease activity, using single agent or doublet combinations of 5-FU, oxaliplatin, and gemcitabine, this has not been validated in a randomised trial<sup>[16]</sup>. Results from the ABC-06 (NCT01926236) phase III trial, investigating FOLFOX chemotherapy compared to supportive care in the second line setting are awaited.

### Pathophysiology

**Desmoplastic stroma:** The tumour microenviron-

ment plays an important role in CCA pathogenesis. CCA bile ducts are typically surrounded by a dense hypovascular desmoplastic stroma, consisting of cancer associated fibroblasts (CAF) expressing  $\alpha$ -smooth muscle actin (SMA), activated macrophages and a fibrotic collagen rich extracellular matrix<sup>[17]</sup>.  $\alpha$ -SMA positive CAFs are involved in CCA progression and tumours of patients expressing high levels of  $\alpha$ -SMA have poorer survival<sup>[18]</sup>. CAFs produce a range of factors involved in autocrine and paracrine signalling, promoting oncogenic processes such as proliferation, invasion, metastasis and apoptosis evasion. Specifically, the factors produced by CAFs include periostin, tenascin-c, thrombospondin 1, stromal cell derived factor 1 (SDF-1), hepatocyte growth factor (HGF) and Wnt-inducible signalling protein-1v (WISP1)<sup>[18]</sup>. These factors interact with CCA cells to manipulate cell-signalling pathways. For instance periostin interacts with tenascin-C, HGF and SDF-1, which bind to their respective receptors, integrin, MET and CXCR4 on CCA cells, leading to activation of the PI3K/AKT signalling pathway. Cancer associated macrophages are also important in the stromal microenvironment and appear to have prognostic significance. In one study, high numbers of CD163<sup>+</sup> macrophages in the stroma of resected ICC correlated with poor disease free survival<sup>[19]</sup>. Inflammatory macrophage infiltrates in CCA are also associated with increased WNT signalling, and abrogation of WNT signalling in preclinical models inhibits CCA growth<sup>[20]</sup>. In cholangiocarcinoma, sustained interleukin-6 (IL-6) signalling which promotes tumour growth *via* autocrine mechanisms is also associated with increasing fibrosis and dense stroma formation; it is recognised that this dense hypovascular stroma poses a challenge to cytotoxic drug delivery<sup>[18]</sup>. Therefore targeting stromal factors involved in cholangiocarcinogenesis or improving drug delivery through the desmoplastic stroma are attractive targets for novel therapeutics.

## MOLECULAR CHARACTERISATION AND POTENTIAL FOR TARGETED THERAPIES

With recent technological advances in genomic sequencing, the mutational landscape of CCA is increasingly understood. Careful evaluation is needed to determine which genetic aberrations are true drivers of CCA. This section will review recent data on key genetic abnormalities thought to be implicated in CCA pathogenesis. There are clear differences in the prevalence of known oncogenic driver mutations between ICC and ECC, implicating distinct processes of oncogenesis for these tumour subtypes (Table 1). However it is also noteworthy that the prevalence of mutations is highly variable across studies, this heterogeneity may be reflective of regional variation, small sample size, or differences in the pathological classification of ICC and ECC prior to sequencing.

**Table 1 Mutation frequency for intrahepatic and extrahepatic cholangiocarcinoma**

Genetic mutation	Frequency (%) in all tumours tested		Ref.
	Intrahepatic CC	Extrahepatic CC	
IDH 1/2	14%-36%	0%	[27,59,67,99-101]
BAP 1	9%-25%	4%-10%	[66,67,101]
KRAS	9%-24%	40%-47%	[62-64,96]
TP53	3%-38%	18%-45%	[62-64,96]
PBRM1	11%-17%	4%-11%	[63,97]
ARID1A	11%-36%	5%-16%	[62-64,95]
EGFR amplification	7%	0%	[101]
HER2	0%-2%	0%-20%	[67,101]
VEGF overexpression	42%	31%	[46,47]
PIK3CA	4%-6%	9%	[66,101]
BRAF	4%-22%	6%	[57,101-103]
FRGR translocation	6%-50%	0%-5%	[66]
MCL1 amplification	16%-21%	NR	[66]
PTEN	1%-11%	4%	[59,101]
FBXW7	1%-6%	4%-15%	[67]
CDK6	7%	NR	[66]
CDKN2A	7%	15%	[66]
BRCA 1/2	4%	NR	[66]
SMAD4	1%-4%	11%-25%	[59,67,101]
mTOR	26%	40%	[67]

HER2: Human epidermal growth factor 2; CC: Cholangiocarcinoma; NR: Not reported.

Also of interest is that liver fluke related CCA is associated with a differing pattern of genetic mutations compared to non-fluke CCA. In one of the first studies to sequence CCA, 8 liver fluke CCA were analysed, revealing novel mutations in SMAD4 (17%), MLL3 (15%), ROBO2, GNAS and RNF (9%) each and CKDN2A and PEG3 (5%) each<sup>[21]</sup>. In a subsequent follow up study of 208 CCA cases (108 caused by liver fluke *O. viverrini*), TP53 mutations were more often seen in fluke related CCA, whilst IDH1/2 and BAP1 mutations were more common in non-fluke CCA<sup>[22]</sup>. This highlights the impact of environmental risk factors on the pattern of somatic mutations. The prognostic value of several somatic mutations seen in CCA has been evaluated, however results are conflicting.

Interestingly IDH1/2 (Isocitrate dehydrogenase) mutations are seen almost exclusively in ICC. The IDH mutation results in reduced normal function of this enzyme and leads to increased production of 2-hydroxyglutarate (2-HG) from alpha-ketoglutarate. 2-HG is considered an oncometabolite and causes epigenetic changes, including histone and DNA methylation, which promotes tumour development<sup>[23]</sup>. In one study of 326 patients with resected ICC, IDH1/2 mutations were associated with improved overall survival<sup>[24]</sup>, however another whole exome sequencing study ( $n = 32$ ) suggested worse overall survival for patients with these aberrations (3 year OS 33% mutant vs 81% wildtype,  $P = 0.003$ )<sup>[25]</sup>, however in this study a higher proportion of patients with IDH mutations had advanced disease (50% vs 15%). Two other studies

**Table 2** Clinical trials of novel agents in cholangiocarcinoma

Title	Target	Phase	Estimated sample size	Expected completion date	Trial number
CX-4945 in combination with gemcitabine and cisplatin for frontline treatment of CCA	Casein kinase 2	I/II	100	Dec 2016	NCT02128282
BGJ398 in patients with advanced CCA and <i>FGFR</i> gene fusion	<i>FGFR</i> gene fusion	II	55	Jul 2018	NCT02150967
Dasatanib in IDH-mutant advanced ICC		II	19	Sep 2022	NCT02428855
RRx-001 in second line treatment of advanced CCA prior to readministration of first line therapy	Epigenetic modifications	II	30	May 2018	NCT02452970
ASLAN001 in advanced CCA who progressed on at least 1 line of therapy	Pan-HER inhibitor	II	25	Dec 2017	NCT02609958
Regorafenib as single agent in advanced CCA who failed first line	Multi-kinase inhibitor (VEGF, KIT, PDGF, FGFR, BRAF)	II	37	Feb 2018	NCT02053376
Copanlisib in combination with gemcitabine and cisplatin in advanced CCA	PI3K inhibitor	II	25	Dec 2018	NCT02631590
LDK378 in <i>ROS1</i> /ALK overexpressed advanced CCA	<i>ROS1</i> and/or ALK	II	34	Jul 2018	NCT02374489
AG120 in advanced solid tumours with IDH1 mutation	IDH1	I	145	May 2016	NCT02073994
Study of LY2801653 in advanced cancer	MET inhibitor	I	190	Nov 2017	NCT01285037
ABC-08: Acelarin in combination with cisplatin in locally advanced/metastatic biliary tract cancers	Nucleotide analogue	I	24	Sep 2018	NCT02352765
Ramucirumab for advanced pre-treated biliary cancers	VEGFR2 antagonist	II	50	Dec 2019	NCT02520141
Keynote-158: Pembrolizumab in participants with advanced solid tumours	PD1 inhibitor	II	1100	Mar 2021	NCT02628067
Immunotherapy using TILs for patients with metastatic cancer	Adoptive T-cell therapy	II	33	Dec 2019	NCT01174121

CCA: Cholangiocarcinoma; EGFR: Epidermal growth factor receptor; FGFR: Fibroblast growth factor receptor; IDH: Isocitrate dehydrogenase; PD1: Programmed death 1; TIL: Tumour infiltrating lymphocytes; VEGF: Vascular endothelial growth factor.

examining the effect of IDH mutations in patients with resected and advanced ICC demonstrated no significant association with prognosis<sup>[26,27]</sup>. Pre-clinical data suggesting oncogenic addiction to IDH signalling can be pharmacologically abrogated resulting in control of cancer cell growth has been demonstrated in IDH mutant glioma lines<sup>[28]</sup>. Inhibitors of mutant IDH1 and IDH2 are currently in clinical trials (Table 2); early results for AG-120 which is an IDH1 inhibitor demonstrated tolerable toxicity with evidence of pharmacodynamic endpoint modulation with reduced circulating levels of 2-hydroxyglutamate were observed in most patients<sup>[29]</sup>. Of twenty CCA patients treated, one (5%) had a partial response whereas 11 (55%) had stable disease.

The genes *BAP1*, *ARID1A* and *PBRM1*, which are involved in chromatin remodelling, have been found to be frequently mutated in ICC and in one study had inactivating mutations in just under half ( $n = 15/32$ ) of ICC cases<sup>[25]</sup>. Whether these mutations can predict sensitivity to the histone deacetylase inhibitor vorinostat, which targets chromatin regulation has not yet been determined. However in a preclinical study vorinostat did show anti-cancer activity against the HuCC-T1 human CCA cell line<sup>[30]</sup>.

### Epidermal growth factor receptor alterations as a target in CCA

The epidermal growth factor receptor (EGFR) is abnormally activated in a number of human cancers and is

therapeutically targeted using monoclonal antibodies or tyrosine kinase inhibitors<sup>[31]</sup>. EGFR targeted agents have demonstrated clinical efficacy in non-small cell lung cancer (NSCLC), colorectal cancer and SCC of the head and neck, where they are now established standard therapies. In NSCLC *EGFR* gene mutation, predicts response to EGFR inhibition<sup>[32]</sup>. Both activating mutations and amplifications of the *EGFR* gene have been observed in CCA. *EGFR* mutations have been observed in 10%-15% of CCA<sup>[33-35]</sup>, however due to small sample numbers data are conflicting regarding whether prevalence is higher in ICC or ECC. *EGFR* overexpression appears to be more prevalent in ICC (11%-27%) compared to ECC (5%-19%)<sup>[36,37]</sup>. Prognostically, EGFR expression has been found to be a negative predictor of overall survival in CCA<sup>[37,38]</sup>, making this an attractive target for drug intervention.

There have been 3 reported phase II trials investigating anti-EGFR monoclonal antibodies (mABs) in CCA but none have demonstrated survival advantage in this patient group. In a single arm phase II trial of 30 patients with advanced biliary tract cancer (aBTC) treated with first-line GEMOX-cetuximab, Gruenberger et al reported an objective response rate (CR + PR) of 63%, mOS of 15.2 mo (9.9-20.5) and 9 patients were able to undergo potentially curative resection following systemic therapy. Whilst this trial did not have a control arm and patients were unselected for *EGFR* expression or *KRAS* status, the reported response rate was considered encouraging<sup>[39]</sup>. Subsequently in the



phase II BINGO trial, 150 patients were randomised to first-line gemcitabine and oxaliplatin (GEMOX) with or without the addition of cetuximab<sup>[40]</sup>. Median overall survival was numerically shorter in the GEMOX-cetuximab group at 11.0 mo compared to 12.4 mo in the GEMOX group indicating lack of benefit from cetuximab. Neither *EGFR* overexpression (18 of 77 cases, 23%) nor *KRAS* mutation (14 of 75 cases, 19%) was associated with patient outcome in either treatment group. More recently, Leone *et al* showed that the anti-*EGFR* mAb panitumumab when used in combination with GEMOX chemotherapy in patients with *KRAS*-WT aBTC produced no improvement in overall survival when compared to chemotherapy alone (9.9 mo vs 10.2 mo,  $P = 0.42$ )<sup>[41]</sup>.

The small molecule *EGFR* tyrosine-kinase inhibitor erlotinib has also been investigated in advanced CCA. In a randomised phase III trial of 268 patients with aBTC (CCA, gallbladder and ampullary cancer) there was no significant difference in the primary end-point, progression free survival (PFS) in patients receiving GEMOX with or without erlotinib (5.8 mo vs 4.2 mo, HR = 0.80, 95%CI: 0.61-1.03,  $P = 0.83$ )<sup>[42]</sup>. However the objective response rate (CR + PR) was higher in the erlotinib group (40 vs 21 patients,  $P = 0.005$ ) and in the subgroup of patients with CCA, PFS was longer in the erlotinib group (5.9 mo vs 3.0 mo, HR = 0.73, 95%CI: 0.53-1.0,  $P = 0.049$ ). *EGFR* overexpression was reported in 12 of 60 patients (43%) in the erlotinib group and of these there was 1 PR and 7 cases of SD. In summary trials investigating anti-*EGFR* therapy in CCA have to date failed to demonstrate any clinically meaningful benefit over standard of care chemotherapy. The caveat to interpretation of these trials is that the inclusion of heterogenous, non-biomarker selected groups of biliary tract cancers may obscure any real survival benefit in smaller patient subsets; biomarker selected studies might be preferred for this reason.

Human epidermal growth factor 2 (HER2) or Erb-B2 Receptor Tyrosine Kinase 2 (ERBB2) amplification is rare in ICC, but may be present in up to 20% of ECC<sup>[37,43]</sup> and this target has been successfully targeted in breast and gastroesophageal cancer patients. Currently only anecdotal reports<sup>[44]</sup> are present in the literature regarding responses to anti-HER2 therapy in CCA, however a molecularly selected trial of trastuzumab in conjunction with GEMOX is ongoing (NCT02836847), and it remains to be seen whether this approach will be beneficial.

### Angiogenesis as a target in CCA

Vascular endothelial growth factor (VEGF) has been targeted therapeutically in a number of malignancies, with anti-VEGF monoclonal antibodies demonstrating efficacy in colorectal, breast and ovarian cancers, whilst TKIs targeting the VEGF receptor are in clinical use in renal cell carcinoma and hepatocellular

carcinoma<sup>[45]</sup>.

VEGF expression is reported in around 30%-40% of CCAs and correlates with increased lymph node metastasis and poorer survival<sup>[46,47]</sup>, making angiogenesis an attractive target in CCA. Bevacizumab, a humanised monoclonal antibody against VEGF-A has been investigated in CCA. Zhu *et al* conducted a single arm phase II trial and treated aBTC patients with first-line GEMOX plus bevacizumab<sup>[48]</sup>. They reported a median PFS of 7.0 mo, mOS of 12.7 mo and ORR of 40%, which was considered favourable compared to historical controls. Similar results were found in a phase II trial combining gemcitabine and capecitabine with bevacizumab in patients with aBTC where a median PFS of 8.1 mo and OS of 11.3 mo was reported<sup>[49]</sup>. Combining VEGF and *EGFR* inhibition has not significantly improved outcomes. Lubner *et al* used a combination of bevacizumab and erlotinib in a phase II trial of 49 patients with aBTC<sup>[50]</sup>. This trial reported a PR in 6 (12.2%) patients in whom the median duration of response was 8.4 mo and the reported mOS was 9.9 mo. However, overall survival in these studies (10-13 mo) did not reasonably exceed expectations for a phase II population and no control arm was available for comparison.

More recently disappointing results were reported from the randomised phase II ABC-03 trial, which investigated gemcitabine-cisplatin with either cediranib or placebo as first-line treatment of patients with aBTC<sup>[51]</sup>. Cediranib, a tyrosine kinase inhibitor of VEGFR1-3 and with additional activity against PDGF and c-KIT, did not improve PFS compared to the control group (PFS 8.0 mo vs 7.4 mo, HR = 0.93, 95%CI: 0.65-1.35,  $P = 0.72$ ) or overall survival (14.1 mo vs 11.9 mo, HR = 0.86,  $P = 0.44$ ). Response rates were higher for cediranib treated patients (44% vs 19% control). An interaction between baseline PDGFbb levels and overall survival benefit from cediranib was noted; patients with PDGFbb concentrations above the median derived an overall survival benefit from cediranib ( $P$  value for interaction 0.002).

Sorafenib, a multi-targeted TKI of VEGFR-2/3, PDGFR, BRAF and CRAF which may also be considered an anti-angiogenic agent has been investigated in CCA with disappointing results. As single agent therapy no clinically meaningful benefit was observed in 2 phase II trials, with reported PFS of 2.3 (range 0-12) and 3 (95%CI: 2-4) mo respectively<sup>[52,53]</sup>. When sorafenib was added to gemcitabine and cisplatin chemotherapy, PFS and OS of 6.5 and 14.4 mo respectively was reported which was similar to historical controls using chemotherapy alone<sup>[54]</sup>. Finally a phase II trial combining sorafenib with erlotinib was closed early due to failure to meet pre-determined survival criteria and reported a disappointing PFS and OS of 2 (95%CI: 2-3) and 6 (95%CI: 3-8) mo respectively<sup>[55]</sup>.

In summary, despite encouraging early trial results, therapeutic targeting of angiogenesis has not been

**Table 3** Fibroblast growth factor receptor fusions according to reported frequency in cholangiocarcinoma

FGFR fusion partner	Frequency	Ref.
FGFR2-AHCYL	7/102 (7%)	[56]
FGFR2-BICC1	2/102 (2%)	[56]
	41/107 (38%)	[57]
	1/28 (4%)	[66]
FGFR2-PPHLN1	17/107 (16%)	[57]
FGFR2-MGEA5	1/6 (17%)	[62]
FGFR2-TACC3	1/6 (17%)	[62]
	1/28 (4%)	[66]
FGFR-KIAA1598	1/28 (4%)	[66]

FGFR: Fibroblast growth factor receptor.

successful in CCA, although using biomarkers such as PDGFbb may improve patient selection in future.

### Fibroblast growth factor receptor fusions

The *FGF* pathway is involved in a number of cellular processes including proliferation, migration and angiogenesis. Abnormalities of this pathway have been implicated as driver events in carcinogenesis. In CCA, fibroblast growth factor receptor (*FGFR*) chromosomal translocations producing *FGFR*-fusion genes have been reported in both ICC and ECC, but are much more frequent in ICC (6%-50%) than ECC (0%-6%) (Table 3). The fusion protein is constitutively activated leading to downstream signalling through mitogen-activated protein kinase (MAPK) and PI3K/mTOR pathways<sup>[56]</sup>. Sia *et al*<sup>[57]</sup> demonstrated in a cohort of 107 ICC patients that *FGFR2* translocations represented the most common actionable target detected; these occurred in 16% of patients screened; this prevalence has been confirmed in other series<sup>[58,59]</sup>. One United States study has suggested that *FGFR2* fusion in ICC is more common in females, and a Japanese study has implicated viral hepatitis infection in this pathway<sup>[56,60]</sup>, however these findings require validation. *FGFR2* translocation in CCA may confer a prognostic benefit. Cancer specific survival in one dataset for patients harbouring *FGFR2* translocations was superior to non-translocated tumours (123 mo vs 37 mo respectively)<sup>[60]</sup>. Preclinical work in cell lines and patient derived xenografts supports blockade of *FGFR2* signalling in CCA as a potential effective therapy<sup>[56,61]</sup> and early anecdotal reports of *FGFR* inhibitor therapy in *FGFR2* translocated CCA patients have been encouraging<sup>[62]</sup>. These promising results were also reflected in an interim report from a phase II clinical trial examining the efficacy of the pan-*FGFR* inhibitor BGJ398 in CCA patients with an *FGFR* abnormality (NCT02150967)<sup>[63]</sup> in which of 36 patients eligible for assessment of response, 8 (22%) had a partial response and the disease control rate was 75%. These results compare very favourably to second line chemotherapy for CCA and serve to highlight the potential benefit of targeted therapy in appropriately

selected patients.

### Other potential targets in CCA

*ROS1* gene rearrangements are seen in a number of cancers and result in a fusion protein with a constitutively activated kinase domain that promotes oncogenesis. In NSCLC, *ROS1* rearranged tumours have shown encouraging response to the *ALK/MET/ROS* inhibitor Crizotinib. In CCA the prevalence of *ROS1* rearrangement is reported to be around 8%-9%<sup>[58,64]</sup>. Larger series are needed to determine whether prevalence is higher in ICC or ECC, however Neia *et al* found that in a cohort of 65 biliary tract cancer patients *FIG-ROS1* fusions were found in 4/25 ECC, 2/14 gallbladder carcinoma, 0/26 ICC<sup>[58]</sup>. In a murine allograft model of ICC the *FIG-ROS* fusion protein was shown to promote tumorigenesis and *FIG-ROS* inactivation resulted in inhibition of tumour growth<sup>[65]</sup>. Clinical trials are in progress to assess the efficacy of targeting *ROS1* rearrangement in CCA (NCT02374489, NCT02034981).

The *RAS/RAF/MEK/ERK* MAPK pathway is a key regulator of cellular proliferation and is defective in a number of malignancies.

*KRAS* mutations are frequent in CCA and have a reported incidence of 9%-47%<sup>[59,66,67]</sup>. In mouse models, tissue specific activation of *KRAS* in the hepatic parenchyma was found to lead to development of ICC<sup>[68]</sup> and this process was accelerated by the presence of simultaneous *P53*<sup>[68]</sup> or *PTEN* loss<sup>[69]</sup>. There are currently no available drugs to directly target *KRAS*, however downstream proteins can be targeted, for instance using *MEK* inhibitors. In a phase II trial of the *MEK 1/2* inhibitor selumetinib in aBTC, 12% (3/28) had objective response and 56% (14/28) prolonged stable disease (> 16 wk) resulting in a PFS of 3.7 mo and OS of 9.8 mo<sup>[70]</sup>.

*BRAF* mutations in CCA are reported to occur at a rate of 0-22%<sup>[71,72]</sup>. In one large study, *BRAF* V600E mutation was detected in 3% (5/159) of ICC cases but in no (0/149) ECC cases<sup>[71]</sup>. *BRAF* mutation showed no correlation with prognosis in this study. Due to the infrequent occurrence of *BRAF* mutation in CC, molecularly targeted clinical trials are difficult to conduct, however a phase II trial of combination *BRAF* and *MEK* inhibition in rare cancers is in progress (NCT02034110).

### Noncoding RNA abnormalities in CCA

MicroRNAs (miRNAs) are small non-coding RNAs that act as negative regulators of gene expression at the post transcription level. They bind to the 3' untranslated region of target mRNAs causing inhibition of translation and mRNA degradation. miRNAs can regulate a number of cellular processes and their abnormal expression is recognised in human cancers, including CCA. The abnormal expression of miRNA in CCA has been found to impact on a number of cellular processes

involved in cell cycle progression, apoptosis and cell signalling. In one of the first reported studies on this topic Meng *et al* demonstrated that miR-21, miR-141, and miR-200b were highly over-expressed in malignant CCA cells, and whereas inhibition of miR-21 and miR-200b increased sensitivity to gemcitabine, inhibition of miR-141 decreased cell growth<sup>[73]</sup>. Chronic inflammation is an important risk factor for CCA. The inflammatory cytokine, IL-6 has been identified as a driver of cholangiocarcinogenesis and has been shown to alter the expression of a number of miRNAs including miRNA 7a, 370, 148a and 152<sup>[74-76]</sup>. IL-6 signalling is associated with increased expression of DNA methyltransferase (DNMT), which promotes silencing of tumour suppressor genes through DNA hypermethylation. The miRNAs 148a and 152 are believed to regulate DNMT expression, as demonstrated by decreased levels of these miRNAs in malignant cholangiocytes in in-vitro and xenograft models. Crucially in cells transfected with these miRNAs DNMT levels were shown to decrease leading to reduced cellular proliferation<sup>[74]</sup>. In another study, in a cell culture model, miRNA 29b was under-expressed in CCA cell lines compared to normal cholangiocytes, resulting in up-regulation of the anti-apoptotic protein MCL-1 and allowing tumour cells to evade apoptosis<sup>[77]</sup>. In a further study, miRNA 494 was shown to induce G1/S transition cell cycle arrest, through downregulation of cyclin dependent kinase 6. In cell-based and xenograft models of CCA, miRNA494 expression was found to be reduced and its upregulation reduced cellular proliferation<sup>[78]</sup>. miRNA 26a was shown to promote proliferation of CCA cells by lowering levels of glycogen synthase kinase 3 $\beta$  (GSK-3 $\beta$ ) and preventing the degradation of  $\beta$ -catenin, leading to upregulation of transcription of target genes involved in carcinogenesis<sup>[79]</sup>. Other miRNAs may be related to chemoresistance; higher levels of miR-21 and miR-200b are associated with resistance to gemcitabine in cell lines whereas the converse is true for miR-29b, miR-205 and miR-221<sup>[80]</sup>. Selaru *et al*<sup>[81]</sup> have also demonstrated that miR-21 may be oncogenic in CCA through inhibition of programmed cell death 4 (PDCD4) and tissue inhibitor of metalloproteinases 3. As pharmacological manipulation of noncoding RNAs develops into a viable therapeutic option<sup>[82]</sup>, these processes could conceivably be targeted in future to benefit CCA patients.

### Circulating miRNAs

Pathological diagnosis of biliary tract tumours is frequently challenging due to the limited cellularity of specimens available post ERCP and also the desmoplastic stroma associated with biliary tract cancers, making the concept of a "liquid biopsy" attractive. Differential expression of several miRNAs has been demonstrated between patients with CCA and healthy controls in both tissue and blood, miR21 is known to be expressed at higher levels in biliary tract cancers, and increasing levels are also associated with

more advanced clinical stage and fall following surgical resection<sup>[83]</sup>. Wang *et al*<sup>[84]</sup> found that miR150 was significantly elevated in the plasma of ICC patients compared to clinical controls and could be used to differentiate ICC patients from volunteers with a sensitivity of 81% and a specificity of 58%, which was enhanced when CA19.9 was used in conjunction with miRNA analysis. Other circulating miRNAs of interest include miRNA192<sup>[85]</sup> which was also linked to more advanced disease and a negative prognosis and miR106a which is downregulated in CCA compared to healthy controls and has similar prognostic value<sup>[86]</sup>.

As bile secreted by the liver through the biliary ducts is more freely available to sample than tissue this also represents a potentially useful diagnostic material for CCA. In bile, miRNAs are contained in extracellular vesicles which maintain miRNA stability. Li *et al* designed a microvesicle based miRNA panel which was able to differentiate CCA from other causes of biliary disease or obstruction with a sensitivity of 67% and specificity of 96%<sup>[87]</sup>. The panel consisted of miR-191, miR-486-3p, miR-16, and miR-484, of which the last is the most sensitive for CCA. A Japanese study evaluating a larger panel of miRNAs in bile found ten to be upregulated in biliary tract cancer compared to benign biliary disease, and selected a combination of two (miR-9 and miR-145) as a proposed diagnostic biomarker with a specificity of 100% and high sensitivity<sup>[88]</sup>. Voigtländer *et al*<sup>[89]</sup> performed a study comparing miRNA expression in both serum and bile in German patients with primary sclerosing cholangitis and CCA, in addition to the serum of healthy controls. Interestingly, distinct miRNA profiles differentiated PSC and CCA in bile and in serum. In serum, lower levels of miR-1281, miR-126, miR26a, miR30b and miR-122 were found in CCA patients compared to PSC and healthy controls, whereas in bile changes in miR-412, miR-640, miR-1537 and miR-3189 predominated. Unfortunately as paired samples from each patient were not available a predictive panel containing blood and bile biomarkers was unable to be generated, however in future such an approach could be of significant utility. Although the use of circulating miRNAs is of significant interest, these studies are relatively small and require validation before becoming clinically applicable. Furthermore consideration of the geographic region of origin of each of the above studies (impacting on CCA aetiology and biology) must be considered before generalising these findings.

### Immunotherapy

**Immune checkpoint inhibitors:** Immune checkpoints, which provide co-stimulatory and co-inhibitory signals to T-cells are exploited by a number of cancers to evade the host immune system and checkpoint inhibition has been used therapeutically, most notably in melanoma and non-small cell lung cancer amongst other malignancies. There may also be a role for

checkpoint inhibition in CCA. Ye *et al*<sup>[90]</sup> studied the expression of the co-inhibitory immune checkpoint, Programmed Death Ligand 1 (PD-L1) in 31 surgically resected ICC samples from Asian patients and found PD-L1 expression to be upregulated in tumour tissue compared to adjacent tissue. Tumours with high levels of PD-L1 expression were associated with poor differentiation, higher TNM stage and higher levels of apoptotic CD8<sup>+</sup> tumour infiltrating lymphocytes (TIL). Poorer survival has also been demonstrated in Western patients with ICC with positive tumour PD-L1 expression<sup>[91,92]</sup>. Sabbatino *et al*<sup>[92]</sup> also found that downregulation of HLA class I antigen expression by tumour cells was associated with poorer clinical outcome. These data indicate PD-L1 upregulation and HLA class I antigen downregulation may be mechanisms of immune escape in CCA and could be potential biomarkers of response to anti-PD1/PDL1 immunotherapy. Chemotherapy may also have a role in modulating the immune system *via* inducing immunogenic cell death and upregulating expression of tumour associated antigens. Koido *et al*<sup>[93]</sup> found treatment of ICC cells with gemcitabine resulted in upregulation of the tumour antigen WT1, calreticulin (a protein that provides a pro-phagocytic signal) and PD-L1. Thus there may be a rationale for combining standard chemotherapy drugs with immune checkpoint inhibitors.

Trials investigating immune checkpoint inhibitors in CCA are in progress but early signals of efficacy have recently been reported. Keynote-028 is a multicohort phase Ib trial of pembrolizumab in PD-L1 positive pre-treated advanced solid tumours<sup>[94]</sup>. Early data from the biliary tract cohort of this trial reported an objective response rate of 17% ( $n = 4/17$ , all partial responses) and a further 17% ( $n = 4/17$ ) achieved stable disease. Responses appeared to be durable with all responding patients remaining on treatment at 40-42 wk. Le *et al*<sup>[95]</sup> also reported data from a phase II trial of 17 patients with mismatch repair deficient non-colorectal gastrointestinal cancers treated with the PD-1 inhibitor pembrolizumab. Of the 3 patients with CCA there was one complete response, one partial response and one stable disease, with durable and ongoing responses at median follow up of 5.3 mo. More mature data with larger sample sizes are eagerly awaited but mismatch repair deficiency appears to be a promising predictive biomarker for checkpoint inhibition (although of relatively rare prevalence). The Keynote-158 phase II trial is recruiting 1100 patient with advanced solid tumours to be treated with pembrolizumab and will include a cohort of patients with biliary tract cancer (NCT02628067).

**Mutation specific adoptive T-Cell therapy:** The use of T-cells with specificity to cancer antigens is an emerging field and efficacy has been demonstrated in metastatic melanoma<sup>[96]</sup> and B cell leukaemia<sup>[97]</sup>. T-cell based therapy for epithelial malignancies, such as CCA

is under investigation.

Tran *et al*<sup>[98]</sup> treated a female patient with metastatic CCA who had progressed on multiple lines of chemotherapy, with autologous T<sub>H</sub>1 tumour infiltrating lymphocytes (TILs) specific to a mutated antigen expressed by the patient's cancer. In this novel approach, TILs from the patient's lung metastases were retrieved and whole exome sequencing performed on tumour tissue to identify somatic mutations present. Further testing revealed that CD4<sup>+</sup> T<sub>H</sub>1 TILs recognised mutated *erbb2* interacting protein (ERBB2IP) in the tumour tissue. These mutation specific TILs were clonally expanded and the patient underwent lymphodepletive chemotherapy, before receiving 42.4 billion TILs (25% ERBB2IP-mutation reactive T cells). There was impressive reduction in size of metastatic lesions and prolonged stable disease for more than 1 year. When the disease progressed after 13 mo the patient was retreated and again achieved disease response. Whilst this demonstrated an important proof of concept for T-cell based therapy in CCA, reproducibility in further patients is needed. Furthermore the highly personalised nature of this approach has high cost implications.

## CONCLUSION

CCA is a molecularly heterogeneous malignancy with currently limited treatment options beyond first line systemic chemotherapy. Genomic profiling studies have highlighted differing patterns of mutation signatures between ICC and ECC, helping to stratify patients for targeted therapies. FGFR fusions and IDH mutations appear to be frequently mutated in ICC and hold promise as therapeutic targets. Immunotherapy also has considerable potential but requires a validated biomarker to guide selection of patients for this approach. Circulating miRNAs are of interest in improving early diagnosis and detecting disease relapse. However given the relative rarity of this cancer and the molecular heterogeneity, multi-centre collaboration is essential in order to design adequately powered clinical trials of targeted agents.

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## Evolving treatment landscape for early and advanced pancreatic cancer

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### Abstract

Pancreatic ductal adenocarcinoma is an infrequent

cancer with a high disease related mortality rate, even in the context of early stage disease. Until recently, the rate of death from pancreatic cancer has remained largely similar whereby gemcitabine monotherapy was the mainstay of systemic treatment for most stages of disease. With the discovery of active multi-agent chemotherapy regimens, namely FOLFIRINOX and gemcitabine plus nab-paclitaxel, the treatment landscape of pancreatic cancer is slowly evolving. FOLFIRINOX and gemcitabine plus nab-paclitaxel are now considered standard first line treatment options in metastatic pancreatic cancer. Studies are ongoing to investigate the utility of these same regimens in the adjuvant setting. The potential of these treatments to downstage disease is also being actively examined in the locally advanced context since neoadjuvant approaches may improve resection rates and surgical outcomes. As more emerging data become available, the management of pancreatic cancer is anticipated to change significantly in the coming years.

**Key words:** Cancer; Neoplasm; Pancreas; Adjuvant treatment; Systemic treatment; Gemcitabine; FOLFIRINOX; Nab-paclitaxel

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**Core tip:** Pancreatic ductal adenocarcinoma is an infrequent cancer with high disease mortality. The focus on management of the disease has been mainly palliation for the past decade. Recently, the discovery of active multi-agent chemotherapies such as FOLFIRINOX and gemcitabine plus nab-paclitaxel has changed the management of the disease. In our current review, we will highlight some of the advances, particularly with respect to systemic therapy options, in the management of different stages of pancreatic cancer.

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## INTRODUCTION

Pancreatic cancer is an uncommon cancer with 85% of cases being adenocarcinomas arising from the ductal epithelium and the remainder originating from endocrine islet cells. The estimated incidence of pancreatic cancer is 53070 cases per year in the United States<sup>[1]</sup>. The incidence has been increasing slowly, at an average of 0.6% per year over the past decade<sup>[1,2]</sup>. Mortality from pancreatic cancer is high, with a 5-year survival rate of only 8% in all patients, irrespective of stage<sup>[1,2]</sup>. Pancreatic cancer is more common in the Western world. Globally, it is the seventh leading cause of death<sup>[3]</sup>. Until 2004, mortality from pancreatic cancer has remained unchanged, indicating a significant need for novel advances in both detection and treatment of this disease.

Surgical resection is the only potentially curative treatment for pancreatic cancer. However, only about 20% of patients present at a point in time when the disease is still considered resectable. Advances in imaging techniques such as endoscopic ultrasound, magnetic resonance imaging and positron emission tomography can better help identify patients who can be managed possibly with surgery. Improvements in surgical techniques as well as a trend for centralization of care to highly specialized surgical centers have also increased the scope of what is defined as surgically resectable<sup>[4]</sup>. Unfortunately, the 5-year survival rate even among patients with an R0 resection remains poor at about 20%. In the past several years, the discovery of new active systemic therapeutic agents against pancreatic cancer has changed the outlook and paradigm of pancreatic cancer management. While the focus of treatment in the past has been mainly palliation and symptom control, new approaches may now offer survival benefits for patients with either early or advanced pancreatic cancer. In the current review article, we will highlight some of these advances, particularly with respect to systemic therapy options, in the management of pancreatic ductal adenocarcinoma.

## EARLY STAGE PANCREATIC CANCER

Early stage pancreatic cancer with disease localized to the primary site is uncommon at diagnosis (Figure 1)<sup>[5]</sup>. The difficulty in early detection is due in part to the challenges associated with identifying high risk groups and a lack of effective screening strategies. Pancreatic cancer is only weakly associated with risk factors such as chronic pancreatitis<sup>[6-8]</sup>, diabetes mellitus<sup>[9-11]</sup>, obesity<sup>[12,13]</sup>, smoking<sup>[14,15]</sup> and specific genetic syndromes<sup>[16,17]</sup>.

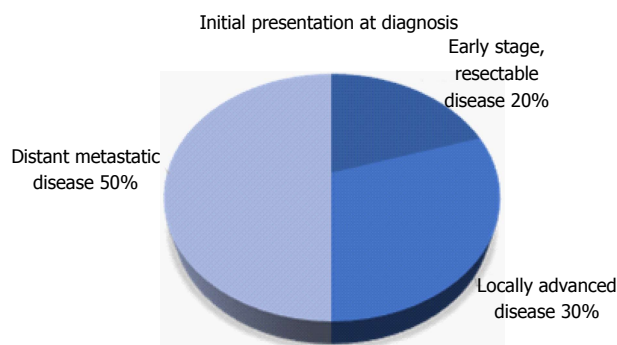


Figure 1 Distribution of stage at time of diagnosis of pancreatic cancer.

## ADJUVANT SYSTEMIC THERAPY

For patients who present sufficiently early to be candidates for surgery, several large randomised trials have demonstrated that adjuvant chemotherapy significantly improves survival outcomes after macroscopic resection of pancreatic cancer. A recent meta-analysis that included ten different studies concluded that adjuvant chemotherapy with 5-fluorouracil (5-FU)/leucovorin (LV) or gemcitabine after resection of pancreatic cancer reduces mortality<sup>[18]</sup>.

Fluoropyrimidine-based regimens were among the first to show activity in the adjuvant setting. In 1993, the combination of 5-FU plus doxorubicin plus mitomycin C in patients with resected pancreatic or ampullary cancers were observed to improve median overall survival (OS) but not 5-year survival rates<sup>[19]</sup>. In the ESPAC-1 study, LV modulated 5-FU adjuvant treatment improved the median overall survival from 14.0 to 19.7 mo (Table 1)<sup>[20]</sup>. The 5-year survival benefit persisted in the chemotherapy group in an updated follow up analysis<sup>[20,21]</sup>. It is important to note that the benefit of chemotherapy in the ESPAC-1 trial may be underestimated since a proportion of patients also received chemoradiation, which has since been shown to pose a detrimental effect on outcomes in this particular trial. As such, a combined analysis of the ESPAC-1 and ESPAC-3 studies was conducted on patients receiving adjuvant 5-FU/LV alone compared to observation<sup>[20-23]</sup>. The results confirmed a statistically significant benefit from receiving 5-FU/LV, with a pooled HR of 0.70<sup>[20-23]</sup>.

Gemcitabine is another agent that improves overall survival in early pancreatic cancer. In the CONKO-001 trial conducted in Germany and Austria, 6 cycles of gemcitabine given weekly compared to observation alone resulted in an improvement in disease free survival (DFS) from 6.9 to 13.4 m<sup>[24]</sup>. An updated analysis of the CONKO-001 study confirmed that the improvement persisted at 5 and 10-years (20.7% vs 10.4% and 12.2% vs 7.7% respectively)<sup>[25]</sup>. The JSAP-02 study was performed around the same time. Unlike the CONKO-001, investigators examined three cycles of adjuvant gemcitabine compared to observation in a Japanese population with resected pancreatic

**Table 1 Summary of adjuvant studies for early stage pancreatic cancer**

Adjuvant systemic therapy									
Study <sup>[20,22-24,26,27,29]</sup>	Treatment	Treatment group				Control group			
		DFS (mo)	OS (mo)	2 yr survival	5 yr survival	DFS (mo)	OS (mo)	2 yr survival	5 yr survival
5-FU based treatments									
ESPAC-1 Neoptolemos <i>et al</i> <sup>[20]</sup> , 2001	5-FU/LV <i>vs</i> observation	-	19.7	-	-	-	14.0	-	-
ESPAC-1 and 3 pooled analysis Neoptolemos <i>et al</i> <sup>[22]</sup> , 2009	5-FU/LV <i>vs</i> observation	-	23.2	49.0%	24.0%	-	16.8	37.0%	14.0%
Gemcitabine based treatments									
CONKO-001, Oettle <i>et al</i> <sup>[24]</sup> , 2007	Gemcitabine <i>vs</i> observation	13.4	22.1	-	16.5%	6.9	20.5	-	5.5%
JSAP-02, Ueno <i>et al</i> <sup>[26]</sup> , 2009	Gemcitabine <i>vs</i> observation	11.4	22.3	48.3%	23.9%	5.0	18.4	40.0%	10.6%
Gemcitabine compared to 5-FU									
ESPAC-3, Neoptolemos <i>et al</i> <sup>[23]</sup> , 2010	Gemcitabine <i>vs</i> 5-FU/LV	14.3	23.6	29.6%	-	14.1	23.0	30.7%	-
RTOG 97-04, Regine <i>et al</i> <sup>[27]</sup> , 2008	Gemcitabine <i>vs</i> 5-FU/LV in patients receiving CRT	-	20.5	-	-	-	16.9	-	-
Combination treatments									
ESPAC-4, Neoptolemos <i>et al</i> <sup>[29]</sup> , 2017	Gemcitabine plus capecitabine <i>vs</i> Gemcitabine	13.9	28.0	53.8%	-	13.1	25.5	52.1%	-

5-FU: 5-fluorouracil; LV: Leucovorin; DFS: Disease free survival; OS: Overall survival.

cancer<sup>[26]</sup>. This study revealed an improvement in DFS (11.4 mo *vs* 5.0 mo), thus providing further evidence for the activity of gemcitabine in this patient population<sup>[26]</sup>.

The activity of 5-FU/LV has been compared directly to gemcitabine in the ESPAC-3 trial<sup>[23]</sup>. It was originally designed as a 3-arm study with a control group, which was subsequently discontinued when evidence showing the benefit of adjuvant chemotherapy became available. This study demonstrated that the median OS for patients treated with 5-FU/LV was 23.0 mo compared to 23.6 mo in patients treated with gemcitabine<sup>[23]</sup>. Patients given gemcitabine had more hematologic adverse events but treatment was generally better tolerated with significantly less grade 3 or 4 toxicities<sup>[23]</sup>. The RTOG-9704 study was designed to compare 5-FU/LV and gemcitabine given before and after receiving concurrent chemoradiotherapy with 5-FU<sup>[27]</sup>. There were no differences in OS between the two groups<sup>[27]</sup>. Grade 4 hematologic adverse effects were significantly worse in the gemcitabine arm of this study, which could be explained by the radiosensitizing effects of gemcitabine. A meta-analysis performed by Yu *et al*<sup>[28]</sup>, which included four of the largest randomized adjuvant pancreatic studies (ESPAC-3, RTOG 9704, CONKO-001 and JSAP-02), found that gemcitabine improved overall survival over the comparator arm of either observation or 5-FU/LV, with a HR 0.88. More importantly, further sensitivity analysis in this meta-analysis indicated that the results remained unchanged even when any one of the studies were removed, thereby providing evidence that the survival improvement was not driven by the placebo arm alone<sup>[28]</sup>. In clinical practice, gemcitabine monotherapy is often preferred due to ease of administration and tolerability.

Because adjuvant chemotherapy offers benefits to some patients, there have been efforts to determine

if intensification of the regimens can increase their effectiveness. The recently published ESPAC-4 study compared a combination of gemcitabine plus capecitabine over gemcitabine alone<sup>[29]</sup>. A larger number of patients included in this study had evidence of nodal disease or locally advanced disease that was deemed upfront resectable. The primary endpoint of OS was significantly improved in the combination group with a median OS of 28.0 mo compared to 25.5 mo in the monotherapy group. Interestingly, there was no difference in the relapse free survival between the two groups. Grade 3-4 adverse events of diarrhea, neutropenia and hand foot syndrome were more common in the gemcitabine plus capecitabine group. However, overall quality of life measures were similar between the two groups. Given the tolerability of gemcitabine plus capecitabine and the demonstrated benefits in survival, combination adjuvant therapy is now considered the standard in clinical settings. Clinical studies are currently underway to examine if there are additional benefits to further treatment intensification. Marsh *et al*<sup>[30]</sup> published preliminary findings of a pilot study where twenty-one patients with early stage pancreatic cancer were given four cycles of modified FOLFIRINOX before and after surgery and found a median OS of 33.4 mo. To this end, regimens such as gemcitabine plus nanoparticle albumin bound paclitaxel (nab-paclitaxel) and a combination of 5-FU, irinotecan and oxaliplatin (FOLFIRINOX) are actively being evaluated in the adjuvant setting.

The ESPAC-4 study also highlights some the challenges with adjuvant systemic treatment in pancreatic cancer patients. Despite most patients having a good performance status at the time of randomization, only 54% and 65% of patients were able to complete all planned cycles of treatment in the gemcitabine plus capecitabine and gemcitabine groups respectively. A neoadjuvant approach with chemotherapy delivered



prior to patients undergoing a major operation may improve rates of systemic treatment completion. Some groups also believe that earlier chemotherapy is important to eradicate micrometastatic disease. The SWOG group is currently recruiting patients with resectable disease to six cycles of neoadjuvant FOLFIRINOX or nine cycles of gemcitabine plus nab-paclitaxel followed by surgical resection<sup>[31]</sup>.

## ADJUVANT CHEMORADIOOTHERAPY

While the benefits of adjuvant chemotherapy are widely accepted and broadly used in clinical practice, the role of adjuvant chemoradiotherapy is more controversial. Prospective evidence regarding the value of chemoradiotherapy is frequently older and underpowered. The GITSG study published in 1985 was one of the first large studies to suggest a benefit of adding radiation to chemotherapy<sup>[32]</sup>. Forty-nine patients were randomized to observation alone or split course radiotherapy to a total of 40 Gy plus concurrent 5-FU. Although median OS was reported to be longer in the chemoradiotherapy group (20 mo vs 11 mo), this study was closed early due to poor accrual and was considered underpowered<sup>[32]</sup>. An updated analysis which included an additional 30 randomized patients revealed similar results<sup>[33]</sup>. The authors concluded that there was a significant survival advantage with the use of adjuvant chemoradiotherapy. As there were some smaller studies with conflicting results published at the same time, the EORTC GI cooperative group pursued another trial with a similar design as the GITSG trial across multiple centers in Europe. Patients were randomized to observation or to the same split course radiotherapy plus infusional 5-FU<sup>[34]</sup>. However, the benefit of chemoradiotherapy seen in this later study was much smaller and only borderline significant<sup>[34]</sup>. In contrast, these authors concluded that there was insufficient evidence to recommend the routine use of chemoradiation after resection of pancreatic cancer<sup>[34]</sup>. Long term follow up of these patient did not identify any differences in outcomes over time<sup>[35]</sup>.

The ESPAC-1 study examined the effect of chemoradiation compared to chemotherapy alone vs observation and concluded that the chemoradiation group had a trend towards worse OS<sup>[20,21]</sup>. A meta-analysis performed by Liao *et al.*<sup>[18]</sup> supported the observation that chemoradiation is less effective than chemotherapy alone. However, the results of this meta-analysis were likely driven by the patients in the ESPAC-1 study. Flaws in the study design of the ESPAC-1 trial, including a pooled analysis of its three different sub-studies, continue to be a major source of controversy. In clinical practice, the patterns of use of chemoradiotherapy differ significantly among clinicians and across cancer centers.

The uncertainty regarding the utility of adjuvant chemoradiotherapy is ongoing. Several contemporary

retrospective studies suggest that there is a survival benefit<sup>[36-39]</sup>. Rutter *et al.*<sup>[36]</sup> reviewed the national cancer database in the United States and identified 6165 patients from 1998 to 2009 who were treated with adjuvant chemotherapy or chemoradiotherapy. The mean radiotherapy dose received was 50.4 Gy, which was higher than the doses used in older prospective studies. They found that chemoradiotherapy was associated with an improved overall survival over chemotherapy alone with an adjusted hazard ratio of 0.89. Although retrospective analyses have their limitations, it is difficult to discount several large population-based studies that suggest a possible survival improvement with chemoradiotherapy. Changes in surgical and modern radiotherapy planning techniques may account for differences in survival over time. New prospective randomized studies that incorporate the use of modern radiation techniques and current chemotherapy regimens are still needed to determine whether adjuvant chemoradiotherapy is actually beneficial.

## LOCALLY ADVANCED PANCREATIC CANCER

About 30% of patients present with non-metastatic locally advanced disease<sup>[40]</sup>. This cohort represents a heterogeneous group of patients whose management differs depending on surgical resectability. Prior to the advent of active systemic therapies, locally advanced tumors were most commonly managed akin to advanced metastatic disease. Gemcitabine, an agent that has been considered the standard of care in distant advanced disease for years, is also used for locally advanced pancreatic cancer<sup>[41]</sup>. One phase II study performed among locally advanced patients reported a median OS of 15 mo<sup>[42]</sup>. Use of multiagent chemotherapy, such as FOLFIRINOX or gemcitabine in combination with other cytotoxic agents, is increasingly common in the first line setting for locally advanced disease albeit there is little prospective evidence. A recent small phase II study along with other observational studies indicate that FOLFIRINOX has a survival benefit in locally advanced disease when compared to historical controls<sup>[43-45]</sup>. A systematic review of studies involving first line FOLFIRINOX in locally pancreatic cancer reported a median overall survival of 24.2 mo<sup>[46]</sup>.

The use of more active systemic treatments has also created the potential that some tumors may be sufficiently downstaged to become resectable. The definitions of locally advanced unresectable disease or borderline resectable disease continue to be vague and highly dependent on surgical expertise and discretion. There is generally a lack of prospective randomized data in this area. Induction chemotherapy is occasionally used in clinical practice and recommended by some consensus-driven guidelines<sup>[47,48]</sup>. There are several

options for systemic therapy with no single regimen being considered the standard. Use of FOLFIRINOX as neoadjuvant therapy is of particular interest given its response rate of 32% in advanced disease<sup>[49]</sup>. Multiple observational analyses on neoadjuvant FOLFIRINOX have been published with encouraging results that show FOLFIRINOX improves R0 resection rates to up to 70% in some studies<sup>[50-52]</sup>. At the current time, there are few published studies examining the use of gemcitabine doublets as neoadjuvant therapy for locally advanced disease. A number of small studies focusing on the neoadjuvant combination of gemcitabine plus oxaliplatin showed that the regimen is feasible, with reports that up to 40%-60% of patients eventually proceed onto surgery<sup>[53,54]</sup>. Gemcitabine in combination with capecitabine or docetaxel have also been described as feasible and potentially effective as neoadjuvant therapy for locally advanced disease<sup>[55,56]</sup>. There is interest in investigating the combination of gemcitabine plus nab-paclitaxel as neoadjuvant treatment given its efficacy in metastatic disease. Early results from observational cohorts suggest a favorable response rate when gemcitabine and nab-paclitaxel was used as induction treatment<sup>[57,58]</sup>.

In contrast to neoadjuvant chemotherapy, the use of concurrent chemoradiotherapy has not been shown to improve survival. The LAP-07 study randomized patients with locally advanced disease to gemcitabine with or without erlotinib for four cycles followed by a second randomization to further chemotherapy or chemoradiation<sup>[59]</sup>. Unfortunately, the study was stopped early due to futility. Concurrent chemoradiotherapy did not show any survival benefits over chemotherapy alone. It is still unclear whether the addition of radiotherapy improves surgical outcomes. Thus, there is continued interest in studying whether radiotherapy after multi-agent induction chemotherapy would improve the rates of R0 resection<sup>[60-62]</sup>. Katz *et al.*<sup>[60]</sup> investigated the combination of modified FOLFIRINOX for 4 cycles followed by concurrent chemoradiation with capecitabine in 22 patients with borderline resectable disease and reported that 60% of patients received a surgical resection with 93% of those achieving an R0 resection.

## ADVANCED PANCREATIC CANCER

More than 50% of patients present with advanced stage disease and experience a dismal prognosis. Patients with locally advanced unresectable disease and distant metastatic disease are frequently treated in a similar fashion. Until recently, single agent chemotherapy was the mainstay of treatment offering only a very modest benefit in survival. Newer approaches with combination chemotherapy have finally shown an improvement in survival when compared to monotherapy.

Before the introduction of combination treatment, gemcitabine monotherapy was the cornerstone of

treatment. At present, it remains the standard first line option for patients with poor performance status who are unable to tolerate combination chemotherapy. In 1997, a phase III trial was published which compared gemcitabine to 5-FU, the latter of which was the standard therapy based on studies in the 1950-1960s with highly variable results (Table 2)<sup>[41]</sup>. The primary endpoint of the trial was clinical benefit, defined as a sustained improvement in symptoms related to pancreatic cancer, which was significantly better in the gemcitabine arm. Secondary endpoints of survival were also improved with median OS of 5.7 mo in the gemcitabine group compared to 4.4 mo in the 5-FU group. Based on results of this trial, gemcitabine became the standard of care for advanced disease for the subsequent 20 years.

There were multiple attempts to combine gemcitabine with other agents to improve survival. Studies involving gemcitabine plus 5-FU, capecitabine, and S1 uniformly failed to demonstrate benefit over gemcitabine alone<sup>[63-65]</sup>. Results of gemcitabine in combination with newer agents targeting the EGFR or VEGF pathway were also disappointing. A phase III study combining gemcitabine plus erlotinib did show a modest improvement in survival by 2 wk<sup>[66]</sup>. However, this regimen has not been widely accepted into clinical practice because the magnitude of benefit was marginal. Furthermore, a study using a combination of gemcitabine and cetuximab, a monoclonal antibody against EGFR, failed to demonstrate any benefit over gemcitabine alone<sup>[67]</sup>. Likewise, gemcitabine plus bevacizumab in a phase III study also failed to show a survival benefit over gemcitabine alone<sup>[68]</sup>.

Because treatment results from initial gemcitabine doublets were generally disappointing, investigations into other active agents were made. Agents such as 5-FU, irinotecan and oxaliplatin have shown activity in pancreatic cancer and a combination of these three were shown to be safe in phase I studies<sup>[69]</sup>. As such, a phase II/III trial was conducted to study the effects of FOLFIRINOX compared to standard gemcitabine monotherapy<sup>[49]</sup>. Surprisingly, the results demonstrated a significant overall survival advantage of 11.0 mo compared to 6.8 mo in the gemcitabine group. Quality of life measured at 6 mo was also significantly better in the FOLFIRINOX group, likely secondary to better disease control. However, toxicity is greater in the FOLFIRINOX group and patients included in the study were required to have a baseline ECOG performance of 0-1. FOLFIRINOX is now considered a first line option in patients with unresectable or advanced pancreatic cancer with a good performance status.

In contrast to other gemcitabine doublets, a recent study demonstrated a clinically significant antitumor effect when gemcitabine was combined with nab-paclitaxel. Molecular profiling of pancreatic cancer show that the tumor often overexpresses an albumin-binding protein suggesting that this formulation may increase

**Table 2 Summary of first line studies for advanced pancreatic cancer**

First line treatment for metastatic disease		Treatment group				Control group			
Study <sup>[41,49,63-67,71]</sup>	Treatment	ORR	PFS (mo)	OS (mo)	1 yr Survival	ORR	PFS (mo)	OS (mo)	1 yr Survival
Standard of care									
Burris <i>et al</i> <sup>[41]</sup> , 1997	Gemcitabine <i>vs</i> 5-FU/LV	-	9 wk	5.65	18.0%	-	4	4.01	2.0%
Conroy <i>et al</i> <sup>[49]</sup> , 2011	FOLFIRINOX <i>vs</i> Gemcitabine	31.6%	6.4	11.1	48.4%	9.4%	3.3	6.8	20.6%
Von Hoff <i>et al</i> <sup>[71]</sup> , 2013	Nab-paclitaxel plus gemcitabine <i>vs</i> gemcitabine	23.0%	5.5	8.5	35.0%	7.0%	3.7	6.7	22.0%
Gemcitabine doublets									
Berlin <i>et al</i> <sup>[63]</sup> , 2002	Gemcitabine plus 5-FU <i>vs</i> gemcitabine	6.9%	3.4	6.7	-	5.6%	2.2	5.4	-
Herrmann <i>et al</i> <sup>[64]</sup> , 2007	Gemcitabine plus capecitabine <i>vs</i> gemcitabine	10.0%	4.3	8.4	32.0%	7.8%	3.9	7.2	30.0%
Moore <i>et al</i> <sup>[66]</sup> , 2007	Gemcitabine plus erlotinib <i>vs</i> gemcitabine	8.6%	3.8	6.2	23.0%	8.0%	3.6	5.9	17.0%
Philip <i>et al</i> <sup>[67]</sup> , 2010	Gemcitabine plus cetuximab <i>vs</i> gemcitabine	12.0%	3.4	6.3	-	14.0%	3.0	5.9	-
Ueno <i>et al</i> <sup>[65]</sup> , 2013	Gemcitabine plus S1 <i>vs</i> gemcitabine	29.3%	5.7	10.1	40.7%	13.3%	4.1	8.8	35.4%

5-FU: 5-fluorouracil; LV: Leucovorin; DFS: Disease free survival; OS: Overall survival; ORR: Overall response rate.

the intratumoral concentrations of gemcitabine<sup>[70]</sup>. The phase III data published in 2013 described that the combination of nab-paclitaxel plus gemcitabine was superior to gemcitabine alone with a median OS of 8.5 mo *vs* 6.7 mo<sup>[71]</sup>. The superiority in survival persisted with long term follow up at 3 years<sup>[72]</sup>. The combination of gemcitabine plus nab-paclitaxel has also been recently approved for first line treatment of advanced pancreatic cancer.

There are currently no studies that directly compare the activity of FOLFIRINOX to gemcitabine plus nab-paclitaxel and both are approved for use in the first line setting. In clinical practice, the choice of regimen is often dependent on the toxicity profiles. FOLFIRINOX has more toxicities and is usually reserved for patients with good performance status. Gemcitabine plus nab-paclitaxel has been studied in patients with a KPS  $\geq$  70, which approximates ECOG 2. Population based studies revealed that few real world patients actually meet the eligibility criteria used in the clinical trials with only about 25% of patients able to receive FOLFIRINOX and 45% able to receive gemcitabine plus nab-paclitaxel<sup>[73,74]</sup>. In patients with borderline performance who may not be able to tolerate combination cytotoxic therapy, gemcitabine monotherapy remains an option. Unfortunately, there are limited data from large prospective randomized data investigating second line therapies upon progression. With the use of more active first line treatments, patients are now faring better to the degree that warrants consideration of second line therapy. Nonetheless, second line treatment represents an area of clinical unmet need. Systemic therapy is still often used for patients with good performance status who wishes to receive treatment. Agents that are considered active in pancreatic cancer such as 5-FU, oxaliplatin, irinotecan and gemcitabine are

reasonable to be used in the second line setting with no single regimen that can be currently considered as the standard of care. Retrospective studies suggest that use of second line therapies is feasible with a potential survival benefit<sup>[75]</sup>. Patients enrolled into the MPACT study were followed prospectively and results were published on the outcomes of second line therapy<sup>[76]</sup>. The authors reported a significant benefit to receiving any second line therapy with an adjusted hazard ratio of 0.47<sup>[76]</sup>. However, the total number of patients was small and results may be confounded. The combination of 5-FU/LV and oxaliplatin has been studied in two phase III trials with conflicting results. The German CONKO study group conducted a trial comparing FF (weekly infusional 5-FU and folinic acid) to OFF (oxaliplatin 85 mg/m<sup>2</sup> on days 8 and 22 plus FF followed by a 2 wk break) in patients who progressed after first line gemcitabine monotherapy<sup>[77,78]</sup>. A significant benefit was seen in the OFF group with a median OS of 5.9 mo compared to 3.3 mo<sup>[77,78]</sup>. The PANCREOX study performed by the Canadian group compared second line biweekly bolus plus infusional 5-FU/LV to mFOLFOX6 (biweekly bolus plus infusional 5-FU/LV plus oxaliplatin 85 mg/m<sup>2</sup>). Contrary to the findings in the German study, patients receiving mFOLFOX6 suffered an inferior survival with more toxicity compared to 5-FU/LV alone (6.1 mo *vs* 9.9 mo)<sup>[79]</sup>. Conflicting results of the two studies may be explained by differences in the inclusion criteria and treatment regimens. The NAPOLI-1 study is a phase III trial investigating the use of nanoliposomal irinotecan with or without 5-FU/LV compared to 5-FU/LV alone in heavily pretreated patients<sup>[80]</sup>. The study demonstrated a median OS of 6.1 mo in patients who received nanoliposomal irinotecan plus 5-FU/LV compared to 4.2 mo in patients receiving 5-FU/LV alone. This combination may become the

standard second line treatment in the future.

## FUTURE DIRECTIONS

The outcomes of pancreatic cancer remain poor despite recent advances. Therefore, research into novel and different ways of targeting this tumor is still ongoing.

One of the reasons why pancreatic cancer is so difficult to treat with conventional cytotoxic therapy is thought to be related to the desmoplastic response in tumor stroma, which promotes tumor growth and compromises chemotherapy delivery<sup>[81-83]</sup>. The JAK/STAT signalling transduction pathway mediates the tumor and host inflammatory response. Ruxolitinib, a JAK inhibitor, in combination with capecitabine has demonstrated efficacy in patients who progressed after gemcitabine in a phase II study<sup>[84]</sup>. The intense stromal reaction is also often associated with tissue hypoxia. Evofosfamide, a prodrug activated under hypoxic conditions could increase drug delivery to the tumor. Unfortunately, the phase III results did not show a survival benefit<sup>[85]</sup>. Pancreatic cancer stroma has also been shown to accumulate hyaluronan and a novel approach using a recombinant human hyaluronidase together with gemcitabine and nab-paclitaxel has shown promising preliminary results, specifically improving response rates and progression free survival in the phase II setting<sup>[86]</sup>. Ibrutinib, an agent commonly used in the treatment of chronic lymphocytic leukemia is thought to inhibit mast cell degranulation in the stroma and subsequent angiogenesis and collagen deposition. This agent is also being investigated<sup>[87]</sup>.

Molecular profiling may further help us gain a better understanding of the molecular pathways in pancreatic cancer<sup>[88,89]</sup>. While mutations in KRAS, TP53 and CDKN2A are common in pancreatic cancer, they have proven to be challenging to target. However, there is mounting evidence of genomic alterations in TGF- $\beta$  signaling and studies investigating the utility of TGF- $\beta$  inhibitors are actively underway<sup>[90]</sup>.

The identification of specific subtypes of pancreatic cancers or special patient populations based on molecular profiles is a significant area of interest. For example, the presence of microsatellite instability may predict response to immunotherapy even though it has not been shown to be a very active type of treatment in an unselected population of pancreatic cancer. A special group of patients are those with mutations in BRCA-1/2. Emerging data from other cancer sites associated with BRCA mutations such as breast and ovarian cancer suggest hypersensitivity to platinum agents<sup>[91-95]</sup>. Oxaliplatin has already demonstrated activity in pancreatic cancer<sup>[49]</sup>, but it is unknown if BRCA mutated patients will demonstrate a superior response compared to an unselected population. PARP inhibitors have been shown to improve treatments outcomes in BRCA mutated ovarian cancer. A germline mutation in BRCA-2 is known to be correlated with the development of pancreatic cancer, but the prevalence

is unknown. It has been reported that up to 5%-9% of pancreatic cancer patients harbor the mutation<sup>[96,97]</sup>. Studies of PARP inhibitors in BRCA mutated pancreatic cancer patients are in development with some early data indicating promising efficacy<sup>[97,98]</sup>.

## CONCLUSION

Pancreatic cancer is a systemic disease since even the majority of patients with early disease eventually develop metastases. While gemcitabine poses some anti-tumor activity and improves survival in the adjuvant setting, the focus of management for most patients with pancreatic cancer has, to date, been palliative. The discovery of active multi-agent chemotherapy regimens such as FOLFIRINOX and gemcitabine plus nab-paclitaxel has changed the recent landscape in the management of this disease in many aspects. In early stage disease, multi-agent chemotherapies are being investigated for their potential benefit in overall survival. The PRODIGE and APACT studies are ongoing and hopefully will provide us with new data in the next several years. The potential for multi-agent chemotherapy to downstage locally advanced disease to improve resection rates is a significant area of interest. In fit patients with metastatic disease who can tolerate combination treatment, FOLFIRINOX as well as gemcitabine plus nab-paclitaxel are considered standards of care. Advances in molecular profiling and gene sequencing will likely help us better understand the biology of pancreatic cancer. Novel targets for drug development as well as new methods of drug delivery are areas of active clinical research. Finally, identification of specific subgroups of patients such as BRCA mutation carriers may also allow clinicians to better individualize care for future patients.

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## Clinical significance of tumor-infiltrating lymphocytes for gastric cancer in the era of immunology

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### Abstract

Immunotherapy has begun to revolutionize cancer treatment, by introducing therapies that target the host immune system instead of the tumor, therapies that possess unique adverse event profiles, and therapies that may cure certain types of cancer. The immune microenvironment of tumors is emerging as the most important means of understanding the relationship between a patient's immune system and their cancer, informing prognosis, and guiding immunotherapy, such as an antibody blockade of immune checkpoints. For some solid tumors, simple quantitation of lymphocyte infiltration would seem to have prognostic significance, suggesting that lymphocyte infiltration is not passive but may actively promote or inhibit tumor growth. For gastric cancers, several studies have provided strong evidence that immune cells contribute to determining prognosis. However, the exact role of immune cells in gastric cancer remains unclear. Therefore, this review focuses on the clinical significance of immune cells, especially tumor-infiltrating lymphocytes, in gastric cancer.

**Key words:** Gastric cancer; Tumor-infiltrating lymphocytes; Immunotherapy

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**Core tip:** Tumor-infiltrating lymphocytes (TILs) are considered a manifestation of the host immune response against tumor cells, and several studies have already reported the potential of TILs as a prognostic parameter for various human malignancies. However, only a few studies have investigated the prognostic impact of TILs in gastric cancer. Based on a comprehensive molecular characterization of gastric cancer, TILs could be a potential biomarker. Accordingly, this review focuses on the clinical significance of immune cells, especially TILs, in gastric cancer.

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## INTRODUCTION

Gastric cancer is a major public health issue and the leading cause of cancer-related deaths. Despite numerous advances in treatment options, the prognosis for gastric cancer remains dismal, as most patients are in an advanced stage at the time of diagnosis<sup>[1]</sup>. To improve the survival outcome, a better understanding of the mechanisms of disease progression is crucial, along with elucidating effective predictive or prognostic factors as therapeutic targets. Yet, while many predictive factors have already been evaluated, including clinicopathologic factors, biomarkers, genes, and microsatellite instability, their prognostic accuracies remain controversial<sup>[2]</sup>.

Meantime, immunotherapy has begun to revolutionize cancer treatment by introducing therapies that target the host immune system rather than the tumor, therapies that possess unique adverse event profiles, and therapies that may even cure certain types of cancer. Thus, the immune microenvironment of tumors is emerging as the most important means of understanding the relationship between a patient's immune system and their cancer, informing prognosis, and guiding immunotherapy, such as an antibody blockade of immune checkpoints<sup>[3]</sup>. For some solid tumors, simple quantitation of lymphocyte infiltration would seem to have prognostic significance, suggesting that lymphocyte infiltration is not passive but may actively promote or inhibit tumor growth<sup>[3]</sup>. For example, a meta-analysis showed a significant correlation between tumor-infiltrating lymphocytes (TILs) and clinical traits in breast cancer patients. Thus, higher value of total TILs not only predicts a neoadjuvant chemotherapy response, but also implies a better prognosis<sup>[4]</sup>. For gastric cancers, several studies have provided strong evidence that immune cells contribute to determining the prognosis. It has been reported that regulatory T cells can play a role of immunosuppression and tumor progression in patients with gastric cancer, leading to a worse prognosis<sup>[5]</sup>. Plus, an intratumoral high regulatory T cell/CD8<sup>+</sup> T cell ratio has been shown as an independent predictor of a poor prognosis for gastric cancer<sup>[6]</sup>. However, the exact role of immune cells in gastric cancer remains unclear. Accordingly, this review focuses on the clinical significance of immune cells, especially TILs, in gastric cancer.

## TILS IN GASTRIC CANCER

### *Cancer immunity and the role of TILs*

The evolution of cancers reflects intricate cellular and

molecular interactions between tumor cells and constituents of the tumor microenvironment<sup>[7]</sup>. In the first step, neoantigens created by oncogenesis are released and captured by dendritic cells for processing. Next, dendritic cells present the captured antigens on major histocompatibility class (MHC) molecules to T cells, resulting in the priming and activation of effector T cell responses against the cancer-specific antigens. Finally, the activated T cells toward to and infiltrate the tumor bed, and destroy their target cancer cells<sup>[8]</sup>. These may be occurred in the tumor core, invasive margin, or adjacent tumor stroma. The functional activity of lymphoid infiltrates, such as T cells, B cells, and natural killer (NK) cells, depends upon MHC complexes or surface antigen that can be recognized specific manner. These cells can be induced to secrete different types of cytokines based on effector functions<sup>[9]</sup>. Many cytokines also have the potential to enhance nonspecific inflammatory responses which by themselves may have anti-tumor activity. Plus, the potential of various cytokines to enhance both specific and innate immune responses against tumors has been demonstrated in experimental models and has been realized in clinical practice<sup>[10]</sup>. Surprisingly, this process is highly regulated through various genes, such as STAT3, High-mobility group protein B1, calreticulin, and endothelial cell adhesion protein<sup>[11]</sup>. Thus, TILs are incorporated into these multi-factorial interactions and their presence has proved to be a major determinant of tumor characteristics and patient outcome.

### *Stromal TILs and intratumoral TILs*

Several recent studies have evaluated the prognostic and predictive importance of TILs in gastric cancer<sup>[12]</sup>. TILs are the major type of infiltrating immune cells, and are represented by T cells, B cells, and NK cells. These cells can infiltrate stroma and tumor cells, and are considered a manifestation of the host immune response against tumor cells<sup>[13]</sup>. Previous studies of TILs in gastric cancer have evaluated stromal and intratumoral lymphocytes separately, where a visual assessment of standard hematoxylin and eosin (H and E)-stained sections is the most commonly used approach to measure TILs<sup>[3,14]</sup>. Based on a histopathologic analysis of TILs using H and E-stained slides, Kang *et al.*<sup>[15]</sup> suggested that stromal TILs can be defined as a tumor stroma area containing infiltrating mononuclear inflammatory cells, while intratumoral TILs can be defined as intraepithelial lymphocytes or mononuclear cells within tumor cells. As a result, they documented that stromal TILs can be used to predict recurrence-free survival (RFS) and disease-free survival (DFS). In contrast, another study found that increasing intratumoral TILs was significantly associated with improved cancer-specific survival (CSS)<sup>[16]</sup> (Table 1). In fact, stromal TILs are well known as a superior and more reproducible parameter in breast cancer<sup>[14]</sup>. Notwithstanding, there is no current consensus on the

**Table 1** Tumor-infiltrating lymphocytes associated with the prognosis of gastric cancer

Ref.	Sample size	Patient group	Location	Criteria (cut-off)	Prognostic role
Kang <i>et al</i> <sup>[15]</sup>	120	EBVaGC	Stromal	High infiltration	Decreased DFS and RFS
Grogg <i>et al</i> <sup>[16]</sup>	110	G	Intratumoral	High infiltration	Increased CSS
Lee <i>et al</i> <sup>[17]</sup>	220	G	Intratumoral	High density	Increased OS

EBVaGC: Epstein-Barr virus-associated gastric cancer; DFS: Disease-free survival; RFS: Recurrence-free survival; G: Gastric cancer; CSS: Cancer-specific survival; OS: Overall-survival.

**Table 2** Lymphocyte subtypes associated with the prognosis of gastric cancer

Ref.	Lymphocyte subtypes	Sample size	Patient group	Criteria (cut-off)	Prognostic role
Lee <i>et al</i> <sup>[17]</sup>	CD3 <sup>+</sup> , CD8 <sup>+</sup> , CD45RO <sup>+</sup>	220	G	High density	Increased OS
Thompson <i>et al</i> <sup>[18]</sup>	CD8 <sup>+</sup>	43	G/GEJ	High density	Decreased PFS and OS
Kawazoe <i>et al</i> <sup>[33]</sup>	CD8 <sup>+</sup>	487	G	High density	Increased OS
Wakatsuki <i>et al</i> <sup>[30]</sup>	CD45RO <sup>+</sup>	101	G	High numbers	Increased PFS and OS
Chiaravalli <i>et al</i> <sup>[34]</sup>	CD3 <sup>+</sup> , CD8 <sup>+</sup>	96	MSI-H G	High numbers	Increased OS
Kim <i>et al</i> <sup>[22]</sup>	CD8 <sup>+</sup> , FOXP3 <sup>+</sup>	99	MSI-H G	High density	Increased OS
Liu <i>et al</i> <sup>[23]</sup>	CD8 <sup>+</sup> /FOXP3 <sup>+</sup> ratio	166	G	High ratio	Increased OS
Shen <i>et al</i> <sup>[26]</sup>	FOXP3 <sup>+</sup> /CD8 <sup>+</sup> ratio	133	G	High ratio	Decreased OS
Wang <i>et al</i> <sup>[5]</sup>	FOXP3 <sup>+</sup>	107	G	High expression	Increased OS
Haas <i>et al</i> <sup>[20]</sup>	FOXP3 <sup>+</sup>	52	G	High numbers	Increased OS
Mizukami <i>et al</i> <sup>[24]</sup>	FOXP3 <sup>+</sup>	120	G	Diffuse pattern	Decreased OS
Perrone <i>et al</i> <sup>[25]</sup>	FOXP3 <sup>+</sup>	110	G	High numbers	Decreased RFS and OS
Zhou <i>et al</i> <sup>[27]</sup>	FOXP3 <sup>+</sup>	133	G	High numbers	Decreased OS
Choi <i>et al</i> <sup>[19]</sup>	FOXP3 <sup>+</sup> /CD4 <sup>+</sup> ratio	28	G	High ratio	Increased OS
Kim <i>et al</i> <sup>[21]</sup>	FOXP3 <sup>+</sup> /CD4 <sup>+</sup> ratio	180	G	High ratio	Decreased OS
Dong <i>et al</i> <sup>[35]</sup>	CD20 <sup>+</sup>	100	G	High density	Increased OS
Ishigami <i>et al</i> <sup>[31]</sup>	NK cells	146	G	High numbers	Increased OS
Rosso <i>et al</i> <sup>[36]</sup>	NK cells	72	G	High concentration	Increased DFS and OS
Ishigami <i>et al</i> <sup>[37]</sup>	NK cells	169	G	High numbers	Increased OS
Ubukata <i>et al</i> <sup>[28]</sup>	Th1/Th2 ratio	157	G	High ratio <sup>1</sup>	Increased OS
Liu <i>et al</i> <sup>[29]</sup>	Th22, Th17	32	G	High numbers <sup>1</sup>	Decreased OS

<sup>1</sup>Peripheral blood. G: Gastric cancer; OS: Overall-survival; G/GEJ: Gastric/gastro-esophageal junction cancer; PFS: Progression-free survival; MSI-H: Microsatellite instability-high; RFS: Relapse-free survival; DFS: Disease-free survival.

best TILs distribution for predicting survival in gastric cancer. Therefore, the methodology of interpreting TILs and cut-off values for gastric cancer needs to be standardized.

### Composition of TILs and their clinical significance

TILs are represented by T cells, B cells, and NK cells. The subset of T cells include CD8<sup>+</sup> cytotoxic T cells, CD4<sup>+</sup> T helper cells, CD45RO<sup>+</sup> memory T cells, FOXP3<sup>+</sup> regulatory T cells, and NK cells<sup>[12]</sup>. In gastric cancer, the prognostic role of each lymphocyte is summarized in the Table 2<sup>[5,17-38]</sup>. A high-density of CD3<sup>+</sup>, CD8<sup>+</sup>, and CD45RO<sup>+</sup> cells has been strongly associated with patient survival and regional lymph node metastasis<sup>[17]</sup>. Recently, Thompson *et al*<sup>[18]</sup> reported that the increasing CD8<sup>+</sup> infiltration was correlated with impaired survival and higher programmed death-ligand 1 (PD-L1) expression, indicating an adaptive immune resistance mechanism. Meanwhile, the presence of FOXP3<sup>+</sup> regulatory T cells has been associated with both good and bad prognosis<sup>[5,19-27]</sup>. Among the other CD4<sup>+</sup> T cell subpopulations, a high T helper 1/T helper 2 ratio has been implicated as a favorable prognostic factor in gastric cancer<sup>[28]</sup>. T helper 17 and T helper

22 cells, producers of proinflammatory interleukin, also appear to have an effect on tumor progression in gastric cancer, while high CD45RO<sup>+</sup> memory T cells are associated with better survival of gastric cancer patients<sup>[29,30]</sup>. Furthermore, the precise role of B cells and NK cells is currently not well defined and remains controversial<sup>[31,32]</sup>.

### Impact of TILs on subtypes of gastric cancer

The Cancer Genome Atlas Research Network recently provided a comprehensive molecular characterization of 295 gastric cancers using various platforms, and proposed four distinct subtypes, as follows: Epstein-Barr virus (EBV)-positive tumors, microsatellite unstable tumors, genomically stable tumors, and tumors with chromosomal instability<sup>[38]</sup>. Among these, EBV-positive tumors and microsatellite unstable tumors often show immune cell signaling activation. Therefore, these findings point to the possibility of TILs as prognostic and predictive markers in gastric cancer patients with EBV or mismatch repair-deficient tumors, suggesting the pivotal role of the immune mechanism in these subsets of gastric cancer. Significant correlations have also been found between microsatellite instability (MSI)



and TIL positivity<sup>[39]</sup>. Plus, higher densities of both CD8<sup>+</sup> and FOXP3<sup>+</sup> TILs have been associated with good prognosis in MSI-high gastric cancer<sup>[22]</sup>. Interestingly, Chiaravalli *et al.*<sup>[34]</sup> reported that a high number of CD3<sup>+</sup> and CD8<sup>+</sup> TILs is a characteristic of gastric cancer with MSI and EBV, correlating with a favorable prognosis. In a separate study, MSI and EBV tumors showed significantly increased TILs compared with non-MSI and non-EBV tumors, and the number of TILs was significantly associated with CSS in EBV tumors<sup>[16]</sup>. Meanwhile, recent data showed an independent association between high TILs and favorable RFS or DFS in 120 patients with EBV-associated gastric cancer (EBVaGC), suggesting that TILs exhibit a host cellular immune response against tumors and immunotherapy may have a potential role in patients with EBVaGC<sup>[15]</sup>. Plus, although their mechanisms and effects on cancer are still unknown, previous reports have indicated that local triggering of cellular immune responses, like activated cytotoxic T cells in EBVaGC, prevents lymph node metastasis, and various molecules, such as chemokines, interleukins, integrins, and adhesion molecules, may contribute to immune surveillance and immunogenic apoptosis<sup>[11,40]</sup>.

### **Roles of programmed cell death protein in immune cells of gastric cancer**

Immune evasion is now recognized to play a key role in carcinogenesis. The strong growth potential and invasive nature of malignant tumors are at least partially attributed to the ability of the tumor cells to escape the host immune surveillance<sup>[41]</sup>. In particular, the effector T-lymphocyte recognizes the tumor cell through interaction between the T-cell receptor and MHC on the tumor cell. After the immune response has been mounted, the tumor is able to express PD-L1 on its surface. The subsequent binding between PD-L1 and programmed cell death-1 (PD-1) will shut down the immune response and allow the tumor cells to escape death<sup>[8]</sup>. PD-1, which belongs to the CD28 family of proteins, is a receptor expressed on a number of immune cells, including T cells, B cells, monocytes, NK cells, and dendritic cells. It has two ligands, PD-L1 and PD-L2. PD-L1 is broadly expressed<sup>[42]</sup>. Several studies have already demonstrated that PD-L1 or PD-1 is highly expressed on tumor cells in gastric cancer patients<sup>[43-46]</sup>. A recent study reported that 53.8% of patients were positive for PD-1 expression which was mainly restricted to TILs and 30.1% were positive for PD-L1 expression in the tumor cells, respectively<sup>[47]</sup>. Although expression of PD-L1 and PD-1 in gastric cancer is closely linked to the prognosis, the results remain inconsistent<sup>[41]</sup>. A recent meta-analysis by Zhang *et al.*<sup>[48]</sup> evaluated the prognostic value of PD-L1 in gastric cancer. Based on 1,901 patients in 10 studies, the final hazard ratio for overall-survival (OS) of 1.64 showed a significant difference in terms of PD-L1 expression (95%CI: 1.11-2.43, *P* = 0.01). Interestingly, this meta-analysis indicated that PD-L1

had no correlation with gender, age, cancer location, differentiation, depth of invasion, and tumor stage. Therefore, this study provided evidence to support benefit from targeted therapy against PD-L1 in the case of gastric cancer. Indeed, we already evaluated the tissue samples that were obtained from patients included in a previous study of EBVaGC<sup>[15]</sup>. We found that intratumoral PD-L1 was significantly associated with DFS in these patients group. These observations have given rise to the hypothesis that specific inhibitors for PD-L1 or PD-1 would be potential therapeutic candidates that can affect a variety of gastric cancer.

Several therapeutic antibodies against this pathway have been developed and clinical trials are ongoing. KEYNOTE-012 was a phase 1b that evaluated pembrolizumab, a humanized IgG4 monoclonal antibody against PD-1, in patients with PD-L1-positive recurrent or metastatic adenocarcinoma of the stomach or gastro-oesophageal junction. In this trial, pembrolizumab had a 22% response rate and manageable toxicity<sup>[49]</sup>. Recently, nivolumab, a human IgG4 anti-PD-1 monoclonal antibody, has been clinically explored following the failure of standard of care. This trial (ONO-4538-12), which compared nivolumab to placebo in patients with unresectable advanced or recurrent gastric cancer, including gastroesophageal junction cancer, refractory to, or intolerant of, standard therapy, also showed a significantly prolonged OS for the nivolumab arm<sup>[50]</sup>. Therefore, the success of these agents has prompted its clinical investigation in a first-line setting and clinical trials for first-line treatment are now ongoing.

## **CONCLUSION**

This article summarized the association of TILs with the prognosis of gastric cancer. While TILs can be easily detected by analyzing slides of tumor sections stained with H&E, methodologic improvements are needed for more accurate determining the density and distribution of immune effectors within and around gastric cancer cells. With the development of more precise methods for analyzing immune infiltrates, it is becoming clearer that distinct infiltrating cell types have different prognostic and predictive significance. In particular, the presence of TILs may be an important biomarker for the treatment of TIL-rich tumors, such as EBV-positive or MSI-high gastric cancer, while immunotherapy including an immune checkpoint blockade can become an important part of the cancer armamentarium. Plus, specific inhibitors for PD-L1 or PD-1 would be potential therapeutic candidates that can affect a variety of gastric cancer. Therefore, understanding the effect of TILs on the natural outcome of gastric cancer will herald new opportunities for personalized therapy.

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## Retrospective Cohort Study

**Prognostic efficacy of inflammation-based markers in patients with curative colorectal cancer resection**

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**Abstract****AIM**

To evaluate the prognostic significance of neutrophil-to-lymphocyte ratio (NLR), platelet-to-lymphocyte ratio (PLR), lymphocyte-to-monocyte ratio (LMR), and prognostic nutritional index (PNI) and other clinico-pathological factors in patients undergoing curative resection of colon cancer.

**METHODS**

183 patients with histologically proven colorectal cancer who had undergone potentially curative resection between 2010 and 2016 at Ankara Numune Training and Research Hospital were retrospectively analyzed and clinicopathological characteristics included age, sex, tumor type, grade, size and localization, the number of metastatic and total number of lymph nodes removed, vascular and perineural invasion of the tumor, TNM stages, tumor marker levels (CEA, CA19-9, AFP, CA-125, CA15-3), complete blood counts, albumin levels, overall survival (months), NLR, PLR, LMR and PNI ratios were retrospectively reviewed and analyzed from the electronic database. The primary outcome measure was overall survival.

**RESULTS**

Regarding overall survival, on univariate analysis the following variables were significantly associated with poor outcome following resection: T-stage ( $P = 0.037$ ), lymph node invasion ( $P = 0.037$ ), cancer stage ( $P = 0.034$ ), CEA ( $P = 0.042$ ), CA19-9 ( $P = 0.004$ ), and PNI

( $P = 0.001$ ). To evaluate the independent prognostic value, multivariate Cox proportional hazard analysis to control for other prognostic factors was used. Using cancer-specific death as an end point for NLR, PLR, LMR, PNI and CA19-9 the optimal cut off values were calculated by ROC analysis. Regarding overall survival, on multivariate analysis high CA19-9 (HR = 1.001, 95%CI: 1.00-1.002,  $P = 0.012$ ) and low PNI (HR = 0.938, 95%CI: 0.891-0.987,  $P = 0.014$ ) were the only variables independently associated with shortened overall survival. Patients with a PNI < 35 had a median OS of 52.25 mo. In contrast, patients with an PNI > 35 had a median OS of 66 mo. Patients with a CA 19-9 < 17 had a median OS of 66 mo and in patients with a CA19-9 > 17 had a median OS of 53.76 mo.

### CONCLUSION

This study shows that decrease in the PNI and increase in CA 19-9 is associated with poor survival in patients with resectable colon cancer.

**Key words:** Colorectal cancer; Prognosis; Overall survival; Prognostic nutritional index; CA19-9

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**Core tip:** Predictors of colorectal cancer (CRC) that may determine overall survival are extremely important. Inflammation is now widely recognised to be a key element of disease advancement and survival in CRC. Aim of this study was to evaluate the prognostic significance of clinicopathologic factors and the indicators of systematic inflammatory response by using neutrophil-to-lymphocyte ratio, platelet-to-lymphocyte ratio, lymphocyte-to-monocyte ratio, and prognostic nutritional index in patients undergoing curative resection of colon cancer. In the present study, we report, for the first time, a longitudinal comparison of the four systemic inflammation-based prognostic scores and CA19-9 in patients with resectable CRC.

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### INTRODUCTION

Colorectal cancer (CRC) is the third most common malignancy and the fourth leading cause of cancer-related deaths worldwide. Five-year overall survival (OS) remains unsatisfactory due to local recurrence or metastasis<sup>[1]</sup>. Predictors that may determine overall survival and patient outcome are extremely important, as the capability to distinguish patients more likely to

acquire poor outcome following surgery would enable surgical and chemotherapeutic treatment personalized appropriately for each individual case<sup>[2]</sup>. To date, prognostic factors, such as tumor, node, and metastasis (TNM) stage, cell differentiation grade, Dukes' stage, tumor type, have commonly been applied<sup>[3]</sup>. The TNM stage system is a gold standard for clinical management as it aids in directing the clinician towards appropriate treatment selection and also predicts the patient's prognosis. However, what the current system lacks is the ability to predict the response and outcome individually<sup>[4]</sup>. For this reason the development of economical and readily available prognostic markers for risk determination in CRC treatment, is a must to thereby deliver a more personalized form of cancer care<sup>[5]</sup>. Recent studies have clearly shown the relationship between host inflammatory response to tumor carcinogenesis and the important role it plays in cancer development, progression and metastasis<sup>[6,7]</sup>.

Since the first observation by Virchow who described the link between inflammation and tumorigenesis, the impact of inflammation on cancer development has been shown in an extensive number of tissues through processes that involve genomic destabilization and initiation of invasion and metastasis<sup>[8]</sup>. Inflammation is now widely accepted to be a key element of disease advancement and survival in CRC<sup>[9]</sup>. The local inflammation that is caused by the tumor is mirrored in a systemic inflammatory response (SIR) that may be straightforwardly measured preoperatively<sup>[2]</sup>. Many studies have shown the SIR to be a key factor in determining outcomes and survival in CRC, by measuring various circulating markers of systemic inflammation<sup>[9]</sup>. Peripheral blood neutrophil-to-lymphocyte ratio (NLR), platelet-to-lymphocyte ratio (PLR), lymphocyte-to-monocyte ratio (LMR), and prognostic nutritional index (PNI) which have been shown to be indicators of systematic inflammatory response, are widely used as valuable predictors for prognosis of cancer patients. However, the results of studies concerning the relationship of these biomarkers and prognosis of CRC patients remained inconsistent<sup>[10,11]</sup>. For this reason the aim of this study was to evaluate the prognostic significance the indicators of systematic inflammatory response by using PLR, NLR, LMR and PNI in patients undergoing curative resection of colon cancer<sup>[2]</sup>.

### MATERIALS AND METHODS

Two hundred and sixty-three patients with histologically proven CRC who, on the basis of laparotomy findings and pre-operative abdominal computed tomography, were considered to have undergone potentially curative resection between 2010 and 2016 at Ankara Numune Training and Research Hospital were retrospectively analyzed and clinicopathological characteristics included age, sex, tumor type, grade, size and localization, the number of metastatic and total number of lymph

nodes removed, vascular and perineural invasion of the tumor, TNM stages, tumor marker levels (CEA, CA19-9, AFP, CA-125, CA15-3), complete blood counts, albumin levels, overall survival (months), NLR, PLR, LMR and PNI ratios were recorded. We included blood count data available within 1 mo of surgical resection for this study. According to the following exclusion criteria: (1) emergency surgery; (2) death within 30 d of surgery; (3) clinical evidence of infection or other inflammatory conditions, such as inflammatory bowel disease or rheumatoid arthritis; (4) lack of preoperative complete blood counts and biochemical analysis; and (5) metastatic disease at diagnosis, 80 patients were excluded from the study. The records of 183 patients were retrospectively reviewed and analyzed from the electronic database. Measurements of a white cell count, absolute neutrophil, lymphocyte and monocyte counts and albumin were recorded within 1 mo before surgery. Tumors were staged using the conventional tumor, node, metastasis (TNM) staging system, 7<sup>th</sup> Edition, 2010 (AJCC, 2010). Patients were seen every 3 mo for the first 2 years, every 6 mo for the next 3 years, and once annually thereafter. NLR was determined by dividing the absolute neutrophil count by the absolute lymphocyte count; PLR by dividing the absolute platelet count by the absolute lymphocyte count; LMR by dividing the absolute lymphocyte count by the absolute monocyte count and PNI by the formula: Serum albumin (g/L) + 5 × total lymphocyte count × 10<sup>9</sup>/L<sup>[12]</sup>. The primary outcome measure was overall survival.

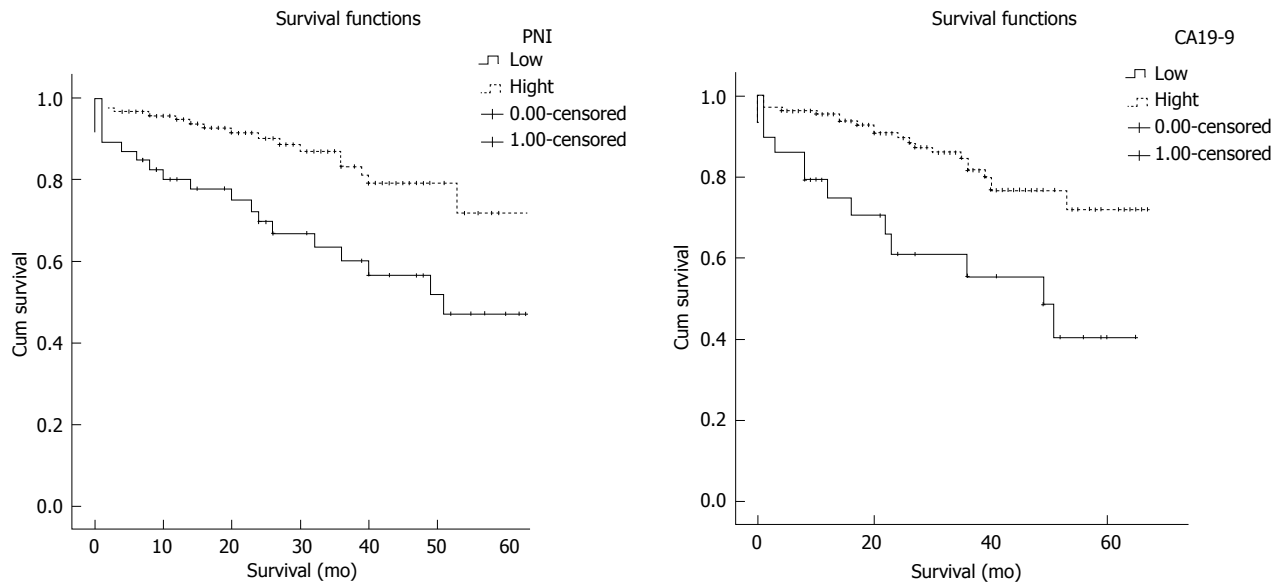
## RESULTS

All of the patients included in the present study were elective resections. Of the 183 colorectal cancer patients, 111 (60.7%) were men and 72 (39.3%) were women. The mean patient age was 63.5 ± 13.1 years (range 33-89 years). Diagnosis was adenocarcinoma in all cases. Two patients (1.1%) were in stage 0, 20 patients were in stage 1 (10.9%), 23 patients were in stage 2A (12.6%), 22 patients were in stage 2B (12%), 54 patients were in stage 2C (29.5%), 3 patients were in stage 3A (1.6%), 10 patients were in stage 3B (5.5%), 49 patients were in stage 3C (26.8%). Only 23 patients (12.6%) had Tis, T1 or T2 lesions, and 120 patients (65.6%) were lymph node negative. All patients underwent surgical resection, and 45 (24.6%) had lymphovascular invasion. Tumor size ranged from 10 to 140 mm, with a median size of 50 mm. One hundred and eighteen (64.5%) patients received adjuvant therapy following resection of the primary tumor. Thirty-day mortality rate following resection was 3.8% (*n* = 7). The median value of neutrophil was 5.1 × 10<sup>6</sup>/mL (range 3.9-67.85), lymphocyte was 1.7 × 10<sup>6</sup>/mL (range 1.3-2.22), platelet was 273 × 10<sup>6</sup>/mL (range 219.5- 354), monocyte was 0.6 × 10<sup>6</sup>/mL (range 0.47-0.8), and albumin was 39 g/L (range 34-43).

The median 5-year OS rate was 61%. The relationship between clinicopathological characteristics, NLR, PLR, LMR, PNI, CA19-9 and overall survival in patients undergoing potentially curative resection for CRC is shown in Table 1. The data were tested for normality by the Kolmogorov-Smirnov test. Normally distributed variables were expressed as mean ± SD and non-parametric variables were expressed as median (interquartile range). Categorical variables were presented as numbers and percentages. A receiver operating characteristics (ROC) curve was generated to calculate the optimal cutoff value of inflammation parameters. OS was the study end point. Survival analysis was drawn using the Kaplan-Meier method, and the differences were compared using the log-rank test. Univariate and multivariate analysis using a Cox proportional hazards model was used to test independent significance. A two-tailed *P*-value < 0.05 was considered to be statistically significant. Statistical analyses were performed using the SPSS 21.0 software (IBM Corporation, Armonk, NY, United States). Regarding overall survival, on univariate analysis the following variables were significantly associated with poor outcome following resection: T-stage (*P* = 0.037), lymph node invasion (*P* = 0.037), cancer stage (*P* = 0.034), CEA (*P* = 0.042), CA 19-9 (*P* = 0.004), and PNI (*P* = 0.001). To evaluate the independent prognostic value, multivariate Cox proportional hazard analysis to control for other prognostic factors was used. Using cancer-specific death as an end point for NLR, PLR, LMR, PNI and CA19-9 the optimal cut off values were calculated by ROC analysis. In analysis of all patients, a cutpoint of 3.3 for the LMR, 35 for the PNI, 3.5 for the NLR, 180 for the PLR and 17 for the CA19-9 was found respectively. Regarding overall survival, on multivariate analysis high CA 19-9 (HR = 1.001, 95%CI: 1.00-1.002, *P* = 0.012) and low PNI (HR = 0.938, 95%CI: 0.891-0.987, *P* = 0.014) were the only variables independently associated with shortened overall survival. Patients with a PNI < 35 had a median OS of 52.25 mo. In contrast, patients with an PNI > 35 had a median OS of 66 mo. Patients with a CA19-9 < 17 had a median OS of 66 mo and in patients with a CA19-9 > 17 had a median OS of 53.76 mo. Kaplan-Meier survival curves demonstrating the associations of the PNI and CA19-9 with overall survival are shown in Figure 1.

## DISCUSSION

In the present study, we report a longitudinal comparison of the four-systemic inflammation-based prognostic scores and CA19-9 in patients with resectable CRC. To our knowledge this is the first time these 5 markers have been used. Our findings demonstrate that the preoperative PNI and CA19-9 are independent predictors of OS for patients with CRC undergoing curative surgical resection. In the present



**Figure 1** Kaplan-Meier survival curve for overall survival in 183 patients undergoing potentially curative resection of colorectal cancer according to the prognostic nutritional index (A) and cancer antigen 19-9 (B). PNI: Prognostic nutritional index; CA19-9: Cancer antigen 19-9.

study PNI and CA19-9 are superior to NLR, PLR and LMR in predicting OS as inflammatory markers. To our knowledge this is the first study in which PNI, LMR, NLR, PLR and CA19-9 were compared as preoperative inflammatory and tumor markers to predict the OS for the patients who have undergone curative resection for colorectal cancer.

The association between cancer progression and systemic inflammatory response and markers of inflammation on malignancy progression and survival been explored extensively<sup>[13]</sup>. There are several reports that have shown factors specific to the individual, such as loss of weight, performance status, and a systemic inflammatory response, to be important indicators of the outcome of clinical treatment<sup>[14,15]</sup>. Both biochemical and hematological markers have been used in oncological malignancies to enumerate the impact of SIR upon outcomes such as the elevation in C-reactive protein (CRP) concentration, increased white cell, neutrophil and platelet counts, and hypoalbuminemia<sup>[16]</sup>. Although studies have shown that a relationship exists between tumor progression and systemic inflammation, the exact mechanism remains unclear<sup>[17]</sup>. The role different leukocytic infiltrates may play can differ in and around neoplasms. While granulocytes promote tumor development, adaptive immune cells such as T lymphocytes induce an antitumor response<sup>[18]</sup>. In colorectal cancer, the lymphocytes play a major role in human immune response, whereas systematic inflammation significantly depressed cellular immunity, resulting in a significantly decrease of CD4<sup>+</sup> T lymphocytes and an increasing of CD8<sup>+</sup> suppressor T lymphocytes<sup>[1]</sup>. A chronically inflamed microenvironment can effect cell proliferation which can lead to the cells losing their

ability to control growth resulting in hyperproliferation and tumorigenesis<sup>[19]</sup>. Consequently, the inflammatory response plays a critical role in carcinogenesis and a series of inflammatory cells and innate immune system signaling molecules are involved in tumor progression, such as neutrophil, lymphocyte, platelet and monocyte. Thus, NLR, PNI, PLR and LMR that represent systematic inflammatory response are potential prognostic factors for CRC<sup>[20]</sup>.

Zahorec<sup>[21]</sup> was the first to report the relationship between NLR and disease severity as a prognostic factor in critically ill patients. Studies evaluating the relationship between NLR and CRC have also shown it to be a strong prognostic factor. For patients with CRC it is presumed that NLR is a combined indicator of both inflammation and the immune status. However, it has not been clearly described whether there is an association between elevated NLR and poor oncologic outcome<sup>[22,23]</sup>. There are studies which have confirmed the potential prognostic utility of the NLR in patients with CRC and other solid tumors<sup>[24,25]</sup>. A study by He *et al* aimed to evaluate the prognostic and predictive value of the NLR and PLR in 243 patients with initially metastatic CRC patients. Their study showed NLR and PLR to be statistically significant poor prognostic factors<sup>[26]</sup>. Our results do not support those findings. A reason for this could be that those studies that found a negative correlation between NLR and OS assessed patients with locally advanced or metastatic colorectal cancers where inflammation may be more advanced due to the extensive nature of the cancer<sup>[27]</sup>. Therefore, it is possible that higher levels of NLR are more probable to predict overall poor outcome when the cancer is advanced<sup>[28]</sup>. Another debate concerning NLR is determining the optimal ratio that has the



**Table 1** Univariate and multivariate analysis of clinicopathologic variables in relation to overall survival in patients with colorectal cancer undergoing curative resection

Clinicopathologic characteristics	Univariate analysis, HR (95%CI)	P value	Multivariate analysis, HR (95%CI)	P value
Sex		0.798		
Tumor location		0.685		
The depth of invasion		0.877		
Perineural invasion		0.931		
T-stage	1.404 (1.021-1.930)	0.037		
Lymph Node	0.234 (0.080-0.683)	0.037		
Cancer Stage	1.173 (1.012-1.359)	0.034		
CEA	1.005 (1.00-1.009)	0.042		
CA19-9	1.001 (1.00-1.002)	0.040	1.001 (1.00-1.002)	0.012
NLR		0.135		
LMR		0.127		
PLR		0.064		
PNI	0.906 (0.865-0.948)	0.001	0.938 (0.891- 0.987)	0.014

CEA: Carcinoembryonic antigen; CA19-9: Cancer antigen 19-9; NLR: Neutrophil-to-lymphocyte ratio; LMR: Lymphocyte-to-monocyte ratio; PNI: Prognostic nutritional index; PLR: Platelet-to-lymphocyte ratio.

greatest prognostic significance. The cut-off value for NLR used in the present study is different from that reported in previous studies. In previous reports, different values have been used; however, in our study, the best cut-off value based on the ROC analysis was 3.5<sup>[24]</sup>. Similarly to NLR evidence for a prognostic role for PLR in colorectal cancer is also conflicting. A study by Kwon *et al*<sup>[22]</sup> demonstrated that an elevated PLR was independently associated with decreased overall survival, in CRC patients that underwent curative resection. Comparably Emir *et al*<sup>[33]</sup> found a statistically significant association between an elevated PLR and decreased overall 5-year survival in uni and multivariate analysis in 140 patients with resectable CRC<sup>[22,29]</sup>. In contrast, similar to our study Baranyai *et al*<sup>[30]</sup> assessed the PLR in patients with non-metastatic CRC of different stages and liver-only metastatic CRC and found PLR not to be a prognostic factor in either group.

Lymphocytes play a vital role in cytotoxic cell death and cytokine production which in turn prevent proliferation and metastatic activity of malignant cells<sup>[31]</sup>. Studies concerning lymphocyte ratio also have conflicting results. Some studies have shown that lymphocytes declined in patients with more advanced colon cancer<sup>[32]</sup>. However, tumor infiltrating lymphocytes have been identified to be associated with a better overall survival in early stage CRC patients<sup>[33]</sup>. The prognostic value of the novel LMR in malignancy, reporting independently significant associations with poor outcome across a range of cancer types including stage III primary colon cancer<sup>[2]</sup>. In a study by Stotz *et al*<sup>[34]</sup> evaluating the prognostic significance of the preoperative LMR in patients with stage III CRC also establish that a decreased LMR envisaged for shorter disease free survival and OS, and indicated that patients with decreased LMR may not profit from 5-FU-based adjuvant treatment. However, in our study,

similar to the studies of Ying *et al*<sup>[1]</sup> and Neal *et al*<sup>[2]</sup>, we found no statistically significant relationship between LMR and OS<sup>[1,2]</sup>. In previous studies, results were obtained in the form of NLR was superior to PLR in predicting OS<sup>[1,22]</sup>, however similar to Chan *et al*<sup>[32]</sup> we found neither the NLR nor the PLR to be independently prognostic when studied together with the LMR.

The nutritional and immunologic conditions of patients apparently effect the postoperative outcomes associated with malignancies. The PNI was also shown recently to be a predictive marker for both postoperative complications and prognosis in patients with CRC<sup>[35]</sup>. The prognostic nutritional index (PNI), calculated from serum albumin levels and peripheral lymphocyte count, reflects both the nutritional and immune status of the patient<sup>[36]</sup>. Studies have shown that the PNI is very similar to the systemic inflammation-based prognostic scores (mGPS, NLR, PLR and PI), but the optimal cut-off value remain unclear. Similar to others in this study a significant relationship between poor prognosis of CRC and PNI was confirmed<sup>[12,36]</sup>. The present result therefore supports the debate that the systemic inflammatory response plays a major role in the relationship between nutritional decline and poor outcome in patients with cancer.

CA19-9 was first defined by Ozawa *et al*<sup>[37]</sup> as a monoclonal antibody against human CRC cell line, and it has been admitted as a molecule that contributes to tumor metastasis by mediating the adhesion of tumor cells to the endothelial cells of blood vessels. In the consideration of guideline suggestions and published documents, the function of CA19-9 as a prognostic marker is still a debated subject<sup>[38]</sup>. Many reports have suggested that CA19-9 might be a clinically valuable prognostic marker in patients with metastatic CRC<sup>[39]</sup>. In our study, multivariate analysis also indicated that a high preoperative serum CA19-9 level was an independent prognostic factor for poor OS in patients

who had undergone curative resection. As far as we know, our study is the second to suggest the predictive power of the preoperative serum CA19-9 level for tumor prognosis in patients with CRC who have undergone curative resection.

The major limitations of the present paper are; its retrospective nature and exclusion of approximately one third of the initial number of patients for various reasons. Furthermore, CRP is not routinely measured prior to CRC resection at our centre, and, therefore, comparison of CRP with the other inflammatory predictors was not possible.

On the other hand being in a single center ensures a more homogeneous evaluation and less loss of data compared with a multicentric study. Besides this, the evaluation of these prognostic factors separately in different disease stages may be a different study subject.

CRC apparently has many molecular markers and studies for new molecular target drugs are presently ongoing, but although these molecular markers may be useful for predicting prognosis, many are limited in terms of the need for resected or biopsied specimens and high prices<sup>[35]</sup>. Furthermore, patients undergoing CRC surgery all undergo preoperative full blood counts and biochemical evaluation. The PNI and CA19-9 can be easily analyzed from routinely available data and does not require any further expenditure. The evaluation of these prognostic factors separately in different disease stages may also be a valuable different study subject.

In conclusion, this study shows that decrease in the PNI and increase in CA19-9 is associated with poor survival in patients with resectable colon cancer. Further investigation with larger datasets is required to confirm our findings.

## COMMENTS

### Background

In colorectal cancer (CRC) patients, the current survival and risk classification is basically based on the pathology result, and the gold standard is tumor node metastasis (TNM) staging. However, different survival results can be found in different patients with the same stage. For this reason, this prognosis prediction can be improved by using different methods. Cancer progression was also found to be associated not only with the tumor's local characteristics but also with the systemic host response. Inflammation-based prognostic scores, such as peripheral blood neutrophil-to-lymphocyte ratio (NLR), platelet-to-lymphocyte ratio (PLR), lymphocyte-to-monocyte ratio (LMR), and prognostic nutritional index (PNI), produced by components of the systemic inflammatory response were found to be associated with patient survival. The effect of these markers on patient overall survival was investigated in various studies in the literature, but inconsistencies were observed between the results. Studies comparing these inflammatory markers in the same patient population are lacking in the literature.

### Research frontiers

Patients with metastasis, infection, chronic systemic inflammatory disease, and patients who were operated under emergency conditions were excluded in this study, which may have an effect on the inflammatory response. The authors investigated the prognostic significance of patient survival in patients with curative

colorectal cancer resection in a single center by comparing parameters such as NLR, PLR, LMR and PNI together with other clinicopathological features. It has been found that preoperative low PNI and high CA 19-9 values are associated with low survival in patients with resectable colorectal cancer. The PNI has been shown to be superior to other inflammatory markers.

### Innovations and breakthrough

Predictors that may determine overall survival and patient outcome are extremely important, as the capability to distinguish patients more likely to develop poor outcome succeeding surgery would enable surgical and chemotherapeutic treatment tailored appropriately for each individual case. In the present study, authors' report, for the first time, a longitudinal comparison of the four systemic inflammation-based prognostic scores and CA19-9 in patients with resectable CRC. Their findings demonstrate that the preoperative PNI and CA19-9 are independent predictors of overall survival for patients with CRC undergoing curative surgical resection. In the present study PNI and CA19-9 are superior to NLR, PLR and LMR in predicting OS as inflammatory markers.

### Applications

The presence of low levels of PNI and high levels of CA19-9, detected during preoperative evaluation in colorectal cancer patients may help them to predict postoperative patient overall survival. Furthermore, patients undergoing CRC surgery all undergo preoperative full blood counts and biochemical evaluation. The PNI and CA19-9 can be easily analyzed from already routinely available data and does not require any further expenditure.

### Peer-review

It's a well-written manuscript about prognostic efficacy of inflammation-based markers in colorectal cancer resection patients. This study is interesting and the objective very clear.

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## Goblet cell carcinoid of the appendix and mixed adenoneuroendocrine carcinoma: Report of three cases

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### Abstract

Neuroendocrine neoplasms are the most common epithelial tumors among appendix tumors. Appendix tumors that are completely or partially composed of neuroendocrine cells are divided into two categories: Classic carcinoid tumors and goblet cell carcinoid tumors (GCCT). They are known to progress more aggressively than classic (neuro) endocrine tumors. In this study, three cases with acute appendicitis symptoms are presented, including their clinical and histopathological findings. Microscopic examination detected GCCT in two cases and mixed adenoneuroendocrine carcinoma in one case, in addition to acute appendicitis.

**Key words:** Appendix vermiformis; Mixed adenoneuroendocrine carcinoma; Goblet cell carcinoid tumors

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**Core tip:** Goblet cell carcinoid is a much more aggressive tumor than classic carcinoid, particularly if the tumor shows transmural involvement or if it has extended to the cecum at the time of the operation. This paper presents three cases clinical and histopathological features. Two were diagnosed as goblet cell carcinoid tumor and one was diagnosed as mixed adenoneuroendocrine carcinoma.

Karaman H, Şenel F, Güreli M, Ekinci T, Topuz Ö. Goblet cell carcinoid of the appendix and mixed adenoneuroendocrine carcinoma: Report of three cases. *World J Gastrointest Oncol* 2017; 9(7): 308-313 Available from: URL: <http://www.wjgnet.com/1948-5204/full/v9/i7/308.htm> DOI: <http://dx.doi.org/10.4251/wjgo.v9.i7.308>

## INTRODUCTION

Of appendix tumors, the most common epithelial tumors are neuroendocrine neoplasias<sup>[1]</sup>. Appendix tumors composed completely or partially of neuroendocrine cells are divided into two categories: Classic carcinoid tumors and goblet cell carcinoid tumors (GCCT) and their variants. GCCT of the appendix are rare and constitute approximately 6% of appendix carcinoids<sup>[1,2]</sup>. This kind of appendix tumor was first identified by Gagne in 1969<sup>[1,3]</sup>. Goblet cell carcinoids originate from pluripotent intestinal stem cells that show both neuroendocrine and mucinous differentiation<sup>[3-5]</sup>. Goblet cell carcinoid is known by various names such as adenocarcinoid, mucinous carcinoid, crypt cell carcinoid, and mucin-producing neuroendocrine tumor<sup>[3,6,7]</sup>. According to the neuroendocrine neoplasm classification published by the World Health Organization (WHO) in 2010, GCCTs are in the neuroendocrine tumor group<sup>[8-10]</sup>. They are known to progress more aggressively than classic (neuro)endocrine tumors<sup>[9,10]</sup>.

In 2010, the WHO and the European Neuroendocrine Tumor Society (ENETS) divided the grade of neuroendocrine tumors (NET) into three groups based on the Ki-67 proliferation index and the number of mitoses. If there are less than two mitoses and a Ki-67 index of less than 3% in 10 HPF, it is called "NET, low grade (Grade 1)". If there are 2-20 mitoses and a Ki-67 index of 3%-20%, it is called "NET, moderate-grade (Grade 2)". If there are more than 20 mitoses with a Ki-67 index greater than 20%, it is classified as "neuroendocrine carcinoma, high grade (Grade 3)"<sup>[4,9]</sup>. The most important prognostic factor is the stage of disease. Appendectomy and right hemicolectomy are the main modalities of treatment, followed by adjuvant chemotherapy in select cases.

## CASE REPORT

This paper presents three cases clinical and histopathological features. Two were diagnosed as goblet cell carcinoid and one was diagnosed as mixed adenoneuroendocrine carcinoma.

### Case 1

A 76-year-old male patient was admitted to the emergency room with abdominal pain and a fever. An appendectomy was performed with a pre-diagnosis of acute abdomen. The appendectomy material was submitted, which was 9 cm × 3 cm × 2 cm. There was a perforated area 2 cm in diameter. In section from the perforated area, tumour is composed of small, rounded nests of signet ring-like cells resembling normal intestinal goblet cells, except for the nuclear compression. The cells display mild-to-moderate atypia, low mitotic activity with a Ki 67 proliferation index < 20%, and infiltrated individually and in groups up to the subserosa and serosa by crossing the muscle layer from the mucosa on the appendix wall (Figure

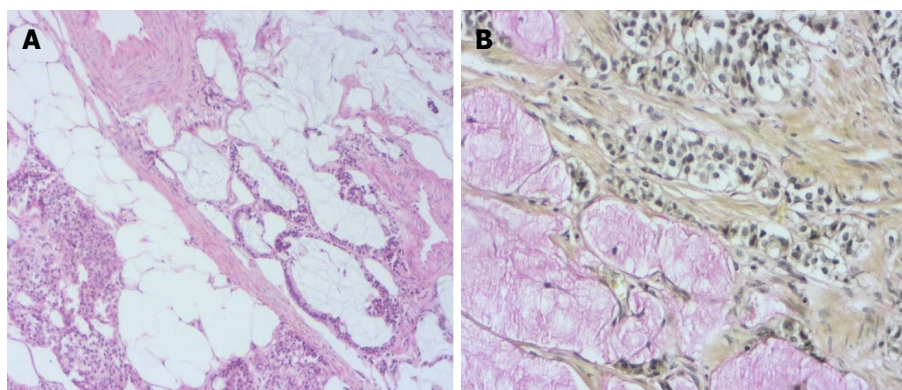
1A). During the histochemical staining, mucicarmine and intra- and extra-cellular mucin deposition were identified in the tumor tissue (Figure 1B). Epithelial membrane antigen (Figure 2A), chromogranin-A (Figure 2B) and NSE (neuron-specific enolase) from the neuroendocrine markers, focal and CEA [monoclonal carcinoembryonic antigen (mCEA)], and wide spread immunoreaction were detected in the tumor cells *via* immunohistochemistry. Ten percent positive staining was obtained with a Ki 67 proliferation marker. There was no vascular and perineural invasion. Based on these findings, the diagnosis was goblet cell carcinoid and (pT3). Because of the tumor was potentially malignant right hemicolectomy were performed.

### Case 2

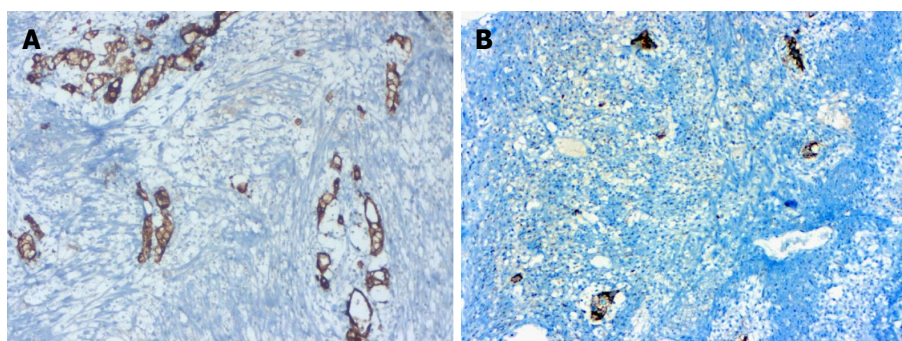
A 71-year-old man patient was admitted to the hospital emergency room with abdominal pain associated nausea and vomiting. The patient was operated for acute appendicitis. During surgery, the appendix was observed to be hyperemic and edematous. No significant tumor structure was observed. The appendix was completely removed for examination. In the examination of microscopic sections from an area of 5 cm, mucosa, submucosa, muscle layer, and tumor tissue invading the subserosa were identified, which were completely covering the tissue, starting from the basal part of the appendix lamina propria crypts (Figure 3). No tumor was identified on the proximal end of the incision. The tumor was wide, eosinophilic, had granular cytoplasm, and was characterized by small uniform nests formed by eccentric nuclei goblet cells and microglandular development. Perineural invasion was monitored. PAS-Alcian Blue pH 2.5 (Figure 4) and intra and extra-cellular mucin deposition were detected in the tumor tissue. In the immunohistochemical studies tumor was positive with the epithelial markers CK-20 and mCEA (Figure 5), and neuroendocrine cell component marker synaptophysin, chromogranin-A, CD56 and S100. With Ki-67, the proliferation index was 3%. There were acute appendicitis findings in the case as well. Overall the histological and immunohistochemical features were those of a goblet cell carcinoid tumor of the appendix tip with co-existing acute appendicitis. Because of the diffuse propagation of the tumor in an area of 5 cm on the appendix wall and the meso-appendix and its potential to be malignant, a right hemicolectomy was performed.

### Case 3

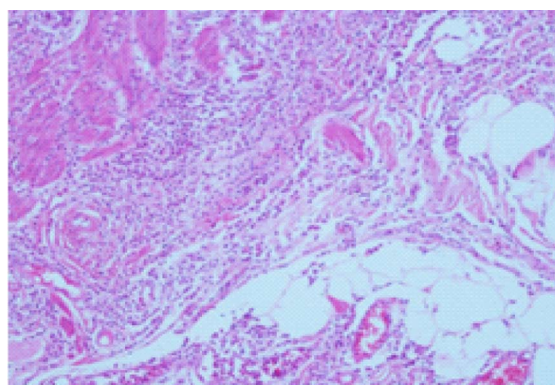
A 56-year-old man patient referred to the hospital with right lower quadrant pain. Physical examination revealed right lower quadrant abdominal tenderness and localized rebound tenderness. No palpable abdominal mass was present. In the emergency abdominal ultrasonography, the appendix diameter was 8.5 mm and markedly edematous. The neighboring mesenteric tissue was also edematous. Due to signs and symptoms typical of acute appendicitis, the patient



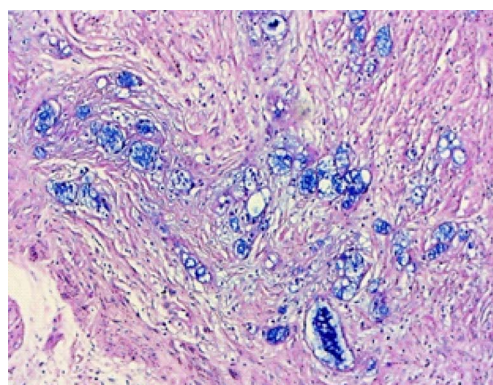
**Figure 1** Heamatoxylin and eosin stained sections showing the tumor itself is composed of small uniform tumor nests of goblet cells and signet ring cells, often arranged in a microglandular fashion and sometimes accompanied by extracellular mucus (A: H and E;  $\times 200$ ) and adenocarcinoid tumor of goblet cell type showing positivity intra- and extra-cellular mucin deposition (B: Mucicarmine;  $\times 400$ ). H and E: Heamatoxylin and eosin.



**Figure 2** Tumor cells exhibit strong immunoreactivity for epithelial membrane antigen (A) and for neuroendocrine markers (B). A: EMA, IHC;  $\times 200$ ; B: Chromogranin-A, IHC;  $\times 200$ .



**Figure 3** Adenocarcinoid tumor of goblet cell type tumor showing strong acute appendicitis findings (H and E;  $\times 100$ ).



**Figure 4** PAS-Alcian Blue pH 2.5 deposition were detected in the tumor tissue (PAS/AB;  $\times 200$ ).

underwent a simple appendectomy. Gross examination of the specimen showed the material measured 6 cm  $\times$  2 cm  $\times$  2 cm. The outer surface of the appendix was fibrinated in many areas. The area showing beige wall thickening was notable in macroscopic cross-section and measured 1.1 cm in diameter. In the surface of the cross-section, mucosa, submucosa, muscle layer, and tumor tissue infiltrated the subserosa were monitored, starting from the basal part of the appendix lamina propria crypts. No tumor was identified on the

proximal end of the section. The tumor was significant cytologic atypia, wide, eosinophilic, had granular cytoplasm forming small nests and cords, and was characterized by small uniform nests with eccentric nuclei, signet-ring with micro glandular development, and cells in goblet cell appearance (Figure 6). No central lumen was observed in the tumor cell groups. In the serial sections, five mitoses were detected in 10 high-power fields (HPF) in the tumor. Perineural invasion was observed. PAS-Alcian Blue pH 2.5 and



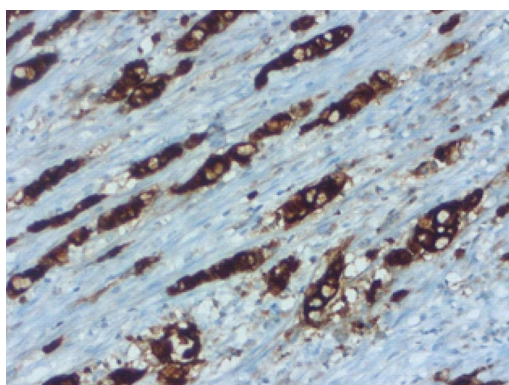


Figure 5 Adenocarcinoid tumor of goblet cell type showing positivity for immunoreactivity monoclonal carcinoembryonic antigen (IHC;  $\times 200$ ).

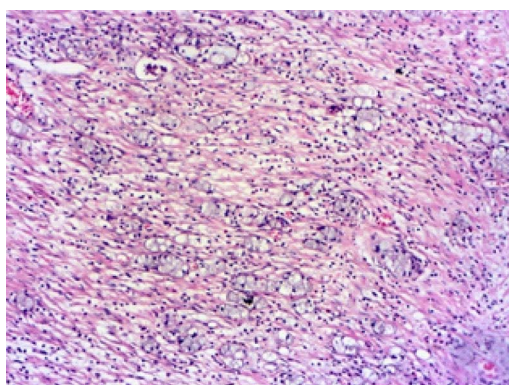


Figure 6 Heamatoxylin and eosin stained sections showing the tumor was wide, eosinophilic, had granular cytoplasm forming small nests and cords, and was characterized by small uniform nests with eccentric nuclei, signet-ring with micro glandular development and cells in goblet cell appearance (H and E;  $\times 200$ ).

intra and extra-cellular mucin deposition were detected in the tumor tissue. In the immunohistochemical studies, mCEA, keratin 7, keratin 20, synaptophysin, chromogranin-A, CD56 and S100 from the common cytoplasmic and neuroendocrine markers, and strong focal positivity were detected in the tumor (Figure 7). With Ki-67, the proliferation index was 5%-10%. There were strong acute appendicitis findings in the case. Based on these findings, the case was diagnosed as "mixed adenoneuroendocrine carcinoma and acute appendicitis". A right hemicolectomy was performed.

We considered these cases worth presenting, given their rarity, and for the opportunity to discuss malignancy criteria.

## DISCUSSION

There are four types of appendix tumors: Epithelial tumors, mesenchymal tumors, lymphomas, and secondary tumors<sup>[1]</sup>. Epithelial tumors are grouped categorized as premalignant lesions, carcinoma, neuroendocrine tumors<sup>[2]</sup>. The most common epithelial tumors are neuroendocrine neoplasias. Appendix tumors that are completely or partially composed of

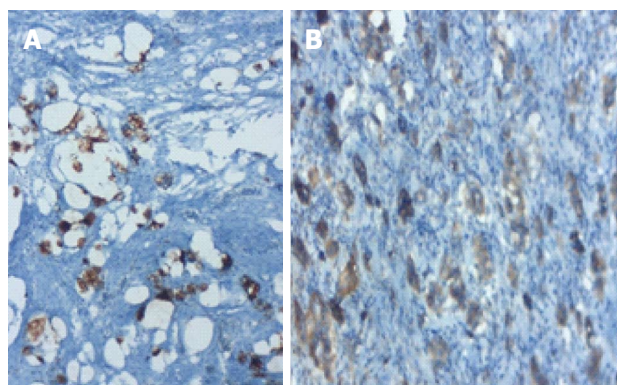


Figure 7 In the immunohistochemical studies neuroendocrine cell component markers, synaptophysin and chromogranin-A strong focal positivity were detected in the tumor (IHC;  $\times 200$ ).

neuroendocrine cells are divided into two different categories: Classic carcinoid tumors and GCCT, and their variants. Tumors showing both glandular and endocrine differentiation are called "amphicrine tumors". The amphicrine tumor of the appendix is goblet cell carcinoid. In the WHO classification, this type of tumors that advance aggressively are called "mixed carcinoid adenocarcinoma" in the "mixed adenoneuroendocrine carcinoma" group, and "adenocarcinoma", which develops on the goblet cell carcinoid<sup>[9]</sup>. The goblet cell carcinoid is called adenocarcinoid of goblet cell type<sup>[11]</sup>, mucinous carcinoid tumor<sup>[12]</sup>, and microglandular carcinoma<sup>[13]</sup>, as well as crypt cell carcinoma<sup>[14]</sup>. Microscopically, it is characterized predominantly by submucosal growth. Extending into the muscle and serosa is common. However, the mucosa is typically preserved except tumor islets and obvious connections between crypt bases. The tumor itself is composed of small uniform goblet cell islets, which are generally arranged in the microglandular style and sometimes accompanied by extracellular mucus. The typical lesion consist of small uniform nests of goblet cells, often arranged in a microglandular fashion and sometimes accompanied by extracellular mucus<sup>[3]</sup>.

### Goblet cell carcinoids rare

GCCs usually occur in older patients, generally median age was 58 years (range 31-73) and no definite sex predominance<sup>[3]</sup>. Clinical presentation followed two distinct patterns: Acute appendicitis or chronic symptoms associated with a pelvic mass. The pathogenesis is unclear however the tumor likely arises from pluripotent intestinal epithelial crypt base stem cells. Loss of Notch signaling may be the driver mutation with other successive downstream mutations likely favors them into progressing and behavior similar to poorly differentiated adenocarcinoma with minimal neuroendocrine differentiation<sup>[9,15,16]</sup>.

Goblet cell carcinoid is a more aggressive tumor than classic carcinoid, if the tumor shows transmural involvement or if it has extended to the cecum at the time of the operation<sup>[3,11,13]</sup>. In 2008, Tang *et al.*<sup>[15]</sup>, GCC



patients are divided into three groups (A, B, C). Typical GCC (Group A); adenocarcinoma ex GCC, signet ring cell type (Group B); adenocarcinoma ex GCC, poorly differentiated carcinoma type (Group C). Typical GCC (Group A) was defined as well-defined goblet cells arranged in cluster or in a cohesive linear pattern, with minimal cytologic atypia and architectural distortion of the appendical wall, and minimal to no desmoplasia. Adenocarcinoma ex GCC, signet ring cell type (Group B) was defined as goblet cells or signet ring cells arranged in irregular large clusters, with the lack of confluent sheets of cells in a discohesive single file or single cell infiltrating pattern with significant cytologic atypia, and desmoplasia and associated destruction of the appendiceal wall<sup>[15,16]</sup>. Adenocarcinoma ex GCC, poorly differentiated carcinoma type (Group C) was defined with the least focal evidence of goblet cell morphology and a component ( $> 1$  low power field or  $1 \text{ mm}^2$ ) that is not otherwise distinguishable from a poorly differentiated adenocarcinoma<sup>[15,16]</sup>.

The tumor itself, as in our case study, is composed of small uniform goblet cell islets, which are generally arranged in the microglandular style and sometimes accompanied by extracellular mucus. The histochemical stainings, mucicarmine, and CEA stainings are always positive. There is generally CK20 in all cases and the focal style CK7 positivity in approximately 70% of the cases. In our case, in agreement with the literature, tumor cells were positive for CEA, CK7, CK20, and neuroendocrine markers.

Fifty percent of patients with these tumors, who are rarely seen, often go to hospitals in the 50s or 60s with abdominal pain and palpable masses<sup>[2]</sup>. Others cases, like ours, were detected incidentally in the appendectomy specimens removed from the patients, who arrived at the hospital with acute abdominal pain and symptoms of acute appendicitis and were taken to surgery diagnosed with acute appendicitis<sup>[3,4,12,14-18]</sup>. In addition to the characteristic histological appearance, as seen in our cases, they showed widespread invasion of the mesoappendix and perineural<sup>[19]</sup>.

Adenocarcinoma developing on the goblet cell carcinoid ground constitutes a significant portion of mixed adenoneuroendocrine carcinoma. The adenocarcinoma component is in the form of signet ring cell carcinoma or poorly differentiated carcinoma. In these tumors, the goblet cell carcinoid tumor area should cover at least low-power fields (or  $1 \text{ mm}^2$ ) of the tumor. The signet ring cell type is composed of cells aligned in a single row or that form irregular groups with the appearance of a signet ring as well as goblet cell carcinoid areas<sup>[4]</sup>. Our case was diagnosed as "mixed adenoneuroendocrine carcinoma diagnosis" because of the presence of the signet ring cell component developing from the goblet cell carcinoid.

In our case, the Ki-67 labeling index was 3%-10%. In the ENETS-2007 pathologic TNM staging,  $< 1 \text{ cm}$  tumors are T1,  $\leq 2 \text{ cm}$  tumors with subserosa or mesoappendix invasion of less than  $3 \text{ mm}$  are T2,  $> 2$

cm tumors with subserosa or mesoappendix invasion of  $> 3 \text{ mm}$  are T3, and tumors with the peritoneum or other organ invasion are T4. GCCT are more aggressive than classical carcinoid tumors. Metastases have been documented in 8%-20% of the cases<sup>[11,13,15,16]</sup>. There are liver, ovarian, and peritoneal metastases during the diagnosis in approximately 10% of patients. While five-year survival is between 50%-80% for the disease when limited to the appendix, it is less than 20% in the presence of distant metastases<sup>[16]</sup>. In the literature, it is reported that an appendectomy should be sufficient for the treatment of tumors smaller than  $1 \text{ cm}$  that do not have no serious mesoappendix involvement and a low Ki-67 index<sup>[4,20-22]</sup>.

The recommended treatment for goblet cell carcinoid, whether pure or combined, is a right hemicolectomy, especially if the tumor has spread beyond the appendix and/or shows a high mitotic count. Although many authors suggest routine right hemicolectomy for goblet cell carcinoids, this recommendation has been questioned, and some authors believe that appendectomy alone may be sufficient if the appendical margin is clear, there is no evidence of spread into the periappendiceal soft tissue, the mitotic count is no more than two mitoses per 10 HPF, and there are no features of mixed goblet cell carcinoid-adenocarcinoma<sup>[3,15,16]</sup>. Both European and North American Neuroendocrine tumor societies guidelines recommend right hemicolectomy after appendectomy due to the high rate of metastases and its impact on prognosis. Our cases underwent a right hemicolectomy after the diagnosis of GCC and mixed adenoneuroendocrine carcinoma and have been made. As a result, because it is known that GCCT are more aggressive than classical carcinoid tumors but they do not show malignant behavior like adenocarcinomas, their histological identification is important.

Finally, GCCs are unique to the appendix. Appendiceal NETs asymptomatic. Carcinoid syndrome is very uncommon. The symptoms resemble acute appendicitis. Since the diagnosis is usually established post appendectomy, appendectomy materials should be examined carefully. NET and adenocarcinomas developing on the goblet cell carcinoid should definitely be kept in mind in terms of malignancy. Grading and staging play an important role in treatment and prognosis.

## COMMENTS

### Case characteristics

Abdominal pain.

### Clinical diagnosis

Acute abdomen.

### Differential diagnosis

Appendiceal neoplasm.

**Laboratory diagnosis**

All labs were within normal limits.

**Imaging diagnosis**

Acute appendicitis.

**Pathological diagnosis**

Goblet cell carcinoid.

**Treatment**

Hemicolectomy mixed adenoneuroendocrine carcinoma.

**Related reports**

Grading and staging play an important role in treatment and prognosis.

**Term explanation**

GCCT: Goblet cell carcinoid tumors.

**Experiences and lessons**

Neuroendocrine tumors and adenocarcinomas developing on the goblet cell carcinoid should definitely be kept in mind in terms of malignancy.

**Peer-review**

The authors presented a rare pathology of appendix. The manuscript was well structured and written.

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*WJGO* covers topics concerning carcinogenesis, tumorigenesis, metastasis, diagnosis, prevention, prognosis, clinical manifestations, nutritional support, molecular mechanisms, and therapy of benign and malignant tumors of the digestive tract. The current columns of *WJGO* include editorial, frontier, diagnostic advances, therapeutics advances, field of vision, mini-reviews, review, topic highlight, medical ethics, original articles, case report, clinical case conference (Clinicopathological conference), and autobiography. Priority publication will be given to articles concerning diagnosis and treatment of gastrointestinal oncology diseases. The following aspects are covered: Clinical diagnosis, laboratory diagnosis, differential diagnosis, imaging tests, pathological diagnosis, molecular biological diagnosis, immunological diagnosis, genetic diagnosis, functional diagnostics, and physical diagnosis; and comprehensive therapy, drug therapy, surgical therapy, interventional treatment, minimally invasive therapy, and robot-assisted therapy.

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Retrospective Study

# S-1 induced hepatic steatosis in patients with pancreatic cancer: Retrospective analysis

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## Abstract

### AIM

To determine whether S-1 induces hepatic steatosis in patients being treated for pancreatic cancer.

### METHODS

This retrospective study evaluated 22 patients who received oral S-1 as a first-line treatment for pancreatic cancer between January 2008 and July 2015 at the Ishikawa Prefectural Central Hospital. Patients underwent abdominal computed tomography (CT) scans before chemotherapy and within 3 mo from the start of treatment. CT numbers of the liver and spleen were measured before and after S-1 administration. Steatosis was diagnosed when the ratio of the CT number of the liver to that of the spleen (liver/spleen ratio) was  $< 0.9$ .

### RESULTS

Median patient age was 68 years (range, 48-85 years), and median body mass index was  $21 \text{ kg/m}^2$  (range, 18-27  $\text{kg/m}^2$ ). Of the 22 patients, six (27%) regularly consumed alcohol, and five (23%) had liver metastases. The mean ratio of CT number of the liver to the spleen was significantly higher before administration of S-1 ( $1.27 \text{ vs } 1.09$ ,  $P = 0.012$ ) compared with after. Of the 22 patients, five (23%) had hepatic steatosis and 17 (77%) did not. The pretreatment demographic and clinical characteristics of these two groups showed no significant differences. The relationship between liver/spleen ratio and alanine transaminase activity in these patients. A statistically significant inverse correlation was observed ( $r = -0.417$ ,  $P < 0.027$ ).

### CONCLUSION

Of the 22 patients with pancreatic cancer, five (23%) experienced S-1 induced hepatic steatosis. Care should be taken during S-1 treatment of patients with pancreatic cancer.

**Key words:** S-1; Hepatic steatosis; Pancreatic cancer;

Drug induced hepatitis; 5-fluorouracil

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**Core tip:** Drug induced hepatic steatosis is a rare form of liver injury. Although hepatic steatosis has been observed in some patients with pancreatic cancer who were administered S-1, the ability of 5-fluorouracil alone to induce hepatic steatosis has not been evaluated systematically. The purpose of our study was to determine whether S-1 induces hepatic steatosis in patients being treated for pancreatic cancer. After analyzing a total of 22 patients, we found that S-1 chemotherapy induced hepatic steatosis in some patients with pancreatic cancer within three months and the correlation between the development of hepatic steatosis and liver function was weak in these patients.

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## INTRODUCTION

S-1 is an oral antitumor drug that combines tegafur, a prodrug of 5-fluorouracil (5-FU), with 5-chloro-2,4-dihydropyridine and potassium oxonate<sup>[1]</sup>. Following S-1 administration, fluorouracil concentrations in blood remain high for long periods of time<sup>[1]</sup>. Several phase III trials have shown the efficacy and safety of S-1 in cancer patients<sup>[2-5]</sup>, and S-1 has been approved in Japan for the treatment of various cancers, including gastric, head and neck, colorectal, lung, breast, pancreatic, and biliary tract cancers.

Nonalcoholic fatty liver disease is the most common form of chronic liver disease in Western countries, and its incidence is increasing, partly owing to the increasing prevalence of diabetes and obesity<sup>[6]</sup>. In Japan also, nonalcoholic fatty liver diseases has become one of the most frequent types of liver disease<sup>[7]</sup>.

In contrast to nonalcoholic fatty liver diseases, drug induced hepatic steatosis is a rare form of liver injury. Drugs found to induce hepatic steatosis include amiodarone, tamoxifen, irinotecan, and valproic acid, with their toxicities due to their effects on hepatocyte mitochondria<sup>[8,9]</sup>. 5-FU, when combined with interferon (IFN) and folinic acid, has also been reported to induce hepatic steatosis<sup>[10,11]</sup>, but the ability of 5-FU alone to induce hepatic steatosis has not been evaluated systematically.

Although hepatic steatosis has been observed in some patients with pancreatic cancer who were administered S-1 (Figure 1), it is not clear whether S-1 was responsible for drug induced hepatic steatosis in

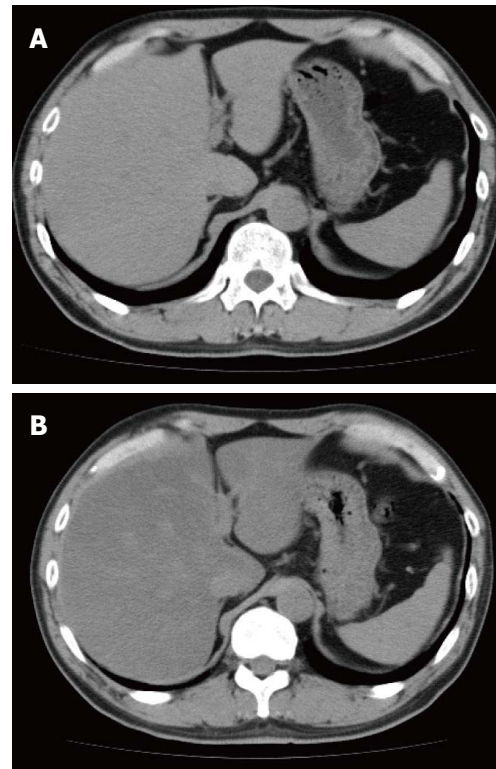


Figure 1 Abdominal computed tomography scans of a pancreatic cancer patient before (A) and during treatment (B) with S-1, showing that the liver/spleen ratio decreased after start of treatment.

these patients. This study therefore evaluated whether S-1 induced hepatic steatosis in patients with pancreatic cancer.

## MATERIALS AND METHODS

### Study populations

This retrospective study analyzed patients with pancreatic cancer who underwent chemotherapy for pancreatic cancer at Ishikawa Prefectural Central Hospital between January 2008 and July 2015. Of the 107 pancreatic cancer patients who received chemotherapy during the study period, 37 received oral S-1 as first-line treatment. Patients were included if they: (1) were aged > 20 years; (2) had an Eastern Cooperative Oncology Group performance status (ECOG PS) of 0-2; (3) underwent regular computed tomography (CT) examinations; and (4) had no other cancer or serious complications, such as active infectious disease or serious heart disease. Twenty-two patients were deemed eligible. This study was performed in accordance with the principles of the Declaration of Helsinki and was approved by the Institutional Review Board of Ishikawa Prefectural Central Hospital.

### CT

All patients underwent abdominal CT scans before the start of chemotherapy and within three months from the start of treatment. CT numbers of the liver and



**Table 1** Demographic and clinical characteristics of patients with pancreatic cancer receiving S-1 treatment

	Number of patients (n = 22)
Age (yr), median (range)	68 (48-85)
Sex	
Male	9
Female	13
Body mass index (kg/m <sup>2</sup> ) median (range)	21 (18-27)
ECOG PS	
0	15
1	5
2	2
Drinking habit	
Yes	6
No	16
Diabetes mellitus	
Yes	6
No	16
Disease status	
Locally advanced	14
Metastatic	8
Liver metastasis	
Yes	5
No	17
Biliary drainage	
Yes	7
No	15
Combination with radiotherapy	
Yes	12
No	10
Alanine transaminase concentration (U/L), median (range)	21 (8-73)

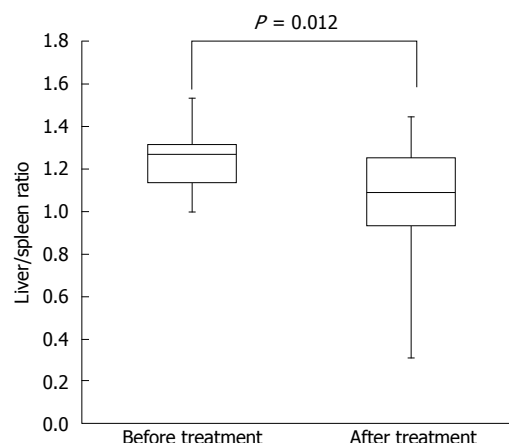
spleen were evaluated before and after administration of S-1. Steatosis was diagnosed when the ratio of the CT number of the liver to that of the spleen (liver/spleen ratio) was  $< 0.9$ <sup>[12]</sup>. Blood was collected and blood tests performed at the time of each CT examination.

### Statistical analysis

Continuous variables were reported as median (range) and analyzed using non-parametric Mann-Whitney *U* tests. Categorical variables were reported as number (percentage) and analyzed using Fisher's exact tests or Wilcoxon signed rank tests. A *P*-value  $< 0.05$  was considered statistically significant. All statistical analyses were performed with EZR software (Saitama Medical Center, Jichi Medical University, Saitama, Japan), a graphical user interface for R (The R Foundation for Statistical Computing, Vienna, Australia). More precisely, EZR is a modified version of R commander designed to add statistical functions frequently used in biostatistics<sup>[13]</sup>.

## RESULTS

The clinical characteristics of the 22 included patients are shown in Table 1. Their median age was 68 years (range, 48-85 years), and their median body mass index was 21 kg/m<sup>2</sup> (range, 18-27 kg/m<sup>2</sup>). Of the 22 patients, six (27%) regularly consumed alcohol, and



**Figure 2** Median liver/spleen ratios in pancreatic cancer patients before and after treatment with S-1. The ratio decreased significantly, from 1.27 before treatment to 1.09 after S-1 treatment ( $P = 0.012$ ).

five (23%) had liver metastases.

Figure 2 shows the median liver/spleen ratios in these patients before and after administration of S-1. This ratio was significantly higher before than after administration of S-1 (1.27 vs 1.09,  $P = 0.012$ ).

Of the 22 patients, five (23%) had hepatic steatosis and 17 (77%) did not. The pretreatment demographic and clinical characteristics of these two groups showed no significant differences (Table 2).

Figure 3 shows the relationship between liver/spleen ratio and alanine transaminase activity in these patients. A statistically significant inverse correlation was observed ( $r = -0.417$ ,  $P < 0.027$ ).

## DISCUSSION

This study showed that S-1 chemotherapy induced hepatic steatosis in some patients with pancreatic cancer within three months. This study also found that the correlation between the development of hepatic steatosis and liver function was weak in these patients.

Recognition of steatosis in patients receiving chemotherapy is important. The liver is of higher attenuation than the hepatic metastases, but it is difficult to delineate making assessment of size of the hepatic metastases as the liver becomes steatosis<sup>[10]</sup>. Obesity, insulin resistance, and the metabolic syndrome have been found to induce nonalcoholic fatty liver disease, a fairly common entity. In contrast, drug induced steatosis is a rare form of liver injury. Agents found to induce steatosis include amiodarone, tamoxifen, irinotecan, and valproic acid<sup>[8,9]</sup>. Drug induced steatosis is largely due to mitochondrial damage. In addition, mitochondrial damage can be induced by the inhibition of fatty acid beta oxidation, oxidative phosphorylation, and mitochondrial respiration<sup>[14]</sup>.

In this study, 23% of patients with pancreatic cancer developed hepatic steatosis within three months of starting the oral chemotherapeutic agent S-1. S-1 is a combination of tegafur, a prodrug of 5-FU; 5-chloro-

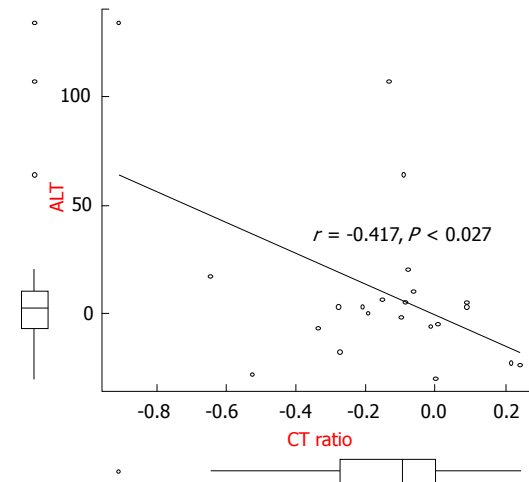
**Table 2** Demographic and clinical characteristics of pancreatic cancer patients

	Without hepatic steatosis ( <i>n</i> = 11)	With hepatic steatosis ( <i>n</i> = 5)	<i>P</i> value
Age (yr), median (range)	70 (57-85)	67 (48-78)	0.39
Sex			
Male	7	2	1
Female	10	3	
Body mass index (kg/m <sup>2</sup> ), median (range)	21.5 (17.9-26.9)	22.6 (19.0-25.3)	0.75
ECOG PS			
0	11	4	1
1	4	1	
2	2	0	
Drinking habit			
Yes	5	1	1
No	12	4	
Diabetes mellitus			
Yes	5	1	1
No	12	4	
Disease status			
Locally advanced	10	4	0.61
Metastatic	7	1	
Liver metastasis			
Yes	5	0	0.29
No	12	5	
Biliary drainage			
Yes	4	3	0.27
No	13	2	
Combination with radiotherapy			
Yes	9	3	1
No	8	2	
Alanine transaminase concentration (U/L), median (range)	24 (8-57)	20.5 (11-73)	0.97

2,4-dihydropyridine, an inhibitor of dihydropyrimidine dehydrogenase, the enzyme responsible for generating 5-FU from tegafur; and potassium oxonate<sup>[1]</sup>. 5-FU has been linked to the development of steatosis<sup>[8]</sup>. For example, one study reported that 47% of patients receiving 5-FU-based therapy for advanced colorectal cancers developed steatosis during treatment<sup>[10]</sup>, and a second study found that 30% of patients treated with interferon-alfa 2a and 5-FU developed steatosis<sup>[11]</sup>. To date, however, hepatic steatosis had not been found to be induced by 5-FU alone.

Although a statistically significant inverse correlation was observed between the development of hepatic steatosis and the loss of liver function, this correlation was weak ( $r = -0.417$ ,  $P < 0.027$ ). A previous study reported no correlation between liver function test results and the degree of steatosis in patients receiving 5-FU-based therapy<sup>[10]</sup>. Our findings are not strong enough to conclude that liver damage and the degree of steatosis are related.

In general, S-1 is safe for the liver, with few reports of S-1 induced fatal liver dysfunction. Indeed, the GEST trial, which compared S-1 and gemcitabine treatment in patients with mild to moderate pancreatic cancer,

**Figure 3** Correlation between liver/spleen ratio and alanine transaminase activity in pancreatic cancer patients treated with S-1. A significant inverse correlation was observed between these parameters ( $r = -0.417$ ,  $P < 0.027$ ).

reported that the percentages of patients with grade  $\geq 3$  elevated AST and ALT levels were significantly lower in the S-1 than in the gemcitabine group<sup>[3]</sup>. Steatosis and liver damage may be underdiagnosed because the laboratory abnormalities are slight. Further investigations are needed to assess the relationship between liver damage and the degree of steatosis in patients receiving S-1.

This study had at least four limitations. First, it was performed at a single institution. Second, this study had a retrospective design. Prospective studies are needed to show the rate of S-1 induced hepatic steatosis in patients with pancreatic and other cancers. Third, none of our patients was histologically evaluated by liver biopsy because all had mild liver damage. CT, however, is reliable in diagnosing hepatic steatosis, with significantly superior diagnostic sensitivity and accuracy than other modalities in diagnosing hepatic steatosis. Fourth, this study was limited to patients with unresectable pancreatic cancer. We experienced the S-1 induced steatohepatitis to patients with gastric cancer in adjuvant therapy. However, Even gastrectomy alone has been found to induce hepatic steatosis<sup>[15]</sup>. Further study is needed to assess the effects of S-1 on steatosis in other types of cancer.

In conclusion, this study found that 23% of patients with pancreatic cancer who were treated with first-line S-1 chemotherapy developed hepatic steatosis. Because adjuvant S-1 chemotherapy has become a standard treatment in patients resected for pancreatic cancer, clinical use of S-1 and the risk of S-1-induced steatosis are expected to increase. The liver function of patients treated with S-1 should therefore be closely monitored during follow-up.

## COMMENTS

### Background

S-1 is an oral chemotherapeutic agent, consisting of tegafur, a prodrug of

5-fluorouracil, 5-chloro-2,4-dihydropyridine, and potassium oxonate. Recent randomized controlled trials in Japan have shown the clinical utility of S-1 treatment in patients with pancreatic cancer. Liver dysfunction, primarily steatohepatitis, has been observed in some S-1-treated patients, but it is not clear whether S-1 induced steatohepatitis.

### Research frontiers

It is not clear whether S-1 was responsible for drug induced steatohepatitis.

### Innovations and breakthroughs

The literature suggests 5-FU has been linked to the development of steatosis, but the ability of 5-FU alone to induce hepatic steatosis has not been evaluated systematically. The current study adds that S-1 chemotherapy induced hepatic steatosis in some patients.

### Applications

This study serves as additional evidence supporting the closely investigation of liver function in patients treated with S-1.

### Terminology

S-1 is a combination of tegafur, a prodrug of 5-FU; 5-chloro-2,4-dihydropyridine, an inhibitor of dihydropyrimidine dehydrogenase, the enzyme responsible for generating 5-FU from tegafur; and potassium oxonate.

### Peer-review

The paper is well-written and contributes important information.

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Retrospective Study

# Does the discrepancy in histologic differentiation between a forceps biopsy and an endoscopic specimen necessitate additional surgery in early gastric cancer?

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## Abstract

### AIM

To investigate the clinicopathological variables in early gastric cancer (EGC) patients in relation to differentiation discrepancy.

### METHODS

The data of 265 specimens from 240 patients with EGC, who had undergone radical operation at Hallym University Sacred Heart Hospital from 2010 to 2015, were retrospectively analyzed. We evaluated clinical, endoscopic, and histopathological data according to histological discrepancy.

### RESULTS

Clinically significant discrepancy rate showed the difference in differentiated type (well and moderately differentiated) and undifferentiated type (poorly differentiated and signet ring cell) between endoscopic biopsies and postoperative specimens was 9.4% (25/265). There were no differences in tumor location, size, gross pattern, and number of biopsies. Specimens having histological discrepancy



showed more submucosal invasion (72.0% *vs* 49.6%,  $P = 0.033$ ) and lymph node involvement (24.0% *vs* 7.9%,  $P = 0.009$ ) than specimens having non-discrepancy. The rate of a positive epidermal growth factor receptor status was higher in specimens having discrepancy than in specimens having non-discrepancy (81.0% *vs* 55.4%,  $P = 0.035$ ).

### CONCLUSION

The discordance of histologic differentiation is associated with higher submucosal invasion and lymph node metastases in EGC. Patients having histological discrepancy may require additional surgical treatments.

**Key words:** Early gastric cancer; Histological discrepancy; Differentiation; Clinicopathological factor; Endoscopic treatment; Surgical treatment

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**Core tip:** The discordance of differentiation between forceps biopsies and endoscopically resected specimens may necessitate a radical gastrectomy and predict poor outcomes. We analyzed clinicopathological variables of early gastric cancer patients in relation to differentiation discrepancy. Clinically significant discrepancy rate between endoscopic biopsies and postoperative specimens was 9.4%. Specimens having histological discrepancy showed more submucosal invasion and lymph node metastases than specimens having non-discrepancy. Patients who have histological discrepancy detected in endoscopically resected specimens may require additional surgical treatments.

Soh JS, Lim H, Kang HS, Kim JH, Kim KC. Does the discrepancy in histologic differentiation between a forceps biopsy and an endoscopic specimen necessitate additional surgery in early gastric cancer? *World J Gastrointest Oncol* 2017; 9(8): 319-326 Available from: URL: <http://www.wjgnet.com/1948-5204/full/v9/i8/319.htm> DOI: <http://dx.doi.org/10.4251/wjgo.v9.i8.319>

## INTRODUCTION

Endoscopic resection is widely used to treat early gastric cancer (EGC), accompanying the development of techniques for endoscopic submucosal dissection (ESD). The *en-bloc* resection method for a large superficial lesion by using a needle knife requires the application of appropriate indications due to fear of lymph node metastasis and incomplete submucosal dissection. The standard and expanded indications for endoscopic treatment of EGC are determined based on the size, depth of invasion, ulcer, and histology of the lesion<sup>[1,2]</sup>. The histopathological type, which is divided into two types; differentiated and undifferentiated, according to the presence or absence of tubular structures, is one of the important factors for choosing ESD, and the histologic diagnosis based on a forceps biopsy is critical.

The discrepancy in histologic differentiation between a forceps biopsy and an endoscopic resection specimen necessitates further treatment such as additional radical gastrectomy in EGC patients. Previous studies showed a 1.5%-8.0% rate of histologic discrepancy between the differentiated and undifferentiated types after endoscopic treatment<sup>[3-7]</sup>. The need for additional surgery in cases of histologic discrepancy is based on the likelihood of deep submucosal invasion and lymph node metastasis<sup>[8,9]</sup>. However, little is known about whether histologic discrepancy between a pre-treatment forceps biopsy and a surgical specimen is associated with more submucosal invasion and lymph node metastasis in EGC.

The aim of the present study is to investigate the clinicopathological factors for histological discrepancy in differentiation between preoperative endoscopic biopsies and surgical specimens in EGC patients who underwent gastrectomy and lymph node dissection, and to identify the prognostic factors according to the presence or absence of histological discrepancy.

## MATERIALS AND METHODS

### Patients

We initially included the patients who underwent curative radical gastrectomy with extended lymphadenectomy for EGC at the Hallym University Sacred Heart Hospital in Anyang, South Korea, from 2010 to 2015. All patients received an esophagogastroduodenoscopy (EGD) with forceps biopsy before treatment. We excluded five patients who were found to have advanced gastric cancer with EGC, four patients who had undergone operation for recurrent cancer, and one patient in whom there was lack of data for evaluating the surgical tissues. Finally, 265 EGC specimens from 240 patients were included and retrospectively analyzed. Information on clinical characteristics, including age at operation, sex, underlying disease, pathologic, and outcome data, was collected by reviewing the patient medical records. Underlying diseases included hypertension, diabetes, and cardiovascular, cerebrovascular, and pulmonary diseases. The local ethics committee at Hallym Sacred Heart Medical Center approved the use of clinical data for this study (IRB 2016-I129).

### Endoscopic evaluation

The following endoscopic findings were reviewed by two experienced endoscopists: Tumor location, gross pattern, ulceration, erythema, fold change, easy friability, exudate, and number of biopsies. Tumor location was determined based on the Japanese Classification of Gastric Cancer as upper, middle, or lower third of the stomach<sup>[10]</sup>. The gross pattern was classified into six types: Elevated (types I and IIa), flat (type IIb), depressed (types IIc and III), mixed elevated (types IIa + IIb and IIa + IIc), mixed flat (types IIb + I, IIb + IIa, IIb + IIc, and IIb + III), and mixed depressed (types IIc + IIb, IIc + I, and III).

**Table 1 Tumor differentiation between preoperative biopsies and postoperative specimens**

After surgery		Before surgery		
		Differentiated		Undifferentiated
		WD	MD	PD
Differentiated	WD	56	12	0
	MD	24	45	2
Undifferentiated	PD	4	19	103

PD: Poorly differentiated; MD: Moderately differentiated; WD: Well differentiated.

+ II c), considering the dominant pattern. Ulceration was defined as discontinuity of gastric mucosa with a creator, which is not a superficial erosion. Fold change was defined as a change in the folds including cutting, fusion, and clubbing. Easy friability was defined as bleeding on slight touch or aeration.

### Histopathological evaluation

A gastrointestinal pathologist from our hospital evaluated and reviewed the histological slides of tissues obtained by endoscopic forceps biopsy before the operation and those of the entire resected specimens obtained by radical operation. The histologic type was determined according to the World Health Organization (WHO) classification of gastrointestinal tumors<sup>[11]</sup>. The differentiation of the tumor was determined according to the proportion of the tumor that exhibited glandular structures between differentiated (well and moderately differentiated adenocarcinoma) and undifferentiated types (poorly differentiated adenocarcinoma, signet ring cell carcinoma, and mucinous adenocarcinoma). After surgery, the following histopathological parameters were evaluated: Tumor size, tumor staging of tumor confined to mucosa (I a) or submucosal invasion (I b), lymph node metastasis, lymphatic invasion, vascular invasion, and Ki-67, p53, human epidermal growth factor 2 (HER2), and epidermal growth factor receptor (EGFR) status. Based on the hematoxylin and eosin-stained slide review, the available formalin-fixed, paraffin-embedded (FFPE) tumor tissue blocks from the 243 available specimens were subjected to immunohistochemical (IHC) staining for D2-40, Ki-67, p53, HER2 and EGFR. HER2 positivity was regarded as tumor score of  $\geq 2+$  on HER2 IHC staining.

### Outcome data

Abdominal computed tomography (CT) and EGD with a biopsy were scheduled at 6 mo after surgery to detect recurrence. After the initial evaluation, abdominal CT was performed every 6 mo and EGD was performed annually for 5 years. Recurrence and death were evaluated during the follow-up period.

### Statistical analysis

Clinical and histopathological characteristics were

compared between the discrepancy and non-discrepancy groups. Categorical variables were analyzed with the  $\chi^2$  test or Fisher's exact test, and continuous variables were compared by the Student *t*-test. The Statistical Package for the Social Sciences (SPSS) version 21.0 (SPSS Inc., Chicago, IL, United States) was used for all statistical analyses. A *P* value of  $< 0.05$  was considered statistically significant.

## RESULTS

### Comparison of the characteristics of patients and tumors between the discrepancy and non-discrepancy groups

Of the 265 specimens, 137 (51.7%) showed the same pathological results of differentiated type in both the preoperative endoscopic biopsy and the postgastrectomy specimen, and 103 (38.9%) showed undifferentiated results on both histological examinations. Of the remaining 25 specimens (9.4%), 23 showed differentiated histology on preoperative biopsy, but they showed undifferentiated histology after surgery. Conversely, two specimens having poorly differentiated histology on preoperative biopsy exhibited moderately differentiated histology in the postoperative specimen (Table 1). There was excellent agreement between preoperative and postoperative histology (kappa coefficient = 0.809,  $P < 0.001$ ). Twenty-five specimens from 24 patients were included in the discrepancy group, and 240 specimens from 216 patients were included in the non-discrepancy group.

Table 2 shows a comparison of the characteristics of patients and tumors between the discrepancy and non-discrepancy groups. The median ages of the discrepancy group and the non-discrepancy group were 58 years (range, 31-83 years) and 61 years (range, 35-90 years), respectively. Sex, underlying disease, and tumor location were not significantly different between the two groups. Depressed feature was the most common gross pattern in both discrepancy and non-discrepancy groups (40.0% vs 38.8%, respectively,  $P = 0.668$ ). With respect to endoscopic tumor characteristics, fold change was significantly higher in the discrepancy group than in the non-discrepancy group (80.0% vs 45.4%,  $P = 0.001$ ). The remaining characteristics including ulcer, erythema, easy friability, and exudate were not different between the two groups. The median number of biopsies in the two groups did not show any difference. The median follow-up duration in the discrepancy group was 41 mo (range, 2-72 mo), and the median follow-up in the non-discrepancy group was 36 mo (range, 2-76 mo) ( $P = 0.629$ ). Recurrence was detected in one patient of the discrepancy group and in two patients of the non-discrepancy group during the follow-up period without statistical significance ( $P = 0.272$ ). All three patients developed recurrence at the anastomosis site and underwent additional surgery with chemotherapy. Death occurred in four patients of the non-discrepancy

**Table 2 Clinical characteristics of 240 patients and tumor characteristics in 265 specimens *n* (%)**

Variables	Discrepancy	Non-discrepancy	<i>P</i> value
Age ( <i>n</i> = 240)			
Median, yr (range)	58 (31-83)	61 (35-90)	0.073
Sex ( <i>n</i> = 240)			
Male	17 (70.8)	147 (68.1)	0.781
Female	7 (29.2)	69 (31.9)	
Underlying disease <sup>1</sup> ( <i>n</i> = 240)			
Yes	12 (50.0)	100 (46.3)	0.730
No	12 (50.0)	116 (53.7)	
Location ( <i>n</i> = 265)			
Upper	4 (16.0)	17 (7.1)	0.776
Middle	10 (40.0)	130 (54.2)	
Lower	11 (44.0)	93 (38.8)	
Gross pattern ( <i>n</i> = 265)			
Elevated	1 (4.0)	22 (9.2)	0.668
Flat	5 (20.0)	42 (17.5)	
Depressed	10 (40.0)	93 (38.8)	
Mixed elevated	2 (8.0)	24 (10.0)	
Mixed flat	4 (16.0)	31 (12.9)	
Mixed depressed	3 (12.0)	28 (11.7)	
Ulcer ( <i>n</i> = 265)			
Positive	12 (48.0)	100 (41.7)	0.542
Negative	13 (52.0)	140 (58.3)	
Erythema ( <i>n</i> = 265)			
Positive	5 (20.0)	52 (21.7)	0.847
Negative	20 (80.0)	188 (78.3)	
Fold change ( <i>n</i> = 265)			
Positive	20 (80.0)	109 (45.4)	0.001
Negative	5 (20.0)	131 (54.9)	
Easy friability ( <i>n</i> = 265)			
Positive	9 (36.0)	84 (35.0)	0.921
Negative	16 (64.0)	156 (65.0)	
Exudate ( <i>n</i> = 265)			
Positive	5 (20.0)	36 (15.0)	0.511
Negative	20 (80.0)	204 (85.0)	
Number of biopsies ( <i>n</i> = 265)			
Median (range)	3 (2-6)	3 (1-10)	0.332
Follow-up period (mo) ( <i>n</i> = 240)			
Median (range)	41 (2-72)	36 (2-76)	0.629
Recurrences during follow-up ( <i>n</i> = 240)	1 (4.2)	2 (0.9)	0.272
Death during follow-up ( <i>n</i> = 240)	0 (0.0)	4 (1.9)	1.000

<sup>1</sup>Underlying diseases include hypertension, diabetes, and cardiovascular, cerebrovascular, and pulmonary diseases.

group during the follow-up period, but it was not EGC-related death. Two patients died of lung cancer, one patient died of infection, and the remaining one patient died of cardiomyopathy.

#### **Comparison of histopathological parameters between the discrepancy and non-discrepancy groups**

The median size of specimens in the discrepancy group was larger than that of specimens in the non-discrepancy group, but this difference was not statistically significant (3.0 cm vs 2.2 cm, *P* = 0.252). The proportion of submucosal involvement was significantly higher in the discrepancy group than in the non-discrepancy group (72.0% vs 49.6%, *P* = 0.033). In addition, the rate of positivity of lymph node metastasis was significantly higher in the discrepancy group (24.0% vs 7.9%, *P* = 0.009). The rate of lymphatic invasion was slightly higher in the discrepancy group without

statistical significance (28.0% vs 17.1%, *P* = 0.177); however, the rate of vascular invasion was similar between the two groups. The rate of positive EGFR status was significantly higher in the discrepancy group than in the non-discrepancy group (81.0% vs 55.4%, *P* = 0.035) (Table 3).

#### **Comparison of histopathological parameters between the discrepancy and non-discrepancy groups with undifferentiated postoperative histology**

We performed a subgroup analysis in patients with poorly differentiated histology of postoperative specimens. There were 23 specimens in the discrepancy group and 103 specimens in the non-discrepancy group. The median size of specimens, number of biopsies, tumor location, and gross pattern were not different between the two groups. The rates of submucosal involvement and lymph node metastasis were signi-

**Table 3** Histopathological characteristics of surgical specimens *n* (%)

Variables	Discrepancy	Non-discrepancy	<i>P</i> value
Tumor size in the specimen ( <i>n</i> = 265)			
Median, cm (range)	3.0 (0.8-5.5)	2.2 (0.4-8.5)	0.252
Tumor staging ( <i>n</i> = 265)			
I a	7 (28.0)	121 (50.4)	0.033
I b	18 (72.0)	119 (49.6)	
Nodal staging ( <i>n</i> = 265)			
Positive	6 (24.0)	19 (7.9)	0.009
Negative	19 (76.0)	221 (92.1)	
Lymphatic invasion ( <i>n</i> = 265)			
Positive	7 (28.0)	41 (17.1)	0.177
Negative	18 (72.0)	199 (82.9)	
Vascular invasion ( <i>n</i> = 265)			
Positive	2 (8.0)	17 (7.1)	0.697
Negative	23 (92.0)	223 (92.9)	
Ki-67 ( <i>n</i> = 243)			
High	20 (95.2)	186 (83.8)	0.215
Low	1 (4.8)	36 (16.2)	
p53 ( <i>n</i> = 243)			
Positive	15 (71.4)	138 (62.2)	0.401
Negative	6 (28.6)	84 (37.8)	
HER2 ( <i>n</i> = 243)			
Positive (2+ and 3+)	7 (33.3)	41 (18.5)	0.102
Negative (0 and 1+)	14 (66.7)	181 (81.5)	
EGFR ( <i>n</i> = 243)			
Positive	17 (81.0)	123 (55.4)	0.035
Negative	4 (19.0)	99 (44.6)	

HER2: Human epidermal growth factor 2; EGFR: Epidermal growth factor receptor.

ificantly higher in the discrepancy group than in the non-discrepancy group (73.9% vs 49.5%,  $P = 0.034$ , and 26.1% vs 8.7%,  $P = 0.020$ , respectively). The rate of lymphatic invasion was also significantly higher in the discrepancy group than in the non-discrepancy group (30.4% vs 11.7%,  $P = 0.023$ ). The rates of positive HER2 status and EGFR status were significantly higher in the discrepancy group than in the non-discrepancy group (36.8% vs 15.8%,  $P = 0.033$ , and 84.2% vs 51.5%,  $P = 0.011$ ) (Table 4).

## DISCUSSION

In the present study, discrepancy between an endoscopic forceps biopsy and a postgastrectomy specimen was associated with higher submucosal invasion, lymph node metastases, and positive EGFR status than non-discrepancy in EGC. In the subgroup analysis performed in undifferentiated post-surgical specimens, the discrepancy group showed a higher rate of lymphatic invasion, positive EGFR, and HER2 status, along with a higher proportion of submucosal invasion and lymph node metastases. These results suggested that discordance between an endoscopic biopsy and a surgical specimen could be a predictive factor related to poor outcome in EGC.

Currently, histologic diagnosis of gastric cancer is determined according to the WHO classification. An EGC lesion consisting of both differentiated and undifferentiated carcinomas is classified based on

the quantitatively predominant type. Histological heterogeneity presenting a mixture of differentiated and undifferentiated components is the most important factor for histological discrepancy between a preoperative biopsy and a post-procedural specimen. Cases of a mixed predominantly undifferentiated type showed higher lymph node metastases than cases of a pure undifferentiated type in EGC patients (19.0% vs 6.0%)<sup>[12]</sup>. In a study of predominantly differentiated type of EGC, the mixed type was significantly associated with large tumor size, more frequent submucosal invasion, and lymphovascular invasion compared to the pure type<sup>[13]</sup>. Therefore, EGC with a mixed histologic type affects the therapeutic outcomes and the consequent clinical course<sup>[14,15]</sup>. In our study, mixed type specimens according to the Lauren classification were more frequently found in the discrepancy group than in the non-discrepancy group (45.0% vs 11.6%,  $P < 0.001$ ). Although all specimens in the discrepancy group were not of the mixed histology type, the results showing more submucosal invasion and lymph node metastases in histological discordance between a biopsy sample and a resected specimen corresponded with those of the above studies.

The rate of discrepancy between a forceps biopsy and an endoscopically resected specimen in EGC was 2.3%-5.2%<sup>[3,5,6,16]</sup>. In a study that evaluated post-surgical specimens of mucosal gastric cancer, the discrepancy rate was 11.9%<sup>[17]</sup>, which was slightly higher than the rate of 9.4% in our study. These studies



**Table 4 Comparison of histopathological characteristics between 23 specimens having discrepancy and 103 specimens having non-discrepancy along with undifferentiated postoperative histology *n* (%)**

Variables	Discrepancy	Non-discrepancy	<i>P</i> value
Tumor size in the specimen ( <i>n</i> = 126)			
Median, cm (range)	3.0 (0.8-5.5)	2.5 (0.5-8.0)	0.343
Number of biopsies ( <i>n</i> = 126)			
Median, No. (range)	3 (2-6)	3 (1-8)	0.374
Location ( <i>n</i> = 126)			
Upper	3 (13.0)	10 (9.7)	0.481
Middle	10 (43.5)	62 (60.2)	
Lower	10 (43.5)	31 (30.1)	
Gross pattern ( <i>n</i> = 126)			
Elevated	1 (4.3)	1 (1.0)	0.615
Flat	4 (17.4)	20 (19.4)	
Depressed	9 (39.1)	51 (49.5)	
Mixed elevated	2 (8.7)	6 (5.8)	
Mixed flat	4 (17.4)	16 (15.5)	
Mixed depressed	3 (13.0)	9 (8.7)	
Tumor staging ( <i>n</i> = 126)			
I a	6 (26.1)	52 (50.5)	0.034
I b	17 (73.9)	51 (49.5)	
Nodal staging ( <i>n</i> = 126)			
Positive	6 (26.1)	9 (8.7)	0.020
Negative	17 (73.9)	94 (91.3)	
Lymphatic invasion ( <i>n</i> = 126)			
Positive	7 (30.4)	12 (11.7)	0.023
Negative	16 (69.6)	91 (88.3)	
Vascular invasion ( <i>n</i> = 126)			
Positive	2 (8.7)	2 (1.9)	0.152
Negative	21 (91.3)	101 (98.1)	
Ki-67 ( <i>n</i> =120)			
High	48 (94.7)	77 (76.2)	0.119
Low	1 (5.3)	24 (23.8)	
p53 ( <i>n</i> = 120)			
Positive	13 (68.4)	59 (58.4)	0.414
Negative	6 (31.6)	42 (41.6)	
HER2 ( <i>n</i> = 120)			
Positive (2+ and 3+)	7 (36.8)	16 (15.8)	0.033
Negative (0 and 1+)	12 (63.2)	85 (84.2)	
EGFR ( <i>n</i> = 120)			
Positive	16 (84.2)	52 (51.5)	0.011
Negative	3 (15.8)	49 (48.5)	

HER2: Human epidermal growth factor 2; EGFR: Epidermal growth factor receptor.

reported that the factors associated with histological discrepancy were lesion location in the upper or middle third of the stomach, easy friability, depressed type, and large tumor size. This indicated that the likelihood of mixed histology or misdiagnosis on biopsy could increase according to tumor location, morphology, gross pattern, or size. Our study did not show significant differences in the above factors between the groups with or without discrepancy. However, in the present study, fold change and positive EGFR status were predictable factors related to discordance. The surrounding fold change in the malignant lesion was an associated factor of invasion of the deeper layer than the confined mucosal layer<sup>[5]</sup>. The discrepancy group showed more fold change than the non-discrepancy group, resulting in more submucosal invasion. EGFR, a group of transmembrane tyrosine kinase receptors that regulate cellular proliferation, survival migration, and differentiation, was expressed in 30%-50% of

gastric cancer cases and it is known to be correlated with poor prognosis<sup>[18]</sup>. More positive EGFR status in the discrepancy group could be a factor related to poor outcomes such as submucosal invasion and lymph node metastasis. Moreover, the rate of HER2 overexpression was higher in the discrepancy group than in the non-discrepancy group in the subgroup analysis performed in specimens having an undifferentiated postoperative histology. HER2 is one of the EGFR family members and it is associated with decreased survival and clinicopathological features of tumor progression in gastric cancer<sup>[19]</sup>. Higher rate of positive HER2 and EGFR status might predict a poor prognosis, and therefore, patients showing discrepancy can be treated with monoclonal antibodies directed against these receptors.

The 5-year overall survival rates of EGC patients who underwent endoscopic resection or surgical resection were 93.6%-97.5%<sup>[20,21]</sup>. Because of high survival rates and good prognosis of EGC, recurrences were observed

in one patient of the discrepancy group and in two patients of the non-discrepancy group and disease-related deaths did not occur in both groups during the median 36-mo follow-up period.

Our study had several limitations. First, the analysis was retrospective and it was a small sized study conducted in a single center. There may be unrecognized or unmeasured biases and we could not generalize the property of discrepancy between an endoscopic biopsy and a surgical specimen based on these results. Second, HER2 overexpression was regarded as  $\geq 2+$  on IHC staining. Other studies defined HER2 overexpression as 3+ on IHC staining and positivity of fluorescence in situ hybridization in 2+ on IHC staining<sup>[22,23]</sup>.

In conclusion, the discrepancy in histologic differentiation between a forceps biopsy and a postoperative specimen was associated with submucosal invasion and lymph node metastases in EGC patients. The discordance was also associated with a more positive EGFR and HER2 status. Accordingly, patients who have histological discrepancy could be predicted to achieve a poor outcome and patients who have histological discrepancy detected in an endoscopically resected specimen might be considered to require additional surgical treatments.

## COMMENTS

### Background

Endoscopic resection is widely used for the treatment of early gastric cancer (EGC). The histologic differentiation type is one of important factor for deciding endoscopic procedure and the discordance of differentiation between a forceps biopsy and endoscopically resected specimen can need a radical gastrectomy. In addition, histologic discrepancy may a predictive factor for predicting poor outcomes. Therefore, it is needed to investigate clinicopathological variables of EGC patients in relation to differentiation discrepancy.

### Research frontiers

The discordance of histological differentiation between a forceps biopsy and an endoscopic specimen is associated with higher submucosal invasion and lymph node metastases in EGC patients.

### Innovations and breakthroughs

In previous studies, the factors associated with histological discrepancy in EGC were tumor location, morphology, gross pattern, and size. In the present study, histological discrepancy was associated with higher submucosal invasion, lymph node metastases, and positive epidermal growth factor receptor (EGFR) status. In the subgroup analysis performed in undifferentiated post-surgical specimens, the discrepancy was associated with higher lymphatic invasion, positive EGFR, and human epidermal growth factor 2 (HER2) status, along with higher submucosal invasion and lymph node metastases. These results suggested that discordance of histological differentiation could be a predictive factor related to poor outcome in EGC.

### Applications

The histological discrepancy between a forceps biopsy and an endoscopic specimen could be a predictive factor for a poor outcome. Patients who have histological discrepancy detected in an endoscopically resected specimen might be considered to require additional surgical treatments.

### Terminology

EGFR is a group of transmembrane tyrosine kinase receptors that regulate cellular proliferation, survival migration, and differentiation. HER2 is one of

the EGFR family members and it is associated with decreased survival and clinicopathological features of tumor progression in gastric cancer.

### Peer-review

The present study showed that discrepancy between an endoscopic forceps biopsy and a postgastrectomy specimen was associated with higher submucosal invasion, lymph node metastasis, and positive EGFR status than non-discrepancy in EGC. The authors concluded that discordance between an endoscopic biopsy and a surgical specimen could be a predictive factor related to poor outcome in EGC. Based on their findings, the authors suggested that patients who have histological discrepancy detected in an endoscopically resected specimen might be considered to require additional surgery.

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## Observational Study

# Gastric xanthoma is a predictive marker for metachronous and synchronous gastric cancer

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**Author contributions:** Shibukawa N, Ouchi S, Wakamatsu S, Wakahara Y and Kaneko A contributed equally to this work; Shibukawa N designed the research; Shibukawa N, Ouchi S, Wakamatsu S, Wakahara Y and Kaneko A performed the research; Shibukawa N and Kaneko A analyzed the data and wrote the paper.

**Institutional review board statement:** This study was reviewed and approved by the NTT West Osaka Hospital Ethics Committee.

**Informed consent statement:** Because of the anonymous nature of the data obtained after each patient had provided written informed consent for ESD, the requirement for informed consent was waived.

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## Abstract

### AIM

To investigate predictive markers for metachronous and synchronous gastric cancer (GC), which can develop after endoscopic submucosal dissection (ESD).

### METHODS

A total of 352 patients underwent ESD for early GC at NTT West Osaka Hospital between June 2006 and February 2016. Exclusion criteria were as follows: Remnant stomach, unknown *Helicobacter pylori* status, and endoscopic observation of the whole stomach outside our hospital. We analyzed data from 192 patients comprising 109 patients with solitary GC (Group A) and 83 with metachronous and synchronous GC (Group B). We retrospectively investigated the clinicopathological and endoscopic characteristics, and endoscopic risk score as predictive markers for GC.

### RESULTS

The median age of Group B [72 years (interquartile range 63-78)] was significantly higher than that of Group A [66 years (interquartile range 61-74), respectively,  $P = 0.0009$ ]. The prevalence of intestinal metaplasia in Group B tended to be higher than that in Group A (57.8% vs 45.0%,  $P = 0.08$ ). The prevalence of gastric xanthoma (GX) in Group B was significantly higher than that in Group A (54.2% vs 32.1%,  $P = 0.003$ ). The atrophy score in Group B was significantly higher than that in Group A ( $P = 0.005$ ). Multivariate analysis revealed that higher age and the presence of GX were independently related to metachronous and synchronous GC [OR = 1.04



(1.01-1.08),  $P = 0.02$ ; and OR = 2.11 (1.14-3.99),  $P = 0.02$ , respectively].

## CONCLUSION

The presence of GX is a useful predictive marker for metachronous and synchronous GC.

**Key words:** Gastric cancer; Metachronous neoplasms; Synchronous neoplasms; Xanthoma; Biomarker

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**Core tip:** This was a retrospective observational study to identify predictive markers for metachronous and synchronous gastric cancer (GC). Multivariate analysis revealed that higher age and the presence of gastric xanthoma were independently related to the development of metachronous and synchronous GC. Additional large prospective studies are necessary to investigate this important issue further.

Shibukawa N, Ouchi S, Wakamatsu S, Wakahara Y, Kaneko A. Gastric xanthoma is a predictive marker for metachronous and synchronous gastric cancer. *World J Gastrointest Oncol* 2017; 9(8): 327-332 Available from: URL: <http://www.wjgnet.com/1948-5204/full/v9/i8/327.htm> DOI: <http://dx.doi.org/10.4251/wjgo.v9.i8.327>

## INTRODUCTION

Endoscopic submucosal dissection (ESD) is currently accepted as an effective and minimally invasive treatment for early gastric cancer (GC)<sup>[1-7]</sup>. However, the development of metachronous and synchronous GC remains a possibility after ESD<sup>[8-14]</sup>. Predictive markers for the development of metachronous and synchronous GC have not been extensively studied. In addition, it is often difficult to clearly distinguish metachronous GC from synchronous GC because of missed detection of synchronous GC.

The cumulative incidence of metachronous GC after endoscopic resection for early GC ranges from 5.2% to 22.7%<sup>[8-14]</sup>. The incidence of missed detection of synchronous GC was reported to be between 1.2% and 7.7%<sup>[15,16]</sup>. In 2015, the Kyoto global consensus report on *Helicobacter pylori* (*H. pylori*) gastritis was published<sup>[17]</sup>. At the same time, an endoscopic score for GC risk was also announced<sup>[18]</sup>. Also, gastric xanthoma (GX) has been reported as the predictive marker of early GC<sup>[19,20]</sup>.

The usefulness of endoscopic score for GC risk as an accurate predictor of GC risk remains unclear. And the utility of GX as a predictive marker of metachronous and synchronous GC also remains unknown.

Therefore, we performed a retrospective study to investigate predictive markers of the development of metachronous and synchronous GC, including GX and

endoscopic score for GC risk.

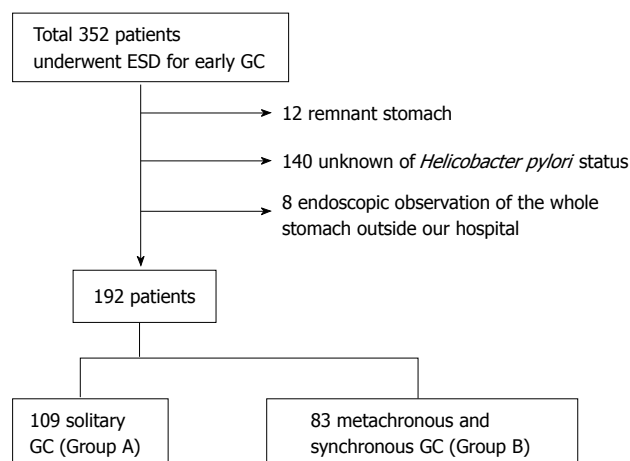
## MATERIALS AND METHODS

### Patients

This study was a retrospective, single-center, observational study. Between June 2006 and February 2016, a total of 352 patients underwent ESD for early GC at NTT West Osaka Hospital. Exclusion criteria for this study were as follows: Remnant stomach, unknown *H. pylori* status, and endoscopic observation of the whole stomach outside our hospital. Finally, 192 patients including 109 with solitary GC (Group A) and 83 with metachronous and synchronous GC (Group B), were included in the study (Figure 1). Solitary GC was defined as no past history of GC and only one GC that developed during the study period. Metachronous and synchronous GC were defined as a new GC that developed in an area other than the site of primary GC and multiple GC that developed at the same endoscopic examination. The following factors were examined: Age, sex, complicated with diabetes mellitus, complicated with other malignant disease, *H. pylori* status, tumor location, tumor size, macroscopic type, histological type, tumor depth, endoscopic score for GC risk, and presence of severe atrophy, intestinal metaplasia, and GX. Presence of *H. pylori* infection was determined by serum antibody, rapid urease test, immunohistochemistry, <sup>13</sup>C urea breath test, or stool antigen test. Endoscopic images were evaluated at the time of GC diagnosis or ESD. Endoscopic images were reviewed by one expert endoscopist. We classified the severity of gastric atrophy according to the criteria of Kimura and Takemoto<sup>[21]</sup>. Severe atrophy was classified as O-2, 3, and P. O-P was the state that gastric atrophy progressed in the whole stomach. We diagnosed presence of GX as yellowish-white flat or slight elevated lesion by white light imaging. Endoscopic score for GC risk was calculated as follows: (1) atrophy: 0 if C-0 and 1, 1 if C-2 and 3, 2 if O-1, 2, 3, and P; (2) intestinal metaplasia: 0 if absent, 1 if present at gastric antrum, 2 if present at gastric antrum and body; (3) fold swelling: 0 if absent, 1 if present; (4) nodular gastritis: 0 if absent, 1 if present; and (5) diffuse redness: 0 if absent, 1 if mild, 2 if severe. For assessment of intestinal metaplasia, image-enhanced endoscopy with distinct white light imaging was necessary. In this study, we evaluated intestinal metaplasia as grayish-white flat elevated lesion by white light imaging, without image-enhanced endoscopy. This study was carried out with the approval of the NTT West Osaka Hospital Ethics Committee. Because of the anonymous nature of the data obtained after each patient had provided written informed consent for ESD, the requirement for informed consent was waived.

### Statistical analysis

All statistical analyses were performed with EZR (Saitama Medical Center, Jichi Medical University,



**Figure 1** Flow diagram for the study. ESD: Endoscopic submucosal dissection; GC: Gastric cancer.

Saitama, Japan), which is a graphical user interface for R (The R Foundation for Statistical Computing, Vienna, Austria). More precisely, it is a modified version of R commander designed to add statistical functions frequently used in biostatistics<sup>[22]</sup>. Fisher's exact test was performed to investigate the relationships between the two groups. Differences between the two groups were analyzed by Mann-Whitney *U* test when the data was not parametric. Multivariate logistic analysis was used to identify predictive markers of metachronous and synchronous GC. Age, sex, and baseline variables with  $P < 0.2$  in univariate analysis were included in the multivariate logistic analysis. The threshold for significance was  $P < 0.05$ .

## RESULTS

### Clinicopathological characteristics of patients

The characteristics of the two patient groups are shown in Table 1.

The median ages in Group A and B were 66 [interquartile range (IQR) 61-74] and 72 (IQR 63-78) years, respectively ( $P = 0.0009$ ). The proportion of male patients was high in both groups (84.4% vs 86.7%,  $P = 0.7$ ). *H. pylori* status was positive for many patients in Group A and B (75.2% vs 68.7%, respectively,  $P = 0.4$ ).

Regarding tumor location, lesion of the upper part of the stomach was least frequent. Median tumor size was 15 mm in both groups. The most frequent macroscopic type was 0-II a and the most frequent histological type was the differentiated type.

### Endoscopic characteristics

Endoscopic characteristics are shown in Table 2. Although the difference was not significant, the prevalence of severe atrophy and intestinal metaplasia in Group B tended to be higher than that in Group A (89.2% vs 79.8%,  $P = 0.1$  and 57.8% vs 45.0%,  $P = 0.08$ ). The prevalence of GX in Group B was significantly higher than that in Group A (54.2% vs

**Table 1** Clinicopathological characteristics of the two groups *n* (%)

	Group A ( <i>n</i> = 109)	Group B ( <i>n</i> = 83)	<i>P</i> value
Age, median [IQR], yr	66 [61-74]	72 [63-78]	0.0009
Male	92 (84.4)	72 (86.7)	0.7
Diabetes mellitus	24 (22.0)	12 (14.5)	0.2
Complicated other malignant disease	27 (24.8)	22 (26.5)	0.9
<i>Helicobacter pylori</i> status			0.4
Positive	82	57	
Post eradicated	23	26	
Negative	4	0	
Tumor location			0.9
Upper	10	11	
Middle	57	39	
Lower	42	33	
Tumor size, median [IQR], mm	15 [10-20]	15 [12-22]	0.3
Macroscopic type			0.5
0-I	9	3	
0-II a	67	52	
0-II b	26	25	
0-II c	7	3	
Differentiated type	109 (100)	81 (97.6)	0.2
Tumor depth			0.1
M	97	80	
SM	12	3	

IQR: Interquartile range; M: Mucosal; SM: Submucosal.

32.1%,  $P = 0.003$ ). The number and size of GX were not significantly different between the two groups.

### Endoscopic score for GC risk

The endoscopic score for GC risk is reported in Table 3. The atrophy score in Group B was significantly higher than that in Group A ( $P = 0.005$ ). The scores for intestinal metaplasia, fold swelling, and diffuse redness, and the total score were not significantly different between the two groups. Nodular gastritis was absent in all patients in this study.

### Multivariate logistic analysis

Age, male sex, severe atrophy, presence of intestinal metaplasia, and presence of GX were subjected to multivariate logistic analysis. As shown in Table 4, higher age and the presence of GX were independently related to metachronous and synchronous GC.

## DISCUSSION

In the present study, we compared the characteristics of 109 patients with solitary GC to those of 83 patients with metachronous and synchronous GC in order to identify predictive markers for metachronous and synchronous GC. Multivariate logistic analysis revealed that high age and the presence of GX were independently related to metachronous and synchronous GC.

The results of recent studies indicated that male sex, multiple initial GC, severe atrophy, and multiple GC before successful *H. pylori* eradication were independent risk factors for metachronous GC<sup>[13,14]</sup>. After performing

**Table 2** Endoscopic characteristics of the two groups *n* (%)

	Group A ( <i>n</i> = 109)	Group B ( <i>n</i> = 83)	<i>P</i> value
Severe atrophy	87 (79.8)	74 (89.2)	0.1
Intestinal metaplasia	49 (45.0)	48 (57.8)	0.08
Gastric xanthoma	35 (32.1)	45 (54.2)	0.003

**Table 3** Endoscopic score of risk for gastric cancer of the two groups

	Group A ( <i>n</i> = 109)	Group B ( <i>n</i> = 83)	<i>P</i> value
Atrophy			0.005
0	4	0	
1	6	0	
2	99	83	
Intestinal metaplasia			0.1
0	60	35	
1	33	34	
2	16	14	
Fold swelling			0.6
0	99	73	
1	10	10	
Nodular gastritis			1.0
0	109	83	
1	0	0	
Diffuse redness			0.5
0	4	0	
1	23	25	
2	82	58	
Total score, median [IQR]	4 [4-5]	4 [4-5]	0.1

IQR: Interquartile range.

univariate analysis, we carried out multivariate logistic analysis using male sex and severe atrophy as variates, but our results revealed that these two markers were not predictive of metachronous and synchronous GC. This finding may reflect the fact that most patients in this study were male and showed severe atrophy in both groups.

Endoscopic findings related to the development of GC have been previously reported<sup>[23-27]</sup>. Of these, five endoscopic findings were confirmed; atrophy, intestinal metaplasia, fold swelling, nodular gastritis, and diffuse redness. A subsequent study reported the endoscopic score for GC risk<sup>[18]</sup>; however, the usefulness of this score remains unclear. After performing univariate analysis, we carried out multivariate logistic analysis using severe atrophy and intestinal metaplasia as variates. However, our results indicated that these two markers were not predictive of metachronous and synchronous GC. Further investigations are necessary to evaluate the usefulness of the endoscopic score for GC risk.

GX, a localized non-neoplastic accumulation of foamy histiocytes in the lamina propria of the inflamed gastric mucosa, is occasionally found during EGD<sup>[28]</sup>. GX is a positive indicator of *H. pylori* and persists after

**Table 4** Multivariate analysis of predictive marker of metachronous and synchronous gastric cancer

	Odds ratio	<i>P</i> value
Age	1.04 (1.01-1.08)	0.02
Male	1.38 (0.57-3.34)	0.47
Severe atrophy	1.68 (0.70-4.05)	0.25
Intestinal metaplasia	1.35 (0.71-2.54)	0.36
Gastric xanthoma	2.11 (1.14-3.99)	0.02

*H. pylori* eradication therapy. GX has received little attention clinically, perhaps because it is considered a benign entity<sup>[20]</sup>. A retrospective cohort study reported that the presence of GX was significantly associated with the presence of GC<sup>[19]</sup>. Another cohort study performed at the same hospital reported that GX was a useful marker for predicting the development of GC by performing endoscopic follow-up examinations<sup>[20]</sup>. However, both these studies did not investigate GX as a predictive marker for metachronous and synchronous GC. In our study, univariate analysis revealed that GX was significantly more prevalent in Group B than in Group A. In addition, results of multivariate logistic analysis indicated that GX was a predictive marker for metachronous and synchronous GC. To our knowledge, this is first report of the presence of GX as a useful predictive marker for metachronous and synchronous GC.

Why does GC develop more frequently in patients with GX? Increased release of oxygen free radicals may be involved in the formation of GX<sup>[29]</sup>. On the other hand, the presence of GX may reflect the severity and long duration of chronic gastritis<sup>[20]</sup>, which is a risk factor for GC development. Because of the same reason, GX may be more frequent in Group B than Group A. However, further studies are required to elucidate this link.

Our study has some limitations. First, it was a retrospective single-center study. Second, the sample size was small. Finally, we did not analyze inter-observer variability in the assessment of endoscopic images.

In summary, our results revealed that higher age and the presence of GX were independently related to metachronous and synchronous GC. These findings, especially the predictive value of the presence of GX, could improve the timely detection and treatment of metachronous and synchronous GC. Further investigations are necessary to confirm the predictive value of these markers.

## COMMENTS

### Background

Predictive markers for the development of metachronous and synchronous gastric cancer (GC) have not been extensively studied. In addition, it is often difficult to clearly distinguish metachronous GC from synchronous GC because of missed detection of synchronous GC. And the usefulness of endoscopic score for GC risk and gastric xanthoma (GX) as the predictive markers of metachronous and synchronous GC remains unknown.

## Research frontiers

This study investigated the predictive markers for metachronous and synchronous GC.

## Innovations and breakthroughs

Higher age and the presence of GX were independently related to the development of metachronous and synchronous GC.

## Applications

GX is a useful predictive marker for metachronous and synchronous GC.

## Terminology

Solitary GC is defined as no past history of GC and only one GC that developed during the study period. Metachronous and synchronous GC are defined as a new GC that developed in an area other than the site of primary GC and multiple GC that developed at the same endoscopic examination.

## Peer-review

This is a retrospective study to investigate predictive markers for metachronous and synchronous GC developing after endoscopic submucosal dissection. This is good idea for clinics.

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Randomized Controlled Trial

# Impact of tumour histological subtype on chemotherapy outcome in advanced oesophageal cancer

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## Abstract

### AIM

To investigate the impact of histology on outcome in advanced oesophageal cancer treated with first-line fluoropyrimidine-based chemotherapy.

### METHODS

Individual patient data were pooled from three randomised phase III trials of fluoropyrimidine-based chemotherapy  $\pm$  platinum/anthracycline in patients with advanced, untreated gastroesophageal adenocarcinoma or squamous cell carcinoma (SCC) randomised between 1994 and 2005. The primary endpoint was overall survival of oesophageal cancer patients according to histology. Secondary endpoints were response rates and a toxicity composite endpoint.

### RESULTS

Of the total 1836 randomised patients, 973 patients (53%)

were eligible (707 patients with gastric cancer were excluded), 841 (86%) had adenocarcinoma and 132 (14%) had SCC. There was no significant difference in survival between patients with adenocarcinoma and SCC, with median overall survivals of 9.5 mo *vs* 7.6 mo (HR = 0.85, 95%CI: 0.70-1.03,  $P = 0.09$ ) and one-year survivals of 38.8% *vs* 28.2% respectively. The overall response rate to chemotherapy was 44% for adenocarcinoma *vs* 33% for SCC ( $P = 0.01$ ). There was no difference in the frequency of the toxicity composite endpoint between the two groups.

## CONCLUSION

There was no significant difference in survival between adenocarcinoma and SCC in patients with advanced oesophageal cancer treated with fluoropyrimidine-based chemotherapy despite a trend for worse survival and less chemo-sensitivity in SCC. Tolerance to treatment was similar in both groups. This analysis highlights the unmet need for SCC-specific studies in advanced oesophageal cancer and will aid in the design of future trials of targeted agents.

**Key words:** Oesophageal cancer; Adenocarcinoma; Chemotherapy; Squamous; Pooled analysis

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**Core tip:** There is a lack of published data on differential treatment response according to histology in oesophageal cancer. This paper shows improved response rates with first-line chemotherapy and a trend towards improved survival in adenocarcinoma compared to squamous cell carcinoma (SCC). It is increasingly recognised that these histological subtypes represent discrete disease entities with divergent treatment pathways in both the early stage and advanced settings. Novel treatments in SCC remain sparse and there are few dedicated trials in this subtype. This data highlights the poor outcomes seen with chemotherapy alone and the need for further research, particularly for SCC.

Davidson M, Chau I, Cunningham D, Khabra K, Iveson T, Hickish T, Seymour M, Starling N. Impact of tumour histological subtype on chemotherapy outcome in advanced oesophageal cancer. *World J Gastrointest Oncol* 2017; 9(8): 333-340 Available from: URL: <http://www.wjgnet.com/1948-5204/full/v9/i8/333.htm> DOI: <http://dx.doi.org/10.4251/wjgo.v9.i8.333>

## INTRODUCTION

Oesophageal cancer is the eighth most common malignancy worldwide with an estimated 456000 new cases and 400000 deaths worldwide in 2012, ranking it the sixth most common cause of cancer-related deaths<sup>[1]</sup>. Despite recent advances in genetic and molecular characterisation and the development

of novel targeted agents survival rates for oesophageal carcinoma have changed little for many decades, and outcomes for advanced disease remain poor. Worldwide, squamous cell carcinoma (SCC) is the predominant histological subtype however in North America and Northern Europe the incidence of oesophageal adenocarcinoma has increased in the last 20 years whereas that of SCC has decreased<sup>[2,3]</sup>. This is likely to reflect the distinct aetiological factors implicated in the development of the two diseases. SCC is strongly correlated with excessive alcohol consumption, cigarette smoking and poor socioeconomic status whereas adenocarcinoma is associated with obesity and gastroesophageal reflux disease (GORD)<sup>[3,6]</sup>. Thus the rise in adenocarcinoma may be in part due to changing lifestyle factors in Western populations<sup>[3]</sup>. Genomic technology has been applied to both gastric and oesophageal cancer in an effort to improve understanding and stratification on a genetic and molecular level, with emerging differences in the genetic landscape between the two histological subtypes suggesting a need for more tailored therapeutic strategies<sup>[7,8]</sup>. Historically however treatment patterns for both subtypes have been similar, with many clinical trials evaluating chemotherapy conducted since the mid-1990s including patients with gastric, oesophageal, or oesophagogastric junction (OGJ) cancer, regardless of histology. Similarly, studies in early stage oesophageal cancer often include both histological subtypes, such as the recent CROSS trial evaluating neoadjuvant chemo-radiation. This identified disparities in outcome according to histology, with a statistically significant overall survival benefit seen only in the smaller SCC cohort<sup>[9]</sup>.

In the advanced disease setting cisplatin/fluorouracil with the possible addition of a third drug - either epirubicin or a taxane - is commonly used as a first-line chemotherapy, and second-line agents include irinotecan, docetaxel and paclitaxel<sup>[10,11]</sup>. More recently treatment patterns have diverged, with the introduction of novel molecularly-targeted therapy for gastroesophageal adenocarcinomas. Notably effective therapies targeting *HER2* (trastuzumab) and the vascular endothelial growth factor receptor 2 (ramucirumab) are applicable only to adenocarcinomas<sup>[12-14]</sup>.

Three randomised phase III studies of fluoropyrimidine-based combination chemotherapy have been published in patients with advanced gastroesophageal cancer including oesophageal SCC and adenocarcinoma<sup>[10,15,16]</sup>. In multivariate Cox regression analysis histology was not identified as a variable impacting on survival, however patients with SCC accounted for less than 10% of the patients in each trial. Although SCC normally represent a small minority of patients enrolled on most clinical trials it is not clear what influence histologic subtype exerts on response rate or survival duration in patients treated with cytotoxic chemotherapy regimens for metastatic disease, and SCC has been associated with both worse, better or similar outcomes to adenocarcinoma<sup>[17-19]</sup>.

The distinct epidemiological, genetic and molecular characteristics of SCC as compared to adenocarcinoma could potentially influence response to therapies administered in the advanced disease setting. In this pooled analysis of the three randomised phase III studies which included patients with both advanced oesophageal SCC and adenocarcinoma, we aimed to evaluate whether there was a differential treatment effect according to histology.

## MATERIALS AND METHODS

### *Patients and treatments*

Between 1994 and 2005, 1836 patients were randomised predominantly from the United Kingdom in three multi-centre randomised controlled trials of fluoropyrimidine-based chemotherapy in patients with untreated locally advanced or metastatic carcinoma of oesophagus, OGJ, or stomach<sup>[10,15,16]</sup>. Similar eligibility criteria were applied in the three trials; patients had histologically confirmed inoperable adenocarcinoma, SCC or undifferentiated carcinoma of the oesophagus, OGJ or stomach, adequate haematological, renal and hepatic function and an Eastern Co-operative Oncology Group performance status (PS) of 0-2. Written informed consent was obtained from all patients and all three studies were approved by the Scientific and Research Ethics Committees of the participating institutions.

The first study randomised 580 patients between 1995 and 1998 to treatment with ECF [epirubicin 50 mg/m<sup>2</sup> intravenously (IV) and cisplatin 60 mg/m<sup>2</sup> IV infusion with hydration on day 1 plus 5-FU 200 mg/m<sup>2</sup> per day by protracted venous infusion (PVI)] or MCF [mitomycin C (MMC) 7 mg/m<sup>2</sup> on day 1 every six weeks, cisplatin 50 mg/m<sup>2</sup> IV day 1 and PVI-5-FU 300 mg/m<sup>2</sup> per day]<sup>[15]</sup>. The second study randomised 254 patients between 1994 and 2001 to PVI 5-FU (300 mg/m<sup>2</sup> per day) or the same dose of PVI 5-FU plus MMC (7 mg/m<sup>2</sup> every six weeks)<sup>[16]</sup>. The third study conducted randomised 1002 patients between 2000 and 2005 to ECF, ECX (X denotes capecitabine given at a dose of 625 mg/m<sup>2</sup> twice a day continuously), EOF (O denotes oxaliplatin 130 mg/m<sup>2</sup> on day 1 every three weeks replacing cisplatin) and EOX<sup>[10]</sup>.

A maximum of 8 cycles of chemotherapy (24 wk) with response assessment computed tomography (CT) scans at 12 and 24 wk was stipulated in the three study protocols. Overall survival (OS) was the primary outcome measure in these trials and toxicity data was recorded at each treatment visit every three weeks.

Only eligible patients with squamous carcinoma or OGJ adenocarcinoma who received at least one dose of chemotherapy were included in this analysis which was based on individual patient data from these trials.

### *Statistical analysis*

OS was the primary endpoint of this pooled analysis and was calculated from the date of randomisation until

death from any cause, or censored at the date of last follow-up for surviving patients according to the Kaplan-Meier method. Survival analyses were performed on the eligible population and compared between patients with SCC and adenocarcinoma using the log rank test. Multivariate survival analysis was performed using Cox proportional hazard model and stratified for treatment centres. The following factors were included: Histology, gender, primary site (oesophagus vs OGJ), liver or peritoneal metastases (presence vs absence), serum alkaline phosphatase (< 100 U/L vs ≥ 100 U/L) and performance status (0-1 vs 2) based on previously-identified prognostic factors in advanced OG cancer<sup>[20,21]</sup>, as well as treatment arm and trial.

Objective response rates between SCC and adenocarcinoma were compared using  $\chi^2$  test. A chemotherapy-specific toxicity composite endpoint (TCE) was constructed as a surrogate for undesirable cytotoxic-related toxicities. TCE was defined as the first occurrence of grade 3 or 4 diarrhoea, neutropenia, febrile neutropenia, fever, infection, nausea and vomiting or grade ≥ 2 renal or neurotoxicity. TCE was compared between the two histological subtypes using  $\chi^2$  test. Time to TCE was compared between SCC and adenocarcinoma using log rank test.

Two-sided *P* value of less than 0.05 were considered significant for the overall survival endpoint, and 95%CI quoted. Analyses were performed using SPSS package version 23 (SPSS Inc, Chicago, IL, United States).

## RESULTS

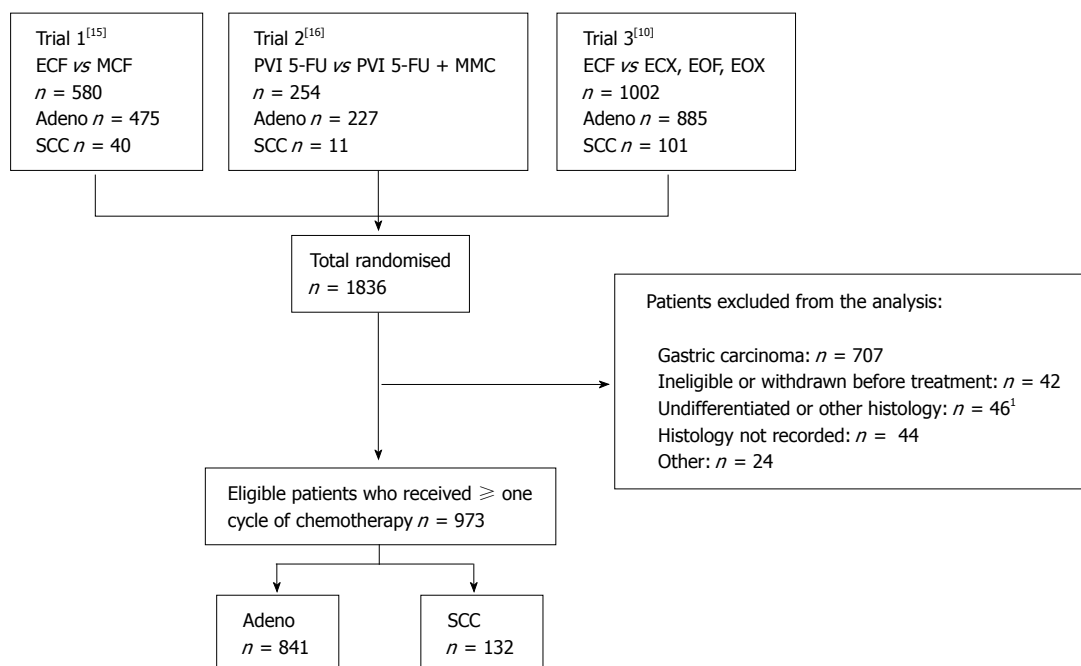
### *Patient characteristics*

Of the 1836 patients randomised to the three trials, 973 patients (53%) were eligible for this pooled analysis as indicated in Figure 1. Seven hundred and seven of the 1836 patients (39%) were excluded due to the primary tumour origin being gastric. Of the 973 eligible patients 841 (86%) had adenocarcinoma and 132 (14%) had SCC. Baseline patient characteristics are shown in Table 1. These were broadly balanced between the two histological sub-types except that predictably a greater proportion of adenocarcinoma occurred at the OGJ with metastases to the liver/peritoneum, and there were more males with adenocarcinoma.

### *Survival*

At the time of the data cut-off, 863 of the 973 patients (88%) had died and the median follow-up for surviving patients was 19 mo. The median survival for the whole cohort of 973 eligible patients was 9.4 mo (95%CI: 8.82-9.99). One year survival was 37.3% (95%CI: 37.27-37.33) and 2 year survival was 13.5% (95%CI: 13.48-13.52). There was no significant difference in survival between patients with adenocarcinoma and SCC, with median OS of 9.5 mo vs 7.6 mo (HR = 0.85, 95%CI: 0.70-1.03, *P* = 0.09), although the curves did appear to separate between 6 mo to 2





**Figure 1** CONSORT diagram indicating the derivation of eligible patients in this analysis. <sup>1</sup>Includes carcinoma, undifferentiated carcinoma, adenosquamous carcinoma. SCC: Squamous cell carcinoma; PVI: Protracted venous infusion.

**Table 1** Patient characteristics *n* (%)

	Adeno	SCC	Total
No. of patients	841	132	973
Median age (range)	62 (22-84)	60 (37-77)	61 (22-84)
Gender			
Male	730 (87)	95 (72)	825 (85)
Female	111 (13)	37 (28)	148 (15)
Performance status <sup>1</sup>			
0	223 (27)	33 (25)	256 (26)
1	489 (58)	75 (57)	564 (58)
2	127 (15)	23 (18)	150 (15)
Sub-site			
Oesophagus	438 (52)	117 (89)	555 (57)
OGJ	403 (48)	15 (11)	418 (43)
Extent of disease <sup>2</sup>			
Locally advanced	219 (26)	36 (27)	255 (26)
Metastatic	622 (74)	95 (72)	717 (74)
Location of metastases			
Liver	340 (46)	46 (35)	386 (40)
Peritoneum	41 (5)	4 (3)	45 (4.5)
Lung	136 (16)	18 (14)	154 (16)

<sup>1</sup>PS unknown or 3 in < 1% of all patients; <sup>2</sup>Data was unavailable for one patient in the SCC group. SCC: Squamous cell carcinoma; OGJ: Oesophagogastric junction.

years suggestive of a poorer survival for SCC during this period (Figure 2A). One and two year survival figures for adenocarcinoma were 38.8% (95%CI: 38.77-38.83) and 13.6% (95%CI: 13.57-13.63) respectively and for SCC were 28.2% (95%CI: 28.12-28.28) and 12.3% (95%CI: 12.24-12.36). When considering “true” oesophageal cancer patients only and excluding those with junctional tumours, there was again no significant difference in survival

between patients with adenocarcinoma (*n* = 438) and SCC (*n* = 117), with median OS of 9.5 mo vs 7.7 mo (HR = 0.91, 95%CI: 0.73-1.13, *P* = 0.38) (Figure 2B). In multivariate analysis, previously identified known prognostic factors of performance status, liver/peritoneal metastases and alkaline phosphatase were all significant. Histology and site of primary tumour were not shown to be significant prognostic factors. For effect of treatment received there was a significant association of treatment within trial 2<sup>[16]</sup>-which did not incorporate a platinum component into either treatment arm-with poorer outcome (Table 2).

### Response and toxicity

The objective response rate to chemotherapy (Table 3) was significantly higher for patients with adenocarcinoma compared to SCC (44% vs 33%, *P* = 0.01). A greater proportion of patients with SCC compared to adenocarcinoma progressed during chemotherapy (29% vs 19%, *P* = 0.01) and the proportion of patients with stable disease was similar for both histological subtypes. There was no difference in the proportion of patients experiencing the toxicity composite endpoint (TCE) for adenocarcinoma as compared to SCC (45% vs 44%, *P* = 0.77) (Table 3). Similarly there was no difference in the time to development of TCE (Figure 3) between the histological subtypes (HR = 0.98, 95%CI: 0.74-1.29, *P* = 0.9).

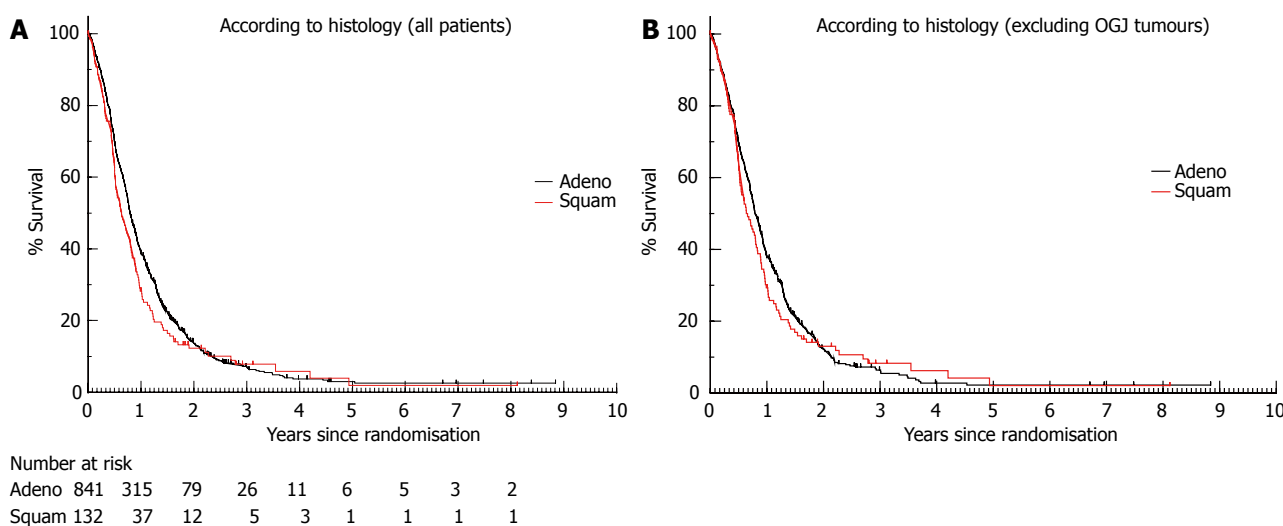
## DISCUSSION

This study represents the largest pooled analysis of differential chemotherapy effects in patients with

**Table 2 Multivariate analyses of overall survival**

Factors		Univariate analysis			Multivariate analysis		
		HR	95%CI	P value	HR	95%CI	P value
Overall survival							
Histological arm	Adenocarcinoma (r)	1.000					
	SCC	1.196	0.972-1.471	0.089			
Sex	Female (r)	1.000					
	Male	0.983	0.807-1.196	0.983			
Subsite	Oesophagus (r)	1.000					
	OGJ	0.988	0.853-1.145	0.876			
Liver mets	No (r)	1.000			1.000		
	Yes	1.671	1.433-1.948	< 0.001	1.581	1.341-1.863	< 0.001
Peritoneal mets	No (r)	1.000			1.000		
	Yes	2.290	1.583-3.314	< 0.01	2.190	1.503-3.191	< 0.001
ALP	< 100 U/I (r)	1.000			1.000		
	≥ 100 U/I	1.608	1.357-1.908	< 0.001	1.287	1.072-1.544	0.007
Performance score	0-1 (r)	1.000			1.000		
	2-3	2.140	1.754-2.611	< 0.001	1.703	1.374-2.110	< 0.001
Treatment arm	EOX (r)	1.000					
	EOF	1.122	0.848-1.484	0.420			
	ECX	1.139	0.862-1.505	0.361			
	ECF	1.175	0.916-1.506	0.204			
	MCF	1.176	0.870-1.589	0.291			
	PVI 5FU + MMC	2.107	1.461-3.040	< 0.001			
	PVI 5FU	2.132	1.481-3.067	< 0.001			
	Overall			< 0.001			
Study	Trial 3 <sup>[10]</sup> (r)	1.000			1.000		
	Trial 1 <sup>[15]</sup>	0.993	0.804-1.228	0.951	1.034	0.830-1.288	0.763
	Trial 2 <sup>[16]</sup>	1.850	1.432-2.390	< 0.001	1.736	1.326-2.271	< 0.001
	Overall			< 0.001			< 0.001

All variables with a *P*-value of less than 0.2 in univariate analyses were entered into a multivariate model in a forward stepwise manner. r: Reference; mets: Metastases; SCC: Squamous cell carcinoma; OGJ: Oesophagogastric junction; PVI: Protracted venous infusion; MMC: Mitomycin C.



**Figure 2 Overall survival.** A: Overall survival according to histology (adenocarcinoma = 841 patients, SCC = 132 patients). The HR for death in the adenocarcinoma group compared to the SCC group was 0.85 (95%CI: 0.70-1.03, *P* = 0.09); B: Overall survival according to histology excluding OGJ tumours (adenocarcinoma = 438 patients, SCC = 117 patients). The HR for death for the adenocarcinoma group compared to the SCC group was 0.91 (95%CI: 0.73-1.13, *P* = 0.38). SCC: Squamous cell carcinoma; OGJ: Oesophagogastric junction.

advanced oesophageal adenocarcinoma and SCC undergoing fluoropyrimidine-based chemotherapy in randomised phase III controlled trials with mature survival data. All three analysed trials incorporated a fluoropyrimidine in each treatment arm, and two of the

trials included a platinum agent in each arm. In this pooled analysis there was no significant difference in overall survival between patients with adenocarcinoma compared to those with SCC with median overall survivals of 9.5 mo vs 7.6 mo (HR = 0.85, *P* = 0.09)

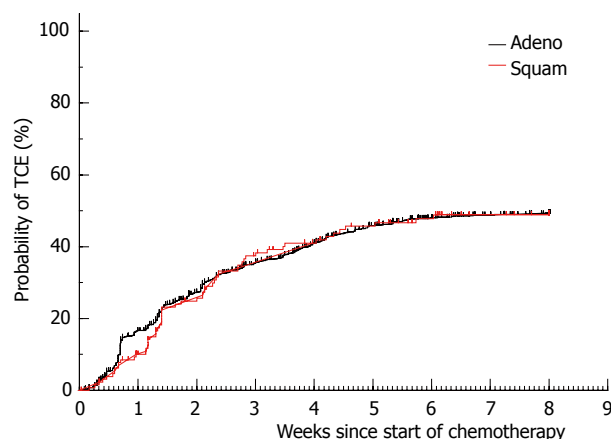
**Table 3** Objective response rates and toxicity composite endpoint *n* (%)

	Adeno	SCC
No. of patients	841	132
Complete response	48 (6)	7 (5)
Partial response	323 (38)	36 (27)
Stable disease	224 (30)	35 (27)
Progressive disease	157 (19)	38 (29)
Objective response rate	371 (44)	43 (33)
(95%CI)	41%-48%	25%-41%
		<i>P</i> = 0.01
Toxicity composite endpoint	381 (45)	58 (44)
(95%CI)	42%-49%	35%-53%
		<i>P</i> = 0.77

SCC: Squamous cell carcinoma.

respectively. A possible limitation of interpretation of this data is the imbalanced distribution of histological subtype between oesophageal and OGJ cancers. As expected, the proportion of SCC histology was higher in the oesophageal only group as compared to the total cohort of oesophageal and OGJ patients (21% vs 14%). A further analysis excluding OGJ patients however also did not show a significant difference in median OS between adenocarcinoma and SCC (9.5 mo vs 7.7 mo, HR = 0.91, *P* = 0.38). Histology and site of primary tumour were not shown to be predictors of survival in multivariate analysis, consistent with previously reported prognostic variables in oesophageal and gastric cancer based on smaller analyses<sup>[20,22,23]</sup>. The survival curves did appear to separate between 6 mo and 2 years, with SCC patients appearing to have worse survival during this period, but the curves then overlapped from two years onwards. Lack of a statistically significant difference in survival in the presence of a trend could reflect that this pooled analysis remains underpowered. Although the potential for heterogeneity may confound interpretation of data from pooled analyses, the eligibility criteria for these three trials were similar, individual patient data were used to strengthen the analysis, treatment arms and trials were incorporated in the multivariate analysis and survival outcomes from ECF, evaluated in the two largest trials<sup>[10,15]</sup>, were consistent. Inclusion of patients with advanced SCC in these studies was controversial in terms of potentially creating a heterogeneous study population however based on the current analysis survival outcomes with standard chemotherapy are not significantly different with SCC compared to adenocarcinoma, although there may be a trend towards worse survival. The only differential treatment effect noted was a significant difference in objective response rates between adenocarcinoma and SCC (44% vs 33% respectively). A greater proportion of SCC patients also progressed during treatment (29% vs 19%), suggesting that oesophageal SCC may be less chemo-sensitive than adenocarcinoma.

There was no difference in time to development of TCE or of the proportion of patients with TCE



**Figure 3** The time to development of the toxicity composite endpoint is shown for patients with adenocarcinoma (*n* = 841) vs squamous cell carcinoma (*n* = 132). TCE: Toxicity composite endpoint.

between the two histological sub-types. A difference might have been expected given the association of co-morbid conditions with SCC. However, within clinical trials there may be selection bias favouring inclusion of fitter patients (patients with a performance status of 2 comprised only 15% of the pooled patient population in this analysis). Although this does potentially limit extrapolation of the results of this analysis to patients with SCC in the general population this would apply to most randomised controlled trials in this disease.

Application of genomic technology is revealing increasing differences between the histological subtypes of oesophageal cancer on a genetic and molecular level. In an analysis performed by the Cancer Genome Atlas, four gastric cancer subtypes have been proposed: Tumours positive for Epstein-Barr virus (EBV), microsatellite unstable tumours (MSI), genomically stable (GS) tumours and tumours with chromosomal instability (CIN)<sup>[24]</sup>. Each subtype was found throughout the stomach, but CIN tumours showed elevated frequency in the OGJ and cardia. In CIN tumours genomic amplifications of receptor tyrosine kinases such as *VEGFA* and cell cycle mediators such as *CCND1* and *CDK6* with potentially relevant clinical implications were found with increased frequency. Specific to oesophageal adenocarcinomas, a sequencing study of 149 tumours by a United States group published in *Nature Medicine* in 2013 confirmed recurrent mutations in known cancer-driving genes including *TP53*, *CDKN2A*, *SMAD4*, *ARID1A* and *PIK3CA*<sup>[25]</sup>. Similarly, a number of recent studies have applied NGS to the study of oesophageal SCC, demonstrating recurrent mutations in known oncogenic drivers including *TP53*, *NOTCH1*, *PIK3CA* and *FAT1*, as well as amplifications in *CCND1* and *CDKN2A*<sup>[26]</sup>. The cell cycle regulation pathway is one of the most consistently altered in oesophageal SCC, where mutations are observed at a high frequency and are associated with poor prognosis and metastasis<sup>[27,28]</sup>. A recent study has compared the genomic profiles of 71 SCC and 231 oesophageal adenocarcinomas, focusing

on the identification of therapeutically relevant genomic alterations in both groups<sup>[8]</sup>. Similarly high frequencies of clinically relevant genomic alterations were found in both histological subtypes; however the profiles of genomic alterations in the two diseases differed substantially. *KRAS* and *HER2* were more frequently altered in adenocarcinoma, while *MTOR* pathway genes (*PIK3CA*, *PTEN*) and *NOTCH1* were more frequently altered in SCC. Exploitation of the molecular differences between the two histological sub-types may help direct optimal application of targeted therapies in this disease.

Although our data is historical, the chemotherapy landscape for oesophageal cancers has not changed significantly in the intervening years. Targeted treatments for oesophageal adenocarcinomas are now in routine clinical use and starting to provide tangible improvement to patient outcomes, however there remains a relative lack of both applied research and effective treatments for advanced SCC. Given small patient numbers and apparently declining incidence, further randomised SCC-specific phase III trials of systemic therapy in advanced oesophageal cancer in Western populations will be challenging. Future improvements in outcome are likely to come from smaller studies investigating cohorts of patients enriched for discrete genetic aberrations, or from the use of combination immunotherapeutic approaches. Optimising the design of such studies using appropriate chemotherapies as either comparators or backbones to newer investigative agents requires an understanding of differential effectiveness and toxicity of standard chemotherapy regimes. This analysis demonstrated no significant difference in survival or tolerance to chemotherapy between patients with adenocarcinoma or SCC. Given the poor outcomes seen with chemotherapy it reinforces the need for SCC-specific trials in advanced oesophageal cancer.

## COMMENTS

### Background

The two main histological subtypes of oesophageal cancer, adenocarcinoma and squamous cell carcinoma (SCC), are increasingly regarded as discrete disease entities with divergent treatment pathways. This is reflected in recently updated international clinical practice guidelines from both the National Comprehensive Cancer Institute and European Society of Medical Oncology, which recommend differing treatment approaches in early stage and, to a lesser extent, late stage disease dependent on histology.

### Research frontiers

Although the chemotherapy landscape for advanced oesophageal cancer has not changed in recent years, improved understanding of the molecular and genomic underpinnings of the disease have led to tangible improvements in outcome, with effective biological targeted agents such as trastuzumab and ramucirumab making a tangible difference to patient outcomes. The clinical application of such targeted agents has so far however been limited to the adenocarcinoma subtype. Emerging data on the use of immunotherapy suggests that it will also play a role in this condition. Recent preliminary data from trials of immunotherapy agents such as the KEYNOTE 028 study evaluating use of the anti-PD1 agent pembrolizumab in advanced oesophageal cancer have reported promising signal in both adenocarcinoma and SCC patients, and studies of immunotherapy in both histological subtypes are

ongoing. Although SCC remains a significant health problem on a global scale, incidence in Western populations is declining and further large scale randomised trials restricted to this subtype are unlikely.

### Innovations and breakthroughs

There is a lack of randomised data on differential chemotherapy response according to histology in oesophageal cancer. This paper shows that adenocarcinomas had a significantly higher response rate to first line fluoropyrimidine-based chemotherapy than SCC. Although there was also a trend towards improved survival outcomes this did not reach statistical significance. This data confirms the generally poor outcomes seen with chemotherapy in advanced oesophageal cancer and suggests that oesophageal SCC may be a less chemotherapy-sensitive disease than adenocarcinoma.

### Applications

Given the now established role of targeted agents in the management of advanced oesophageal adenocarcinoma and an emerging potential role for immunotherapeutic approaches it is possible that treatment pathways for the two subtypes will further diverge. Improvements in outcome are likely to come from smaller studies investigating targeted agents or combination immunotherapeutic approaches. Optimising the design of such studies using appropriate chemotherapies as either comparators or backbones to newer investigative agents requires knowledge of the differential effectiveness and toxicity of chemotherapy.

### Peer-review

The study is interesting and relevant.

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## Epigenetics of gastroenteropancreatic neuroendocrine tumors: A clinicopathologic perspective

Brendan M Finnerty, Katherine D Gray, Maureen D Moore, Rasa Zarnegar, Thomas J Fahey III

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NETs) are a heterogeneous group of rare tumors whose site-specific tumor incidence and clinical behavior vary widely. Genetic alterations associated with familial inherited syndromes have been well defined; however, the genetic profile of sporadic tumors is less clear as their tumorigenesis does not appear to be controlled by classic oncogenes such as *P53*, *RB*, or *KRAS*. Even within GEP-NETs, there are no common oncogenic drivers; for example, *DAXX/ATRX* mutations are strongly implicated in the tumorigenesis of pancreatic but not small bowel NETs. Accordingly, the dysregulation of epigenetic mechanisms has been hypothesized as a potential regulator of GEP-NET tumorigenesis and has become a major focus of recent studies. Despite the heterogeneity of tumor cohorts evaluated in these studies, it is obvious that there are methylation patterns, chromatin remodeling alterations, and microRNA and long non-coding RNA (lncRNA) differential expression profiles that are distinctive of GEP-NETs, some of which are correlated with significant differences in clinical outcomes. Several translational studies have provided convincing data identifying potential prognostic biomarkers, and some of these have demonstrated preliminary success as serum biomarkers that can be used clinically. Nevertheless, there are many opportunities to further define the mechanisms by which these epigenetic modifications influence tumorigenesis, and this will provide better insight into their prognostic and therapeutic utility. Furthermore, these findings form the foundation for future studies evaluating the clinical efficacy of epigenetic modifications as prognostic biomarkers, as well as potential therapeutic targets.

**Key words:** Epigenetics; Carcinoid; Neuroendocrine; MicroRNA; Methylation; Histone modifications; Chromatin remodeling; lncRNA

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### Abstract

Gastroenteropancreatic neuroendocrine tumors (GEP-

**Core tip:** Herein, we describe a review of the literature addressing known epigenetic changes which are thought to lead to the development of gastroenteropancreatic

neuroendocrine tumors (GEP-NETs). Through a variety of scientific works, methylation patterns, chromatin remodeling alterations, and microRNA and long non-coding RNA differential expression profiles have been identified and in many cases correlated with GEP-NET malignancy and clinical outcomes. This overview shows the strong foundation which exists and underlines the importance of future work to evaluate the clinical efficacy of epigenetic modifications as prognostic biomarkers, as well as potential therapeutic targets.

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## INTRODUCTION

### Overview of gastroenteropancreatic neuroendocrine tumors

Gastroenteropancreatic neuroendocrine tumors (GEP-NETs) are a heterogeneous group of rare tumors thought to arise from the malignant transformation of neuroendocrine cells located in the digestive tract. The incidence of GEP-NETs has risen from 1.0 per 100000 in 1977 to 3.7 per 100000 in 2007, with site-specific tumor incidence and behavior varying widely<sup>[1]</sup>. These differences may have roots in genomic and epigenetic differences, but the rarity of GEP-NETs has limited the progress of large-scale comprehensive analyses that could provide definitive insight into oncogenic molecular mechanisms. Genetic alterations associated with familial inherited syndromes have been well defined, such as in the multiple endocrine neoplasia syndromes, von-Hippel Lindau syndrome, neurofibromatosis, and tuberous sclerosis. On the other hand, the genetic profile of sporadic tumors, which are more common, is less clear as their tumorigenesis does not appear to be controlled by classic oncogenes such as *P53*, *RB*, or *KRAS*<sup>[2-6]</sup>.

Recent studies have identified key molecular pathways, such as activation of the PI3K/Akt/mTOR cascade secondary to PTEN and TSC2 downregulation in sporadic pancreatic NETs<sup>[7]</sup>, which has led to the clinical success of the targeted mTOR inhibitor everolimus for metastatic pancreatic NETs<sup>[8]</sup>. While everolimus has been shown to extend progression free survival in phase 3 trials, the lack of a complete response for all patients implies there are other factors contributing to malignant transformation, either by alteration of other signal transduction pathways or by dysregulation at the gene expression level. Further investigation into sporadic pancreatic NETs revealed genetic alterations in *MEN1* and *DAXX* / *ATRX* (death-domain-associated protein/  $\alpha$ -thalassemia-mental retardation syndrome X-linked), which impair histone methyltransferase activity and

chromatin stabilization, respectively<sup>[9]</sup>. These findings provide a link between genomic alterations and their control of gene expression, and thus have sparked further investigation into the epigenetic regulation of neuroendocrine tumors.

Epigenetics is the study of heritable modifications that control gene expression without alterations to the gene's DNA sequence itself<sup>[10]</sup>. It provides a deeper level of understanding phenotypic changes in the absence of alterations in classical genetics. This review summarizes the different types of epigenetic mechanisms that have been implicated in GEP-NET tumorigenesis, as well as key developments in the potential for epigenetic alterations to serve as biomarkers or therapeutic targets.

### Epigenetic mechanisms

Table 1 describes the different types of epigenetic mechanisms. Perhaps the most well understood epigenetic alteration is DNA methylation, which occurs at the 5'-position of the cytosine ring within dinucleotide CpG islands typically located in a gene's regulatory region<sup>[11]</sup>. Overall, methylation has a stabilizing effect on cellular function. In cancer development, methylation patterns are tissue- and chromosome-specific - hypomethylation has been associated with chromosomal instability, reactivation of transposable elements, and loss of imprinting, whereas hypermethylation is associated with inactivation of tumor-suppressor genes<sup>[12]</sup>. Methylation is regulated by DNA methyltransferase enzymes (DNMTs); cells with defects in DNMTs are also shown to have marked nuclear abnormalities<sup>[13]</sup>. DNMT inhibitors such as 5-azacytidine (AZA) and decitabine have been shown to effectively induce demethylation in pre-clinical and clinical trials<sup>[14]</sup>.

Another epigenetic change that is frequently seen is histone modification. Histone proteins are the main component of chromatin and form the nucleosome backbone for DNA packaging. Modifications in their structure contribute to the epigenetic regulation of gene expression, including acetylation, methylation, and phosphorylation. The exact regulatory effect is dependent on the type of chemical modification; for example, lysine acetylation is generally associated with transcriptional activation, whereas methylation can either suppress or activate transcription depending on amino acid (arginine vs lysine) and histone site<sup>[12]</sup>. Several enzymes are responsible for its regulation, including histone acetyltransferases and deacetylases (HDACs) as well as histone methyltransferases. Modification of DNA packaging components is crucial to chromatin remodeling, which alters between heterochromatin (tightly packaged, transcriptionally silent) and euchromatin (less condensed, transcriptionally active) states<sup>[15]</sup>.

Finally, noncoding RNAs also play a role in epigenetic changes. Noncoding RNAs account for 98% of the transcribed genome, but do not undergo subsequent translation<sup>[16]</sup>. Instead, they act as pre- and post-translational modifiers, and have gained recent attention

**Table 1 Overview of epigenetic mechanisms**

Mechanism	Effect	Regulation	Ref.
DNA Methylation		DNMTs	
Hypomethylation	Chromosomal instability, reactivation of transposable elements, loss of imprinting		[12]
Hypermethylation	Inactivation of tumor suppressor genes		[12]
Histone modification		HDACs, histone methyltransferases	
Lysine acetylation	Transcription activation		[12]
Methylation	Transcription activation or suppression		[12]
Noncoding RNAs		Variable	
LncRNAs	Pre-transcriptional regulation		[17]
MiRNAs	Post-transcriptional binding to 3'-untranslated regions to inhibit translation or promote mRNA degradation		[18]

DNMTs: DNA methyltransferase enzymes; HDACs: Histone acetyltransferases and deacetylases; LncRNA: Long non-coding RNA.

as having regulatory roles in both normal cellular development and oncogenesis. These include large RNAs (> 200 nucleotides) such as long noncoding RNAs (lncRNA), and small RNAs (< 200 nucleotides) such as microRNAs (miRNA)<sup>[15]</sup>. lncRNAs have a pre-transcriptional function by providing molecular scaffolds for chromatin regulators, and have been implicated as biomarkers for prostate, hepatocellular, and metastatic colorectal carcinomas<sup>[17,18]</sup>. There is also evidence of their involvement of gene regulation at the transcriptional, translational, and post-translational levels<sup>[18]</sup>. On the other hand, miRNAs appear to have a mainly post-transcriptional role by binding to complementary target sites of 3'-untranslated regions (UTRs) of messenger RNAs (mRNA), which then either inhibit translation or promote mRNA degradation. The over- and under-expression of various miRNAs have been implicated in nearly all solid tumors including breast, cervical, colorectal, lung, prostate, pancreatic, and thyroid cancers<sup>[19]</sup>.

### Major advancements in epigenetics

As the functional mechanisms of epigenetic regulation have become better understood, the association between these modifications and malignant transformation has fueled further investigation into the role of epigenetics in tumorigenesis and patient outcomes. Traditional methods of epigenomic analysis, such as bisulfite sequencing for DNA methylation and chromatin immunoprecipitation (ChIP) for analyzing chromatin modifications and DNA-protein interactions, provided the fundamental platform necessary for the development of more efficient and accurate techniques. These methods are becoming integrated with microarray and Next-Generation Sequencing (NGS) technology to provide even more comprehensive analyses; for example, NGS can be used to elucidate a tumor's "methylome" at single-nucleotide resolution<sup>[20]</sup>.

These technologies have enabled significant epigenomic discoveries in other tumors including myelodysplastic syndrome (MDS) and cutaneous T-cell lymphoma. While it is beyond the scope of this review to detail their comprehensive history, it is important to

highlight several therapeutic breakthroughs that have had an impact on clinical practice. Specifically, there are currently four FDA-approved drugs that have an epigenetic mechanism of action: 5-azacytidine and decitabine (DNMT inhibitors), and suberoylanilide hydroxamic acid and romidepsin (HDAC inhibitors). The DNMT inhibitors have demonstrated significantly higher response rates and reduced risk of leukemic transformation in the treatment for high risk myelodysplastic syndrome<sup>[21,22]</sup>, and the HDAC inhibitors have been shown to induce durable responses in patients with cutaneous T-cell lymphoma<sup>[23,24]</sup>. Additionally, there have been promising preliminary *in vivo* data using microRNAs to improve survival in hepatocellular carcinoma<sup>[25]</sup>. While this led to a multicenter Phase I study of a liposome-based miR-34 mimic for patients with advanced HCC (ClinicalTrials.gov identifier: NCT0182997), this trial was withdrawn for immune-related serious adverse events, thus highlighting the need for further research into developing safe epigenetic therapies.

Overall, these advancements have proven the clinical value of using epigenomic modifications not only as oncologic biomarkers, but as potential therapeutic targets as well. Accordingly, current studies are investigating the epigenetic mechanisms that may contribute to GEP-NET tumorigenesis, and hopefully these advancements will serve as the foundation for future therapeutic developments.

## CURRENT STATUS OF EPIGENETICS IN NETS

### Hypermethylation in NETs

Methylation profiles of candidate genes in GEP-NETs has been extensively studied. There are data to suggest that these profiles are uniquely different between pancreatic tumors (PNETs) and gastrointestinal tumors (GI-NETs). Chan *et al.*<sup>[26]</sup> detected a significantly higher degree of promoter methylation of 14 candidate genes in 14 GI-NETs compared to 11 PNETs, including MGMT (25% vs 0%), THSB1 (44% vs 9%), P14 (44% vs 9%), INK4a/P16 (31% vs 9%) and RARb (25% vs 0%).



**Table 2** Methylation profiles in neuroendocrine tumors

Modification	Gene	Gene function	Clinical effect	Tumor	Ref.
Hypermethylation (Inactivation)	<i>RASSF1</i>	Induces cell cycle arrest	Correlated with malignancy, levels highest in metastases	PNET > GI-NET	[32-34]
	<i>INK4a/p16</i>	Induces cell cycle arrest and apoptosis	Decreased 5-yr survival, liver metastases	PNET, gastrinoma	[38,39]
	<i>MGMT</i>	DNA repair	Improved response to temozolomide	PNET	[32]
	<i>TIMP-3</i>	Inhibits metalloproteinases	Correlated with metastases	PNET	[44]
	<i>UCHL-1</i>	Post-translational modifier, de-ubiquitinates proteins marked for lysosomal degradation	Correlated with metastases	GEP-NET	[45-47]
	<i>IGF2</i>	Chromatin packaging	Specific for insulinomas, increased stage	Insulinoma	[51]
	<i>MLH1</i>	DNA repair	Correlated with malignancy	PNET, insulinoma	[52]
Global hypomethylation	<i>LINE-1</i>	Repeating long interspersed nucleotide elements	Correlated with malignancy and lymph node metastases	Ileal NET > GEP-NET	[53-55]
	<i>Alu</i>	Repeating long interspersed nucleotide elements	Correlated with malignancy	GEP-NET	[54]

GEP-NETs: Gastroenteropancreatic neuroendocrine tumors; PNETs: Pancreatic tumors; GI-NETs: Gastrointestinal tumors.

While INK4a/P16 methylation was associated with GEP-NET liver metastasis, the small sample size of this study limited a comprehensive clinical outcomes analysis. A larger, more recent analysis by How-Kit *et al.*<sup>[27]</sup> was able to distinguish between GEP-NET subtypes by evaluating the methylation status of 807 oncogenic genes in 60 tumors using the Illumina GoldenGate technology. This study found unique DNA methylation patterns on hierarchical clustering between small bowel NETs (SB-NETs) and PNETs, as well as between functional PNET subtypes (insulinoma, gastrinoma, non-functioning). The analysis also found that gastrinomas are characterized predominantly by hypomethylated genes including metalloproteinases (MMP1, MMP3, TIMP2, TIMP3) and genes of the serpin family (SERPINA5, SERPINB5), whereas insulinomas and non-functioning PNETs had mixed hyper- and hypo-methylation profiles. Lastly, they reported hypermethylation of tumor suppressors (SMARCB1, CASP8 and NBL1) and hypomethylation of oncogenes (IL2, MCF2 and MOS); gene ontology and network analysis integrating these results detected cellular growth, apoptosis, cellular movement, and cell-cell signaling as the main molecular and cellular functions affected by the differentially methylated gene profiles. Overall, these results describe how epigenetic modifications differ between GEP-NET location and functionality, and have begun to shed light as to which genes and signaling pathways may be responsible for their differentiation.

The promoter regions of several individual genes in GEP-NETs warrant discussion, including RASSF1A, INK4a/P16, TIMP3, MGMT, IGF2, UCHL1, among others (Table 2). Although this section will describe the methylation patterns of individual genes' promoters, it is important to note that studies have correlated hypermethylation of multiple tumor suppressor genes with more advanced disease<sup>[28,29]</sup>. For instance, in GEP-NETs, Arnold *et al.*<sup>[30]</sup>

found that a high degree of methylation across CpG sites of multiple tumor suppressors - known as CpG island methylator phenotype (CIMP) positivity - is correlated with worse clinical outcomes in a mixed cohort of 71 well-differentiated tumors of the foregut and midgut. Specifically, CIMP positivity was found in 74% of foregut and midgut tumors and was associated with higher grade tumors (ki67 > 10%), whereas CIMP negativity had a non-significant trend towards better overall survival (7 years vs 4 years)<sup>[30]</sup>. Additionally, methylation of two or more tumor suppressor genes has been shown to be associated with liver metastases in a separate GEP-NET cohort<sup>[31]</sup>.

Multiple groups have shown clinically relevant methylation patterns in single gene promoter regions in GEP-NETs specifically. One of the initial large cohorts evaluating the methylation patterns of GEP-NETs analyzed 11 selected tumor suppressors in 48 PNETs, 75% of which were well-differentiated<sup>[32]</sup>. RASSF1 was hypermethylated in 75% of cases, followed by INK4a/p16 (40%), O<sup>6</sup>-MGMT (40%), RAR- $\beta$  (25%), and hMLH1 (23%). Interestingly, the methylation patterns were largely preserved when comparing metastatic tumor deposits to the matched primary tumor.

Ras-association domain gene family 1 (RASSF1) is a tumor suppressor that induces cell cycle arrest whose promoter region has been well-studied and is hypermethylated in 57% of GEP-NETs<sup>[31]</sup>. It appears that there are differences in RASSF1 hypermethylation depending on the site of origin of the GEP-NET as 75%-100% of PNETs<sup>[32-34]</sup>, 33% of gastric NETs<sup>[34]</sup>, and 0%-61% of SB-NETs<sup>[34,35]</sup> were found to have RASSF1 hypermethylation. The degree of methylation in PNETs has been found to be higher than adjacent normal pancreas and is expectedly inversely correlated with its level of gene expression<sup>[36]</sup>. Slightly counterintuitive to the statistically similar methylation profiles found

between matched metastatic vs primary PNET cohorts described above, the degree of RASSF1 methylation has been reported to be higher in lymphatic metastases in a GEP-NET cohort<sup>[31]</sup> and distant metastatic deposits in a SB-NET cohort<sup>[35,37]</sup> when compared to primary tumors. Nonetheless, while RASSF1 promoter methylation itself has not been correlated with changes in survival, its gene expression on an mRNA level is correlated with survival in one cohort of SB-NETs<sup>[37]</sup>.

The methylation status of INK4a/p16 (also known as cyclin-dependent kinase inhibitor 2A or CDKN2A) is another well-studied area in GEP-NET epigenetics. Methylation at three or more tumor suppressor genes, specifically at the INK4a/p16 locus, is predictive of decreased 5-year survival as well as tumor recurrence within 2 years of operation on multivariate analysis<sup>[32]</sup>. Additional studies have documented that methylation of the INK4a/p16 locus occurs in up to 58% of PNETs and gastrinomas<sup>[38,39]</sup> but less than 15% of benign insulinomas<sup>[40]</sup>, and is associated with liver metastasis in GEP-NETs<sup>[26,31]</sup> and poorer overall survival rates in poorly differentiated colorectal NETs<sup>[41]</sup>. The gene is located on chromosome 9p21 and is known to induce cell cycle arrest and apoptosis. Its inactivation by homozygous deletion is well-documented in many cancers; however, hypermethylation of its promoter has also been associated with colorectal, lung, breast, renal, and prostate cancers<sup>[42]</sup>. Despite its prevalence and association with cell cycle regulation, further clinical association is unclear as some studies have found the degree of INK4a/p16 promoter methylation to be independent of disease stage in gastrinomas<sup>[39]</sup>. Nevertheless, since INK4a/p16 promoter hypermethylation is a prevalent finding in PNETs, it still is thought to be a driver for early tumorigenesis and should have continued focus as a major epigenetic modification in GEP-NETs.

The silencing of several other individual loci by promoter hypermethylation may have important implications in GEP-NET tumorigenesis. MGMT is a DNA methyltransferase that serves an important role in DNA repair. The MGMT promoter is methylated in up to 40% of PNETs<sup>[32]</sup>. Several studies have correlated hypermethylation of the MGMT promoter (and subsequent protein loss) with improved response to temozolomide, an oral chemotherapeutic agent, specifically longer progression-free and overall survival<sup>[43]</sup>. TIMP-3 is a tumor suppressor involved in the inhibition of proteolytic activity of the matrix metalloproteinases; its decreased expression allows MMPs to contribute to tumor growth, angiogenesis and invasion. Hypermethylation of its promoter occurs in 44% of sporadic PNETs, and appears to be more prevalent in metastatic tumors<sup>[44]</sup>. UCHL-1 is a post-translational modifier that de-ubiquitinates proteins otherwise destined for lysosomal degradation; it is a known tumor suppressor in multiple tumor types and has been shown to stabilize p53 levels and induce cell cycle arrest<sup>[45,46]</sup>. In a cohort of well-differentiated GEP-NETs, loss of UCHL1 expression

by CpG promoter hypermethylation has been shown to be associated with metastatic GEP-NETs in well-differentiated tumors<sup>[47]</sup>. Additionally, hypermethylation of CTNNB1 (beta-catenin), WIF1 (wnt inhibitory factor), TCEB3C (elongin A3), and SEMA3F (semaphorin 3F) have been identified in SB-NETs<sup>[35,37,48,49]</sup>, with CTNNB1 and SEMA3F hypermethylation also having been identified in metastatic tumors<sup>[35,49]</sup>. Most recently, hypermethylation of the tumor suppressor homeobox-only protein has been associated with worse recurrence free survival in a cohort of 36 PNETs<sup>[50]</sup>.

To further illustrate the complexity of NETs, insulinomas have a unique methylation profile compared to other sporadic NETs. In a cohort of insulinomas, gastrinomas, non-functioning pancreatic NETs, and SB-NETs, hypermethylation of the CpG-rich differentially methylated region 2 (DMR2) region of the imprinted gene IGF2 was specific for insulinomas, which results in inhibition of chromatin packaging and thus allows continued expression of IGF2. Furthermore, there was a correlation between decreasing degree of IGF2 methylation with increasing stage of malignancy as defined by the WHO classification across the entire cohort<sup>[51]</sup>. In a separate study, insulinomas had a higher rate of MLH1 promoter hypermethylation, which has a regulatory role in DNA repair; decreased gene expression of MLH1 was further correlated with tumor malignancy<sup>[52]</sup>.

Contrary to many reports of hypermethylated genes described above, global hypomethylation of GEP-NETs has been reported as well. Global hypomethylation is assessed by analyzing the methylation status of long interspersed nucleotide elements (LINE)-1 and Alu, which are heavily methylated repeating elements that comprise 15% and 10% of the human genome, respectively. A recent meta-analysis concluded that global hypomethylation is associated with a worse prognosis in colorectal tumors, melanoma, gastric cancer, hepatocellular carcinoma, amongst others<sup>[53]</sup>. Within GEP-NETs, the significance and incidence of hypomethylation seems to vary by tumor type. Choi *et al.*<sup>[54]</sup> compared a cohort of 35 well-differentiated GEP-NETs to normal tissue, finding that global hypomethylation was found more frequently in tumor samples than in normal tissue. This same study showed that relative tumor hypomethylation of LINE-1 was more prevalent in ileal carcinoid tumors than in non-ileal carcinoid tumors and PNETs, and was also more prevalent in tumors with lymph node metastasis, chromosome 18 loss, and RASSF1 methylation. Interestingly, Alu methylation was inversely correlated with methylation of MGMT<sup>[54]</sup>. There were no survival differences between degrees of LINE-1 and Alu methylation, and more importantly, methylation of LINE-1 and Alu did not appear to be a sensitive marker for generalized CpG methylation of multiple genes of interest (*e.g.*, MGMT).

In contrast to the Choi study<sup>[54]</sup> cited above, a study of 58 GEP-NETs of variable grades showed that LINE-1

hypomethylation was highest in PNETs compared to SB-NETs, and is correlated with worse tumor staging in PNETs<sup>[55]</sup>. The most convincing data regarding global hypomethylation in PNETs was described by Stefanoli *et al.*<sup>[56]</sup> who performed quantitative bisulfite pyrosequencing to determine methylation status on 56 PNETs and 8 normal pancreas samples. LINE-1 methylation was significantly lower in PNETs compared to normal tissue. Additionally, in tumor samples, LINE-1 methylation was significantly lower in stage III and IV disease as compared to stage I and II disease (57.4% vs 61.7%, respectively,  $P = 0.002$ ). Furthermore, PNETs with less than 58% LINE-1 methylation were correlated with worse overall survival ( $P < 0.0001$ ). This study also analyzed the methylation status of 33 tumor suppressor genes, and identified three PNET clusters each with increasing frequency of gene-specific hypermethylation. The PNET cluster with the highest degree of methylation was associated with stage IV disease ( $P = 0.04$ ) and poor overall survival ( $P = 0.004$ ), and implicated ten tumor suppressors: DAPK1, TIMP3, PAX5, HIC1, CADM1, PYCARD, ESR1, VHL, RARB and WT1. Interestingly, most of the LINE-1 hypomethylated PNETs were distributed within the clusters containing a higher frequency of hypermethylated tumor suppressors, thus highlighting that global hypomethylation and gene-specific hypermethylation may be found concurrently in aggressive PNETs.

### Chromatin remodeling in NETs

One of the initial investigations into chromatin remodeling in GEP-NETs was by Jiao *et al.*<sup>[9]</sup>. The authors performed exome sequencing of 10 sporadic PNETs and identified mutations in several genes involved in chromatin remodeling that were subsequently confirmed in a 58-sample validation cohort. The top three mutated genes, *MEN1* (44%), *DAXX* (25%), and *ATRX* (18%) are all recognized to have a prominent role in chromatin remodeling, and may even provide a link between DNA methylation and histone modifications. Interestingly, in this study, patients with mutations in *MEN1*, *DAXX/ATRX*, or a combination of both had improved survival when compared to those without any mutation, particularly in those with metastatic disease<sup>[9]</sup>. While the clinical implications of *MEN1*, *DAXX*, and *ATRX* mutations vary in subsequent studies (discussed below), these findings prompted further investigation into the genes' downstream mechanistic effects. *MEN1* encodes menin, a nuclear scaffold protein that serves as a transcriptional regulator by remodeling chromatin and is also an essential component of a histone methyltransferase complex containing MLL2 and Ash2L<sup>[57]</sup>. *ATRX* is a chromatin remodeling protein that interacts with DNA methyltransferase 3A and 3L - this promotes DNA methylation of histone H3K4 when it is unmodified, which can result in telomere/chromatin changes as well as transcriptional activation<sup>[58]</sup>. *DAXX* is an H3.3-specific histone chaperone, which interacts with *ATRX* for H3.3 incorporation and heterochromatin

assembly at telomeres<sup>[59,60]</sup>. Ultimately, these complex interactions between DNA methylation and chromatin remodeling are essential for maintaining histone methylation patterns in newly replicated chromatin and preserving the stability of gene expression<sup>[58]</sup>.

Further work has defined the role of these mutations in the cellular behavior of GEP-NETs. The defining feature of telomerase-independent telomere maintenance associated with *DAXX/ATRX* gene mutations is alternative lengthening of telomeres (ALT). This phenomenon can be detected by telomere-specific fluorescence *in situ* hybridization (FISH) and was seen in 25/41 (61%) of PNET tumors in one cohort<sup>[61]</sup>. All PNETs with *DAXX/ATRX* mutations ( $n = 19$ ) had positive ALT during FISH analysis in this study, and tumors without mutations that were ALT positive ( $n = 6$ ) had lost nuclear expression of *DAXX/ATRX*. Other studies in GEP-NETs have reported similar findings - specifically that the ALT positive phenotype is associated with loss of *ATRX* or *DAXX* expression, particularly in PNETs<sup>[62]</sup>. Comparable findings have been reported in other tumors as well, including pediatric glioblastomas, where 44% of patients harbor a mutation in the *DAXX/ATRX* pathway resulting in ALT positivity<sup>[63]</sup>.

Several subsequent studies have corroborated the above *DAXX/ATRX* mutation findings in GEP-NETs. de Wilde *et al.*<sup>[64]</sup> evaluated 109 well-differentiated PNETs from 28 patients with MEN-1 syndrome and found that expression of *DAXX/ATRX* was normal and there was no ALT positivity in tumors  $< 0.5$  cm. However, 25% of tumors  $> 3$  cm (6% of tumors  $> 0.5$  cm) had lost expression; in each of these tumors, there was ALT positivity. Additionally, available lymph node metastases (2/3) had the same phenotype, and tumor grading was more likely to be WHO grade 2. The authors concluded that since loss of *DAXX/ATRX* expression was found only in larger, more aggressive tumors, *DAXX/ATRX* defects might be a later event in MEN-1 syndrome PNET tumorigenesis. Unfortunately, the cohort was too small ( $n = 12$  for size  $> 3$  cm) to perform other translational analyses, such as correlating *DAXX/ATRX* expression with survival or other clinicopathologic features.

In addition to these findings, Marinoni *et al.*<sup>[65]</sup> evaluated *DAXX/ATRX* expression in a derivation cohort of 61 well-differentiated PNET tumors and validation cohort of 70 tumors. Their analysis showed that loss of *DAXX* or *ATRX* protein occurred in 42% of the cohort and was correlated with ALT positivity. Furthermore, *DAXX/ATRX* loss of expression was also correlated with chromosome instability, which has previously been associated with poor outcomes in PNETs. Both cohorts in this study also demonstrated that a loss of *DAXX* or *ATRX* correlated significantly with a decreased relapse-free survival; only the derivation cohort detected a correlation with decreased tumor-specific survival. These findings are contrary to the initial report of *DAXX/ATRX* mutations described above, which appeared to be correlated with improved prognosis<sup>[9]</sup>. These discrepancies are likely secondary to the initial report's cohort consisting of all

metastatic PNETs, whereas only 18% of the Marinoni *et al.*<sup>[65]</sup> tumors were metastatic. Therefore, it is more important to appreciate that DAXX/ATRX loss of expression identifies only a subset of PNETs, and might have a different role in localized and distant tumor stages.

Notably, another study by Pipinikas *et al.*<sup>[66]</sup> found similar clinical outcomes as Marinoni *et al.*<sup>[65]</sup> - PNETs that were DAXX-negative (*i.e.*, DAXX-deficient) had the worst progression-free survival at five years (DAXX-negative: 16%,  $P < 0.001$ ; ATRX-negative: 52%,  $P = 0.15$ ; and DAXX/ATRX-positive: 85%). There was a significantly higher proportion of DAXX/ATRX loss in intermediate grade tumors compared to low-grade tumors (68% vs 27%,  $P = 0.008$ )<sup>[66]</sup>. This study also bridged the relationship between DAXX/ATRX, DNA methylation, and histone modifications. Using genome-wide methylation analysis, they found a significantly higher number of methylation-variable positions in DAXX-negative vs ATRX/DAXX-positive tumors compared to ATRX-negative vs ATRX/DAXX-positive tumors. This highlights the functional importance of DAXX (a H3.3-specific histone chaperone) as a driver for genome-wide methylation changes. Lastly, they found a higher incidence of copy number variations in DAXX/ATRX-negative tumors compared to DAXX/ATRX-positive tumors.

These data were definitively confirmed in the largest series evaluating ALT positivity and DAXX/ATRX loss in PNETs. Singhi *et al.*<sup>[67]</sup> performed telomere-specific FISH and DAXX/ATRX immunohistochemistry on 321 patients with resected PNETs, as well as 191 paired distant metastases from 52 patients. ALT-positivity and DAXX/ATRX-loss were present in 31% and 26% of resected PNETs, respectively, and associated with greater tumor size, worse WHO grade, lymph node metastasis, and distant metastasis. The rates of ALT-positivity and DAXX/ATRX-loss were higher in distant metastases (67% and 52%, respectively), and in fact, distant metastases only occurred in the setting of ALT-positive and DAXX/ATRX-negative primary tumors. Comparing long-term outcomes to wild-type PNETs, ALT-positive patients had worse 5-year disease-free survival (40% vs 96%,  $P < 0.001$ ) and 10-year disease-specific survival (50% vs 89%,  $P < 0.001$ ). Similar to the Marioni and Pipinikas studies, ALT was an independent prognostic factor for disease free survival ( $HR = 7.1$ ,  $P < 0.001$ ), but not for disease-specific survival (similar results were found when substituting DAXX/ATRX-loss for ALT positivity). These studies provide strong evidence suggesting that ALT-positivity and DAXX/ATRX-loss are associated with aggressive clinicopathologic features and worse disease-free survival, but not necessarily disease-specific survival.

Another gene of interest in chromatin remodeling of GEP-NETs is NAP1L1 (nucleosome assembly protein 1-like 1), whose function is thought to be in nucleosome assembly and exchange of histone dimers, which may contribute to the regulation of cell proliferation as a

transcriptional modifier<sup>[68,69]</sup>. In a cohort of 43 PNETs, NAP1L1 was significantly overexpressed in metastasis and inversely correlated with expression of p57<sup>Kip2</sup><sup>[70]</sup>. This inverse correlation was confirmed *in vitro* by silencing NAP1L1 in the BON cell line, which correlated with a subsequent increase in p57<sup>Kip2</sup> mRNA and protein levels. Furthermore, there was decreased signaling of the mammalian target of rapamycin (mTOR) pathway, which lead to less aggressive phenotypes *in vitro* and *in vivo*. Most importantly, though, the p57<sup>Kip2</sup> promoter in NAP1L1-silenced BON cells was significantly less methylated, and using ChIP analysis, NAP1L1-bound DNA fragments were found to include the p57<sup>Kip2</sup> promoter. The authors concluded that although NAP1-like proteins typically control gene expression *via* histone H3 acetylation<sup>[71]</sup>, NAP1L1 may have the ability to regulate p57<sup>Kip2</sup> through promoter methylation in GEP-NETs. This finding highlights the overlapping complexity of epigenetic modifications, and while these recent studies have provided a major step in advancing our understanding of chromatin remodelers as PNET prognostic indicators, the mechanisms by which these mutations affect survival on a cellular level remain to be fully elucidated.

### MicroRNAs in NETs

Recent studies have begun to focus on global miRNA profiling of GEP-NETs, and several of these are reviewed here. miRNAs primarily play a post-transcriptional role by either inhibiting translation of mRNA or promoting mRNA degradation. Many different specific miRNAs have been identified with a breadth of clinical implications in GEP-NETs (Figure 1, Supplementary Table 1). While the prognostic and therapeutic implications of these changes are still controversial, these findings provide a strong foundation for future investigations.

Roldo *et al.*<sup>[72]</sup> in one of the first GEP-NET miRNA studies, evaluated 12 insulinomas, 28 nonfunctioning PNETs, and four acinar carcinomas in comparison to normal pancreatic tissue. Their analysis demonstrated that overexpression of miR-103 and miR-107 and underexpression of miR-155 could discriminate the whole tumor cohort from normal tissue. Interestingly, overexpression of miR-204 was specific to insulinomas and correlated with immunohistochemical staining of insulin better than insulin mRNA expression. Lastly, miR-21 was correlated with Ki67 > 2% as well as liver metastasis. While these data do not necessarily explain the downstream mechanistic effects of these expression differences, this initial report gave insight into the miRNA expression differences of PNETs - in addition to those mentioned above, ten microRNAs (miR-125a, miR-99a, miR-99b, miR-125b-1, miR-342, miR-130a, miR-132, miR-129-2, miR-125b-2) were able to differentiate PNETs from normal tissue and acinar tumors independently, and the miRNA profiles between nonfunctioning tumors and insulinomas were noted to be indistinguishable.

miRNA profiling of PNETs has been further analyzed



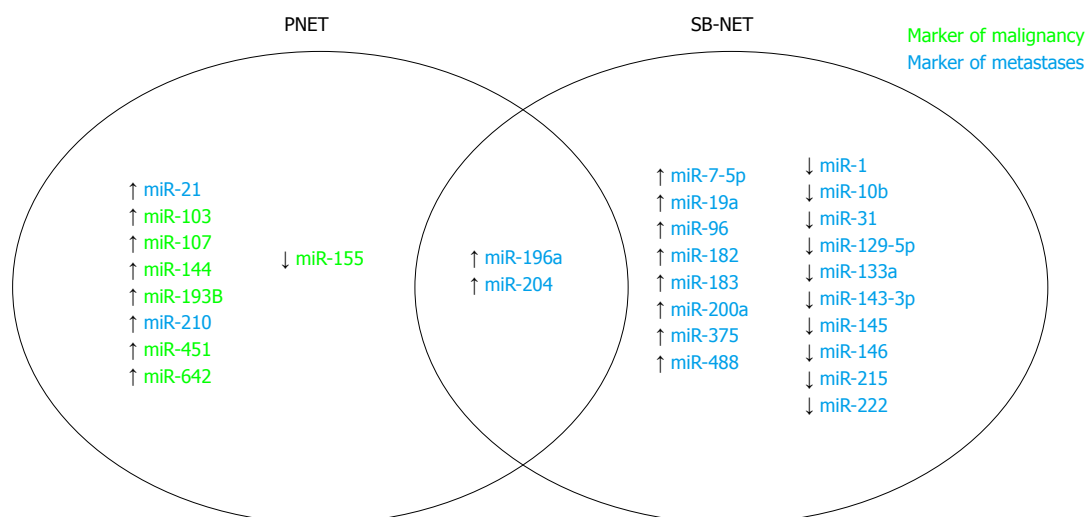


Figure 1 MicroRNA in neuroendocrine tumors. PNET: Pancreatic tumors; SB-NETs: Small bowe neuroendocrine tumors.

by Thorns *et al.*<sup>[73]</sup> in a cohort including PNET tumor samples of various grades, normal tissue, as well as serum. This study found that expression of miR-642 correlated with Ki-67 score, and miR-210 correlated with metastatic disease. Interestingly, 13 miRNAs were more abundant in the serum of patients with PNETs compared to normal subjects; specifically, miR-193b was a marker for PNETs in both tumor and serum samples<sup>[73]</sup>.

Investigation into miRNA profiling in SB-NETs first gained traction in a study by Ruebel *et al.*<sup>[74]</sup> evaluating the differential expression of 95 miRNAs in eight matched primary and metastatic ileal NETs. There was downregulation of miR-133a, miR-145, miR-146, miR-222, and miR-10b in all metastatic samples compared to primary tumors, and up-regulation of miR-183, miR-488 and miR-19a in six of eight metastatic tumors compared to the primary. miR-133a was significantly downregulated in a validation cohort of six additional cases. This study presented an initial understanding of miRNA expression differences in SB-NETs. However, perhaps one of the best designed miRNA profiling studies in GEP-NETs was performed in a cohort of five primary SB-NETs, five lymph node metastases, and five liver metastases<sup>[75]</sup>. In this cohort, the Affymetrix Genechip miRNA array detected 15 upregulated and 18 downregulated miRNAs when comparing mesenteric metastases to primary tumors. These 33 miRNAs were further differentially expressed when comparing liver metastases to lymph node metastases; in fact, 14 of 15 upregulated miRNAs had a further increase in expression in the liver metastases. Furthermore, nine miRNAs of interest were validated in a cohort of three primary tumors, three lymph node metastases, and three liver metastases - miR-96, miR-182, miR-183, miR-196a, and miR-200a were confirmed to be upregulated in metastases, and miR-31, miR-129-5p, miR-133a, and miR-215 were confirmed to be downregulated in metastases. These results provide insight into the differential expression of miRNAs that may be implicated

in disease progression.

A subsequent comprehensive analysis of 90 patients by Miller *et al.*<sup>[76]</sup> profiled the global miRNAome of SB-NETs and their metastases. The authors identified 39 differentially expressed miRNAs between primary tumors and normal tissue. Interestingly, they found significant overlap between upregulated miRNAs in both primary SB-NETs and their metastases compared to their respective normal tissues. While the most differentially expressed microRNAs were upregulation of miR-204, miR-7-5p, and miR-375, the profile included microRNAs identified in the above studies, specifically miR-31, miR-96, miR-129-5p, miR-182, miR-196a, miR-200a and miR-215. When comparing lymph node and liver metastases to their matched primary tumors, several significantly downregulated microRNAs were identified, including miR-1, miR-133a, miR-143-3p, and miR-145-5p - again, several of these were also identified in the above studies. The authors then analyzed the target genes of miR-1 and miR-143 in the existing GSE27162 dataset. They identified both miRNAs are predicted to target the NUAK2 and FOSB oncogenes, which are significantly upregulated in SB-NET lymph node metastasis. The inverse correlation identified in this bioinformatics analysis was confirmed *in vitro*, indicating that this miRNA/mRNA interaction may be a crucial step in metastatic progression of SB-NETs.

miR-196a has been identified in several studies, and has been further investigated by Li *et al.*<sup>[77]</sup>. The authors used miRNA target prediction software, which identified HOXA9, HOXB7, LRP4, and RSPO2 as potential downstream targets of miR-196a. The transcripts of these targets were found to be downregulated in tumor and serum samples of patients with SB-NETs compared to healthy donors. More interestingly, when miR-196a was silenced in the SB-NET cell line CNDT2.5, all four targets' gene and protein expression significantly increased. Further downstream targets of these genes,

including those in the WNT signaling pathway, were also expectedly upregulated. This did not result in any phenotypic changes in cell growth; however, the only *in vitro* assay performed was an MTT (cell viability) assay.

miR-196a has also been implicated in PNET clinicopathologic outcomes in a study by Lee *et al.*<sup>[78]</sup>. The authors analyzed 37 resected PNETs and found that increased expression of miR-196a was significantly associated with advanced pathologic T stage (50% vs 7%), N stage (50% vs 4%), higher mitotic counts (60% vs 4%), and greater ki-67 index (60% vs 22%). Furthermore, patients with increased expression had a higher risk for recurrence (hazard ratio: 16.3, 95% confidence interval: 1.7 to 154,  $P = 0.015$ ), worse disease-free survival ( $P < 0.001$ ), and worse overall survival ( $P = 0.046$ ). These findings demonstrate that miR-196a may have prognostic utility in both pancreatic and small bowel NETs.

miRNAs may also play a role in the development of insulinomas. Jiang *et al* identified 114 differentially expressed microRNAs when comparing four insulinomas to 4 normal pancreatic islet cells - 28 of these belonged to three miRNA families that localized to the epigenetically-regulated imprinted chromosome 14q32<sup>[79]</sup>. The most significant differentially expressed cluster, miR-144/451, was validated in 25 insulinomas and 8 normal pancreatic islets. The authors further demonstrated that in mouse pancreatic beta-cells, overexpression of miR-144/451 increased cell viability and proliferation - miR-144 was found to inhibit the tumor suppressor PTEN leading to increased AKT pathway activation, while miR-451 directly downregulated p19, a cell-cycle regulator. Further investigation into the downstream effects of the other differentially expressed microRNAs in this study could provide even more insight into the tumorigenesis of insulinomas.

Lastly, there is increasing evidence that serum miRNA expression can serve as a biomarker for patients with SB-NETs. The panel of nine miRNAs described above (miR-96, miR-182, miR-183, miR-196a, miR-200a, miR-31, miR-129-5p, miR-133a, and miR-215) was evaluated in the serum of patients with SB-NETs and compared to healthy volunteers by Li *et al.*<sup>[80]</sup>. The study found that miR-96, miR-182, miR-196a, and miR-200a were upregulated and miR-31, miR-129-5p, miR-133a, and miR-215 were downregulated when comparing patients with lymph node metastases compared to healthy volunteers - these findings are largely congruent with the authors' original study in tumor tissue<sup>[75]</sup>, although serum expression of miR-183 did not differ between groups. The study further shows that patients treated with somatostatin analogues (SSA) have even further upregulation of miR-96, miR-182, miR-183, miR-196a, and miR-200a at all stages of disease (*i.e.*, in primary tumors, lymph node and liver metastases), with the exception that miR-200a is not significantly upregulated in patients with liver metastases. Therefore, these results suggest that there are certain serum miRNA profiles that are not only

detectable in patients with SB-NETs, but also have a specific response to somatostatin analogues.

### **lncRNA in NETs**

The current knowledge regarding the pathogenesis and prognostic implications of lncRNAs is still in its preliminary stages. However, one study thus far has described a potentially relevant lncRNA in GEP-NETs<sup>[81]</sup>. Modali *et al.*<sup>[82]</sup> specifically investigated the relationship between menin and MEG3, a monoallelic, maternally-expressed lncRNA whose loss of expression has been described in several tumors. The authors demonstrated that in a mouse insulinoma cell line, overexpression of menin significantly increased MEG3 expression by histone-H3 lysine-4 trimethylation (a marker of transcriptional activation) and CpG hypomethylation at the Meg3 promoter CRE site, which allows binding of the transcription factor cAMP response element-binding protein. Furthermore, MEG3 overexpression was shown to reduce cell proliferation rates, induce cell cycle arrest, and downregulate c-Met proto-oncogene expression. This inverse relationship of menin-Meg3 to c-Met was confirmed in an *in vivo* PNET mouse model, as well as in human MEN1 PNET and sporadic insulinoma samples. In all of these, MEG3 expression was decreased, c-Met expression was elevated, and the MEG3 promoter was hypermethylated in tumors compared to normal islets. This initial study of lncRNA in PNETs has highlighted the potential importance of lncRNA epigenetic regulation in neuroendocrine tumor development.

## **CLINICAL APPLICATIONS AND FUTURE DIRECTIONS**

Several studies discussed above have provided data identifying potential prognostic biomarkers, and some have even hypothesized clinical translation by evaluating their utility as serum biomarkers. Continued investigation is required to identify the mechanisms by which these epigenetic modifications influence tumorigenesis, providing better insight into their prognostic and therapeutic utility.

There has been limited success in developing effective epigenetic therapies, as most of these studies have only evaluated drug treatments in GEP-NET cell lines such as BON (pancreas) and CNDT2.5 (midgut). However, they have shown initial promising effects by demonstrating attenuation of aggressive GEP-NET tumor phenotypes with drug treatments. For instance, overexpression of DNA methyltransferases 1, 3a, and 3b have been implicated in GEP-NET tumorigenesis<sup>[83]</sup>. Accordingly, AZA therapy (*i.e.*, DNMT inhibition) in BON and CNDT2.5 cells has been shown to reduce cell proliferation, induce cell cycle arrest at the G2 to M transition, and decrease expression of the neuroendocrine markers chromogranin A (CgA) and neuron-specific enolase<sup>[84]</sup>. Similarly, HDAC inhibition with trichostatin A (TSA), sodium butyrate (NaB), and MS-275 in BON cells inhibited cell

proliferation, induced apoptosis *via* caspase-3 activation and Bcl-2 downregulation, and promoted cell cycle arrest at the G1-S transition<sup>[85]</sup>. Furthermore, combination treatment of BON cells with both valproic acid (an HDAC inhibitor) and lithium has been shown to suppress CgA expression, upregulate Notch1 signaling, and inhibit glycogen synthase kinase-3 $\beta$  activity, thereby increasing cellular proliferation<sup>[86]</sup>. Currently, no targeted miRNA therapies have been attempted.

Despite these *in vitro* findings with DNMT and HDAC inhibitors, clinical trials have been unsuccessful. Only one published phase 2 clinical trial of any epigenetic therapy for neuroendocrine tumors has been attempted<sup>[87]</sup>. The HDAC inhibitor depsipeptide was administered to 15 patients with metastatic GEP-NET and lung neuroendocrine tumors, but the study was terminated prematurely due to a high rate of adverse cardiac events (ventricular tachycardia, prolonged QTc, sudden death) precluding observation of an objective response rate. More studies evaluating the anti-tumor pharmacology and adverse events in *in vitro* and *in vivo* experiments are necessary before future attempts at clinical trials are indicated.

## CONCLUSION

The field of epigenetics is constantly evolving and major strides have been made to help define its relevance in GEP-NET tumorigenesis. The current body of literature suggests there are methylation patterns, chromatin remodeling alterations, and microRNA and lncRNA differential expression profiles that are distinctive of GEP-NETs, some of which are correlated with poorer clinical outcomes. However, given the variety of GEP-NETs, many studies' results are confounded by heterogeneity of tumor cohorts. Thus, larger studies with more stringent inclusion criteria are required to evaluate the utility of epigenetic modifications as prognostic biomarkers as well as potential therapeutic targets.

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Retrospective Cohort Study

# Stratification of outcomes for mucinous appendiceal adenocarcinoma with peritoneal metastasis by histological grade

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## Abstract

### AIM

To investigate the importance of a three-tiered histologic grade on outcomes for patients with mucinous appendiceal adenocarcinoma (MAA).

### METHODS

Two hundred and sixty-five patients with MAA undergoing cytoreductive surgery and hyperthermic intraperitoneal chemotherapy were identified from a prospective database from 2004 through 2014. All pathology was reviewed by our gastrointestinal subspecialty pathologists and histological grade was classified as well-differentiated, moderately differentiated, and poorly differentiated. Survival analysis was performed using Cox proportional hazards regression.

### RESULTS

There were 201 (75.8%) well-, 45 (16.9%) moderately- and 19 (7.2%) poorly-differentiated tumors. Histological grade significantly stratified the 5-year overall survival (OS), 94%, 71% and 30% respectively ( $P < 0.001$ ) as well as the 5-year disease-free survival (DFS) 66%, 21% and 0%, respectively ( $P < 0.001$ ). Independent predictors of DFS included tumor grade (HR = 1.78, 95%CI: 1.21-2.63,  $P = 0.008$ ), lymph node involvement (HR = 0.33, 95%CI: 0.11-0.98,  $P < 0.02$ ), previous surgical score (HR = 1.31, 95%CI: 1.1-1.65,  $P = 0.03$ ) and peritoneal carcinomatosis index (PCI) (HR = 1.05, 95%CI: 1.02-1.08,  $P = 0.002$ ). Independent predictors of OS include tumor grade (HR = 2.79, 95%CI: 1.26-6.21,  $P = 0.01$ ), PCI (HR = 1.10, 95%CI: 1.03-1.16,  $P = 0.002$ ), and complete cytoreduction (HR = 0.32, 95%CI: 0.11-0.92,  $P = 0.03$ ). Tumor grade and PCI were the only independent predictors of both DFS and OS. Furthermore, histological grade and lymphovascular invasion stratified the risk of lymph node metastasis into a low (6%) and high (40%) risk groups.

### CONCLUSION

Our data demonstrates that moderately differentiated MAA have a clinical behavior and outcome that is distinct from well- and poorly-differentiated MAA. The three-tier grade classification provides improved prognostic stratification and should be incorporated into patient selection and treatment algorithms.

**Key words:** Hyperthermic intraperitoneal chemotherapy; Pseudomyxoma peritonei; Histology; Grade; Prognostic; Outcomes

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**Core tip:** The natural history of mucinous appendiceal adenocarcinoma encompasses a wide spectrum of clinical

outcomes. This study illustrates that classification of these tumors using tumor cellularity, architectural features and cytologic abnormalities into three distinct histological grades; well-, moderately- and poorly-differentiated allows the clinician to better estimate relative risk of recurrence and death. Thus facilitating patient selection, education and comparison of different treatments.

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## INTRODUCTION

Mucinous appendiceal adenocarcinoma (MAA) is a rare tumor. The vast majority of these lesions are thought to arise from low-grade appendiceal neoplasms which have a unique biological predisposition for peritoneal spread, with rare lymphatic and hematogenous metastasis. These tumors can produce abundant mucin causing abdominal distension and the clinical condition known as pseudomyxoma peritonei. The spectrum of biological aggressiveness of these tumors varies widely leading to difficulties in classification of both the primary tumor and secondary peritoneal metastasis. This has led to confusing nomenclature. Ronnett *et al.*<sup>[1]</sup>, in a retrospective review proposed a three-tier staging system according to the histology of the peritoneal disease rather than the primary. Peritoneal tumors with abundant extracellular mucin containing scant, simple to focally proliferative, mucinous epithelium, with little cytological atypia or mitotic activity was classified as diffuse peritoneal adenomucinosis (DPAM). In contrast, peritoneal tumors composed of more abundant mucinous epithelium with the architectural and cytological features of carcinoma were classified as peritoneal mucinous carcinomatosis (PMCA). Peritoneal tumors with a histology intermediate to DPAM and PMCA were classified as PMCA-Intermediate<sup>[1]</sup>. This classification stratified tumor behavior well and has been supported by others<sup>[2-4]</sup>. Others have proposed a two tier system combining either the intermediate and high grade groups or the intermediate and low-grade groups because of their presumed similar prognosis<sup>[5,6]</sup>.

A consensus statement published in 2008 highlighted the controversy as 44% of participants used the Ronnett three-tier classification and the remaining 56% used a two-tier classification system<sup>[5]</sup>. In the seventh edition of the American Joint Committee on Cancer (AJCC) Staging Manual, appendiceal carcinomas are now classified separately from colorectal carcinomas<sup>[7]</sup>. Currently, the histologic grading scheme endorsed by AJCC is unclear. The AJCC staging form allows for



a 2-, 3-, or 4-tier system while the staging criterion to stratify stage IV disease appears to use a three-tier system (designated by "G"). Comparisons of these systems are made under the grade explanation, with G1 and well-differentiated corresponding to "mucinous low grade" and G2/G3 and moderately to poorly differentiated corresponding to "mucinous high grade". G4 corresponds to an undifferentiated tumor. Within the AJCC system currently in use, there is no distinction between mucinous tumors arising from a low-grade appendiceal neoplasm and the less common MAA showing conventional colonic-type features (infiltrating glands associated with destructive invasion and desmoplasia), although, this distinction has begun to be recognized in the 8<sup>th</sup> edition of the AJCC Staging manual<sup>[7]</sup>.

These challenges and controversies were discussed at the 2012 World Congress of the Peritoneal Surface Oncology Group International (PSOGI) in Berlin where an expert panel achieved a consensus using a three-tier system using both histological grade and signet ring cell morphology; low grade, high grade and high grade with signet ring cells<sup>[8]</sup>. However, a recent investigation of the Surveillance Epidemiology and End Result (SEER) database did not support the merging of moderately and poorly differentiated grades into a single high-grade category as moderately differentiated tumors had a survival that was intermediate to well differentiated and poorly differentiated tumors<sup>[9]</sup>. Therefore, we sought to evaluate our outcomes following cytoreductive surgery (CRS) and hyperthermic intraperitoneal chemotherapy (HIPEC) according to the degree of metastatic tumor differentiation.

## MATERIALS AND METHODS

### Patient selection

This study was approved by an Institutional Review Board at University of Texas, MD Anderson Cancer Center. Consecutive patients with MAA, who underwent CRS and HIPEC at our institution between January 2004 and December 2014, were identified from a prospectively maintained database. All patient evaluations included a complete history and physical exam, laboratory studies, assessment of tumor markers (CA 19-9, CA-125 and CEA) and imaging of the chest, abdomen and pelvis. All pathology was reviewed by our gastrointestinal subspecialty pathologists and histological grade was classified as well-differentiated, moderately differentiated, and poorly differentiated based on tumor cellularity, architectural features (strips of cells, clustering, complex architecture such as cribriform structures, papillae, or pseudopapillae), and cytologic abnormalities (nuclear polarity, presence of mitotic figures and/or apoptotic bodies, nuclear size, and chromatin characteristics). If signet ring cells within mucin were not a significant component of the tumor (*i.e.*, less than 10% of the neoplastic cells), these were not incorporated into the grade but were annotated.

In general, well-differentiated MAA are paucicellular, consisting mostly of strips of epithelium with little complexity and bland to slightly atypical cytologic features. Poorly differentiated MAA showed cellular tumors with clusters of tumor cells within (instead of, or in addition to, lining) mucin pools. The neoplastic epithelium has either easily identifiable cytologic atypia (enlarged nuclei, open chromatin, apoptotic bodies, loss of polarity) and/or architectural complexity, and/or loss of cohesion (clusters of neoplastic cells and/or abundant single cells). Moderately differentiated MAA often have features in between these two ends of the spectrum. When tumor heterogeneity is present between different specimens in the same case, the highest grade was utilized for analysis. CRS and HIPEC was performed in our standard closed fashion with Mitomycin C (20–25 mg/m<sup>2</sup>) for 90 min at 40.5 °C, as previously described<sup>[10]</sup>.

### Statistical analysis

Descriptive statistics, including frequencies and proportions for categorical data, and medians and inter-quartile range for continuous outcomes were calculated for all study measures. Associations between categorical variables were compared using the  $\chi^2$  test and Fisher's exact test. Continuous variables were compared using a Wilcoxon rank-sum test. Overall survival (OS) was estimated using the Kaplan-Meier method and compared with the log-rank test. OS was calculated from the date of first treatment to the date of death or last follow up. Disease-free survival (DFS) was calculated from the date of CRS and HIPEC to time of first recurrence identified on imaging. Multivariate analysis was performed with Cox regression analysis in which a backward elimination process was used for variable selection with an entry and removal limit of  $P < 0.1$  and  $P < 0.05$ , respectively. All analyses were performed using SPSS 22 (IBM Corp. Released 2013. IBM SPSS Statistics for Windows, Version 22.0. Armonk, NY: IBM Corp.). All graphs were made using GraphPad Prism version 6.00 for Windows (GraphPad Software, La Jolla, CA, United States, www.graphpad.com). All *P*-values were two-sided and a *P* value of 0.05 or less was considered statistically significant.

## RESULTS

Baseline, clinical and pathological characteristics for the entire cohort are stratified by histological grade and are reported in Table 1. Two hundred and sixty-five patients with MAA undergoing CRS and HIPEC were identified. Preoperative diagnosis was confirmed in all patients. A percutaneous biopsy or laparoscopic biopsy/appendectomy was performed in 104 (39.2%). A pre-referral laparotomy with limited resection was performed in 75 (28.3%) and previous (pre-referral) extended resection was performed in 59 (22.3%) and cytoreduction in 27 (10.2%). The pre-operative pathology was classified as well differentiated in 198 (73.9%) patients, moderately differentiated in 49

**Table 1** Baseline clinical and pathological characteristics of the 265 patients with mucinous appendiceal adenocarcinoma treated with cytoreduction and hyperthermic intraperitoneal chemotherapy stratified by histological grade *n* (%)

Characteristics of the entire cohort ( <i>n</i> = 265)	Well-differentiated ( <i>n</i> = 201)	Moderately-differentiated ( <i>n</i> = 45)	Poorly-differentiated ( <i>n</i> = 19)	Total	<i>P</i> value
Age	53.1 (45-59)	54.9 (46-59)	49.6 (45-60)	53.4 (45-59)	<i>P</i> = 0.96 <sup>3</sup>
Sex					<i>P</i> = 0.62 <sup>2</sup>
Male	78 (39)	21 (47)	8 (42)	107 (40.4)	
Female	123 (61)	24 (53)	11 (58)	158 (59.6)	
Signet ring cell					<i>P</i> < 0.001 <sup>1,2</sup>
> 50%	0	3 (7)	11 (58)	14 (5.3)	
1%-50%	6 (3)	4 (9)	0	10 (3.8)	
None	195 (97)	38 (84)	8 (42)	241 (90.9)	
LVI					<i>P</i> < 0.001 <sup>1,2</sup>
Yes	3 (1)	8 (18)	9 (47)	20 (7.5)	
No	198 (98)	35 (78)	3 (16)	241 (90.9)	
LN involvement					<i>P</i> < 0.001 <sup>1,2</sup>
Yes	11 (5)	4 (9)	8 (42)	23 (8.7)	
No	160 (80)	38 (84)	4 (21)	208 (78.5)	
Neoadjuvant chemo					<i>P</i> < 0.001 <sup>1,2</sup>
Yes	29 (14)	21 (47)	15 (79)	65 (24.5)	
No	172 (86)	24 (53)	4 (21)	200 (75.5)	
Adjuvant chemo					<i>P</i> < 0.001 <sup>1,2</sup>
Yes	12 (6)	3 (7)	8 (42)	23 (8.7)	
No	189 (94)	42 (93)	11 (58)	242 (91.3)	
PSS					<i>P</i> = 0.052 <sup>2</sup>
0	77 (38)	20 (44)	7 (37)	104 (39.2)	
1	59 (29)	13 (29)	3 (16)	75 (28.3)	
2	44 (22)	6 (13)	9 (47)	59 (22.3)	
3	21 (10)	6 (13)	0	27 (10.2)	
Albumin	4.2 (4.0-4.4)	4.3 (4.1-4.5)	4.2 (4.0-4.9)	4.2 (4.1-4.5)	<i>P</i> = 0.77 <sup>3</sup>
CEA	2.8 (1.3-10.7)	4.5 (2.4-15.7)	2.2 (1.4-4.9)	3.4 (1.5-10.5)	<i>P</i> = 0.92 <sup>3</sup>
PCI	17.5 (11-26)	14 (8-24)	15.5 (12-25)	16.5 (10-25)	<i>P</i> = 0.45 <sup>3</sup>
Completeness of cytoreduction					<i>P</i> = 0.32 <sup>2</sup>
CC 0-1	176 (88)	40 (89)	15 (79)	231 (87.2)	
CC 2-3	19 (9)	5 (11)	4 (21)	28 (10.6)	
Unknown				6 (2.3)	
OR time	9.2 (7.7-11.4)	8.7 (6.9-10.0)	7.9 (6.9-10.5)	8.9 (7.5-11.1)	<i>P</i> = 0.82 <sup>3</sup>
Blood loss	600 (350-997)	375 (250-588)	350 (250-550)	500 (300-900)	<i>P</i> = 0.007 <sup>1,3</sup>
LOS	19 (13-27)	13 (10-22)	16 (10-23)	17 (12-26)	<i>P</i> = 0.008 <sup>1,3</sup>
30 d morbidity any grade	108 (54)	28 (68)	11 (58)	149 (55)	<i>P</i> = 0.49 <sup>2</sup>
90 d grade III/IV	35 (17)	14 (31)	2 (10)	51 (19.2)	<i>P</i> = 0.35 <sup>2</sup>
90 d mortality	2 (1)	0	0	2 (0.8)	<i>P</i> = 0.73 <sup>2</sup>

Descriptive statistics are reported as median and interquartile range or number and frequency. <sup>1</sup>Significant *P*-values; <sup>2</sup> $\chi^2$  test; <sup>3</sup>Kruskal-Wallis test. LVI: Lymphovascular invasion; PSS: Previous surgical score; CEA: Carcinoembryonic antigen; PCI: Peritoneal carcinomatosis index; LOS: Length of stay.

(18.3%) patients and poorly differentiated in 18 (6.7%) patients. The pre-operative pathology was discordant with the pathology at the time of CRS and HIPEC in 32 (12.1%) patients. In 14 (43.8%) the pathology was downgraded at the time of CRS and in 18 (56.3%) it was upgraded.

At the time of cytoreduction 34 (12.8%) patients had acellular mucin only; 167 (63.0%) had well differentiated MAA; 45 patients had moderately differentiated MAA and 19 patients had poorly differentiated MAA. Figure 1 illustrates the 5-year OS is 94%, 71% and 20% respectively (*P* < 0.001). Similarly, the 5-year DFS is 66%, 21% and 20%, respectively (*P* < 0.001) (Figure 2). Regardless of the initial pathology, the finding of acellular mucin only at time of CRS and HIPEC was associated with a 93% 5-year DFS and 100% 5-year OS. Important prognostic variables for DFS on multivariate analysis (Table 2) include: Tumor grade (HR = 1.78, 95%CI:

1.21-2.63, *P* = 0.008), lymph node involvement (HR = 0.33, 95%CI: 0.11-0.98, *P* < 0.02), previous surgical score (PSS) (HR = 1.31, 95%CI: 1.1-1.65, *P* = 0.03) and PCI (HR = 1.05, 95%CI: 1.02-1.08, *P* = 0.002). Important prognostic factors for OS on multivariate analysis (Table 2) include: Tumor grade (HR = 2.79, 95%CI: 1.26-6.21, *P* = 0.01), PCI (HR = 1.10, 95%CI: 1.03-1.16, *P* = 0.002), and complete cytoreduction (HR = 0.32, 95%CI: 0.11-0.92, *P* = 0.03). Tumor grade and PCI were the only independent predictors of both DFS and OS.

All three histological grades were significantly different from each other in terms of overall survival (all *P* < 0.001). However, in terms of disease-free survival the moderately differentiated tumors were significantly different (*P* < 0.001) from the well-differentiated but not the poorly differentiated tumors (*P* = 0.31).

The presence of signet rings cells was significant

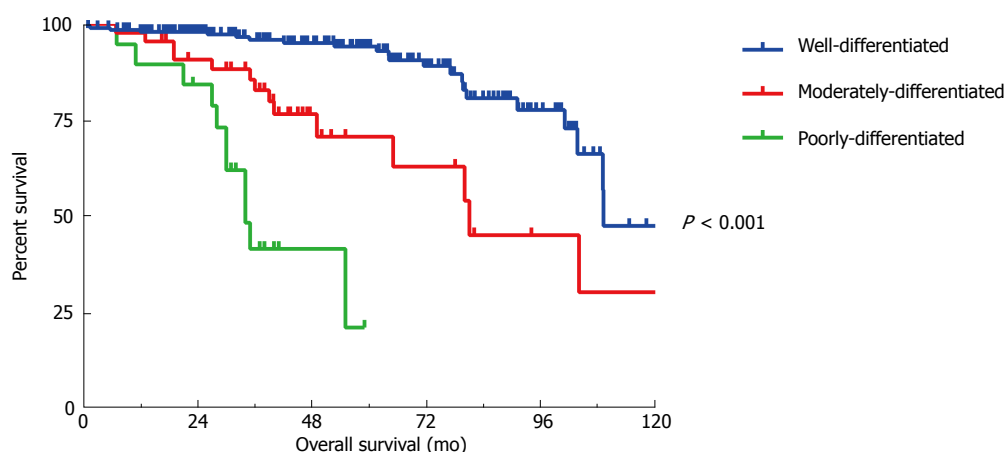


Figure 1 Kaplan Meier graph demonstrating the significant difference in overall survival for mucinous appendiceal adenocarcinoma stratified by histological grade.

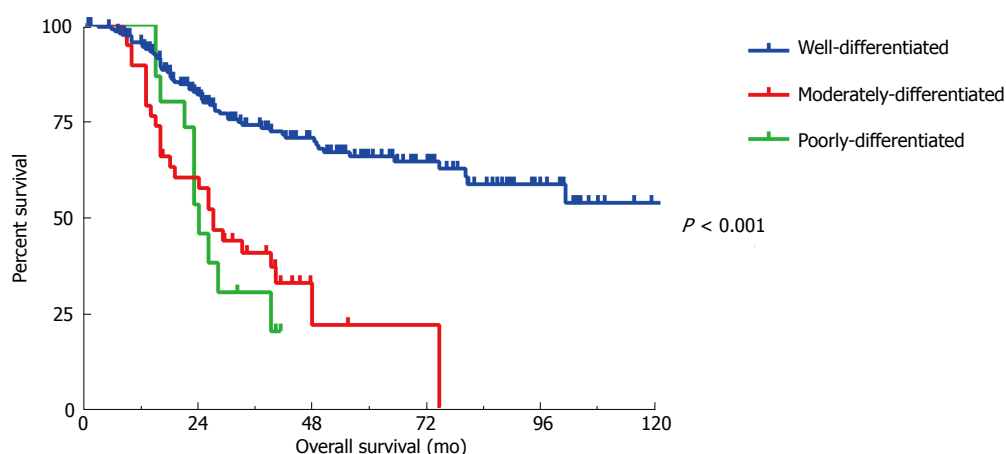


Figure 2 Kaplan Meier graph demonstrating the significant difference in disease-free survival for mucinous appendiceal adenocarcinoma stratified by histological grade.

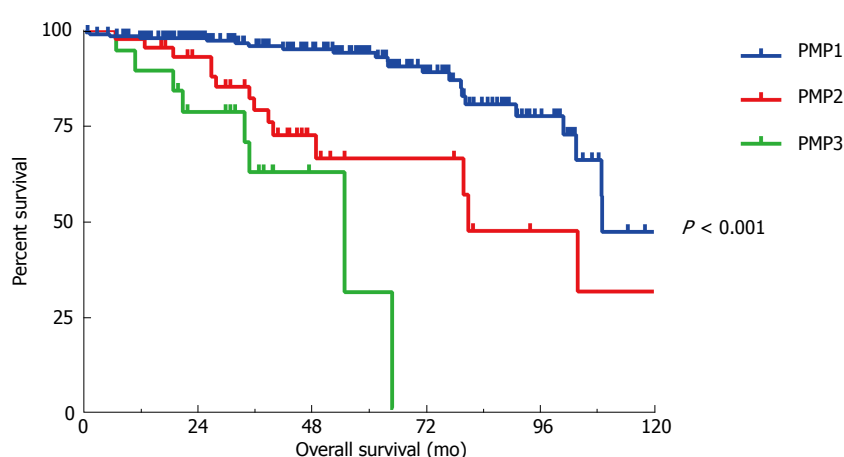


Figure 3 Kaplan Meier graph demonstrating the significant difference in overall survival for mucinous appendiceal adenocarcinoma stratified by the Shetty classification. PMP1: Pseudomyxoma Peritonei 1; PMP2: Pseudomyxoma Peritonei 2; PMP3: Pseudomyxoma Peritonei 3.

predictor of OS on univariate but not multivariate analysis and therefore the tumors were alternatively classified according to Shetty classifications of well differentiated (PMP1) and moderately and poorly

differentiated MAA without signet ring cells (PMP2) and with signet ring cells (PMP3). The median OS for the three groups are 109 mo, 81 mo and 55 mo, respectively ( $P < 0.001$ ) (Figure 3). However,

**Table 2** Univariate and Multivariate models using Cox regression analysis for disease-free and overall survival

Disease-free survival				Overall survival			
Univariate	P value	Multivariate (HR, 95%CI)	P value	Univariate	P value	Multivariate (HR, 95%CI)	P value
Age	0.45			Age	0.35		
Sex	0.05 <sup>1</sup>		0.59	Sex	< 0.001 <sup>1</sup>		0.13
Albumin	0.27			Albumin	0.28		
ECOG status	0.38			ECOG status	0.006 <sup>1</sup>		0.15
Pre-op CEA	0.39			Pre-op CEA	0.07		0.51
Grade	< 0.001 <sup>1</sup>	1.8 (1.2-2.8)	0.008 <sup>1</sup>	Grade	< 0.001 <sup>1</sup>	2.80 (1.26-6.21)	0.01 <sup>1</sup>
Signet ring cell	< 0.001 <sup>1</sup>		0.828	Signet ring cell	< 0.001 <sup>1</sup>		0.22
Lympho-vascular invasion	< 0.001 <sup>1</sup>		0.15	Lympho-vascular invasion	< 0.001 <sup>1</sup>		0.82
Lymph node metastasis	< 0.001 <sup>1</sup>	0.42 (0.20-0.88)	0.02 <sup>1</sup>	Lymph node metastasis	< 0.001 <sup>1</sup>		0.47
PSS	0.09	1.3 (1.1-1.6)	0.03 <sup>1</sup>	PSS	0.14		
PCI	0.03 <sup>1</sup>	1.05 (1.02-1.08)	0.002 <sup>1</sup>	PCI	< 0.001	1.10 (1.03-1.16)	0.002 <sup>1</sup>
				CC0 resection	< 0.001 <sup>1</sup>	0.32 (0.11-0.92)	0.03

<sup>1</sup>Significant variables. ECOG: Eastern Cooperative Oncology Group; CEA: Carcinoembryonic antigen; PSS: Previous surgical score; PCI: Peritoneal carcinomatosis index.

**Table 3** Illustrates the incidence of lymph node metastasis as stratified by histological grade, and the presence or absence of signet ring cells

Incidence of lymph node metastasis		
Histological grade		$P < 0.001$
Well-differentiated	5.50%	
Moderately-differentiated	10.80%	
Poorly-differentiated	42.10%	
Signet ring cell		$P = 0.08$
Absent	7.50%	
Present	20.80%	
Moderately-differentiated and LVI	6%	$P = 0.008$
Moderately-differentiated and LVI <sup>+</sup>	40%	

The incidences of lymph node metastasis for the moderately-differentiated tumors are further stratified by lymphovascular invasion (LVI).

the presence or absence of signet ring cells failed to significantly stratify survival outcomes between the PMP2 and the PMP3 categories in regards to DFS ( $P = 0.76$ ) and OS ( $P = 0.08$ ). Patients with < 50% signet rings cells did similarly as poor as those with > 50% signet ring cells (median OS 43 mo vs 34 mo) when compared to those without signet rings cells (median OS 109 mo) ( $P < 0.001$  when comparing < 50% signet rings cells to no signet ring cells and when comparing > 50% signet ring cells to no signet ring cells. No statistical difference between those with < 50% signet ring cells and those with > 50% signet rings cells,  $P = 0.64$ ).

The presence of lymph node metastasis varied by histological grade ( $P < 0.001$ ) but not by the presence of signet ring cells ( $P = 0.08$ ). Lymphovascular invasion was also a significant predictor of nodal positivity and significantly stratified the risk of lymph node metastasis for the moderately-differentiated MAA (Table 3). Moderately-differentiated MAA without lymphovascular invasion had the same low incidence of lymph node metastasis as the well-differentiated tumors. In contrast,

the moderately-differentiated MAA with lymphovascular invasion had the same high incidence of lymph node metastasis as the poorly-differentiated MAA.

## DISCUSSION

This analysis highlights the important prognostic distinction of an intermediate classification for MAA based on histological grade alone. Although histological grade has previously been demonstrated to have a major prognostic impact on survival, most authors combine either the intermediate and high grade groups or the intermediate and low grade groups because of their presumed similar prognosis. The 7<sup>th</sup> edition of the AJCC Cancer Staging Manual recognized the significance of histological grade and incorporated it into the staging classification of MAA<sup>[11]</sup>. While there is a suggestion by the WHO<sup>[12]</sup> and AJCC<sup>[7]</sup>, as well as other authors<sup>[5,6]</sup>, to use a two-tiered system, this is not supported by a population based study utilizing the SEER database which demonstrated that moderately differentiated MAA have a distinctly different clinical behavior than that of well differentiated and poorly differentiated MAA<sup>[13]</sup>. Similarly, we found moderately differentiated MAA have a survival intermediate to well and poorly differentiated MAA and should be categorized separately.

The unique biological behavior of MAA makes classification of both the primary tumor and metastatic peritoneal spread difficult and there has been considerable debate in the literature about the correct terminology<sup>[1,2,4,6]</sup>. Unfortunately, the end result has been a variety of different proposed classifications that has led to confusion within the literature and among treating clinicians. Issues that plague grading schema include subjectivity, sampling, tumor characteristics, tumor heterogeneity, and even recognition of differing biologic behaviors depending on the appendiceal primary. Unfortunately, grading relies often on subjective observation rather than objective measurement; although attempts have been made to provide objective



criteria, it can be difficult in these tumors<sup>[14]</sup>. Tumor heterogeneity between sites, or even within the same site, in a patient is not uncommon. Because of this, sampling artifact can be problematic both at the clinical level (needle biopsy vs larger biopsy) or at the time of pathologic dissection. Histologic characteristics also play a role. While most of the mucinous tumors arise in a background of low grade appendiceal mucinous neoplasms (LAMN), a smaller percentage of MAA arise either *de novo* (possibly obliterating the precursor lesion) or in association with an adenoma and have the appearance of a mucinous type colonic adenocarcinoma. The latter lesions may have more gland formation, allowing categorization into a “moderately differentiated”/intermediate grade category. These tumors are much more likely to show lymphovascular invasion and lymph node metastases. In an attempt to standardize terminology and clarify histological classification the Peritoneal Surface Oncology Group International (PSOGI) formed an international expert panel to achieve a consensus. Within the expert panel there were 6 different classification systems used. The final recommendations were similar to the Shetty classification in which low grade tumors consist of well-differentiated MAA and the moderately and poorly differentiated MAA are grouped into a high grade category stratified by the presence of signet ring cells<sup>[8]</sup>. However, the definition of signet ring cells is inconsistent. The Shetty classification defined signet ring cells as any percentage of signet ring cells. Other reports suggest that only infiltrative signet ring cells are prognostic and not degenerative cells floating in mucin, which may mimic signet ring cells<sup>[14,15]</sup>. AJCC and the PSOGI consensus utilized the WHO classification of signet ring cell adenocarcinoma which is defined as a tumor composed of > 50% of cells. In the current study, we found that the presence of any degree of signet ring cells was prognostic not just those with > 50% signet ring cells. Furthermore, we found that histological grade not signet ring cells was an independent predictor of DFS. Histological grade also stratified nodal metastasis risk and signet ring cells did not. Our data is unique in that we found a truly intermediate prognosis for the moderately differentiated MAA. Also we demonstrate the importance of histological grade as it was the only independent predictor of both DFS and OS. Therefore, we conclude that histological grade is superior to the presence of signet ring cells in stratifying both DFS and OS and should be incorporated into the AJCC staging criteria for stage IV MAA.

Consistently differentiating and reporting MAA into unambiguous histological classifications is imperative as they reflect the divergent biology of the tumor. This information may be then used to develop the divergent treatment approaches that are employed. For example, well-differentiated MAA are likely to behave in an indolent fashion and therefore, aggressive cytoreductive surgery (CRS) with the intent of removing all tumor burden to  $\leq 2.5$  mm has been demonstrated to substantially prolong survival<sup>[16]</sup>. However, adjuvant systemic chemotherapy has been demonstrated to provide no benefit for well-

differentiated tumors<sup>[17]</sup>. In contrast, poorly-differentiated MAA which are likely to behave aggressively derive survival benefit from adjuvant systemic chemotherapy and the aggressiveness of surgical cytoreduction must be carefully weighed against the morbidity and reduced probability of long-term survival. Moderately-differentiated MAA exhibit an intermediate biology that appears to respond to systemic chemotherapy<sup>[17]</sup> and warrant a more aggressive approach to surgical cytoreduction given the higher likelihood of long-term survival<sup>[18]</sup>.

Furthermore, performing a right hemicolectomy is predicated on the risk of lymph node metastasis. Our data demonstrates that the risk of lymph node metastasis is well stratified by the histological grade and presence of lymphovascular invasion. Well-differentiated MAA have a low risk of nodal metastasis and therefore a right hemicolectomy is unwarranted. In contrast, a poorly-differentiated MAA has a 40% incidence of lymph node metastasis and a right hemicolectomy is indicated. As anticipated a moderately differentiated tumor has an incidence of lymph node metastasis that is intermediate to the well- and poorly-differentiated tumors. However, our data suggests that the presence or absence of lymphovascular invasion further stratifies risk such that moderately-differentiated MAA without lymphovascular invasion demonstrate the same low incidence of nodal metastasis as well-differentiated MAA and therefore no colectomy is warranted. In contrast, moderately-differentiated MAA with lymphovascular invasion have a high incidence of lymph node metastasis similar to poorly-differentiated MAA and therefore a right hemicolectomy is warranted.

We recognize there are significant limitations to the study as in all retrospective single institutional reports including the small sample size, selection bias and non-uniformity of treatment. In addition, while the grade was determined prospectively by our group of subspecialized gastrointestinal pathologists, the cases were not re-reviewed by a single pathologist to eliminate interobserver variability. However, potential strengths of this study include the large sample size, consistent reporting of histological grade, lymphovascular invasion, and presence of signet ring cells in order to inform standardized care, and the long-term follow up.

In conclusion, histological grade is independently associated with DFS and OS and accurately predicts risk of nodal metastasis. Our data supports a three-tier histological grading system for peritoneal carcinomatosis from MAA. This classification best stratifies survival outcomes and should be incorporated into patient selection and treatment algorithms and potentially into future AJCC staging updates. The 8<sup>th</sup> edition of the AJCC staging system currently groups grade G2 and G3 (moderate and poorly differentiated) MAA into the same Stage IVB group. Our data suggests a separate Stage IV for each grade may be more appropriate.

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## COMMENTS

### Background

The authors' understanding of mucinous appendiceal adenocarcinoma (MAA) is plagued by confusing and indelible terminology, which does not capture the heterogeneous biology and outcomes of these tumors. There remains an unmet need for further investigation of the clinical behavior and outcomes of clearly defined histological classifications.

### Research frontiers

Survey results show that even amongst experts at high volume centers there is considerable discrepancy in the terminology and classification of MAA. A consensus conference was unable to agree on a single terminology and the AJCC staging system allows both two and three-tiered grading system. Further evidence is necessary to bring clarification to this field.

### Innovations and breakthroughs

This is one of the largest single institutional investigations of MAAs with consistent and clearly defined histological grading and the associated long-term outcomes following cytoreductive surgery (CRS) and hyperthermic intraperitoneal chemotherapy (HIPEC). The data demonstrates that moderately differentiated carcinomas have a clinical behavior and outcome that is distinct from well- and poorly-differentiated carcinomas.

### Applications

The three-tier grade classification provides improved prognostic stratification and should be incorporated into patient selection and treatment algorithms and potentially into future AJCC staging updates. The 8<sup>th</sup> edition of the AJCC staging system currently groups grade G2 and G3 (moderate and poorly differentiated) MAA into the same Stage IVB group. The data suggests a separate Stage IV for each grade may be more appropriate.

### Terminology

CRS involves a full midline laparotomy and systematic evaluation of the entire peritoneal surface including all recesses. All visible tumor is then removed by means of peritonectomy or resection of underlying organs. HIPEC is the subsequent administration of high dosages of chemotherapy into the peritoneum under hyperthermia (40 °C–42 °C) for 60–90 min under constant agitation.

### Peer-review

This article is to investigate the importance of a three-tiered histologic grade on outcomes for patients with MAA. The study is good.

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## Retrospective Cohort Study

# Characterisation and risk assessment of venous thromboembolism in gastrointestinal cancers

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**Author contributions:** Metcalf RL and Hasan J designed the research; Hopley N, Henry T and McGurk A performed the data collection; Al-Hadithi E analysed the data and co-wrote the paper; all authors contributed to data analysis and manuscript preparation.

**Institutional review board statement:** This study was conducted as part of the Christie NHS Foundation Trust's on-going process of clinical audit including the analysis of the occurrence of thromboembolism and subsequent management and was reviewed and approved by the Institutional Clinical Audit Committee.

**Informed consent statement:** All data were anonymised prior to analysis and no patient identifiable data were included in the analysis or subsequent presentation of the results. This analysis was performed on a retrospective cohort of 2209 patients treated between 2006 and 2012 as part of the Trust's process of clinical audit and the analysis was approved by the department of clinical audit. Individual participant informed consent was therefore not sought for this retrospective analysis of non-identifiable population level data.

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## Abstract

### AIM

To characterise venous thromboembolism (VTE) in gastrointestinal cancer and assess the clinical utility of risk stratification scoring.

### METHODS

We performed a retrospective analysis using electronic patient records of 910 gastro-oesophageal (GO) cancer and 1299 colorectal cancer (CRC) patients referred to a tertiary cancer centre to identify the incidence of VTE, its relationship to chemotherapy and impact on survival.



VTE risk scores were calculated using the Khorana index. Patients were classified as low risk (0 points), intermediate risk (1 to 2 points) or high risk (3 points). Data was analysed to determine the sensitivity of the Khorana score to predict VTE.

## RESULTS

The incidence of VTE was 8.9% for CRC patients and 9.7% for GO cancer patients. Pulmonary emboli (PE) were more common in advanced than in localised CRC (50% *vs* 21% of events respectively) and lower limb deep vein thrombosis (DVT) were more common in localised than in advanced CRC (62% *vs* 39% of events respectively). The median time to VTE from cancer diagnosis was 8.3 mo for CRC patients compared to 6.7 mo in GO cancer. In localised CRC median time to VTE was 7.1 mo compared with 10.1 mo in advanced CRC. In contrast in GO cancer, the median time to VTE was 12.5 mo in localised disease and 6.8 mo in advanced disease. No survival difference was seen between patients with and without VTE in this cohort. The majority of patients with CRC in whom VTE was diagnosed had low or intermediate Khorana risk score (94% for localised and 97% in advanced CRC). In GO cancer, all patients scored either intermediate or high risk due to the primary site demonstrating a limitation of the risk assessment score in discriminating high and low risk patients with GO cancers. Additional risk factors were identified in this cohort including surgery, chemotherapy or hospital admission. Overall, 81% of patients with CRC and 77% of patients with GO cancer had one or more of these factors within 4 wk prior to diagnosis VTE. These should be factored into clinical risk assessment scores.

## CONCLUSION

The Khorana score has low sensitivity for thrombotic events in CRC and cannot discriminate low risk patients in high risk cancer sites such as GO cancer.

**Key words:** Thrombo-embolism; Deep venous thrombosis; Pulmonary embolism; Colorectal cancer; Oesophageal cancer; Gastro-oesophageal cancer

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**Core tip:** Analysis of clinical outcomes in 2209 patients with gastrointestinal cancers demonstrated that the Khorana score to assess venous thromboembolism (VTE) risk may have inadequate sensitivity to be clinically useful beyond short term VTE risk.

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## INTRODUCTION

Approximately 20% of cases of venous thromboembolism (VTE) occur in cancer patients<sup>[1]</sup>. The cumulative 2-year incidence rate of VTE in cancer patients is approximately 1.6%<sup>[2]</sup>, with an elevated risk with gastro-intestinal cancers<sup>[3]</sup>.

In addition to the classical Virchow's triad of venous stasis, thrombophilia and endothelial injury, increased incidence of VTE in cancer patients has been linked to platelet activation, direct factor X activation, decreased hepatic anticoagulant synthesis, reduced hepatic clearance of coagulation factors, and the development of antiphospholipid antibodies<sup>[4]</sup>.

Symptomatic VTE has a detrimental effect on cancer survival, in addition to causing substantial morbidity<sup>[5,6]</sup>. To minimise the occurrence and clinical impact of VTE in patients with cancer, current guidelines recommend the prophylactic anticoagulation of patients during medical admissions and following surgery<sup>[7,8]</sup> which are recognised as periods of high risk of VTE<sup>[9,10]</sup>. However, the majority of cancer patients receive their treatment in the outpatient setting, and VTE is still a frequent occurrence in this group<sup>[4]</sup>. Current ASCO and ESMO guidance<sup>[8,11]</sup> does not recommend routine anticoagulation in ambulatory patients, but that it may be considered in high risk patients. There is some debate, however, on how such high risk patients are identified.

Clinical trials randomising ambulatory patients with cancer to receive placebo or prophylactic anticoagulation have shown inconsistent results<sup>[12-24]</sup>. Some have shown beneficial use of thromboprophylaxis in ambulatory cancer patients<sup>[12,13]</sup>. A meta-analysis<sup>[25]</sup> of 7622 patients found a reduction in the incidence of symptomatic VTE (RR = 0.56, 95%CI: 0.40-0.74) at the expense of an increased risk of minor bleeding (RR = 1.32, 95%CI: 1.02-1.71). Overall, however, these studies have been unable to convincingly demonstrate significantly reduced rates of VTE. This may be related to the fact that these studies have included a large number of patients who have low VTE risk, and the benefit of anticoagulation has been weakened as a result.

Two studies<sup>[20,26]</sup> have shown the use of higher doses of anticoagulation in high risk ambulatory pancreatic cancer patients. The FRAGEM trial<sup>[20]</sup> used therapeutic doses of dalteparin, whilst the CONKO-004<sup>[26]</sup> trial used half therapeutic doses of enoxaparin. They demonstrated significant reduction in VTE incidence (RR = 0.145 and 0.12 respectively) with an increased bleeding risk and no overall effect on survival.

A risk model has been developed to identify patients at higher risk of developing VTE based upon blood results (elevated leukocytes, elevated platelets, and reduced haemoglobin), elevated BMI and the primary cancer site<sup>[27]</sup>. This assessment categorises patients into groups with a VTE incidence of 6.7% for high risk, 2% for intermediate risk and 0.3% for low risk

patients over a median of 2.5 mo follow up, and the use of this risk assessment score has been advocated within ASCO and ESMO guidance<sup>[8,11]</sup> for clinical decision making in the anticoagulation of selected ambulatory outpatients receiving chemotherapy. The AVERT clinical trial (NCT02048865) is currently evaluating the benefit of thrombo-prophylaxis in ambulatory outpatients who have high and intermediate VTE risk scores.

There is also scope for further development of the Khorana scoring system as has been shown with the Vienna prediction score<sup>[28]</sup> which combines the Khorana score with additional lab parameters such as D-dimer; and the Protecht prediction score<sup>[29]</sup> which takes into consideration whether patients have been given chemotherapy associated with additional VTE risk.

In order to determine the applicability of the VTE risk score to the “real world setting”, we sought to characterise the incidence and risk factors for VTE in patients with gastrointestinal cancers treated at a tertiary cancer centre. In the colorectal cancer (CRC) population, we evaluated the sensitivity of a high VTE risk score to detect patients with VTE in their lifetime. Patients with gastro-oesophageal (GO) cancers were not included within this analysis as all GO cancer patients score as very high risk as this is classified as a high risk cancer site in the risk assessment tool<sup>[3]</sup>.

## MATERIALS AND METHODS

The electronic patient records of 2209 patients [1299 with colorectal cancer (CRC) and 910 with gastro-oesophageal (GO) cancers] that were referred to The Christie NHS Foundation Trust between 2006 and 2012 were screened as previously described<sup>[30]</sup> to identify patients diagnosed with VTE. Briefly, the electronic patient record was searched using the search text function for the terms “thromb”, “embol”, “DVT” (deep vein thrombosis) and “PE” (pulmonary embolism) recorded anywhere within the patient notes to identify patients with VTE (cases). Demographic and clinical data including details of previous chemotherapy, surgery, hospital admissions and patient survival was extracted from the records of patients with VTE for analysis. From the patients without VTE, a control cohort was generated matching by site and stage of disease and performance status. Survival data was collected on the matched cohort to evaluate the prognostic effect of VTE in these patients. A VTE risk score<sup>[27]</sup> was calculated for the patients with CRC diagnosed with VTE as previously reported. One point each was allocated for platelets  $> 350 \times 10^9/L$ , haemoglobin  $< 10$  g/dL, leukocytes  $> 11 \times 10^9/L$  and body mass index  $> 35$  kg/m<sup>2</sup> and one point for a high risk cancer site (lung, lymphoma, gynaecologic, bladder, and testicular) and two points for a very high risk site (stomach and pancreas). Patients were classified as low risk (0 points), intermediate risk (1 to 2 points) or high risk (3 points). In view of the fact that GO cancers automatically score 2 points as a very high risk site, this risk score was only calculated for

**Table 1 Site of thromboses in patients with gastrointestinal cancers *n* (%)**

	Colorectal cancer	Gastro-oesophageal cancer
All		
Lower limb thrombosis	51 (44)	44 (50)
Upper limb thrombosis	9 (8)	4 (4)
Visceral thrombosis	6 (5)	2 (2)
Pulmonary embolism	49 (43)	39 (44)
Localised		
Lower limb thrombosis	18 (62)	19 (43)
Upper limb thrombosis	1 (3)	2 (5)
Visceral thrombosis	4 (14)	2 (4)
Pulmonary embolism	6 (21)	21 (48)
Advanced		
Lower limb thrombosis	33 (39)	25 (56)
Upper limb thrombosis	8 (9)	2 (4)
Visceral thrombosis	2 (2)	0 (0)
Pulmonary embolism	43 (50)	18 (40)

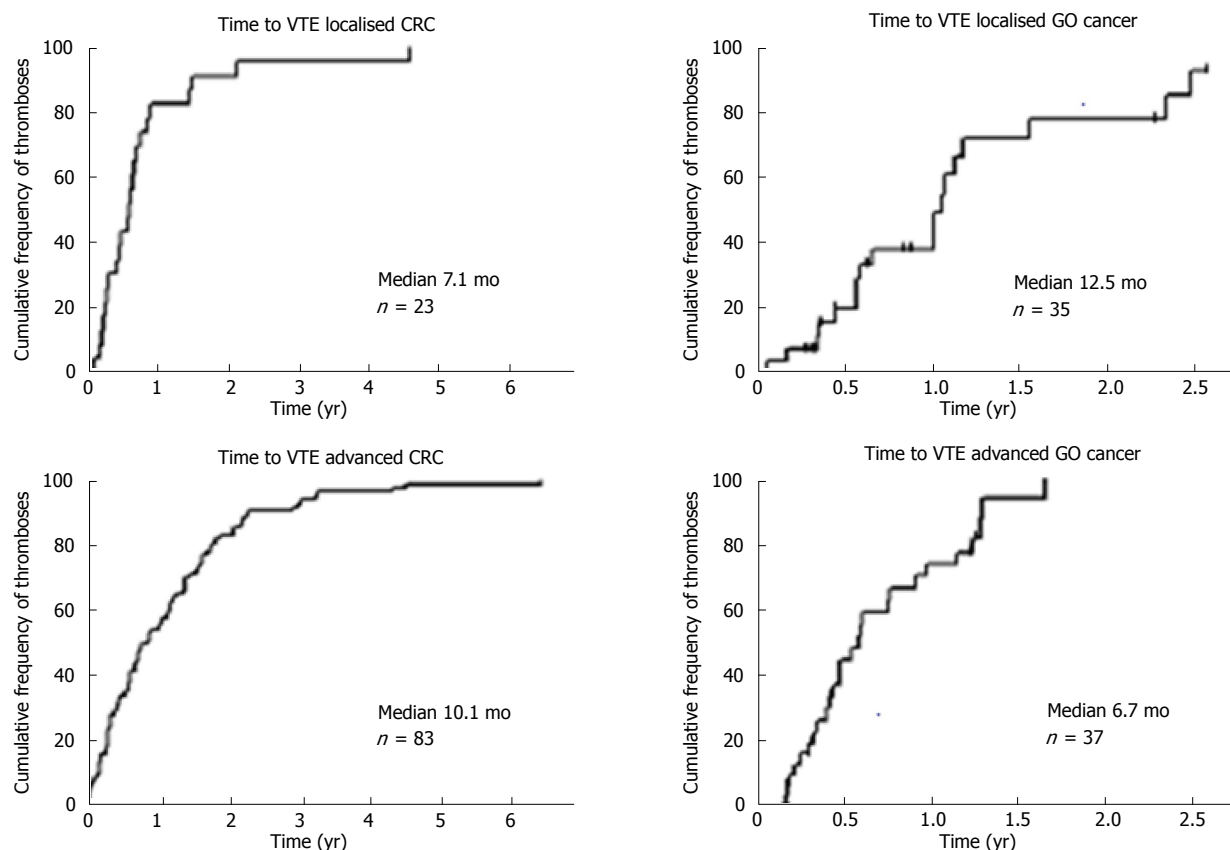
CRC patients in order to evaluate the sensitivity of this approach to identify patients at risk of VTE with a low risk primary site.

Data was exported to Microsoft Excel and analysed using SPSS for Windows (Chicago, IL, United States). To evaluate for the impact of VTE on patient survival, a control cohort matched for primary site, stage of disease and performance status was identified in whom no VTE was diagnosed in the patient lifetime. This data was tested by univariate survival analysis using the Kaplan-Meier method and log-rank test and  $P \leq 0.05$  was considered significant. Survival analysis was performed on all patients and separate analyses performed on patients with localised and advanced disease. Time to event analysis was performed using cumulative incidence plot and median time to thrombosis was calculated. Statistical review of the study was conducted by Clare Hodgson, department of biostatistics, the Christie NHS Foundation Trust. Clinical records were also reviewed to identify additional risk factors for VTE in this cohort in order to establish whether the Khorana score could be modified or used alongside the Khorana score to more accurately identify high risk patients.

## RESULTS

Of the 2209 patients with GI cancers, VTE was diagnosed in 203 patients giving a cumulative incidence rate of 9.2%. The incidence of VTE was 8.9% (115/1299) for CRC patients and 9.7% (88/822) for upper GI cancer patients.

Table 1 shows the site of thromboses in patients with CRC. As a proportion of all events, pulmonary emboli (PE) were more common in advanced than in localised CRC (50% vs 21% of events respectively) and lower limb deep vein thrombosis (DVT) were more common in localised than in advanced CRC (62% vs 39% of events respectively). This difference was not seen in patients with GO cancers. In localised GO



**Figure 1** Cumulative of venous thromboembolism plotted over time. VTE: Venous thromboembolism; CRC: Colorectal cancer; GO: Gastro-oesophageal.

cancers, DVT and PE made up 48% and 44% of VTE and in advanced GO cancers they made up 40% and 51% of VTE respectively. This difference may in part be attributed to the increased patient survival in advanced CRC compared to GO cancer. Patients with longer survival tend to have an increased number of CT scans to evaluate their disease and consequently may have a greater number of PEs detected as incidental findings on routine imaging. To evaluate this further, the proportion of VTE identified as incidental findings on routine clinical imaging was recorded. Consistent with this hypothesis, VTE were more frequently detected as incidental findings on diagnostic imaging in CRC, accounting for 40% of events, compared with 26% in GO cancer.

To determine the relationship between the time of cancer diagnosis and the occurrence of VTE, the cumulative incidence of VTE was plotted over time (Figure 1). For patients with CRC, the median time to VTE from cancer diagnosis was 8.3 mo for all patients. This compares with a median time to VTE for patients with GO cancers of 6.7 mo. Analysing patients with localised and advanced disease separately identified that for patients with localised CRC median time to VTE was 7.1 mo and in advanced CRC, median time to VTE was 10.1 mo. In contrast in GO cancer, the median time to VTE was 12.5 mo in localised disease and 6.8 mo in advanced disease, which may reflect the increased frequency of recurrent disease and the shorter time to progression following first line therapy seen in patients

with GO cancers.

Consistent with previous approaches<sup>[31]</sup> to evaluate for the impact of VTE on patient survival, a control cohort matched for primary site, stage of disease and performance status was identified in whom no VTE was diagnosed in the patient lifetime. Kaplan-Meier survival analyses of cases and controls are summarised in Figure 2. For patients with CRC, median survival was 23.8 mo for cases and 24.7 mo for controls ( $P = 0.20$ ) and for upper gastrointestinal cancer, median survival was 13.9 mo for cases and 10.2 mo for controls ( $P = 0.59$ ). Sub-group analysis of the patients with localised disease for each cancer type showed that although there was no significant difference in median survival between cases and controls (40.3 mo vs 50.7 mo for CRC cases and controls,  $P = 0.41$ ; and 16.9 and 17.0 mo for GO cancer cases and controls,  $P = 0.72$ ), the curves began to separate after the median. A limitation of this approach is an inherent underestimation of the association between VTE and reduced survival. This plateau in the survival curves seen in the control cohorts in both CRC and GO cancer was not evident in the cases with VTE. This observation suggests that a subset of patients with long term survival is present in the control cohort which is not seen in the VTE cohort.

The Khorana VTE risk score<sup>[27]</sup> identifies patients at higher short term risk of VTE and is being incorporated into clinical trials of prophylactic anticoagulation in the ambulatory out-patient setting. To evaluate the broader

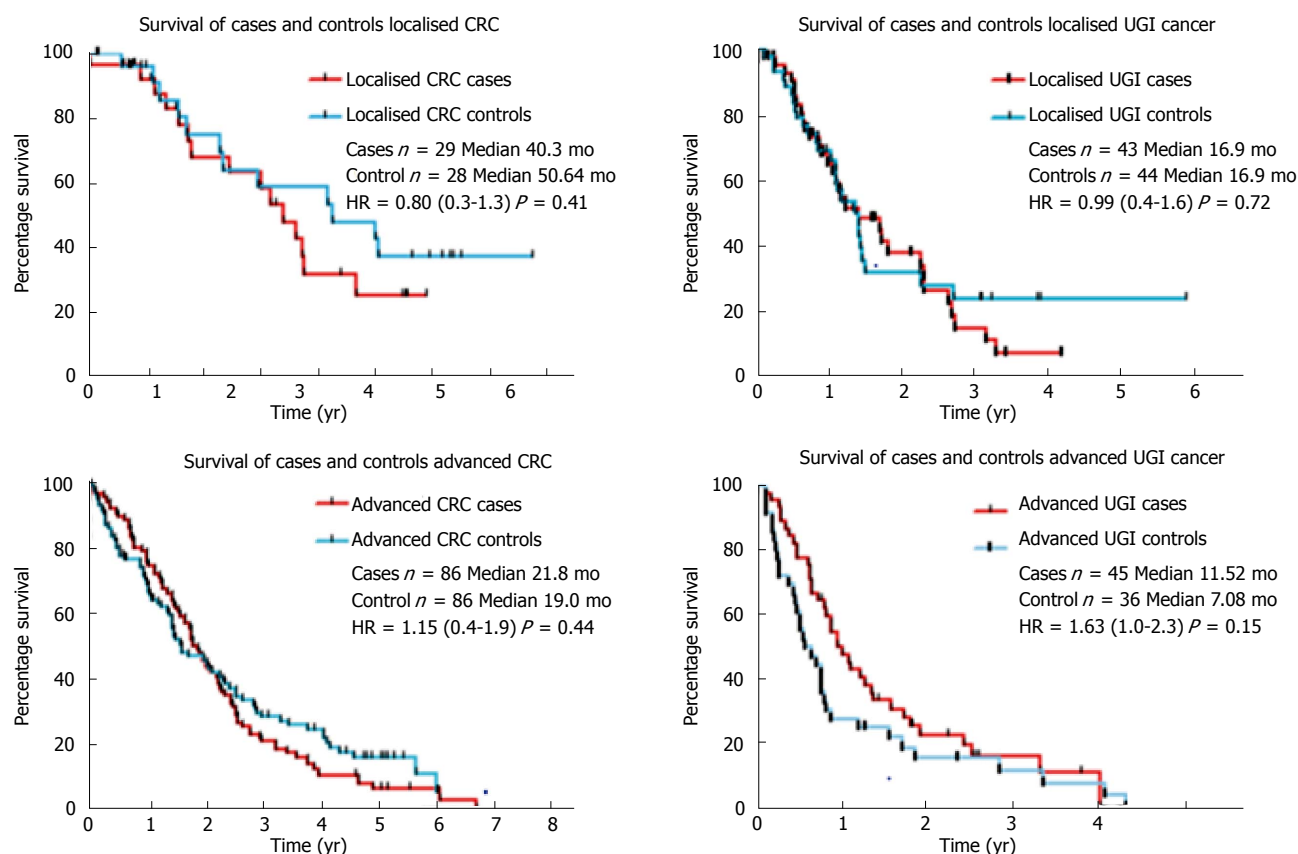


Figure 2 Kaplan-Meier survival analyses of cases and controls. CRC: Colorectal cancer; UGI: Upper gastrointestinal.

clinical utility of Khorana VTE risk score in patients without a “high risk” primary site, we determined the sensitivity of the risk score for predicting subsequent risk of VTE in CRC patients. The risk score was calculated in the patients with CRC in whom VTE had been diagnosed. Sufficient clinical data to complete this assessment were available for 72/115 (63%) patients. Using the cut-off score of  $> 2$  points to classify patients as high risk for VTE, the sensitivity of the risk score was low for prediction of subsequent VTE. The majority of patients with CRC in whom VTE was diagnosed had low or intermediate Khorana risk score (94% for localised and 97% in advanced CRC). This finding demonstrates that although the Khorana risk score has been validated to identify patients at high short term risk of VTE whilst receiving chemotherapy, it fails to identify the majority of VTE which occur in patients with cancer beyond this short term window.

Clinical records were reviewed to identify additional risk factors of surgery, chemotherapy or hospital admission within 4 wk prior to the event for all patients identified to have VTE. Overall, 81% of patients with CRC and 77% of patients with GO cancer had one or more of these factors within 4 wk prior to the diagnosis of VTE. These characteristics, which are well described as risk factors for the occurrence of VTE, may be utilised alongside characteristics included in the Khorana risk score to identify patients at highest risk of developing VTE.

## DISCUSSION

This study has characterised VTE in patients with gastrointestinal cancers treated at a tertiary cancer centre, reporting a cumulative incidence of VTE approaching 1 in 10 in both CRC and GO cancer. The incidence of 9.7% we report in GO cancer is consistent with previous reports identifying a cumulative incidence of 9.4% in advanced disease<sup>[32]</sup> and 12.5% to 13% in patients with localised disease undergoing dual modality treatment<sup>[6,33]</sup>. However, the identification of VTE in 8.9% of patients with CRC is higher than previous reports. Retrospective analyses of cancer registry databases identified VTE incidence of 5.4% in CRC patients in England<sup>[34]</sup> and 3.1% in the Californian Cancer Registry, using data from the 1990s<sup>[35]</sup>. However, the risk of VTE is higher for patients with Stage IV disease than Stages I-III disease (HR = 3.08, 95%CI: 1.95-4.84) and for patients receiving chemotherapy (HR = 1.39, 95%CI: 1.14-1.69)<sup>[34]</sup>, both of which would be over-represented in the patients treated at the tertiary cancer centre included in the current study. In addition, VTE are becoming more common, and more recent United States cancer registry database studies found a VTE incidence of 10.6%<sup>[36]</sup> consistent with the current study.

All the studies described above report a higher incidence of VTE in GI cancers than the 2.2% incidence reported in the development and validation of the VTE risk assessment model<sup>[27]</sup>. This can be explained by the



short follow up (median follow up of 2.5 mo following initiation of chemotherapy) in the development of this model. The current study found that the median time to VTE was 8.3 mo for CRC and 6.7 mo for GO cancers.

Although no statistically significant difference in survival was seen in the VTE cohort compared with the control cohort, an intriguing difference was seen in the long term survivorship in patients with localised disease experiencing VTE. The survival curves separate beyond three years suggesting a sub-group of long term survivors in the control cohort and this was not seen in the VTE cases. This finding suggests that the occurrence of VTE in localised disease is a surrogate for occult micro-metastatic disease, as metastatic relapse is responsible for the majority of the cases of death from cancer. Previous studies of the prognostic impact of VTE in CRC have also found this selective effect in localised disease<sup>[35]</sup>. This hypothesis may also explain why an impact of VTE on survival is not seen in advanced disease.

This work has identified that as a proportion of all thrombotic events, PE are more frequent in advanced than in localised CRC however, representing 50% and 21% of VTE respectively. In contrast, in GO cancer, PE comprises 44% and 51% of VTE in localised and advanced disease respectively. Although incidental PE were a more frequent occurrence in CRC than GO cancer in this series which may be due to the longer survival time in CRC and an increased number of CTC scans performed in the patient lifetime, another explanation for this finding may be that PE are a surrogate for aggressive tumour biology seen in localised GO cancer.

Although no statistically significant difference in survival was detected between cases and controls in this analysis, this may be influenced by a bias in selection of the control cohort. Within the control cohort, some of the patients would be predicted to have died of a non-VTE related cause with short follow up, who may have gone on to develop VTE if they had a longer survival time. Although this could be mitigated by including events which occurred within a short follow up period, for example 3 mo from diagnosis, this approach would miss a large proportion of all clinically meaningful VTE events. However, this study demonstrates that this approach would fail to capture most VTE events occurring. The consequences of this are two-fold. Firstly, the total number of events detected and analysed would be reduced, reducing both the utility of both descriptive and statistical analysis. Secondly, as the majority of VTE events would fall outside this follow up window, the clinical relevance of such an analysis would be reduced.

Retrospective data collection permits a larger number of events to be captured from cancer databases; however it is not without limitations. As VTE may occur after cancer diagnosis, patients with shorter survival are less likely to develop a VTE. This represents an inherent bias underestimating the association between VTE and worse prognosis. In the subjects who did not experience VTE, the time to event analysis can only

include the patients who experienced a VTE, and this approach does not account for censored subjects who died without having an event. This limitation applies to all retrospective analyses of this nature, however, the current study is more powerful as it is able to identify a larger number of events and characterise those in more detail.

Another limitation of the approach to retrospective data collection undertaken in this study and other studies of this type is that we cannot guarantee that all thrombotic events were identified and the actual number may be underestimated. However, the approach to evaluation of both the clinical records and imaging assessments will mitigate this effect. However, the clinical heterogeneity between symptomatic and incidental VTE may impact on the relationship between VTE and survival. Future studies may require the use of an analytical strategy to account for this variation.

A high Khorana risk score has been validated as a predictor of short term risk of VTE in patients receiving chemotherapy, and the sensitivity of the risk score, describing the probability of patients experiencing VTE being attributed a high risk score was reported as 40%<sup>[27]</sup>. We sought to test the sensitivity of this model in a larger cohort of patients with CRC, finding that when looking at a patient's lifetime risk of VTE, almost no patients who sustained VTE had a high risk score. This was performed in patients with CRC as opposed to GO cancer as the latter is considered a very high risk primary site which automatically scores 2 points on the Khorana risk score placing all patients in the upper intermediate risk category as a minimum.

The observation that almost no patients with CRC who sustained a VTE in their lifetime were classified as high risk using the Khorana assessment is significant as the findings of clinical studies using this risk score to enrich for patients with a high risk of thrombosis occurring shortly after diagnosis will not be generally applicable to the majority of thrombotic events which neither occurs in high risk patients nor within a short follow up. The current study suggests that these other events in patients not classified as high risk make up the majority of thrombotic events in the real world setting. This study does not bring into question the validity of the Khorana risk score for the identification of patients at higher risk of developing VTE whilst receiving chemotherapy. However, it does question the applicability of this risk score alone to enrich for patients who may benefit from prophylactic anti-coagulation.

This study also highlights the difficulty of taking a score made for general application and using it in a particular patient group. The Khorana score was not developed to be applied to specific cancer sites but rather cancer patients as a whole. The fact that the Khorana score did not perform as well in colorectal cancer patients specifically suggests that further validation studies in site-specific cancer groups may be needed.

Upper limb thromboses have been included in our

results, something which differs from the original Khorana study. Although upper limb thromboses are strongly associated with indwelling catheters in general, the pro-thrombotic nature of cancer patients may be expected to make these events more frequent in this cohort of patients. Including these patients in the data analysis has allowed us to obtain a complete picture of the clinical burden of VTE in relation to cancer. Overall, the indwelling catheter-associated thromboses represent a small proportion of the overall events in this cohort. However, looking into the incidence of indwelling-catheter associated thromboses in colorectal cancer patients as compared to the general population would present an interesting future study.

Although the classification of patients as high risk failed to identify patients with VTE, the majority of VTE occurred in patients with established risk factors such as surgery, chemotherapy and hospitalisation within 4 wk prior to the event in this study. This finding suggests that an alternative approach to prophylactic anticoagulation is to study extended anticoagulation following these high risk periods. Studies have already begun looking to develop a modified risk score to increase the sensitivity of this approach and reduce the number needed to treat to prevent VTE. For example, data from the Protecht study<sup>[13]</sup>, a prospective placebo controlled randomised trial evaluating anticoagulation with nadroparin in patients receiving chemotherapy was used to generate a modified risk score<sup>[29]</sup> which was more effective at identifying patients at high risk of VTE compared with the Khorana risk score. However, this approach has yet to be validated in a prospective cohort.

Finally, the current clinical guidance<sup>[8,11]</sup> does not recommend the routine prophylactic anticoagulation of ambulatory cancer patients. In addition to efforts to optimise the risk stratification of patients with cancer using datasets such as those evaluated in the current study, developments of novel oral anticoagulant (NOAC) therapies may alter the risk to benefit ratio if these drugs have an improved therapeutic index and do not require daily sub-cutaneous administration.

In summary, we have shown that VTE affects up to 1 in 10 patients with GI cancers and the peak incidence is in the first 6 mo following diagnosis. The prognostic impact of VTE in CRC is more evident in patients with localised disease than with advanced disease, suggesting that VTE may be a surrogate for occult metastasis. Finally, we demonstrate that the VTE risk score may have inadequate sensitivity to be clinically useful for the majority of thrombotic events occurring in a patient's lifetime. The findings of the current study warrant validation in a larger cohort, and this work is on-going. However, the implications of these findings are significant, as for studies enriching patients based upon VTE risk score to be applicable to clinical practice; the score requires a clinically meaningful sensitivity. If the majority of patients who experience VTE have low risk scores, and these events do not occur in the immediate

few months following diagnosis, then most VTE events would remain unaffected by the strategy of treating high and intermediate risk patients being employed in the AVERT clinical trial. This study raises the possibility that risk stratification using the Khorana VTE risk score, whilst an appealing approach to the incorporation in clinical trial design, may not be applicable to reducing the lifetime burden of VTE risk in clinical practice.

## COMMENTS

### Background

Venous thromboembolic disease is a frequent even in patients with cancer and there is a need for predictors of these events to identify patients at high risk occurrence to target prophylactic interventions.

### Research frontiers

This study characterised venous thromboembolism in a large cohort of patients with gastrointestinal cancer and evaluated clinical and biochemical predictors of events in these patients.

### Innovations and breakthrough

This study identified that existing risk scores using clinical and biochemical parameters have a low sensitivity for thrombotic events in colorectal cancer; the majority of thrombotic events occur in patients who are classified as low or intermediate risk. This study identifies the need for refined risk scores to identify groups of patients with cancer at high risk of thrombosis.

### Applications

The identification of high risk groups will enable prospective studies to be planned to formally compare prophylactic anticoagulation in patients with cancer.

### Peer-review

Metcalfe and coworkers present the results of a very interesting manuscript assessing the performance of the Khorana VTE risk score on a cohort of patients with gastrointestinal tumors. This study highlighted the limitations of said score. Overall the paper is well written and the analysis and discussion are sound and balanced.

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## Retrospective Study

**En bloc pancreaticoduodenectomy and right hemicolectomy for locally advanced right-sided colon cancer**

Yuji Kaneda, Hiroshi Noda, Yuhei Endo, Nao Kakizawa, Kosuke Ichida, Fumiaki Watanabe, Takaharu Kato, Yasuyuki Miyakura, Koichi Suzuki, Toshiki Rikiyama

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**Author contributions:** Kaneda Y collected the data and drafted the manuscript; Noda H and Rikiyama T designed the research and supervised the report; Endo Y, Kakizawa N, Ichida K, Watanabe F, Kato T, Miyakura Y and Suzuki K were involved in editing the manuscript; all authors have read and approved the final manuscript.

**Institutional review board statement:** This study was reviewed and approved by the Ethics Committee of Saitama Medical Center, Jichi Medical University.

**Informed consent statement:** Patients were not required to give their informed consent for inclusion in this retrospective study, because we used anonymous clinical data and individual cannot be identified according to the data present. We announced this study on our institution's website and explained about patients' right to refuse inclusion in this study and about the study's publication.

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**Abstract****AIM**

To assess the usefulness of *en bloc* right hemicolectomy with pancreaticoduodenectomy (RHCPD) for locally advanced right-sided colon cancer (LARCC).

**METHODS**

We retrospectively reviewed the database of Saitama Medical Center, Jichi Medical University, between January 2009 and December 2016. During this time, 299 patients underwent radical right hemicolectomy for right-sided colon cancer. Among them, 5 underwent RHCPD for LARCC with tumor infiltration to adjacent organs. Preoperative computed tomography (CT) was routinely performed to evaluate local tumor infiltration into adjacent organs. During the operation, we evaluated the resectability and the amount of infiltration into the adjacent organs without dissecting the adherent organs from the cancer. When we confirmed that radical resection was feasible and could lead to R0 resection, we performed RHCPD. The clinical data were carefully reviewed, and the demographic variables, intraoperative data, and postoperative parameters were recorded.

## RESULTS

The median age of the 5 patients who underwent RHCPD for LARCC was 70 years. The tumors were located in the ascending colon (three patients) and transverse colon (two patients). Preoperative CT revealed infiltration of the tumor into the duodenum in all patients, the pancreas in four patients, the superior mesenteric vein (SMV) in two patients, and tumor thrombosis in the SMV in one patient. We performed RHCPD plus SMV resection in three patients. Major postoperative complications occurred in 3 patients (60%) as pancreatic fistula (grade B and grade C, according to International Study Group on Pancreatic Fistula Definition) and delayed gastric empty. None of the patients died during their hospital stay. A histological examination confirmed malignant infiltration into the duodenum and/or pancreas in 4 patients (80%), and no patients showed any malignant infiltration into the SMV. Two patients were histologically confirmed to have tumor thrombosis in the SMV. All of the tumors had clear resection margins (R0). The median follow-up time was 77 mo. During this period, two patients with tumor thrombosis died from liver metastasis. The overall survival rates were 80% at 1 year and 60% at 5 years. All patients with node-negative status ( $n = 2$ ) survived for more than seven years.

## CONCLUSION

This study showed that the long-term survival is possible for patients with LARCC if RHCPD is performed successfully, particularly in those with node-negative status.

**Key words:** Locally advanced right-sided colon cancer; Right hemicolectomy; Malignant infiltration; Inflammatory adhesion; Pancreaticoduodenectomy

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**Core tip:** In this study, we retrospectively assessed the usefulness of *en bloc* right hemicolectomy with pancreaticoduodenectomy (RHCPD) in five patients with locally advanced right-sided colon cancer (LARCC) with malignant infiltration into adjacent organs. A histological examination confirmed malignant infiltration into the duodenum and/or pancreas in four patients, with no malignant infiltration into the superior mesenteric vein. The OS rates were 80% at 1 year and 60% at 5 years. All patients with node-negative status survived more than seven years without recurrence. The long-term survival is possible for patients, particularly node-negative ones, with LARCC if the RHCPD is performed successfully.

Kaneda Y, Noda H, Endo Y, Kakizawa N, Ichida K, Watanabe F, Kato T, Miyakura Y, Suzuki K, Rikiyama T. *En bloc* pancreaticoduodenectomy and right hemicolectomy for locally advanced right-sided colon cancer. *World J Gastrointest Oncol* 2017; 9(9): 372-378 Available from: URL: <http://www.wjgnet.com/1948-5204/full/v9/i9/372.htm> DOI: <http://dx.doi.org/10.4251/wjgo.v9.i9.372>

## INTRODUCTION

Locally advanced colorectal cancers invading into adjacent organs account for 5.5%-16.7% of all colorectal cancers<sup>[1-3]</sup>. Incomplete resection and separation of colon cancer from adherent organs are considered to lead to tumor recurrence and a poor prognosis<sup>[4,5]</sup>. Locally advanced right-sided colon cancer (LARCC) can invade the duodenum, pancreas, and other organs, and in this situation, *en bloc* right hemicolectomy with pancreaticoduodenectomy (RHCPD) is necessary to achieve R0 resection.

RHCPD for LARCC was first reported in 1953<sup>[6]</sup>, and high-volume centers, including our hospital, have reported acceptable outcomes with RHCPD<sup>[7-9]</sup>. However, the number of reports describing the long-term survival and histological findings is limited given the few cases of LARCC treated with RHCPD<sup>[7,10,11]</sup>.

In the present study, we retrospectively reviewed the preoperative and intraoperative assessments of LARCC with malignant infiltration into adjacent organs and the clinical outcomes of RHCPD in these cases.

## MATERIALS AND METHODS

### Patient characteristics

We retrospectively reviewed the database of Saitama Medical Center, Jichi Medical University, between January 2009 and December 2016. During this period, 299 patients underwent radical right hemicolectomy (RHC) for right-sided colon cancer. Among them, 5 patients underwent RHCPD because of LARCC with direct infiltration to the duodenum and/or pancreas. Preoperative computed tomography (CT) was routinely performed to evaluate local tumor infiltration into adjacent organs. The preoperative carcinoembryonic antigen (CEA) and cancer antigen 19-9 (CA19-9) levels were routinely tested in all patients. Preoperative upper endoscopy, colonoscopy, and histological confirmation of the diagnosis were performed in all patients. The optimal treatment strategies for LARCC were discussed in a multidisciplinary forum including surgeons, oncologists and radiologists.

### Indications for RHCPD

The indications for RHCPD were as follows: (1) histological confirmation of colon cancer; (2) colon cancer that could not be dissociated from the pancreas and/or duodenum because of tumor infiltration; (3) radical resection deemed feasible on preoperative imaging and intraoperative exploration; and (4) no secondary or recurrent tumors.

### Surgical method of RHCPD

After the Cattell-Braasch maneuver, a Kocher maneuver was performed to fully mobilize the duodenum. We evaluated the resectability of the LARCC and the amount of infiltration into the duodenum and/or pancreas without dissecting the adherent organs from

**Table 1** Patients' characteristics

Case	Gender	Age (yr)	Site of colon cancer	Adjacent organ infiltration on preoperative CT	Preoperative CEA (ng/mL)	Preoperative CA19-9 (IU/mL)
1	Female	73	T	Du	120.8	22.5
2	Female	74	A	Du + Pa + St	36.6	20.3
3	Male	70	A	Du + Pa	0.5	49.2
4	Female	57	T	Du + Pa + Gb + SMV	2.6	13.7
5	Male	47	A	Du + Pa + SMV <sup>1</sup>	12.3	196.8

<sup>1</sup>Tumor thrombosis in SMV. A: Ascending colon; T: Transverse colon; CEA: Carcinoembryonic antigen; CA19-9: Cancer antigen 19-9; CT: Computed tomography; Du: Duodenum; St: Stomach; Pa: Pancreas; Gb: Gallbladder; SMV: Superior mesenteric vein.

the cancer. After confirming that radical resection was feasible and could lead to R0 resection, we performed RHCPD.

First, RHC was performed in accordance with the standard procedure. Pancreaticoduodenectomy (PD) was then performed *via* the standard procedure, and if the superior mesenteric vein (SMV) was involved, we performed *en bloc* SMV resection and end-to-end anastomosis of the SMV. Reconstruction was carried out in accordance with the modified Child's reconstruction method. End-to-side anastomosis was made between the proximal stump of the pancreas and jejunum. The stent of the pancreatic duct was routinely used in pancreatojejunostomy. Finally, reconstruction of the bowel was performed *via* stapled side-to-side anastomosis of the ileum and transverse colon. After reconstruction, rubber drains were placed near the biliary and pancreatic anastomoses, and the abdominal wall wounds were closed.

### Primary and secondary outcomes

The overall survival (OS) was considered the primary outcome. The disease-free survival (DFS), 30-d postoperative mortality, and major complications were considered the secondary outcomes. The staging process was based on the tumor-lymph node-metastasis (TNM) classification proposed by the American Joint Committee of Cancer<sup>[12]</sup>. Postoperative pancreatic fistula were categorized according to the International Study Group on Pancreatic Fistula Definition (ISGPF)<sup>[13]</sup>.

### Observation indices

Clinical data were carefully reviewed. Demographic variables, intraoperative data, and postoperative parameters were recorded. Numerical data were presented as the median (range).

### Follow-up system

All patients were examined at three-month intervals at our outpatient department. The patients were followed up in accordance with the standard protocol, including CEA and CA19-9 measurement, abdominal ultrasound or CT, and annual colonoscopy.

### Ethical issues

The study design and procedures were approved by

the Ethics Committee of Saitama Medical Center, Jichi Medical University.

## RESULTS

The characteristics of the patients in this series are shown in Table 1. Between 2009 and 2016, five patients (two male and three female) underwent RHCPD for LARCC with direct infiltration into adjacent organs in our hospital. The median age of the patients was 70 years (range, 47-74 years). The tumors were located in the ascending colon (three patients) and transverse colon (two patients). The histological results confirmed the diagnosis of right-sided colon cancer in all of the patients. The preoperative CEA values were 12.3 ng/mL (range, 0.5-120.8 ng/mL) and CA19-9 values were 22.5 IU/mL (range, 13.7-196.8 IU/mL). None of the patients received preoperative chemotherapy. Preoperative CT revealed infiltration of the tumor into the duodenum in all patients, the pancreas in four patients (Cases 2-5), and the SMV in two patients (Cases 4 and 5). Distant metastasis was not observed on preoperative imaging assessment in four patients (Cases 1-4). Although tumor thrombosis in the SMV was noted in one patient (Case 5), we were unable to administer preoperative chemotherapy because of tumor bleeding and stenosis (Figure 1).

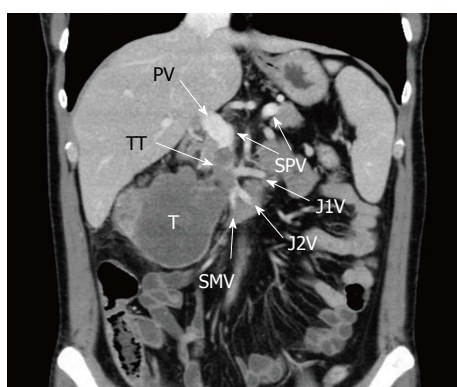
The perioperative data of the patients are listed in Table 2. Because infiltration of the tumor into the SMV was suspected based on preoperative CT findings or surgical exploration, we performed RHCPD plus SMV resection in three patients (Cases 2, 4 and 5) and added removal of tumor thrombosis in one patient (Case 5). In one patient (Case 1), we added distal pancreatectomy to RHCPD because of a neuroendocrine tumor in the pancreatic tail. The median operative time was 506 minutes (range, 304-538 minutes), and the median operative blood loss was 940 mL (range, 200-2760 mL). Major postoperative complications occurred in 3 patients (60%) as pancreatic fistula (grade B and grade C, according to ISGPF) and delayed gastric empty (DGE). After the operation, the postoperative hospital stay was 35 d (range, 27-39 d). The postoperative course was fair in all patients, and none of the patients died during their hospital stay.

The histological characteristics are listed in Table 3.

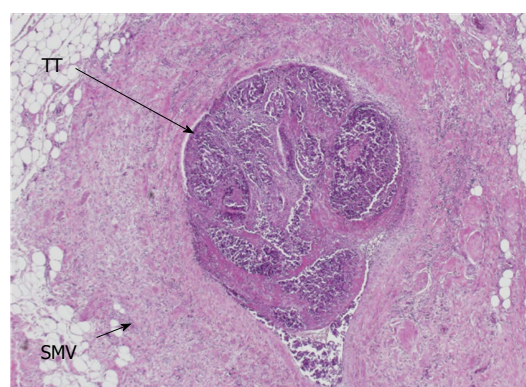
**Table 2 Surgical findings and complications**

Case	Operation	Adjacent organ infiltration in surgical exploration	OT (min)	OBL (mL)	DHS (d)	Complications
1	PD + RHC + DP	Du	406	940	35	PF (B)
2	PD + RHC + SMVR	Du + Pa + St + SMV	524	840	27	PF (C)
3	PD + RHC	Du + Pa + Gb	304	200	33	PF (A), DGE
4	PD + RHC + SMVR	Du + Pa + Gb + SMV	538	2760	36	PF (A)
5	PD + RHC + SMVR	Du + Pa + SMV <sup>1</sup>	506	2470	39	PF (A)

<sup>1</sup>Tumor thrombosis in SMV. OT: Operation time; OBL: Operative blood loss; DHS: Duration of hospital stay; PD: Pancreaticoduodenectomy; RHC: Right hemicolectomy; DP: Distal pancreatectomy; SMVR: Superior mesenteric vein resection; Du: Duodenum; St: Stomach; Pa: Pancreas; Gb: Gallbladder; SMV: Superior mesenteric vein; PF: Pancreatic fistula; DGE: Delayed gastric emptying.



**Figure 1** Preoperative computed tomography showing tumor thrombosis in the superior mesenteric vein in Case 5. T: Tumor; TT: Tumor thrombosis; PV: Portal vein; SPV: Splenic vein; SMV: Superior mesenteric vein; J1V: First jejunal vein; J2V: Second jejunal vein.



**Figure 2** Histological findings revealing tumor thrombosis in the superior mesenteric vein in Case 2 (x4). SMV: Superior mesenteric vein; TT: Tumor thrombosis.

According to the TNM classification, stage T<sub>4b</sub>N<sub>0</sub>M<sub>0</sub> (Cases 3 and 4), stage T<sub>4b</sub>N<sub>0</sub>M<sub>1a</sub> (Case 1, with metastasis of peripancreatic lymph node), stage T<sub>4b</sub>N<sub>1b</sub>M<sub>1b</sub> (Case 2, with metastasis of periduodenal lymph node and tumor thrombosis in SMV), and stage T<sub>4b</sub>N<sub>1a</sub>M<sub>1a</sub> (Case 5, with tumor thrombosis in SMV) were defined. A histological examination confirmed direct infiltration into the duodenum and/or pancreas head in four patients. Even though direct infiltration into the SMV was suspected by preoperative CT in two patients (Cases 4 and 5) or by surgical exploration in three patients (Cases 2, 4 and 5), no malignant infiltration into the SMV was confirmed. However, two patients (Cases 2 and 5) were histologically confirmed to have tumor thrombosis in the SMV (Figure 2). Well-differentiated adenocarcinoma (Cases 3, 4 and 5), moderately differentiated adenocarcinoma (Case 1), and mucinous adenocarcinoma (Case 2) were confirmed histologically. All of the tumors had clear resection margins (R0).

The chemotherapy regimens and outcomes are listed in Table 4. One patient received adjuvant chemotherapy with capecitabine treatment (Case 1). Two patients did not wish to receive adjuvant chemotherapy (Cases 3 and 4). One patient could not receive adjuvant chemotherapy because of appetite loss, but we introduced chemotherapy with cetuximab treatment for liver metastasis (Case 2). One patient had early recurrence with liver metastasis, and we introduced chemotherapy with capecitabine plus

oxaliplatin and bevacizumab treatment (Case 5). The median follow-up time was 77 mo (range, 11-95 mo). No patients were lost to follow-up. During this period, two patients with tumor thrombosis died from liver metastasis (Cases 2 and 5). The OS rates were 80% at 1 year and 60% at 5 years. Three patients survived more than six years with no recurrence (Cases 1, 3 and 4). All patients without lymph node metastasis survived more than seven years (Cases 3 and 4). On the other hand, only one patient with lymph node metastasis achieved a long-term survival (Case 1).

## DISCUSSION

To distinguish between inflammatory adhesion and malignant infiltration is difficult by preoperative imaging assessments or surgical exploration. With the development of CT technology, preoperative CT provides accurate information about the staging of colon cancer and invasion beyond the muscularis propria into adjacent organs<sup>[14]</sup>. However, preoperative CT and surgical exploration often cannot distinguish inflammatory adhesions from malignant infiltration<sup>[10,11,15-18]</sup>. In our series, malignant infiltration to the duodenum was suspected in all 5 cases by preoperative CT and surgical exploration and subsequently confirmed in 4 cases (80%) by a histological examination. However, malignant infiltration into the pancreas was suspected in 4 cases by preoperative CT and surgical exploration but only confirmed in 2 cases (50%)



**Table 3** Histological findings

Case	Stage			Histological infiltration	Histological type	R
	T	N	M			
1	4b	0	1a (LYM <sup>1</sup> )	Du	Mod + NET	0
2	4b	1b	1b (LYM <sup>2</sup> , OTH <sup>3</sup> )	St	Muc	0
3	4b	0	0	Du + Pa	Well	0
4	4b	0	0	Du + Gb	Well	0
5	4b	1a	1a (OTH <sup>3</sup> )	Du + Pa	Well	0

<sup>1</sup>Extra-regional lymph node metastasis (peripancreatic lymph node); <sup>2</sup>Extra-regional lymph node metastasis (periduodenal lymph node); <sup>3</sup>Tumor thrombosis in SMV. Mod: Moderately differentiated adenocarcinoma; Well: Well-differentiated adenocarcinoma; NET: Neuroendocrine tumor; Du: Duodenum; St: Stomach; Pa: Pancreas; Gb: Gallbladder.

**Table 4** Chemotherapy and outcomes

Case	ACT	DFS (mo)	CT	OS (mo)	Status	CD
1	Cape	77	-	77	Alive	
2	-	5	Cetu	11	Dead	LM
3	-	95	-	95	Alive	
4	-	85	-	85	Alive	
5	-	1	Cape + OX + Beva	11	Dead	LM

ACT: Adjuvant chemotherapy; CT: Chemotherapy; DFS: Disease-free survival; OS: Overall survival; CD: Cause of death; LM: Liver metastasis; Cape: Capecitabine; Cetu: Cetuximab; Beva: Bevacizumab; OX: Oxaliplatin.

by a histological examination. Furthermore, malignant infiltration into the SMV was suspected in 2 cases by preoperative CT and 3 cases by surgical exploration but not confirmed in any cases (0%) by a histological examination. Therefore, in our series, both preoperative CT and surgical exploration were found to be unreliable for identifying malignant infiltration, and this phenomenon is in line with the findings of previous studies.

In the right-sided colon cancer, the rate of malignant infiltration in adhesion between the cancer and adjacent organs has been reported to range from 71%-94%<sup>[7,10,11,19]</sup>. In addition, separation of colon cancer from the adherent organs may lead to tumor recurrence rates of 90%-100%<sup>[4,5]</sup>. Therefore, adhesion between the colon cancer and adjacent organs should be assumed to be malignant infiltration. When LARCC is suspected of having infiltrated the adjacent organs, RHCPD should be performed as long as radical operation is possible. While a few authors maintain that SMV invasion is not an indication for RHCPD<sup>[20,21]</sup>, we advise against hesitating to perform RHCPD with SMV resection to achieve R0 resection<sup>[8,22]</sup>.

Previous studies have reported that *en bloc* multivisceral resection can lead to a good prognosis, with a 5-year survival rate ranging from 21%-55%, for patients with LARCC invading adjacent organs<sup>[7,10,11,20]</sup>. In our series, all patients underwent RHCPD, and three additionally underwent SMV resection to achieve R0 resection. In all patients, R0 resection was achieved, and the OS rate at 5 years was favorable (60%). Interestingly, the patients with node-negative status survived for more than seven years without recurrence (Cases 3 and 4). Saiura *et al.*<sup>[7]</sup> reported that patients

with node-negative status achieved a significantly longer survival than node-positive patients. Similarly, in another study, the patients that survived for more than seven years all had node-negative status<sup>[10]</sup>.

Some colorectal cancers may behave in a locally aggressive invasion instead of causing lymphatic or hematogenous spread<sup>[7,11,23,24]</sup>. As such, RHCPD seems to provide a favorable survival for LARCC patients with this condition. In our series, only one patient (Case 1) with node-positive status survived for more than six years without recurrence, and this patient received adjuvant chemotherapy with capecitabine. Even if a patient has node-positive status, *en bloc* multivisceral resection plus adjuvant chemotherapy might be able to improve their prognosis. In our series, all patients with node-negative status had well-differentiated adenocarcinoma (Cases 3 and 4). Furthermore, the patient with mucinous adenocarcinoma had node-positive status in not only the regional lymph nodes but also the periduodenal lymph nodes (Case 2). In a previous report, the rate of lymph node metastasis was significantly lower for well-differentiated adenocarcinoma than mucinous or poorly differentiated adenocarcinoma in LARCC with direct infiltration into adjacent organs<sup>[7]</sup>. These previous results and our findings suggest that the histological type of tumor may affect the lymph node metastasis and prognosis in patients with LARCC.

In our series, two patients with tumor thrombosis in SMV relapsed with liver metastasis soon after operation (Cases 2 and 5). A previous review of colorectal cancer with venous tumor thrombosis found that the incidence of synchronous liver metastasis was as high as 19.5%,

and the incidence of liver metastatic recurrence after complete surgical resection of the tumor was as high as 24.4%, while the liver metastatic recurrence rate of general colorectal cancer was 7.1%<sup>[25]</sup>. Tumor thrombosis in the SMV therefore seems to be a strong risk factor of synchronous and metachronous liver metastasis, leading to a poor prognosis. In the FOxTROT trial, preoperative chemotherapy resulted in significant higher rates of downstaging (55%) and R0 resection (96%) than postoperative chemotherapy for locally advanced colon cancer<sup>[26]</sup>. In another previous study, preoperative chemotherapy achieved a tumor volume reduction (62.5% of volume), R0 resection (100%), and an encouraging prognosis (3- to 5-year DFS of 88.9%-85.6% and OS of 95.3%) for locally advanced colon cancer<sup>[27]</sup>. Given the findings of these recent reports, it may thus be better to perform preoperative chemotherapy for LARCC patients with tumor thrombosis in the SMV than preemptive surgery. The role of induction preoperative chemotherapy, which might be indicated in T4b colon cancer, has not been discussed in previous reports of LARCC. In the era of advanced chemotherapy for colorectal cancer, preoperative chemotherapy might result in a better prognosis for LARCC patients with severe invasion, lymph node metastasis, or tumor thrombosis in the SMV. This issue merits further studies in the near future.

Several limitations associated with the present study warrant mention. First, the number of patients in this study was small. Because cases of LARCC undergoing RHCPD are rare, the number of patients per medical institution tends to be small. Large-scale studies may produce more reliable results. Second, the chemotherapy regimens varied among patients. In this era of advanced chemotherapy, administering the same regimen for a long-term study seems unfeasible. However, the present study also has several strengths. First, all of the patients were followed up, and the assessment of the prognosis proved to be accurate. Second, a histological examination was performed in detail, and we were able to evaluate the relationship between the preoperative CT findings, surgical exploration, and prognosis and histological findings.

In conclusion, we found that a long-term survival was therefore possible for patients with LARCC infiltrating adjacent organs, provided that RHCPD was successfully performed. This aggressive approach may help improve the prognosis, particularly in patients with node-negative status. Large-scale and long-term studies may produce more reliable results.

## COMMENTS

### Background

In locally advanced colorectal cancers invading the adjacent organs, incomplete resection and the separation of colon cancer from any adherent organs are considered to lead to tumor recurrence and a poor prognosis. The number of reports describing the long-term survival and histological findings is limited given the few cases of locally advanced right-sided colon cancer (LARCC)

treated by *en bloc* right hemicolectomy with pancreaticoduodenectomy (RHCPD).

### Research frontiers

This is a retrospective single center experience regarding the long-term survival and histological findings of LARCC treated with RHCPD.

### Innovations and breakthroughs

Long-term survival was possible for patients with LARCC that had successfully undergone RHCPD. RHCPD may help improve the prognosis of patients with LARCC, particularly in patients with a node-negative status.

### Applications

The present study suggests that LARCC patients with a node-negative status are indicated for RHCPD. If a patient has tumor thrombosis in SMV, then the possibility of early recurrence should also be considered.

### Peer-review

This manuscript deals with RHCPD for LARCC. The authors have described the preoperative assessment, intraoperative assessment, histological findings and prognosis.

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## Prospective Study

# Polyethylene glycol microspheres loaded with irinotecan for arterially directed embolic therapy of metastatic liver cancer

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## Abstract

### AIM

To study tumor response, and tolerability of arterially directed embolic therapy (ADET) with polyethylene glycol embolics loaded with irinotecan for the treatment of colorectal cancer liver metastases (CRC-LM). Secondary objectives were to monitor quality of life, time to progression and survival of patients.

### METHODS

Patients were included in the study if they were affected by CRC-LM, refractory to systemic chemotherapy, treated with ADET using polyethylene glycol embolics, and had liver involvement < 50%. Tumor response, performance status (PS), tumor marker antigens, and quality of life (QoL) were monitored at 1, 3 and 6 mo after ADET. QoL was assessed with the Palliative Performance Scale (PPS).

### RESULTS

We treated 50 consecutive CRC-LM patients with ADET using polyethylene glycol embolics. Their tumor response one month after ADET was: 28% of complete response (CR), 48% of partial response (PR), 8% stable disease (SD), and 16% of progression. Tumor response 3 mo after ADET was CR 24%, PR 38%, SD 19% and progression disease (PD) 19%. Tumor response 6 mo after ADET was CR 18%, PR 44%, SD 21% and PD 18%. QoL was 90% PPS at each time point. Median time to progression for patients who progressed was 2.5 mo (range 0.8-6). Median follow-up was 14 mo (0.8-25 range). ADETs were performed with no complications. Observed side effects (mild or moderate intensity) were: Pain in 32% of patients, increase of transaminase levels in 20% and fever in 14%, whereas 30% of patients did not complain any adverse event.

### CONCLUSION

The treatment of unresectable CRC-LM with ADET using polyethylene glycol microspheres loaded with irinotecan was effective in tumor response and resulted in mild toxicity, and good QoL.

**Key words:** Liver metastases; Arterially directed embolic therapy; Colorectal cancer; Polyethylene glycol embolics; Irinotecan

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**Core tip:** Patients with liver metastases from colorectal cancer are in 80% of cases non-indicated for resection.

The standard first line treatment of unresectable liver metastases is systemic chemotherapy, however this method results in progression for 70% of patients. The indicated therapy for refractory patients is the chemoembolization. In this study, we monitored tumor response, and adverse events after chemoembolization of colorectal cancer liver metastases with polyethylene glycol embolics loaded with irinotecan. Chemoembolization with these embolics is effective in terms of tumor response, time to progression, survival and quality of life and resulted in mild toxicity.

Fiorentini G, Carandina R, Sarti D, Nardella M, Zoras O, Guadagni S, Inchingolo R, Nestola M, Felicioli A, Barnes Navarro D, Munoz Gomez F, Aliberti C. Polyethylene glycol microspheres loaded with irinotecan for arterially directed embolic therapy of metastatic liver cancer. *World J Gastrointest Oncol* 2017; 9(9): 379-384 Available from: URL: <http://www.wjgnet.com/1948-5204/full/v9/i9/379.htm> DOI: <http://dx.doi.org/10.4251/wjgo.v9.i9.379>

## INTRODUCTION

Patients with liver metastases from colorectal cancer (CRC-LM) are non indicated for resection in 80% of cases<sup>[1]</sup>. The standard first line treatment of unresectable CRC-LM is systemic chemotherapy, administering 5-fluorouracil in association with oxaliplatin and/or irinotecan, and biologics. This method results in limited tumor control with progression for 70% of patients<sup>[1]</sup>. The treatment of patients refractory to chemotherapy is very challenging, since they will hardly have a response to following chemotherapy lines.

A recent review on CRC-LM therapy methods shows that key strategies are local therapies, including loco-regional and ablative methods<sup>[2,3]</sup>. Hepatic arterial infusion (HAI), arterially directed embolic therapy (ADET), transarterial embolization associated to selective internal radiation therapy (SIRT) are among the most applied locoregional therapies. Radiofrequency ablation (RFA), microwave ablation (MWA) and cryo-ablation are the most used ablative techniques<sup>[3]</sup>.

A recent report shows the efficacy of ADET with irinotecan loaded beads also as neoadjuvant therapy, leading to complete resectability (R0), and resulting in tumor response and survival comparable to those of chemotherapy<sup>[4]</sup>.

The advantages of ADET are several, such as reducing drug leakage, liver and systemic toxicity<sup>[5]</sup>. ADET is widely used for patients with CRC-LM after failure of surgery or systemic chemotherapy, and can be used for both pre- and post-operative downsizing, reducing the time to surgery, and prolonging overall survival<sup>[6]</sup>.

In our last report, we show that ADET with newly introduced polyethylene glycol microspheres loaded with irinotecan are safe and effective for the treatment of primary and secondary liver cancer<sup>[7]</sup>. In this study

**Table 1** Baseline patient characteristics

	<i>n</i>	% (range)
Male	28	56
Female	22	44
Age (yr), median	63	(46-86)
Tumor size (mm), median	35	(5-130)
1-2 nodules	15	30
3-5 nodules	18	36
> 5 nodules	17	34
Tumor antigens		
Ca 19.9 (U/mL), median baseline	14	(1.9-7628)
Ca 19.9 (U/mL), median 1 mo	10.3	(1.8-1558)
Ca 19.9 (U/mL), median 3 mo	20	(5.8-1234)
Ca 19.9 (U/mL), median 6 mo	85	(2.6-1138)
CEA (U/mL), median baseline	31.1	(0.7-453)
CEA (U/mL), median 1 mo	35	(3-370)
CEA (U/mL), median 3 mo	32.5	(3-1057)
CEA (U/mL), median 6 mo	22.85	(0.5-735)
Previous surgery		
Primary tumor resection	48	96
Metastasectomy	17	34
No surgery	2	4

we focused on CRC-LM to assess tumor response, and adverse events after ADET with polyethylene glycol embolics loaded with irinotecan. We also monitor quality of life, time to progression and survival of these patients.

## MATERIALS AND METHODS

### Sample

We enrolled 50 consecutive eligible patients affected by unresectable CRC-LM that were refractory to systemic chemotherapy and were treated with ADET using polyethylene glycol embolics and irinotecan (LIFIRI®). Inclusion criteria for patient selection were: > 18 years, histological diagnosis of CRC-LM; refractory to systemic chemotherapy, Performance status (PS) 0-2; tumor size evaluable according to RECIST version 1.1<sup>[8]</sup>; liver involvement < 50%; life expectancy  $\geq$  3 mo.

Exclusion criteria were: Contraindication to angiographic catheterization; extensive extra-hepatic disease; liver involvement > 50%; pregnancy or breast feeding; other severe clinical impairments.

### Arterially directed embolic therapy procedure

The interventional radiologist performed a diagnostic angiography to assess tumor arterial perfusion before ADET. Distal catheterization was used in order to avoid extra-hepatic leakage.

ADET was performed infusing 2 mL of LifePearl® (Terumo Europe NV, Leuven, Belgium) that were loaded with irinotecan (100 mg), and diluted in 5 mL of non-ionic contrast solution and 5 mL of distilled water<sup>[7]</sup>. The diameter of microspheres was  $100 \pm 25$  micron. Infusion median time was 12 min at a fixed speed of 1 mL/min. It was possible to perform a second ADET after 30 d, according to physician evaluation of tumor response. Periprocedural and supportive therapy

to prevent side effects were administered as in our previous study<sup>[9,10]</sup>.

### Tumor response assessment

RECIST criteria version 1.1<sup>[11]</sup> and European Association for the Study of Liver Disease method<sup>[12]</sup> were used for tumor response assessment from abdomen and pelvis computed tomography (CT) imaging. Tumor response was monitored at 1, 3 and 6 after ADET.

### Adverse events

National Cancer Institute Common Terminology Criteria for Adverse Events (NCI-CTCAE), version 3.0 was applied for adverse events classification and intensity evaluation.

### Quality of life

Palliative Performance Scale (PPS) was used for quality of life (QoL) at 1, 3, and 6 mo after ADET<sup>[13]</sup>. Our hypothesis was that patients would have better physical and social characteristics, and better health perception one month after ADET.

### Statistical analysis

Data analysis of the sample ( $n = 50$ ) was performed. The median was computed for continuous data, and proportions were expressed in percentage. Significance of continuous variables was assessed with  $\chi^2$  and Student's *t*-test ( $P < 0.05$ ).

## RESULTS

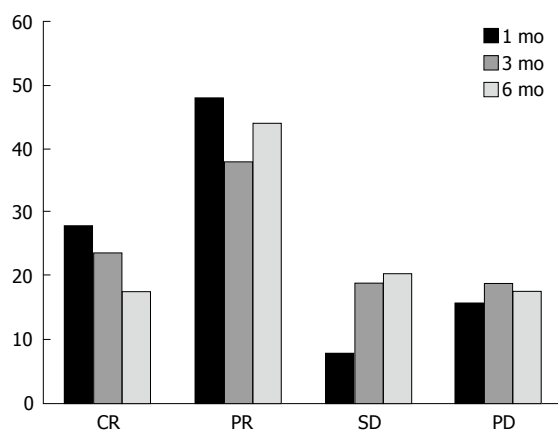
### Sample

The sample included 50 patients affected by CRC-LM that were treated with ADET using polyethylene glycol embolics and irinotecan (LIFIRI®). Twenty-eight (56%) patients were males and 22 (44%) females. Median sample age was 63 years (range 46-86). PS was 0 at baseline in 35 (70%) patients, PS = 1 in 13 (26%) patients and PS = 2 in 2 (4%) patients. Other site of concomitant metastases were: Lung in 2 (4%), lymph nodes in 1 (2%) and lung, omentum and ovary in 1 (2%) patient (Table 1).

Surgery of primary tumor was done in 48 (96%) and metastasectomy in 17 (34%) patients. Tumor markers levels CEA and Ca 19.9, and tumor size were reported in Table 1. Most of the sample 72% received one ADET, whereas 5 (22%) patients received two ADETs.

### Tumor response

One month after ADET we observed 14 (28%) complete response (CR), and 24 (48%) partial response (PR), 4 (8%) stable disease (SD), and 8 (16%) progression disease (PD) (Figure 1). Tumor response 3 mo after ADET was 10 (24%) CR, 16 (38%) PR, 8 (19%) SD and 8 (19%) PD. Tumor response 6 mo after ADET was 6 (18%) CR, 15 (44%) PR, 7 (21%) SD and 6 (18%) PD.



**Figure 1 Tumor response.** CR: Complete response; PR: Partial response; SD: Stable disease; PD: Progressive disease.

Median time to progression for patients who progressed was 2.5 mo (range 0.8-6). Median follow-up was 14 mo (0.8-25 range). Overall survival was (OS) 14 mo (range 1.3-25).

We report the imaging of a particular case that we treated. The imaging showed a voluminous unresectable metastases in the right lobe of one patients at diagnosis (Figure 2). One month after LIFIRI, there was reduction of contrast enhancement and increase of necrotic areas, at 3 mo after LIFIRI, tumor shrinkage and reduction of viable tissue was observed, and at 6 mo after LIFIRI, the metastases appeared almost completely necrotic.

### Adverse events

No complications were observed during ADET. Most reported adverse events after ADET were symptoms correlated to the post-embolic syndrome (PES) (Table 2). Observed side effects were of mild (88%) or moderate intensity (12%). They included: Pain in 16 (32%) and fever in 7 (14%) patients. Transaminase rise > 2.5 upper normal level (ULN) was observed in 10 (20%) patient. Adverse effects were all mild (G1) intensity except one case of moderate (G2) Transaminase rise and two cases of pain, and they were all resolved without complications. Thirty four (68%) patients did not complain any adverse event.

### QoL

QoL was 90% PPS at each time point, 3, 6 and 9 mo after ADET, suggesting improvements in physical and social functions and better health perception.

## DISCUSSION

Systemic therapy for unresectable CRC-LM had an OS of 20-27 mo and patients' deaths were mainly due to disease progression<sup>[14]</sup>. Locoregional therapies were introduced in order to improve survival, and include different methods: HAI, radioembolization (RE), and ADET<sup>[15-18]</sup>.

These intrahepatic arterial therapies were developed because the liver disease mainly exploits the arterial

**Table 2 Adverse events (G1-G2)**

	n (%)
Pain	16 (32)
Transaminase rise	10 (20)
Fever	7 (14)
None	20 (40)

system as source of blood supply, whereas normal liver relies on portal circulation<sup>[19,20]</sup>. A review on their efficacy of these locoregional methods showed that HAI, RE, and ADET had similar tumor response in patients affected by unresectable CRLM, and only small differences in overall survival<sup>[18]</sup>. Other studies reported ADET efficacy also as neo-adjuvant therapy for CRC-LM, obtaining significant surgical down-staging with irinotecan eluting beads<sup>[4,21]</sup>.

Advantages of ADET were based on the application of drug eluting bead that can deliver a high concentrations of toxic chemotherapeutic drug in the liver minimizing the leakage into adjacent tissues, by embolizing the terminal arterial capillaries<sup>[22,23]</sup>. ADET has been increasingly used in the last decades for CRC. LM, and several improvements of the methodology has been applied. Improvements included the direct beads delivery into the tumor without increasing risks, prolonged exposure to new toxic drug, and the application of new types of beads.

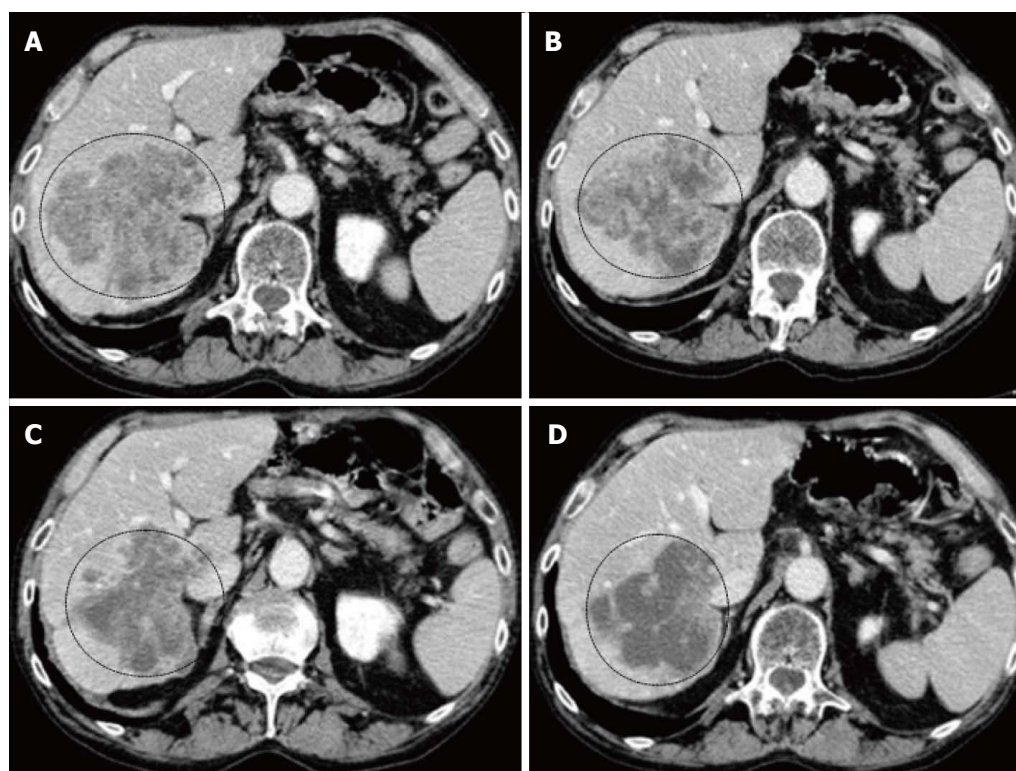
In our last study we reported the use of ADET with PEG embolics for the treatment of primary (HCC and cholangiocarcinoma) and metastatic (CRC, breast and uvea) liver cancer<sup>[7,24]</sup>. We applied ADET with these new type of PEG microspheres loaded with irinotecan (LIFIRI®) to a larger sample of CRC-LM (50 patients), and we collected data on tumor response, adverse events, QoL, time to progression and survival.

We observed 28% of CR and 24% of PR in patients affected by CRC-LM one month after the LIFIRI®. Tumor response 3 mo after ADET was CR 24%, PR 38%, SD 19% and PD 19%. Tumor response 6 mo showed an increase of PR and a decrease of CR, whereas SD and PD were stable: CR 18%, PR 44%, SD 21% and progression disease (PD) 18%. Median time to progression was 2.5 mo (range 0.8-6). These data were in agreement with results reported by other studies showing response rate in the range of 60%-75%<sup>[22-25]</sup>.

ADETs were performed with no complications. Observed side effects were all of mild (G1) or except one of moderate intensity (G2). Adverse events were: Pain (32%) and hypertransaminemia in 20% of patients and fever in 7%. These symptoms were correlated to post embolic syndrome, as reported in other studies<sup>[9,10,25]</sup>. Many patients (40%) did not complain any adverse event.

QoL was measured with PPS, and data analysis showed a PPS of 90% at each time point after ADET, suggesting good physical and social functions, and health perception. These data were reported also when measuring QoL after ADET with DC-Beads<sup>[9]</sup>. This may





**Figure 2** Effects of chemoembolization with polyethylene glycol microspheres loaded with irinotecan. A: Before LIFIR: Voluminous non resectable metastases in the right lobe; B: One month after LIFIR: Reduction of contrast enhancement and increase of necrotic areas; C: Three months after LIFIR: Tumor shrinkage and reduction of viable tissue; D: Six months after LIFIR: Metastases appears almost completely necrotic and reduced in diameter (as shown by circles around tumor mass).

suggest that comparable results may be attained with PEG microspheres in respect of previous available drug-eluting beads.

Our study has several limitations, such as the small number of patients observed and the short time of follow-up. Our results, however, were very interesting because they were the first to report the feasibility and tolerability of ADET with PEG microspheres for the treatment of unresectable CRC-LM that were refractory to systemic chemotherapy. Future multicenter randomized studies with a larger number patients and longer times of observation are required to confirm these data.

The therapy of unresectable CRC-LM with ADET using polyethylene glycol microspheres loaded with irinotecan was effective in tumor response and resulted in mild toxicity, and good QoL, showing non-inferior results than previous drug eluting beads.

toxicity, and good quality of life (QoL), showing non-inferior results than previous drug eluting beads.

### Innovations and breakthroughs

The use of polyethylene glycol (PEG) microspheres allow a high tumor response maintaining low levels of toxicity, and are an important innovation in the treatment of un-resectable liver metastases from colon carcinoma. These microsphere are more resilient to stress and attrition.

### Terminology

ADET: Delivery of embolics directly inside the tumor-feeding vessels by arterial infusion; PEG is a hydrophilic material that allows a good elasticity, compressibility, and maximizes suspension time.

### Peer-review

Overall, this is a very strong prospective study with solid experimental design. The manuscript is well written and the results support the authors' conclusion. The results are novel and provide promising clue to physicians.

## COMMENTS

### Background

Patients with liver metastases from colorectal cancer are in 80% of cases non indicated for resection. The standard first line treatment of unresectable liver metastases is systemic chemotherapy, however this method results in progression for 70% of patients. The indicated therapy for refractory patients is the arterially directed embolic therapy (ADET).

### Research frontiers

ADET of colorectal cancer liver metastases with polyethylene glycol embolics loaded with irinotecan was effective in tumor response and resulted in mild

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## Desmoid type fibromatosis: A case report with an unusual etiology

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**Author contributions:** Obaisi O prepared the manuscript and conducted the literature search; Vergara GG provided the histopathology narrative and photomicrographs; all other authors directly involved in the clinical care of the patient reviewed the manuscript and provided adequate input to finalize the manuscript.

**Institutional review board statement:** The application for Exemption from the IRB review and the research summary as written for the Case Report: Desmoid type fibromatosis: A case report with an unusual etiology was reviewed. It was determined the project is exempt from the IRB review by DHHS regulation 45CFR 46.101(b)(4).

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### Abstract

Desmoid type fibromatosis (DTF) is a rare, locally invasive, non-metastasizing soft tissue tumor. We report an interesting case of DTF involving the pancreatic head of a 54-year-old woman. She presented with intermittent dysphagia and significant weight loss within a 3-mo period. Laboratory findings showed mild elevation of transaminases, significant elevation of alkaline phosphatase and direct hyperbilirubinemia, indicating obstructive jaundice. Computerized tomography of the abdomen revealed a mass in the head of the pancreas, dilated common bile duct, and dilated pancreatic duct. Endoscopic retrograde cholangiopancreatography and endoscopic ultrasound showed a large hypoechoic mass

in the head of the pancreas causing extrahepatic biliary obstruction and pancreatic ductal dilation. The patient underwent a successful partial pancreatectomy and cholecystectomy. She received no additional therapy after surgery, and liver function tests were normalized within nine days after surgery. Currently, surgical resection is the recommended first line treatment. The patient will be followed for any recurrence.

**Key words:** Desmoid type fibromatosis; Desmoid tumor; Aggressive fibromatosis; Pancreas; Painless jaundice

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**Core tip:** Desmoid type fibromatosis (DTF) is a rare, locally invasive, non-metastasizing soft tissue tumor. We report an extremely rare case of DTF in the pancreatic head with an unusual etiology. This case study is valuable for the understanding of the diagnosis and treatment of DTF of the pancreatic head.

Jafri SF, Obaisi O, Vergara GG, Cates J, Singh J, Feeback J, Yandrapu H. Desmoid type fibromatosis: A case report with an unusual etiology. *World J Gastrointest Oncol* 2017; 9(9): 385-389 Available from: URL: <http://www.wjgnet.com/1948-5204/full/v9/i9/385.htm> DOI: <http://dx.doi.org/10.4251/wjgo.v9.i9.385>

## INTRODUCTION

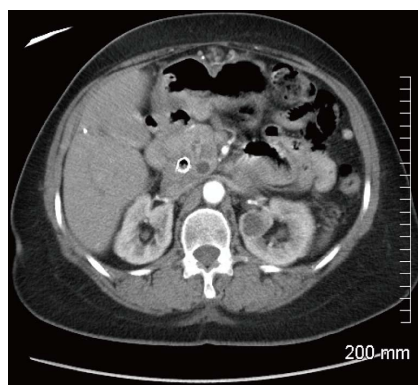
Desmoid type fibromatosis (DTF), also known as a desmoid tumor, deep fibromatosis, or aggressive fibromatosis, is a rare, locally invasive, non-metastasizing soft tissue tumor with a high potential for recurrence after resection. It accounts for 3% of all soft tissue tumors<sup>[1]</sup>. While the etiology is still unknown, it can arise sporadically at any anatomical site throughout the body<sup>[2]</sup>. DTF is categorized by location as extra-abdominal, abdominal wall, and intra-abdominal.

Intra-abdominal DTF is associated with sites of previous trauma, scarring, or irradiation<sup>[3]</sup>. An association occurs in patients with familial adenomatous polyposis (FAP) and Gardner syndrome, showing 7.5% of patients with DTF to have FAP<sup>[4]</sup>. To our knowledge, sporadic intra-abdominal DTF cases are very uncommon with only 10 cases having been previously reported, and cases of pancreatic origin being even more uncommon<sup>[1,3,5-11]</sup>.

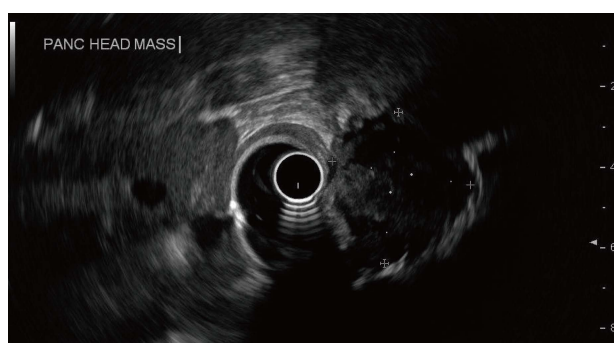
Here, we report the case of a 54-year-old female patient presenting with obstructive jaundice due to a sporadic DTF, located at the pancreatic head. The patient underwent a successful Whipple procedure and will be followed for any possible recurrence.

## CASE REPORT

A 54-year-old female presented to the emergency department with intermittent dysphagia and a 60-pound weight loss within a 3-mo period. The patient denied



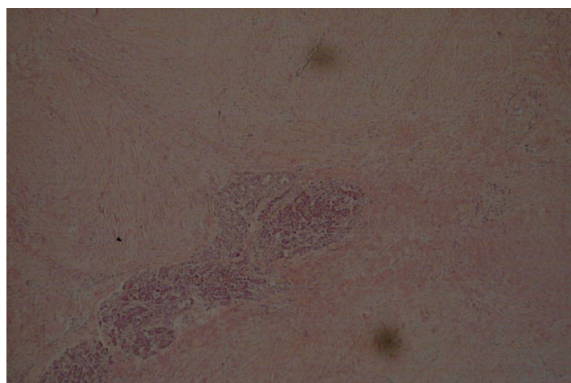
**Figure 1** Computerized of the abdomen: Mass in head of pancreas, a dilated common bile duct with a metallic stent in place and a dilated pancreatic duct.



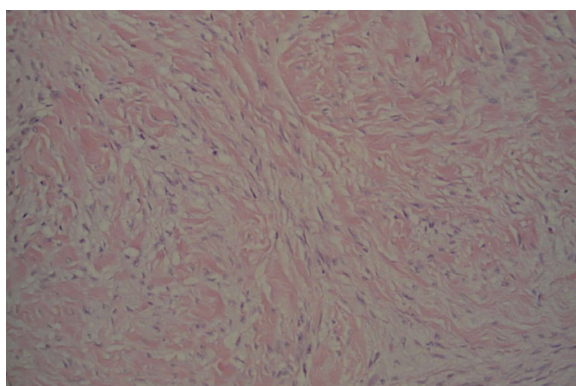
**Figure 2** Endoscopic ultrasound showing a 2.5 cm × 2.2 cm hypoechoic mass in the head of the pancreas causing extrahepatic biliary obstruction and pancreatic ductal dilation.

any complaints of nausea or vomiting. Computerized tomography (CT) (Figure 1) of the abdomen revealed a mass in the head of the pancreas, a dilated common bile duct, and a dilated pancreatic duct. Her past surgical history included a total hysterectomy and bilateral tubal ligation. There was no family history to suggest a genetic hereditary disease. Laboratory tests showed mild elevation of transaminases, significant elevation of alkaline phosphatase and direct hyperbilirubinemia, indicating obstructive jaundice. Carbohydrate antigen 19-9 was elevated (380 IU/mL) and carcinoembryonic antigen was unremarkable. Endoscopic retrograde cholangiopancreatography (ERCP) showed a large 2.5 cm irregular, ill-defined mass at the head of the pancreas causing complete bile and pancreatic duct obstruction. Endoscopic ultrasound (EUS) (Figure 2) showed a 2.5 cm × 2.2 cm hypoechoic mass in the head of the pancreas causing extrahepatic biliary obstruction and pancreatic ductal dilation. Marked edematous and inflammatory changes were noted around this area. A fine needle aspiration of the pancreatic mass showed predominantly fibrotic, bland spindle cells with scattered normal skeletal muscle components. Immunohistochemical stains for CD117, CD34, and Pancytokeratin were negative. No pancreatic epithelial elements or significant inflammatory infiltrates were noted. Surgery was recommended on the sus-





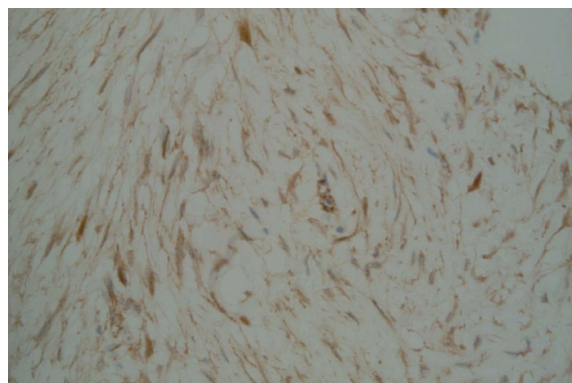
**Figure 3** Histologic section (H and E) of the pancreatic head tumor. Diffusely infiltrative tumor replacing parenchyma with focal remnants of normal appearing epithelial structures.



**Figure 4** Typical DTF histology (H and E): Uniform sweeping fascicles of spindled myofibroblasts with low cellularity, no cellular atypia, minimal inflammation and scattered keloid-like collagen within a collagenous stroma.

picion of a reactive fibroinflammatory pseudotumor. However, surgery was deferred due to underlying pancreatitis.

Two months after the emergency department admission, she underwent a new abdominal CT scan, which showed an enlarging of 5.2 cm × 4.2 cm in the pancreatic head. This had not changed significantly on a repeat CT scan after seven weeks. A repeat EUS guided fine needle aspiration was performed due to high suspicion of malignancy. Cytological examination was again negative for epithelial malignancy. Four months after initial presentation, the patient underwent a Whipple procedure with end-to-end pancreaticojejunostomy, cholecystectomy, an end-to-side choledochojejunostomy, a wedge liver biopsy of segment 3, a biopsy of the superior pancreatic lymph node, and a retrocolic end-to-end gastrojejunostomy. The procedure was successful, and a feeding jejunal tube was placed. On pathologic microscopic examination, the surgical margins of the liver and lymph node biopsies were negative for a tumor. The pancreatic head lesion showed typical features of DTF. A poorly circumscribed uniform fascicular spindle cell proliferation infiltrates the pancreatic parenchyma among remnants



**Figure 5** Beta-catenin immunohistochemistry: Positive, diffuse, plump myofibroblast nuclear staining, faint cytoplasmic staining characteristic of desmoid type fibromatosis.

of normal pancreatic epithelial structures (Figure 3). The spindled myofibroblasts exhibit low cellularity and bland cytology (no atypia, no mitosis) with scattered keloid-like collagen and minimal inflammation within a collagenous stroma (Figure 4). Immunohistochemistry demonstrated positive Vimentin (mesenchymal marker) and Actin (focal, patchy, myofibroblast marker). Markers for myogenic cells (Desmin), stromal cells (CD117, CD34), neural cells (S-100) and epithelial cells (Pancytokeratin) were all negative. An initial pathologic impression of inflammatory myofibroblastic tumor was made after an additional immunohistochemical stain for ALK-1 was interpreted as showing “staining of spindle cells within the lesion”. However, further consultative review (Gerardo G Vergara) disclosed histologic features more consistent with DTF and the ALK-1 immunostain (faint, patchy staining of spindle cells) was essentially negative. An additional immunostain for Beta-catenin was diffusely nuclear positive (Figure 5), supportive and diagnostic of DTF.

Inflammatory Myofibroblastic Tumor exhibits more cellularity, cellular atypia, more inflammation, and ALK-1 positivity. In reactive fibroblastic/myofibroblastic proliferative lesion, cells are cytologically indistinguishable but architecturally different from fibromatosis and are B-catenin negative. The final diagnosis was therefore changed to DTF.

Recognizing and distinguishing between these lesions are clinically important due to different prognostic and treatment implications, which is key in our case. The patient received no additional therapy after surgery. The patient's liver function tests normalized within nine days after surgery.

## DISCUSSION

DTF is a rare mesenchymal neoplasm that develops from muscle connective tissue, fasciae, and aponeuroses<sup>[12]</sup>. Although it is benign, it can be life threatening due to aggressive local invasion, which can result in adverse events through compression and/or obstruction of the digestive system, urinary system, or blood vessels.



While the etiology of DTF is unknown, identified risk factors include surgical scars, FAP, and high-estrogen states<sup>[13]</sup>. And although intra-abdominal DTF is rare, DTF of the pancreas is even rarer, with only 10 cases being reported<sup>[1,3,5-11]</sup>. Of these 10 reported cases, DTF was located in the pancreatic head in only two cases<sup>[3,7]</sup>.

A confirmed diagnosis of sporadic intra-abdominal DTF is not very probable to be reached before surgery. It is very difficult to diagnose DTF symptomatically. Patients are often asymptomatic or have non-specific symptoms such as weight loss or epigastric pain<sup>[4]</sup>. Symptoms depend on the location and extent. Painless jaundice, a classic manifestation of pancreatic head cancer, is rarely seen in patients with pancreatic head DTF, as it usually does not obstruct the common bile duct<sup>[8]</sup>. However, in our case, the patient had painless jaundice due to the obstruction of the common bile duct.

Additionally, the patient had an elevated aspartate transaminase, alanine transaminase, alkaline phosphatase, and total bilirubin. ERCP and EUS both showed a mass in the head of the pancreas causing extrahepatic biliary obstruction and pancreatic ductal dilation. Dilation of the pancreatic duct is usually indicative of pancreatic adenocarcinoma<sup>[14]</sup>. According to Tummala *et al.*<sup>[14]</sup>, in 81.2% of patients with dilation of the pancreatic duct associated with a pancreatic lesion, the lesion was found to be malignant with pancreatic adenocarcinoma comprising the majority (71.6%) of the lesions.

Due to a high suspicion of malignancy, fine needle aspiration of the pancreatic mass was done. However, a definitive diagnosis was not possible. After repeat testing yielded the same results, a reactive fibroinflammatory pseudotumor was suspected. A fibroinflammatory pseudotumor radiographically presents as a mass that resembles a carcinoma or a sarcoma<sup>[15]</sup>. There have been a number of reported inflammatory pseudotumors of the pancreas, which were frequently localized in the head of the pancreas, were fibrous in appearance, and involved the distal bile duct<sup>[15]</sup>. The definitive diagnosis of such pseudotumors is usually obtained through a total or partial surgical resection<sup>[16]</sup>.

Histologically, differentiation of DTF from other soft tissue neoplasms can sometimes be challenging, particularly when it occurs in an uncommon site (in our case, the pancreas) or with a low index of suspicion. DTF typically consists of spindle cells and fibroblasts with a low mitotic rate<sup>[17]</sup>. However, reaching a confirmed diagnosis is still unlikely until surgery is performed with total tumor removal, as in the case of an inflammatory pseudotumor. Immunohistochemistry against specific cell markers in the differential diagnosis of pancreatic DTF is useful, supportive and diagnostic. Positive staining for Vimentin and Actin are indicative of mesenchymal cells and myofibroblasts, respectively, while positive staining for beta-catenin helps distinguish DTF from other fibroblastic and myofibroblastic lesions<sup>[18]</sup>. In 80%-90%

of sporadic cases, somatic mutations of adenomatous polyposis coli (APC) gene and activating mutations in CTNNB1 (beta-catenin gene) usually result in the accumulation of beta-catenin. This accumulation triggers fibroblastic proliferation<sup>[19]</sup>. This was relevant in our case, as the initial diagnosis was inflammatory myofibroblastic tumor was later changed to DTF.

The mainstay treatment for DTF is surgery with wide microscopic resection of the margins<sup>[20]</sup>. However, there is a very high chance of local recurrence with surgical resection. Long-term prognosis is currently unknown. Recurrence of pancreatic DTF has not been seen except in one patient with FAP<sup>[9]</sup>. In the current case, the patient will be followed for any possible recurrence and possible work up for FAP.

In conclusion, sporadic DTF of the pancreas is extremely rare. Although it is benign, it is locally aggressive and can be life threatening. Since it can be asymptomatic or present with non-specific symptoms, diagnosis can be difficult clinically or by imaging studies and requires adequate tissue sample, high index of suspicion, and recognition of typical histopathology and immunohistochemistry. Distinguishing this lesion from other lesions is clinically significant, both for prognosis and treatment decision-making. First line treatment is surgical resection. As there is a high chance of local recurrence and an unknown long-term prognosis, follow-up is necessary.

## COMMENTS

### Case characteristics

A 54-year-old female presenting with intermittent dysphagia and a 60-pound weight loss within a 3-mo period.

### Clinical diagnosis

Desmoid type fibromatosis (DTF) of the pancreatic head.

### Differential diagnosis

Reactive fibroinflammatory pseudotumor, pancreatic adenocarcinoma.

### Laboratory diagnosis

Obstructive jaundice.

### Imaging diagnosis

Endoscopic retrograde cholangiopancreatography showed a large 2.5 cm irregular, ill-defined mass at the head of the pancreas causing complete bile and pancreatic duct obstruction. Endoscopic ultrasound (Figure 2) showed a 2.5 cm × 2.2 cm hypoechoic mass in the head of the pancreas causing extrahepatic biliary obstruction and pancreatic ductal dilation. Computed tomography showed a persistent mass of 5.2 cm × 4.2 cm in the pancreatic head.

### Pathological diagnosis

DTF.

### Treatment

Successful surgical resection with no complications.

### Term explanation

Sporadic DTF of the pancreas is extremely rare.

## Experiences and lessons

Although DTF of the pancreas is benign, it is locally aggressive and can be life threatening. As there is a high chance of local recurrence and an unknown long-term prognosis, follow-up is necessary.

## Peer-review

This manuscript was reported about a rare case of DTF at the pancreatic head.

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## Pancreatic adenosquamous carcinoma and intraductal papillary mucinous neoplasm in a *CDKN2A* germline mutation carrier

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**Author contributions:** Martínez de Juan F and Machado I wrote the paper and contributed equally to this work; Reolid Escribano M, Martínez Lapiedra C and Maia de Alcantara F collected the clinical data and endoscopy ultrasound images and made a critical revision of the paper; Calatrava Fons A collected the cytology images and made a critical revision of the paper; Caballero Soto M collected the data referred to the surgical details and made a critical revision of the paper.

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### Abstract

A 69-year-old woman from a kindred with familial atypical multiple mole melanoma and carrier of a germline mutation in *CDKN2A*, presented with abdominal pain caused by a solid-cystic pancreatic mass. The patient had an abdominal computed tomography three years before in which there was no evidence of pancreatic lesion. The endoscopic ultrasound guided fine needle aspiration showed adenocarcinoma with squamous component. After surgical resection the final diagnosis was adenosquamous pancreatic carcinoma (ASPC) arising in an intraductal papillary mucinous neoplasm (IPMN). Adenosquamous carcinomas are uncommon in the pancreas and have rarely been described in association with IPMNs. It has worse prognosis than the ordinary pancreatic ductal adenocarcinoma and some distinct features. We review the clinical, imaging, pathologic and molecular aspects of ASPC. Differential diagnosis with contamination, squamous metaplasia and pancreatic metastases from a distant squamous carcinoma is discussed. Besides, the case is an accelerated model of the adenoma (IPMN)-carcinoma sequence probably due to the *CDKN2A* germline mutation. Somatic *CDKN2A* mutations are common

events in the early steps of sporadic pancreatic cancer, but germline mutation carriers have a significantly higher risk of pancreatic carcinoma.

**Key words:** Intraductal papillary mucinous neoplasia; Melanoma-pancreatic cancer syndrome; Adenosquamous carcinoma; Pancreatic carcinoma; *CDKN2A*

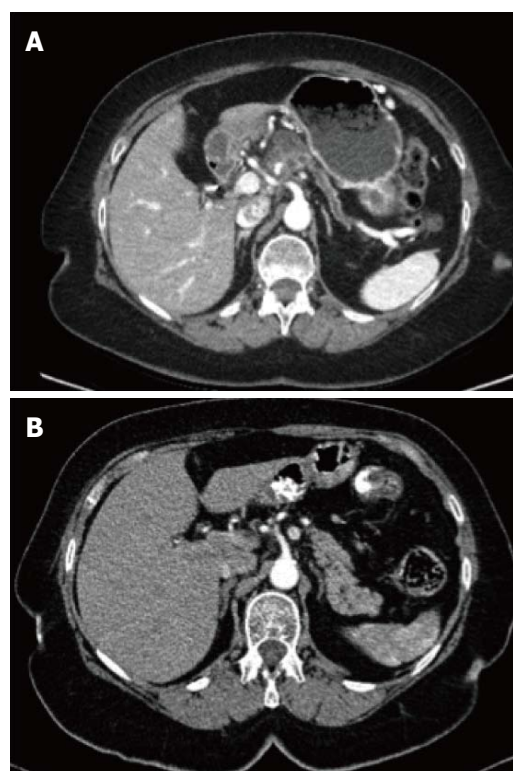
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**Core tip:** We present a rare case of adenosquamous pancreatic carcinoma (ASPC) arising in an intraductal papillary mucinous neoplasm in a *CDKN2A* germline mutation carrier from a kindred with familial atypical multiple mole melanoma. The case is an accelerated model of the adenoma-carcinoma sequence in pancreatic carcinogenesis. We discuss the differential diagnoses of squamous lesions in the pancreas and review the clinical and morphological features of ASPC.

Martínez de Juan F, Reolid Escribano M, Martínez Lapiedra C, Maia de Alcantara F, Caballero Soto M, Calatrava Fons A, Machado I. Pancreatic adenosquamous carcinoma and intraductal papillary mucinous neoplasm in a *CDKN2A* germline mutation carrier. *World J Gastrointest Oncol* 2017; 9(9): 390-396. Available from: URL: <http://www.wjgnet.com/1948-5204/full/v9/i9/390.htm> DOI: <http://dx.doi.org/10.4251/wjgo.v9.i9.390>

## INTRODUCTION

Ductal adenocarcinoma is the most frequent malignant tumour of the pancreas, and with a life expectancy of 5% at 5 years, the prognosis has not improved in the last 20 years<sup>[1]</sup>. Surgical excision, the only potentially curative treatment, yields a 20% chance of 5-year survival<sup>[1]</sup>. Unfortunately, only 15%-20% of patients are candidates for surgical resection due to distant metastases or locally advanced disease at diagnosis<sup>[1]</sup>. Hence, much effort should be focused in recognizing premalignant lesions or early invasive carcinomas. Pancreatic cancer screening risks outweigh benefits in the general population, but it might benefit individuals at high risk of pancreatic cancer<sup>[2]</sup>. However, only 10% of the pancreatic cancers are considered to be caused by inherited germline mutations, sporadically occurring mutations being responsible for the vast majority<sup>[3]</sup>. Pancreatic adenosquamous carcinoma is a rare variant with even worse prognosis than adenocarcinoma with some distinct clinical, imaging and pathological features<sup>[4,5]</sup>. We present the case of a pancreatic cancer predisposing germinal mutation carrier who developed an adenosquamous carcinoma of the pancreas arising in an intraductal papillary mucinous neoplasm. The case illustrates the adenoma-carcinoma sequence in pancreatic cancer, probably accelerated due to the germline mutation.

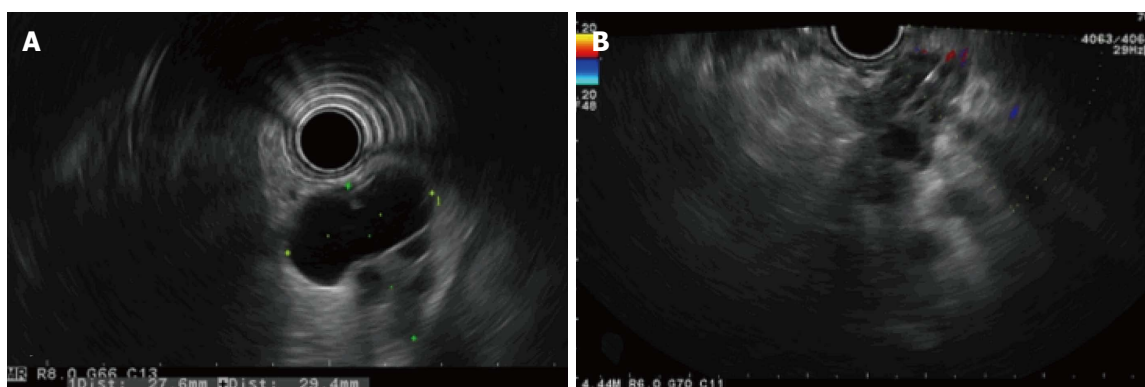


**Figure 1** Computed tomography image. A: Computed tomography (CT) image of the solid-cystic pancreatic mass with distal atrophy of the pancreas and pancreatic duct dilatation; B: CT three years before in which no pancreatic lesions were present.

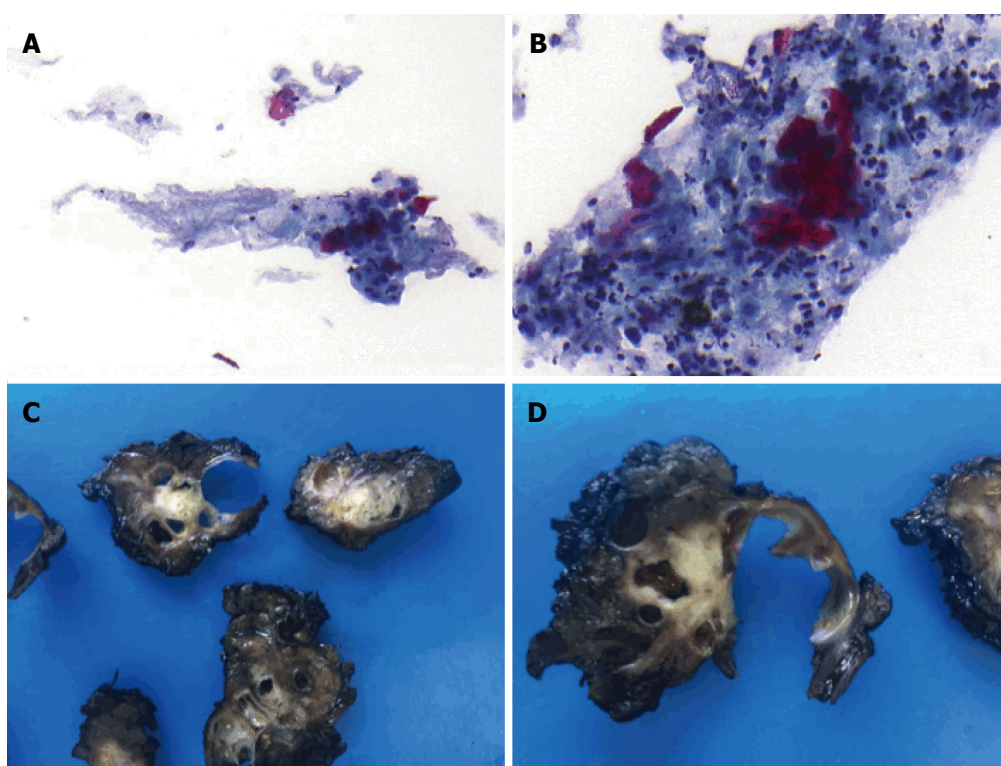
## CASE REPORT

A 69-year-old woman attended the hospital with upper abdominal pain of growing intensity during two months. She had a cutaneous malignant melanoma resected in 1997 and was a carrier of a germline mutation in *CDKN2A* at codon 59 (GTG>GGG) from a kindred with Familial Atypical Mole Melanoma (FAMMM). The computed tomography (CT) revealed a 30-mm solid-cystic mass in the body of the pancreas, with ill-defined borders, pancreatic tail atrophy, splenic vessels encasement and partial superior mesenteric artery and vein involvement (Figure 1A). The celiac trunk and branches were not involved. A CT performed 3 years previously due to a self-limited diffuse abdominal pain, resulted in no evident pancreatic lesion (Figure 1B). Radial and linear endoscopic ultrasonography (EUS) (Figure 2) confirmed the CT findings and the transgastric EUS guided fine needle aspiration (EUS-FNA) showed the presence of malignant cells with both glandular and squamous differentiation (Figure 3A and B). The multidisciplinary oncology board considered the tumour borderline for surgical resection, hence neoadjuvant chemotherapy with FOLFIRINOX (folinic acid, 5-fluorouracil, irinotecan and oxaliplatin) was indicated. After the neoadjuvant treatment, pain was relieved and CT showed reduction in the tumor size and no superior mesenteric artery involvement, therefore the patient was considered for surgical treatment. Partial pancreatectomy including the body and the tail together





**Figure 2** Endoscopic ultrasonography image. A: Radial endoscopic ultrasonography (EUS) view of the mass; B: Lineal guided EUS fine needle aspiration of the solid component of the mass.



**Figure 3** Endoscopic ultrasonography fine needle aspiration biopsies and surgical specimen. A and B: Positive cytology from the pancreatic mass (adenocarcinoma with a significant keratinizing component suggestive of adenosquamous carcinoma), Papanicolaou staining 20 × and 40 ×, respectively; C and D: A solid-cystic pancreatic mass (gross pathology).

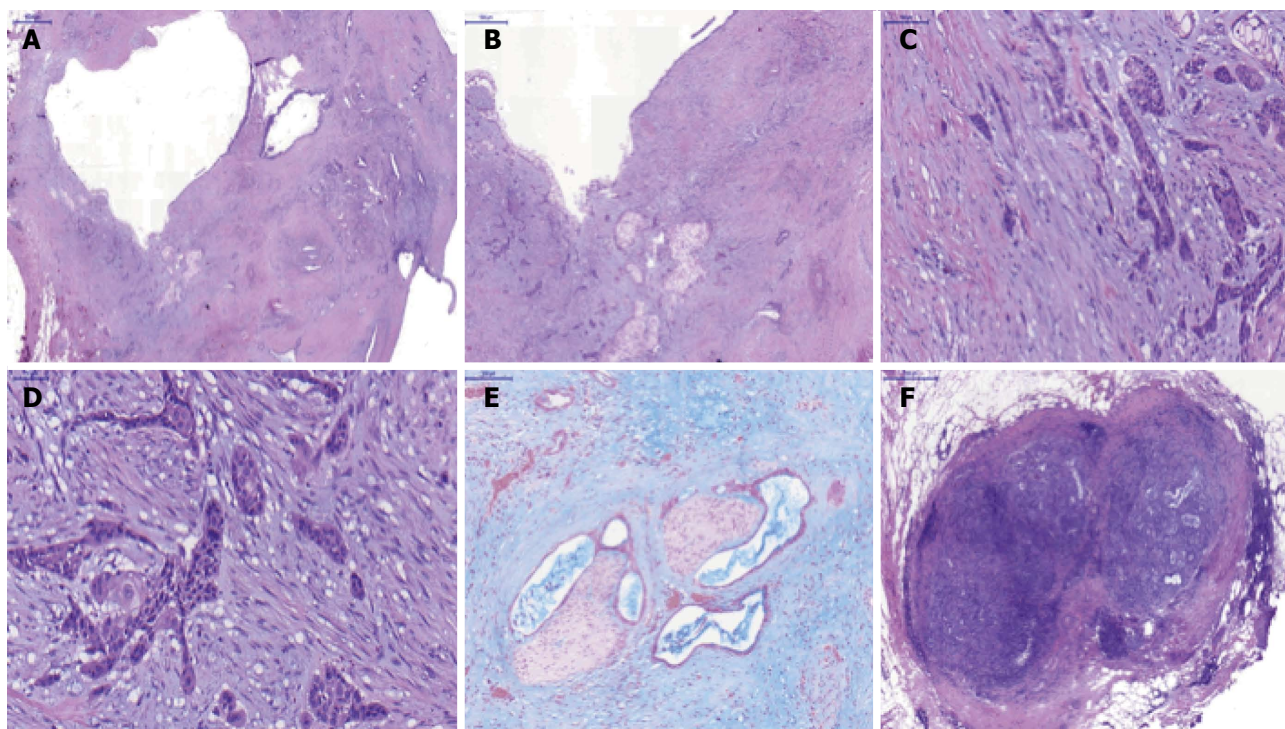
with splenectomy was performed without complications. In the surgical specimen, the pathological study of the solid-cystic mass (Figure 3C and D) showed an intraductal papillary mucinous neoplasm (IPMN) in connection with adenocarcinoma and squamous cell carcinoma components (Figure 4). Squamous metaplasia was also observed. The squamous carcinoma represented one third of the whole malignant component, thus a final diagnosis of pancreatic adenosquamous carcinoma probably arising in an IPMN was rendered. The immunohistochemical study showed CK (34βE12/p63, CEA and CK 5/6 strong positivity in the squamous component. CK 7 and MUC5A revealed strong positivity in the adenocarcinoma area (Figure 5A-C). p53 and EMA were strongly positive in both components (Figure 5D). Ki-67 index was 25%. The

retroperitoneal surgical margin was affected, and 10 out of 27 lymph nodes were metastatic, one of them located between cava and aorta. The final pathological stage was ypT3N1M1. Five months after surgery, the patient is currently ongoing maintenance chemotherapy.

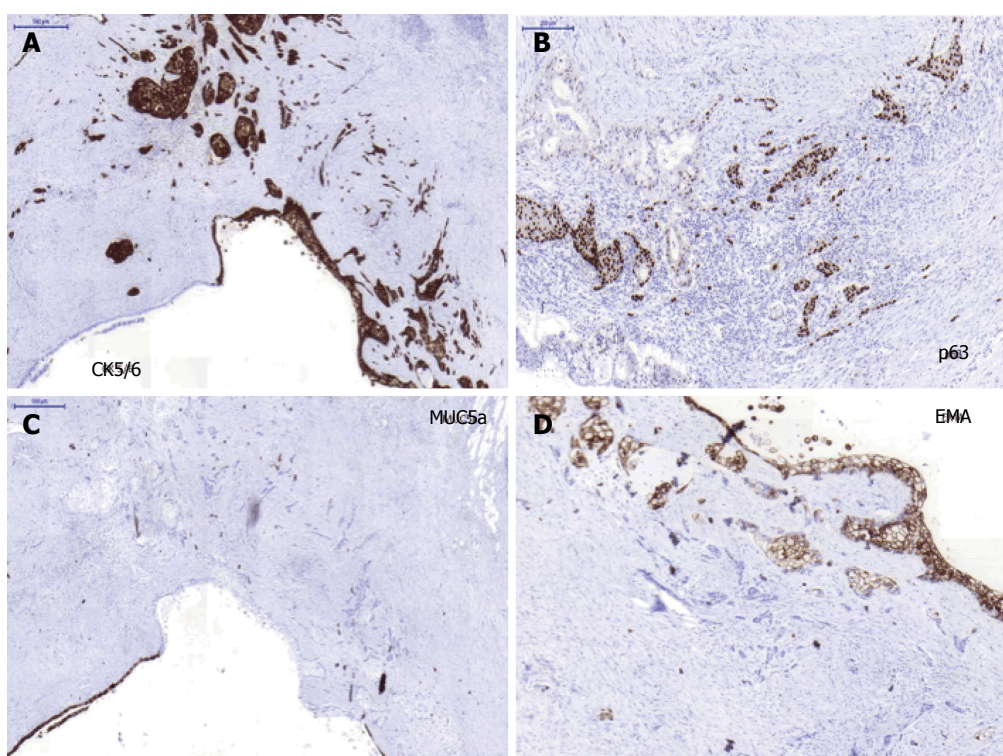
## DISCUSSION

Adenosquamous carcinomas may appear in various locations in the body (gastrointestinal tract<sup>[6,7]</sup> female reproductive organs<sup>[8]</sup>, lung, bladder). Adenosquamous pancreatic carcinoma (ASPC) is rare, accounting for less than 5% of all pancreatic carcinomas in the largest series<sup>[4]</sup>. Areas of squamous differentiation are often seen in a pancreatic ductal adenocarcinoma (PDA), but





**Figure 4** Microscopic pathology of the surgical specimen. A: Intraductal papillary mucinous neoplasm with adenocarcinoma component, hematoxylin and eosin (H and E) 10 ×; B-D: Squamous metaplasia and evident infiltrative squamous carcinoma, H and E 10 ×, 20 × and 40 ×, respectively; E: Adenocarcinoma with perineural invasion, alcian blue 20 ×; F: Peripancreatic lymph node metastasis (adenocarcinoma component), H and E 20 ×.



**Figure 5** Immunohistochemical study in the surgical specimen. A: CK5/6 strong positivity in squamous component (metaplasia and carcinoma), × 20; B: Strong nuclear p63 immunopositivity in the invasive squamous carcinoma. The adenocarcinoma area display poor p63 nuclear positivity, × 20; C: MUC5a negativity in the squamous carcinoma component and strong positive in ductal epithelial cells, × 20; D: MUC1/EMA positivity in the squamous metaplastic component, × 20.

the diagnosis of ASPC is conventionally accepted only if the squamous component is present in at least 30% of

the total volume of the tumour. Exclusively squamous carcinomas of the pancreas are exceptional; moreover,

they are usually regarded as metastases from a primary squamous carcinoma. ASPC symptoms are basically the same as PDA's (mainly abdominal or back pain, but also jaundice, weight loss or diabetes depending on its relation with the biliary tract and the grade of exocrine and endocrine impairment caused). It may be seen more frequently in the head of the pancreas, but ASPC presents less likely in the head and more commonly in the body or tail than PDA<sup>[4]</sup>. It is slightly predominant in white males, around the seventh decade of life<sup>[4]</sup>. Respecting size, ASPC is usually bigger than PDA. The squamous component usually locates in the periphery of the lesion, whilst adenocarcinoma lays in the center. Extensive necrotic areas are more common in ASPC. Poor differentiation grade is frequent<sup>[4,5]</sup>, and tends to grow perineurally. In the same way, vascular and lymph nodes involvement is frequently seen<sup>[5]</sup>. Rhabdoid components<sup>[9]</sup>, osteoclast-like and pleomorphic giant cells, and acantholysis<sup>[10]</sup> have been described in some cases. Immunohistochemical (IHC) analysis show positivity for keratins (AE1/E3, CK1, CK5/6, CK7, and less frequently Cam 5.2 and CK20)<sup>[11]</sup>, p63 (which may be helpful in identifying squamous differentiation in cases with acantholysis), overexpression of EGFR, and reduced or loss of E-cadherin expression<sup>[12]</sup>. p16 and Dpc4 expression are usually lost or reduced and nuclear p53<sup>[12]</sup>, CA 19.9 and CEA are usually positive<sup>[12]</sup> similar to PDA. One study<sup>[13]</sup> used an IHC commercial assay<sup>[14]</sup> of molecular markers which are implicated in anti-tumor drug performance, showing TOPO2A, MRP1, BCRP and MGMT overexpression. There is not a definitive sign of ASPC in imaging tests, but some studies remark that it is frequently round or has a lobulated shape<sup>[15]</sup> with ill-defined borders<sup>[16]</sup>, and characteristically demonstrates central necrotic areas<sup>[15]</sup>. It usually displays peripheral contrast enhancement in the arterial phase, which persists in the venous phase<sup>[17]</sup>, and thrombus in the portal vein system is often present<sup>[15]</sup>. Opposite to PDA, lack of distal glandular atrophy and only mild pancreatic ductal dilatation<sup>[16]</sup> have been noted. Not surprisingly, in view of the aggressive morphologic features, prognosis is even poorer than PDA's: In a large population based study comparing 415 cases of ASPC and 45693 of PDA, 2-year survival after surgery was 29% in ASPC patients and 36% in the PDA group ( $P < 0.0001$ )<sup>[4]</sup>. A matched case control study yielded a significantly worse median overall survival of 8.38 mo in ASPC patients compared with 15.75 mo in PDA patients (HR = 1.94; 95%CI: 1.07-3.51;  $P = 0.026$ )<sup>[18]</sup>.

The etiology of the ASPC is unknown, but there are three hypotheses that try to explain the development of ASPC: (1) Squamous carcinoma metaplasia may be a consequence of metaplastic changes due to the microenvironmental conditions in the ductal lumen obstructed by an already existent PDA; (2) areas of squamous carcinoma might be present since the first steps of carcinogenesis originated at the same time that the adenocarcinoma line from a common tumor stem cell; and (3) two concomitant carcinomas (squamous

and adenocarcinoma) collide and merge forming one tumor mass. There are no studies that demonstrate which, if any, is correct, but some studies find the same KRAS mutation in both tumor lines<sup>[12]</sup> suggesting a common origin, thus making the collision hypothesis less likely.

The molecular alterations in ASPC are similar to PDA<sup>[12]</sup>, being the loss of p16 (the protein coded by the gene *CDKN2A*) a common event in the early steps of pancreatic carcinogenesis. Loss of p16 may be caused by genetic mutations or gene silencing due to epigenetic changes. Our patient, being a carrier of a germline mutation in *CDKN2A*, had a predisposition to pancreatic cancer. As a fact, it has been estimated that carriers of a germline *CDKN2A* mutation in kindreds with FAMMM, have a 38-fold higher risk of pancreatic cancer than the general population<sup>[3]</sup>. Nevertheless, many other genetic, epigenetic or environmental factors and interactions among them must influence, as not all the families with *CDKN2A* mutations have a familial history of pancreatic cancer<sup>[19]</sup>. There are some scientific societies<sup>[20-22]</sup> that have reached some consensus in screening special populations with markedly increased risk of pancreatic cancer, mainly with EUS or MRI. Notwithstanding this, there are still knowledge gaps, lack of standardization in procedures, and differences in availability of resources that hinder the implementation in daily clinical practice, so that this patient was not following a pancreatic cancer screening program.

There are few reports of ASPC arising or associated with IPMN<sup>[23,24]</sup>. IPMNs are premalignant lesions that are estimated to develop a carcinoma in 47% the main duct type, and in 17% the branch duct type<sup>[25]</sup>. It usually takes more than a decade to transform into a malignant invasive lesion, for those who eventually do<sup>[25]</sup>. Our patient had an abdominal CT three years before, in which no lesion was apparent in the pancreas. It could be argued that a lesion of less than 2 cm may have been overlooked; even if this was case, the sequence from premalignant to malignant was clearly accelerated, most probably due to the *CDKN2A* mutation.

The EUS-guided samples in our case showed plenty squamous component, not only in the cell aspirate but also in the cellular block, together with adenocarcinoma, therefore a diagnosis of ASPC was proposed. Preoperative diagnosis of ASPC is difficult: the finding of squamous component in the cytology should be interpreted cautiously, as it could represent contamination<sup>[26]</sup>. Furthermore, even if the specimen allows architectural study and thus contamination is likely ruled out, it is not possible to determine the proportion of squamous carcinoma in the whole malignant mass with the biopsy sampling. Hence, the preoperative diagnosis of ASPC may be suggested, but not definitive. The differential diagnosis when squamous component is found in a pancreatic neoplasia also includes ductal squamous metaplasia, mucoepidermoid carcinoma (which is characterized by the presence of squamoid intermediate cells and the absence of individual cell keratinization



and keratin pearls) and pancreatoblastoma, which is rarely found in adults. In addition, in a patient with a previous history of malignant melanoma the possibility of de-differentiated melanoma with adenocarcinoma-like component should be also excluded<sup>[27]</sup>.

In summary, this case illustrates the accelerated carcinogenesis in a genetically predisposed patient, with the premalignant ground (IPMN) and a rare malignant variant (ASPC), all together in one lesion. The imaging studies (CT) and especially EUS may help to detect this rare entity, although the definitive diagnosis requires surgical resection. Currently there is not a specific strategy for ASPC, but the advances in the molecular profiling and the new targeted therapies might change its management.

## ACKNOWLEDGMENTS

We acknowledge David Thivey for reviewing the language.

## COMMENTS

### Case characteristics

A 69-year-old woman with antecedents of familial melanoma and *CDKN2A* mutation carrier presented with a two months of abdominal pain.

### Clinical diagnosis

The physical exam was normal.

### Differential diagnosis

Although the abdominal pain was unspecific, the personal, familial and genetic antecedents lead to the suspicion of pancreatic carcinoma. The relevant differential diagnosis is mainly pathological. When squamous component is found in a pancreatic tumor, ductal squamous metaplasia, adenosquamous carcinoma, mucoepidermoid carcinoma, pancreatoblastoma, and metastasis of a primary squamous carcinoma or a de-differentiated melanoma with adenocarcinoma-like component, should be considered.

### Laboratory diagnosis

All labs, including CA19.9 were within normal limits.

### Imaging diagnosis

The computed tomography and endoscopic ultrasonography revealed a 30-mm solid-cystic mass in the body of the pancreas, with ill-defined borders, pancreatic tail atrophy, splenic vessels encasement and partial superior mesenteric artery and vein involvement.

### Pathological diagnosis

Pancreatic adenosquamous carcinoma in an intraductal papillary mucinous neoplasm.

### Treatment

Neoadjuvant chemotherapy (FOLFIRINOX) and partial pancreatectomy followed by adjuvant chemotherapy.

### Related reports

Adenosquamous pancreatic carcinomas (ASPCs) are unfrequent have worse prognosis than pancreatic adenocarcinoma, and have rarely been described in a intraductal papillary mucinous neoplasm.

### Term explanation

ASPC is a rare variant of pancreatic adenocarcinoma, characterized by the

presence of squamous component in at least 30% of the tumoral mass. Intraductal papillary mucinous neoplasms (IPMN) are premalignant pancreatic cystic lesions which may need surgical resection. Cyclin dependent kinase inhibitor 2A gene (*CDKN2A*) is a tumor suppressor gene that codes p16 or *CDKN2A* protein; mutations in this gene has been related to melanoma and pancreatic carcinoma, but also to head and neck, breast and lung cancers. Familial Atypical Mole Melanoma (FAMMM) includes families with a high tendency to develop malignant melanoma. Some of this families carry inherited mutations in *CDKN2A*.

## Experiences and lessons

ASPC is a rare variant of pancreatic carcinoma and may develop in intra-papillary mucinous neoplasms as the usual pancreatic adenocarcinoma. *CDKN2A* germinal mutation carriers bear a high risk of pancreatic carcinoma. Although evidence supporting pancreatic cancer screening is limited, the indication of a surveillance strategy might be discussed with the patient.

## Peer-review

The authors report a rare case of ASPC arising in a FAMMM patient who is a carrier of *CDKN2A* germline mutation. Although it is well known that FAMMM patient has a high risk of occurring pancreatic carcinoma, ASPC arising in a background of IPMN has rarely been reported. Therefore the paper may enrich our knowledge on FAMMM associated pancreatic tumor.

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## Cervical Castleman's disease mimicking lymph node metastasis of esophageal carcinoma

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**Institutional review board statement:** This case report was approved by the ethics committee of National Hospital Organization Hakodate Hospital.

**Informed consent statement:** All study participants provided informed written consent prior to the treatment.

**Conflict-of-interest statement:** No benefits in any form have been received or will be received from a commercial party related directly or indirectly to the subject of this article.

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### Abstract

Castleman's disease (CD) is an uncommon benign lymphoproliferative disorder of unknown etiology. A rare case of cervical CD diagnosed at lymph node dissection for esophageal carcinoma is reported. An esophageal tumor was identified in a 67-year-old man during a follow-up examination after surgery for oral carcinoma. Esophagoscopy revealed a type 1 tumor in the cervical esophagus. Histology of esophagoscopy biopsies indicated squamous cell carcinoma. Contrast-enhanced computed tomography revealed swollen lymph nodes of the right cervical region. No distant metastasis was detected. Esophageal carcinoma, T2N2M0, Stage IIIA was diagnosed. Neoadjuvant chemotherapy was recommended, but the patient rejected the chemotherapy. The patient underwent laparoscopic-assisted transhiatal esophagectomy. The histopathological diagnosis was moderately differentiated squamous cell carcinoma with pT1bN0M0, Stage I A. On histology, the swollen lymph nodes of the right cervical region revealed CD. The patient's postoperative course was relatively good.

**Key words:** Castleman's disease; Lymph node metastasis; Esophageal carcinoma

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**Core tip:** The association of Castleman's disease (CD) with epithelial malignancy is rare. To the best of our knowledge, the present case is the first report of a synchronous esophageal carcinoma and cervical CD. In the present case, we clinically diagnosed esophageal carcinoma with right cervical lymph nodes metastasis, T2N2M0, Stage IIIA preoperatively, but the stage was revised to pT1bN0M0, Stage I A on pathological diagnosis. *Via* histology, the swollen lymph nodes of the right cervical region revealed CD. This case demonstrates that cervical CD is rarely associated with an esophageal carcinoma and can clinically mimic nodal metastasis.

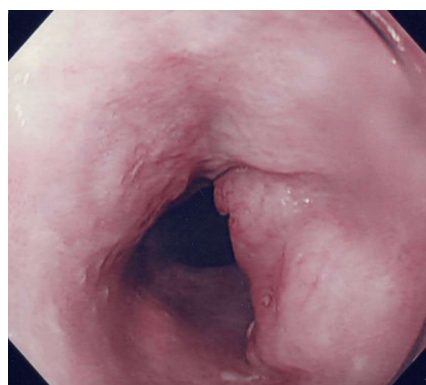
Yamabuki T, Ohara M, Kato M, Kimura N, Shirosaki T, Okamura K, Fujiwara A, Takahashi R, Komuro K, Iwashiro N, Hirano S. Cervical Castleman's disease mimicking lymph node metastasis of esophageal carcinoma. *World J Gastrointest Oncol* 2017; 9(9): 397-401 Available from: URL: <http://www.wjgnet.com/1948-5204/full/v9/i9/397.htm> DOI: <http://dx.doi.org/10.4251/wjgo.v9.i9.397>

## INTRODUCTION

Castleman's disease (CD), which is otherwise known as angiofollicular hyperplasia of the lymph node, is a rare lymphoproliferative disorder that was first described in 1956 by Castleman *et al*<sup>[1]</sup> as a benign proliferation of mediastinal lymphoid tissues of unknown etiology. CD most commonly develops in the mediastinum, and the cervical region is the second most common location. Clinically, CD manifests as localized disease (unicentric) or widespread disease (multicentric). The unicentric type of CD (UCD) typically presents as an asymptomatic single enlarged lymph node. By contrast, the multicentric type of CD (MCD) is associated with systemic symptoms, including hepatosplenomegaly, recurrent fevers, night sweats, and lymphadenopathy<sup>[2]</sup>. The association or coexistence of an epithelial malignancy with CD is an exceptional occurrence. An extremely rare case presenting with synchronous cervical CD and esophageal carcinoma that clinically simulated nodal metastatic disease is reported, and the relevant literature is reviewed.

## CASE REPORT

An esophageal tumor was identified in a 67-year-old man during a follow-up examination after surgery for oral carcinoma. He had comorbid disorders of hypertension, angina pectoris, diabetes mellitus, and hyperlipidemia. Enlarged lymph nodes in the right neck were palpable. Routine laboratory findings were unremarkable, with the exception of a slightly elevated serum squamous cell carcinoma (SCC)-related antigen



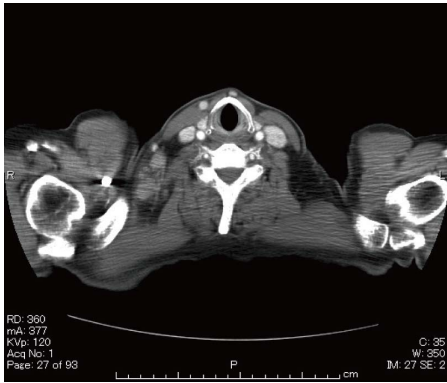
**Figure 1 Esophagoscopy view of the tumor.** Esophagoscopy reveals a type 1 tumor in the cervical esophagus.



**Figure 2 Upper gastrointestinal barium study image.** An upper gastrointestinal barium study reveals a 15-mm filling defect at the cervical esophagus.

(2.4 ng/mL, normal 0-1.5 ng/mL). Esophagoscopy revealed a type 1 tumor in the cervical esophagus (Figure 1). Histological examination of esophagoscopy biopsies revealed squamous cell carcinoma. An upper gastrointestinal barium study indicated a filling defect 15 mm in size at the cervical esophagus (Figure 2). Contrast-enhanced computed tomography (CT) revealed weakly enhanced swollen lymph nodes of the right cervical region (Figure 3). No distant metastasis was demonstrated. Esophageal carcinoma T2N2M0, Stage III A was diagnosed clinically. Neoadjuvant chemotherapy was recommended, but the patient rejected the chemotherapy. The patient underwent laparoscopic-assisted transhiatal esophagectomy with reconstruction using a posterior mediastinal gastric tube. The operative time was 265 minutes, and the amount of blood loss was 140 mL. Macroscopically, the type 1 tumor was located in the cervical esophagus, and it measured 30 mm × 15 mm (Figure 4). The histopathological diagnosis of the esophageal tumor was moderately differentiated squamous cell carcinoma (Figure 5A). In addition to the main tumor, numerous microscopic foci of squamous intraepithelial neoplasia from low grade to high grade were noted throughout the resected esophagus. No tumor metastases were present in the lymph nodes. However, three swollen lymph nodes





**Figure 3 Preoperative computed tomography image.** Contrast-enhanced computed tomography indicates weakly enhanced swollen lymph nodes in the right cervical region.

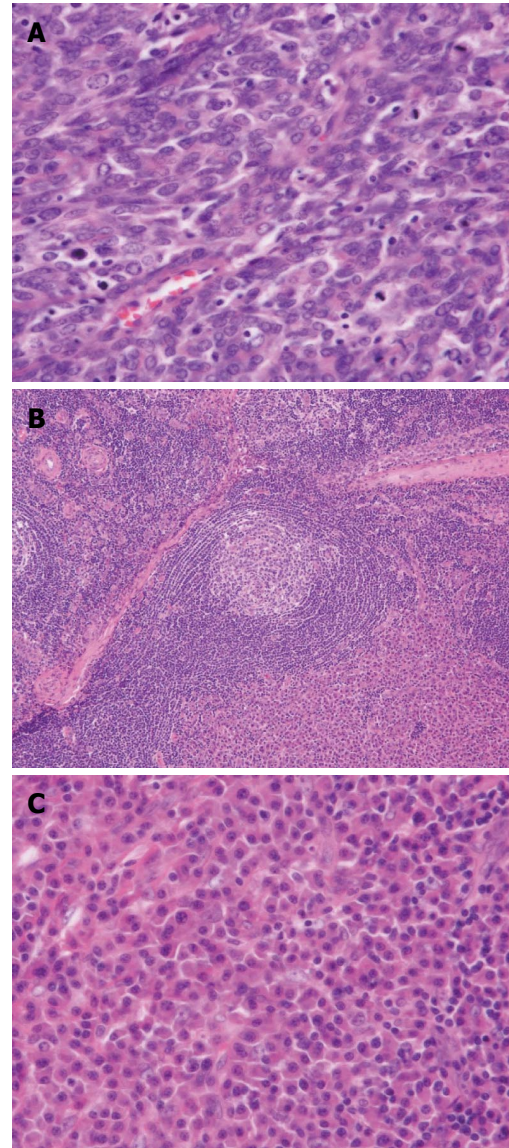


**Figure 4 Surgical specimen of the esophagus.** Macroscopically, the type 1 tumor is located in the cervical esophagus, and it measures 30 mm × 15 mm.

were identified in the right cervical region at #104. Histological examination of these lymph nodes revealed characteristic findings, such as tight concentric layering of lymphocytes at the periphery of the lymph follicles resulting in an onionskin appearance and inter-follicular diffuse proliferation of plasma cells. The findings were compatible with CD, plasma cell type (Figure 5B and C). The final diagnosis was esophageal squamous cell carcinoma without lymph node metastasis, pT1bN0M0, Stage I A and CD of the cervical lymph nodes. The patient had an uneventful postoperative course.

## DISCUSSION

CD is an uncommon benign lymphoproliferative disorder of unknown etiology that was first reported by Castleman *et al*<sup>[1]</sup> in 1956. A variant of the multicentric form was later found to be associated with human herpesvirus 8 (HHV-8), which is the same virus found in Kaposi's sarcoma. Similar to Kaposi's sarcoma, it is often identified in patients with human immunodeficiency virus (HIV)<sup>[3]</sup>. From a systematic case review including 278 patients with unicentric CD (UCD), the main sites of disease are the chest (29%), neck (23%), abdomen (21%), and retroperitoneum (17%). In addition, other lymph node groups (axillary, inguinal) and the pelvis are also potential



**Figure 5 Histopathological results of the resected specimen.** A: The histopathological diagnosis of the resected esophagus is moderately differentiated squamous cell carcinoma (HE × 200); B: Histological examination of the right-sided neck lymph nodes reveals onionskin arrangement of small lymphocytes (HE × 20); C: Interfollicular diffuse proliferation of plasma cells (HE × 200).

sites of involvement<sup>[4]</sup>. With advancements in the understanding of the disease entity, CD is classified into four subtypes: (1) hyaline-vascular CD; (2) plasma cell CD; (3) HHV-8-associated (plasmablastic) multicentric CD (MCD); and (4) MCD, not otherwise specified (NOS). The hyaline-vascular subtype is characterized by widened mantle zones composed of concentric rings of small lymphocytes in an "onion skin" pattern around small atrophic germinal centers with penetrating hyalinized vessels and dysplastic follicular dendritic cells<sup>[3]</sup>. The subtype typically occurs as a unicentric process, involving a single node or local group of nodes. The plasma cell variety, which is characterized by sheets of mature plasma cells in the interfollicular tissue, occurs more often as a multicentric process<sup>[5]</sup>. Patients with HHV-8-



associated (plasmablastic) MCD incur a unique risk of developing HHV-8-positive plasmablastic lymphoma<sup>[6]</sup>. The multicentric NOS variant is a wastebasket term used to classify multicentric cases that are HHV-8 negative and/or those that exhibit intermediate histopathology (such as mixed features of both plasma cell and hyaline-vascular)<sup>[7]</sup>.

The radiographic characteristics of CD are non-specific, but some features could help raise suspicion for the diagnosis of CD. Plain radiographic findings include a mass effect. In addition, in approximately 30% of cases exhibit localized calcifications harboring a radial arrangement or star-shaped calcification, which is considered characteristic of CD. Ultrasonography (US) typically demonstrates a hypoechogenic and homogeneous mass with a quite clear delimitation. CT scans reveal a solid, homogeneous, and well delimited mass that is enhanced with vascular contrast as a result of hypervascularity<sup>[2]</sup>.

A review of current therapeutic strategies for CD revealed that complete surgical resection is curative for UCD, leading to excellent long-term outcomes with 10-year overall survival rates in excess of 95%. Although the available literature is confined to a small number of cases, radiotherapy appears to be a reasonable alternative treatment option in unresectable cases of UCD. A range of systemic therapies has been used in MCD, including cytotoxic chemotherapy, antibodies directed against CD20 (rituximab) and interleukin-6 (IL-6) (siltuximab) and its receptor (tocilizumab), immunomodulators (interferon alpha, thalidomide, and lenalidomide), bortezomib, and antiviral agents (zidovudine and valganciclovir, ganciclovir, and cidofovir). Although these agents inhibit disease activity, the literature documenting their use is mainly confirmed to case reports or small series of patients, limiting overall assessment of efficacy and direct comparisons between regimens<sup>[8]</sup>.

CD is rarely associated with epithelial malignancy. Previously, five case reports observed the synchronous occurrence of carcinoma, two cases of pulmonary carcinoma<sup>[9,10]</sup>, two cases of squamous cell carcinoma of tongue<sup>[11,12]</sup>, and a thymic squamous cell carcinoma<sup>[13]</sup>. To the best of our knowledge, the present case is the first report of a synchronous esophageal squamous cell carcinoma and a unicentric plasma cell type of CD.

IL-6 is related to the pathogenesis of CD in many patients<sup>[14]</sup>. Several human tumors also secrete IL-6, including multiple myeloma<sup>[15]</sup>, renal cell carcinoma<sup>[16]</sup>, lung carcinoma<sup>[17]</sup>, cervical carcinoma<sup>[18]</sup>, and esophageal carcinoma<sup>[19]</sup>. Furthermore, the serum IL-6 concentration is correlated with disease status and prognosis in esophageal squamous cell carcinoma<sup>[19]</sup>. Although further studies are necessary to confirm this association, the present case suggests that IL-6 may serve as an important pathogenic link between CD and esophageal squamous cell carcinoma.

In the present case, the preoperative clinical diagnosis was esophageal carcinoma T2N2M0, Stage III

A, but the stage was revised to pT1bN0M0, Stage I A upon pathological diagnosis. The preoperative diagnosis of CD is very difficult given the lack of disease-specific signs. This case demonstrated that cervical CD could be rarely associated with an esophageal carcinoma when it clinically mimics nodal metastasis.

## COMMENTS

### Case characteristics

An esophageal tumor was identified in a 67-year-old man during a follow-up examination after surgery for oral carcinoma.

### Clinical diagnosis

Enlarged lymph nodes in the right neck were palpable.

### Differential diagnosis

Metastases from head and neck tumors, lymphoma, Kaposi sarcoma, bacterial infection, viral infection or tuberculous cervical lymphadenitis.

### Laboratory diagnosis

Routine laboratory findings were unremarkable, with the exception of a slightly elevated serum squamous cell carcinoma-related antigen.

### Imaging diagnosis

Esophagoscopy revealed a type 1 tumor in the cervical esophagus, and contrast-enhanced computed tomography indicated weakly enhanced swollen lymph nodes of the right cervical region.

### Pathological diagnosis

The histopathological diagnosis of the esophageal tumor was moderately differentiated squamous cell carcinoma, and histological examination of cervical lymph nodes revealed Castleman's disease, plasma cell type.

### Treatment

The patient underwent laparoscopic-assisted transhiatal esophagectomy with cervical lymph node dissection.

### Related reports

Castleman's disease is rarely associated with epithelial malignancy. Previously, five case reports observed the synchronous occurrence of carcinoma: two cases of pulmonary carcinoma, two cases of squamous cell carcinoma of tongue, and a thymic squamous cell carcinoma.

### Term explanation

Castleman's disease is an uncommon benign lymphoproliferative disorder of unknown etiology. The main sites of disease were the chest, neck, abdomen, and retroperitoneum.

### Experiences and lessons

This case demonstrates that cervical Castleman's disease can be rarely associated with an esophageal carcinoma when it clinically mimics nodal metastasis.

### Peer-review

This is an interesting case report demonstrating that swollen lymph nodes during preoperative tumor staging can be due to other uncommon benign diagnoses.

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# World Journal of *Gastrointestinal Oncology*

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## New endoscopy advances to refine adenoma detection rate for colorectal cancer screening: None is the winner

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### Abstract

Colorectal cancer (CRC) is the third most common

cancer in males and second in females, and globally the fourth cause for cancer death worldwide. Oncological screening of CRC has a major role in the management of the disease and it is mostly performed by colonoscopy. Anyway, effectiveness of endoscopic screening for CRC strictly depends on adequate detection and removal of potentially precancerous lesions, and accuracy of colonoscopy in detection of adenomas is still suboptimal. For this reason, several technological advances have been implemented in order to improve the diagnostic sensitivity of colonoscopy in adenoma detection. Among these: (1) Visual technologies such as chromoendoscopy and narrow band imaging; (2) optical innovation as high definition endoscopy, full-spectrum endoscopy or Third Eye Retro-scope; and (3) mechanical advances as Cap assisted colonoscopy, Endocuff, Endoring and G-Eye endoscope. All these technologies advances have been tested over time by clinical studies with mixed results. Which of them is more likely to be successful in the next future?

**Key words:** Colorectal cancer screening; Colonoscopy; Adenoma detection rate; Diagnostic advances

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**Core tip:** Oncological screening of colorectal cancer is mostly performed by colonoscopy and effectiveness of this technique strictly depends on adequate detection and removal of potentially precancerous lesions. Anyway, accuracy of colonoscopy in detection of adenomas is still suboptimal. For this reasons several technological advances have been implemented in order to improve the diagnostic sensitivity of colonoscopy in adenoma detection. Which of them is more likely to be successful in the next future?

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## INTRODUCTION

Colorectal cancer (CRC) is the third most common cancer in males and second in females, and globally the fourth cause for cancer death worldwide<sup>[1,2]</sup>. Oncological screening of CRC has a major role in the management of the disease, since several randomized controlled trials demonstrated an increase in 5-year survival and a reduction in mortality for healthy subject undergoing surveillance, compared to patients who are diagnosed in the clinical phase of the disease<sup>[3]</sup>. To date several tests have been used in CRC screening, among them fecal occult blood test, fecal DNA test, sigmoidoscopy, colonoscopy and computed tomographic colonography. Anyway colonoscopy has a pivotal role in CRC screening, since it can be used both as primary screening test, both as recall strategy after a positive result of a different test in order to confirm diagnosis and provide removal of polyps. Since effective endoscopic screening for CRC strictly depends on adequate detection and removal of potentially precancerous lesions, over time performance measures and quality indicators have been assessed in order to ensure the quality of the examination and improve patient outcomes<sup>[4-6]</sup>.

The European Society of Gastrointestinal Endoscopy and United European Gastroenterology have recently presented a short list of key performance measures for lower gastrointestinal endoscopy<sup>[7]</sup>. Among these, cecal intubation rates, withdrawal times, quality of bowel preparation and adenoma detection rate (ADR).

ADR is the primary quality indicator for colonoscopy and depends by the performance of the endoscopist. It is defined as the proportion of screening colonoscopies in patients aged 50 years or older detecting at least one adenoma, and it should be ideally at least 25%. A first study in 2010 showed that ADR is an independent predictor of the risk of interval CRC after screening colonoscopy<sup>[8]</sup> and a recent prospective study of individuals who underwent screening colonoscopy within a National Colorectal Cancer Screening Program, showed that increased ADR is associated with reduced risk of interval CRC and death<sup>[9]</sup>. Anyway, despite quality measures, the accuracy of colonoscopy in detection of adenomas is still suboptimal<sup>[10]</sup>. Up to date several technological advances have been implemented in order to improve the diagnostic sensitivity of colonoscopy in adenoma detection.

First of all visual and optical enhancement technologies have been introduced with the aim of improve ADR. In the group of visual enhancement advances, chromoendoscopy and narrow band imaging (NBI) have been test over time. As suggested by a Cochrane review, chromoendoscopy can improve detection of polyps, anyway it is a time-consuming technique and it

is not always feasible in real practice<sup>[11]</sup>. Contrariwise, as showed by several studies, NBI does not improve ADR during colonoscopy<sup>[12,13]</sup>. Among optical innovation, high definition endoscopy (HDE), using high definition monitor and a high resolution charge coupled device with up to a million pixels, allows a better image view compared to standard vision endoscopy (SVE). Anyway studies report conflicting results. A recent meta-analysis comparing high definition vs standard video endoscopy showed, in favor of HDE, an incremental yield of 3.8% (95%CI: 1%-6.7%) for the detection of any polyp, an incremental yield of 3.5% (95%CI: 0.9%-6.1%) for detection of adenomatous polyps and no differences between HDE and SVE in the detection of high-risk adenomas<sup>[14]</sup>.

The full-spectrum endoscopy (FUSE, EndoChoice, GA, United States) is a new technology using a colonoscope equipped with two lateral lenses, in addition to the one on the forward tip, so to increase the maximum field of view up to 330°, compared to the ≤ 170° of standard forward-viewing (SFV) colonoscopy. This allows greater visual field and, at least in theory, greater detection rate of polyps.

A multicenter, randomized back-to-back study showed a significantly higher detection rate of adenomas (69% additional adenomas) and a lower adenoma miss rate with FUSE (7%) respect to SFV colonoscopy (41%) ( $P < 0.0001$ )<sup>[15]</sup>.

Despite this good premise, a randomized controlled trial performed on a large population of patients undergoing colonoscopy following a positive fecal immunochemical test, showed no statistically significant difference in detection rates of adenomas (ADR) and advanced adenomas (defined as adenomas ≥ 10 mm and/or with villous component > 20%, and/or high-grade dysplasia) in a per patient analysis<sup>[16]</sup>.

Another recent randomized back to back study compared adenoma miss rates of full-spectrum endoscopy (FSC) with those of conventional colonoscopy complemented by right-colon re-examination using scope retroflexion (CC/R) performed by endoscopists with documented ADRs > 35%. FSC showed, by a per-lesion analysis, a significantly lower adenoma miss rate compared with CC/R [10.9% (95%CI: 3.8-18.1) vs 33.7% (95%CI: 23.4-44.1)] and a lower advanced adenoma miss rate lower with FSC [4.3% (95%CI: -4.0-12.7) vs 25.9% (95%CI: 9.4-42.5)] showing as FSC outperforms conventional colonoscopy even when performed by experienced endoscopists<sup>[17]</sup>. Therefore, despite its good technical result, so far literature data are conflicting and a definite benefit on ADR has not been yet demonstrated.

One more technological solution is the Third Eye Retroscope (TER; Avantis Medical Systems, Inc), a device that can be inserted through a standard colonoscope's working channel, advanced over the tip and bend to 180 degrees before the withdrawal phase, in order to obtain an additional backward view that increases the visibility of blind areas not fully visible on standard view examination.



Studies performed so far showed a gain in ADR from 13.2% to 23.2%<sup>[18,19]</sup>. Despite a quite gain in adenoma detection, however the procedure is time consuming and presents some disadvantages such as an inferior image quality, a reduced suction capacity of the scope and the necessity of removing the third eye retroscope whenever another device need to be inserted through the working channel.

One additional method to enhance ADR is that to obtain a mechanical improvement of endoscopic view by a mechanical flattening of haustral folds and tip stabilization. In this line, several devices have been introduced to refine efficiency of the standard colonoscopy, such as cap, cuff and rings.

Cap assisted colonoscopy (CAC) is a simple technique utilizing a transparent cap mounted on the tip of a standard colonoscopy, with the aim to obtain folds flattening during withdrawal and preventing the collapse of the mucosa against lenses. This device have been originally used during endoscopic submucosal dissection (ESD) and subsequently tested also for diagnostic colonoscopy in order to enhance visibility of blind areas and improve ADR. A recent meta-analysis performed on 4 studies compared CAC vs standard colonoscopy (SC), showed a higher right ADR (23% vs 17%; OR = 1.49, 95%CI: 1.08-2.05;  $I^2 = 79\%$ ;  $P = 0.01$ ), similar to that obtained with TER, and an improved detection rate of flat adenoma (OR = 2.08; 95%CI: 1.35-3.20;  $P < 0.01$ ) for CAC respect to SC<sup>[20]</sup>. Another meta-analysis of 23 RCTs comparing CAC vs SC showed an increase in detection rate of polyps (OR = 1.17,  $P < 0.01$ ), but no statistically significant difference in ADR<sup>[21]</sup>.

One different mechanical solution is Endorings (EndoAid Ltd., Caesarea, Israel), a silicone-rubber device fitted onto the distal end of the colonoscopy and composed by flexible circular rings that allow mechanical stretch of colonic folds during withdrawal and stabilize the tip to the center of the lumen. A recent multicenter, randomized study showed that EndoRings colonoscopy compared with standard colonoscopy allows a lower polyp miss rate (9.1% vs 52.8%;  $P < 0.001$ ) and a significantly lower adenoma miss rate (10.4% vs 48.3%;  $P < 0.001$ )<sup>[22]</sup>.

Similarly to Endorings, Endocuff (Arc Medical, Leeds, United Kingdom) is a plastic mechanical device provided with rows of finger-like projections, which is mounted onto the distal tip of endoscope. During gently insertion of colonoscopy, finger projections collapse back, while during withdrawal they flare out allowing a mechanical grip with flattening of the colonic folds and centering the tip in the lumen. Two RCTs showed that colonoscopy with Endocuff increase by 63% detection of polyps and by 83% detection of adenoma<sup>[23]</sup>, as well as increase significantly ADR (35% vs 21%;  $P < 0.0001$ ) respect to standard colonoscopy<sup>[24]</sup>.

Contrariwise to these results a subsequent RCT performed on a large number of patients, even showing an higher detection of adenomas sized  $< 6$  mm (443 vs 378;  $P = 0.03$ ) and of flat polyps (213 vs 161;  $P =$

0.03), did not found difference in ADR overall between Endocuff and standard colonoscopy<sup>[25]</sup>. Finally, the use of EndoRings and Endocuff is safe since no major adverse events have been registered so far, while minor drawbacks are the possibility of device detachment from the colonoscopy and risk of slight mucosal lacerations.

One of the latest mechanical advances is G-EYE (Smart Medical Systems Ltd). The G-EYE endoscope employs a permanently-integrated balloon at the tip of the standard endoscope, which is moderately inflated at a selected partial pressure during withdrawal, with the aim to straighten colonic folds, centering the tip and enhancing endoscopic visibility. This technique has been assessed in a randomized tandem study showing that G-EYE colonoscopy increased ADR by 81% ( $P < 0.001$ ) and lowered adenoma miss rate (7.5% vs 44.7%;  $P = 0.0002$ ) compared with standard colonoscopy, without significant adverse events<sup>[26]</sup>.

## DISCUSSION

Oncological screening have a key role in the prevention of CRC and strong evidences from literature clearly demonstrated an increase in 5-year survival and a reduction in mortality for healthy subject undergoing surveillance. Although colonoscopy is the gold standard for CRC screening, its accuracy is still suboptimal and a significant number of adenomas are still missed during examination, mostly due to inherent limitations of the technique that does not allow a full visualization of hidden points especially the ones behind colonic folds and flexures.

Today one of the most important challenges is that to increase the quality of the endoscopic technique, with the aim to enhance ADR and consequently the effectiveness of oncological screening. On this line, many innovations have been developed with promising results. Between these, HDE showed excellent results in terms of image definition and will probably replace over time the standard definition technology. Similarly, the FUSE showed a spectacular 330° field of view, but recent evidences proved no difference in ADR and it is unlikely that this technology will be further developed in the future. The use third eye retroscope showed a gain in ADR, but this device is burdened by an inferior quality of image and the procedure is often time consuming and not always comfortable.

Mechanical advances such as CAP assisted colonoscopy, EndoRings and Endocuff showed promising result in terms of ADR. In addition these solutions are simple to use, economical and safe. Anyway, before recommending a widespread use, further randomized controlled trials are needed in order to better assess performance of these devices. Finally, G-EYE endoscope has been recently introduced and needs further studies.

In conclusion, great technological advances have been made so far, but none of these innovations have been proven to be so effective to be strongly recommended right now in clinical practice worldwide.

Currently existing devices require further assessment, and at the same time new technologies need to be developed.

Waiting for that, we recommend the use of high definition image systems ensuring, at the same time, adherence to quality measures for lower endoscopy, including high cecal intubation rates, withdrawal times of 6 min or longer and optimal quality of bowel preparation.

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## Ampullary cancer of intestinal origin and duodenal cancer - A logical clinical and therapeutic subgroup in periampullary cancer

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### Abstract

Periampullary cancers include pancreatic, ampullary, biliary and duodenal cancers. At presentation, the majority of periampullary tumours have grown to involve the pancreas, bile duct, ampulla and duodenum. This can result in difficulty in defining the primary site of origin in all but the smallest tumors due to anatomical proximity and architectural distortion. This has led to variation in the reported proportions of resected periampullary cancers. Pancreatic cancer is the most common cancer resected with a pancreaticoduodenectomy followed by ampullary



(16%-50%), bile duct (5%-39%), and duodenal cancer (3%-17%). Patients with resected duodenal and ampullary cancers have a better reported median survival (29-47 mo and 22-54 mo) compared to pancreatic cancer (13-19 mo). The poorer survival with pancreatic cancer relates to differences in tumour characteristics such as a higher incidence of nodal, neural and vascular invasion. While small ampullary cancers can present early with biliary obstruction, pancreatic cancers need to reach a certain size before biliary obstruction ensues. This larger size at presentation contributes to a higher incidence of resection margin involvement in pancreatic cancer. Ampullary cancers can be subdivided into intestinal or pancreatobiliary subtype cancers with histomolecular staining. This avoids relying on histomorphology alone, as even some poorly differentiated cancers preserve the histomolecular profile of their mucosa of origin. Histomolecular profiling is superior to anatomic location in prognosticating survival. Ampullary cancers of intestinal subtype and duodenal cancers are similar in their intestinal origin and form a logical clinical and therapeutic subgroup of periampullary cancers. They respond to 5-FU based chemotherapeutic regimens such as capecitabine-oxaliplatin. Unlike pancreatic cancers, *KRAS* mutation occurs in only approximately a third of ampullary and duodenal cancers. Future clinical trials should group ampullary cancers of intestinal origin and duodenal cancers together given their similarities and their response to fluoropyrimidine therapy in combination with oxaliplatin. The addition of anti-epidermal growth factor receptor therapy in this group warrants study.

**Key words:** Periampullary cancer; Pancreatobiliary subtype; Intestinal subtype; Ampullary cancer; Duodenal cancer; Epidermal growth factor receptor; Pancreatic cancer; Chemotherapy; Pancreaticoduodenectomy; *KRAS*

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**Core tip:** Periampullary cancers include pancreatic, ampullary, bile duct and duodenal cancers. Pancreatic cancer is the most common cancer resected with a pancreaticoduodenectomy followed by ampullary, bile duct and duodenal cancer. Patients with resected duodenal and ampullary cancers have better prognosis compared to pancreatic cancer. Ampullary cancers can be subdivided into intestinal or pancreatobiliary subtype cancers with histomolecular staining. Histomolecular profiling is superior to anatomic location in prognosticating survival. Ampullary cancers of intestinal subtype and duodenal cancers are similar in their intestinal origin and form a logical clinical and therapeutic subgroup. They respond to 5-FU based chemotherapeutic regimens such as capecitabine-oxaliplatin.

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## INTRODUCTION

Periampullary cancers are defined as cancers arising within 2 cm of the papilla of Vater and include pancreatic, ampullary, biliary and duodenal cancers<sup>[1]</sup>. The region of the ampulla is anatomically complex because it is the area of convergence of the bile duct, pancreatic duct and the duodenum. Conceptually the distinction between pancreatic, biliary, ampullary and duodenal carcinoma is straightforward. The 7<sup>th</sup> edition 2009 AJCC staging manual states that this distinction is based solely on the presumed anatomical primary site of origin<sup>[2]</sup>. However, in practice by the time of presentation, the majority of periampullary tumours have grown to involve the pancreas, bile duct, ampulla and duodenum. Therefore it may be difficult to define the primary site of origin in all but the smallest tumors<sup>[3]</sup>. As a result the distinction between many non-pancreatic periampullary cancers arising in this region from pancreatic cancer is inherently difficult and subjective<sup>[4]</sup>. This has led to variation in the reported proportions of pancreatic, ampullary, biliary and duodenal cancers resected with a pancreaticoduodenectomy (PD)<sup>[5]</sup>. Pancreatic cancers represent the majority of cancers resected with a PD in most series<sup>[6]</sup>. There are fundamental genomic and molecular differences in the four cancer subtypes<sup>[7]</sup>. There is a need to categorise these cancer subtypes in order to treat them in a way that respects their histological, molecular and behavioural differences.

## PROPORTION OF PERIAMPULLARY CANCER SUBTYPES RESECTED WITH A PD

Pancreatic cancer accounts for the majority of periampullary cancers resected with a PD in most series, followed by ampullary 16%-50%, biliary 5%-39%, and duodenal cancer 3%-17%<sup>[6-8]</sup> (Table 1). The wide variation in the reported incidence and proportion of resected periampullary cancers relates partly to difficulties in accurate determination of the primary tissue origin. This is due to close anatomical proximity of the cancer subtypes and architectural distortion at time of presentation.

Review of pathology slides results in reassignment of cancer origin in a significant number of cases and highlights the importance of central pathology review in clinical trials<sup>[9-12]</sup>. The Pomianowska *et al*<sup>[13]</sup> study of 207 resected periampullary cancers, demonstrated that slide review changed the diagnosis in 27% of cases. Inaccurate subtyping of periampullary cancers or the addition of non-pancreatic cancers to pancreatic cancer studies can distort and may inflate survival data and skew tumour size and stage. Indeed, Verbeke *et al*<sup>[5]</sup>

**Table 1** Proportion of periampullary cancer subtypes resected in pancreaticoduodenectomy series

Study (Institution, author, yr)	n	Pancreatic cancer	Ampullary cancer	Biliary cancer	Duodenal cancer
Johns Hopkins, United States He <i>et al</i> <sup>[14]</sup> , 2014	2564	66%	16%	12%	6%
Academic Medical Centre, The Netherlands Tol <i>et al</i> <sup>[8]</sup> , 2015	760	46%	30%	20%	4%
Taipei Veterans General Hospital, Taiwan Chen <i>et al</i> <sup>[15]</sup> , 2013	501	34%	50%	10%	5%
Ohio State University, United States Hatzaras <i>et al</i> <sup>[24]</sup> , 2010	346	72%	23%	5%	0
Oslo University Hospital, Norway Pomianowska <i>et al</i> <sup>[16]</sup> , 2013	207	33%	28%	14%	25%
South Australian Pathology Database, Adelaide, Australia Chandrasegaram <i>et al</i> <sup>[6]</sup> , 2015	115	55%	28%	15%	3%
University Medical Center Groningen, The Netherlands Van Roest <i>et al</i> <sup>[25]</sup> , 2008	121	42%	25%	16%	17%
Leeds Teaching Hospitals NHS Trust, United Kingdom Menon <i>et al</i> <sup>[76]</sup> , 2009	83	33%	29%	39%	N/I
Queen Elizabeth Hospital, Birmingham, United Kingdom Jarufe <i>et al</i> <sup>[28]</sup> , 2004	251	53%	35%	12%	N/I
University of California San Diego, United States Katz <i>et al</i> <sup>[17]</sup> , 2004	120	62%	26%	8%	4%

N/I: May not have been included.

proposed that the failure to accurately distinguish the cancer subtypes represented the most important factor in the variation in clinicopathological and survival data in periampullary cancer studies.

## DIFFERENCES IN SURVIVAL IN PERIAMPULLARY CANCERS

Pancreatic cancer has the poorest survival amongst periampullary cancers. Reported median survival for each cancer subgroup is outlined in Table 2. He *et al*<sup>[14]</sup> study of 2564 patients with resected periampullary cancers from Johns Hopkins, reported that patients with duodenal cancer had the highest estimated 5-year survival (49%), followed by ampullary cancer (45%), distal bile duct cancer (27%), and pancreatic cancer (18%)<sup>[14]</sup>. The recent Dutch study by Tol *et al*<sup>[8]</sup> of 760 cancer resections reported that duodenal cancer patients had the most favourable survival. In the Taiwanese study of 501 patients with periampullary cancer, Chen *et al*<sup>[15]</sup> reported that patients with ampullary cancer formed the majority (76%) of long-term ( $\geq 5$  years) survivors.

## DIFFERENCES IN NODAL, NEUROVASCULAR AND MARGIN STATUS IN PERIAMPULLARY CANCERS

The poorer survival seen with pancreatic cancer has been attributed to differences in tumour behavior and invasiveness<sup>[6,16-18]</sup>. Pancreatic cancers have a higher incidence of nodal, neural and vascular invasion compared to non-pancreatic periampullary cancers<sup>[19-25]</sup>. Pancreatic cancers also tend to have a much higher incidence of margin positivity<sup>[14,22,26,27]</sup>. Multiple studies

have demonstrated that resection margin status, neurovascular invasion, lymph node involvement and lymph node ratio  $> 0.2$  are important prognostic factors for survival with periampullary adenocarcinomas<sup>[8,28,29]</sup>.

Zenali *et al*<sup>[30]</sup>, showed that patients with duodenal and ampullary cancer had lower frequencies of nodal metastasis, margin involvement and had improved survival compared to patients with pancreatic cancer. Interestingly such differences were not demonstrated between patients with ampullary and duodenal cancers.

Historically periampullary tumours have been treated as a single group. There is strong evidence that non-pancreatic periampullary cancers require further stratification in future clinical trials<sup>[7,31]</sup>.

## AMPULLARY CANCER SUBTYPES: INTESTINAL AND PANCREATOBILIARY SUBTYPES

The ampulla of Vater is made up of the union of 2 distinct mucosal tissue types, by virtue of its location at the opening of the bile duct into the duodenum. The ampullo-duodenal part of the papilla is lined by intestinal mucosa and the deeper part of the ampulla is lined by pancreatobiliary ductal mucosa. In 1994 Kimura *et al*<sup>[32]</sup> classified ampullary cancers into two histological subtypes of either intestinal or pancreatobiliary subtype. Differentiating ampullary cancers into these subtypes is aided by the use of histomolecular staining. This method of subtyping ampullary cancers can overcome difficulties in distinguishing these cancers on the basis of histomorphology alone, as even poorly differentiated cancers preserve the histological marker profile of their mucosa of origin<sup>[33]</sup>.

**Table 2** Median survival of patients following resection of periampullary cancers

Study (Institution, author, yr)	n	Median survival, mo			
		Pancreatic cancer	Ampullary cancer	Biliary cancer	Duodenal cancer
Johns Hopkins, United States He <i>et al</i> <sup>[14]</sup> , 2014	2564	19	47	23	54
Academic Medical Centre, The Netherlands Tol <i>et al</i> <sup>[8]</sup> , 2015	760	19	36	29	Not reached
Taipei Veterans General Hospital, Taiwan Chen <i>et al</i> <sup>[15]</sup> , 2013	501	13.7	28.9	24.4	21.7
Ohio State University, United States Hatzaras <i>et al</i> <sup>[24]</sup> , 2010	346	17.1	44.3	17.9	N/I
Queen Elizabeth Hospital, Birmingham, United Kingdom Jarufe <i>et al</i> <sup>[28]</sup> , 2004	251	13.4	35.5	16	N/I

N/I: Subtype not included or reported.

Transcription factor CDX2 is expressed in the nucleus of intestinal epithelium<sup>[34,35]</sup>. Mucin (MUC) 1 is expressed at the apical border of cells of pancreatobiliary ductal origin<sup>[36]</sup>. In addition to CDX2 and MUC 1, other markers have been used to subtype ampullary cancers. CDX2, CK 20 and MUC 2 are expressed in intestinal subtype cancers, whereas CK 7, CK 17, MUC 1 and MUC 4 are expressed in pancreatobiliary subtype cancers<sup>[37]</sup>.

The markers have varying sensitivity and specificity in tissue subtyping and often their reported performance depends more on the gold standard to which they are compared to than the clinical utility of the markers<sup>[38]</sup>. For example, if a very rigid definition is applied so that the term ampullary carcinoma only applies to tumours in which there is absolute certainty of origin from the ampullary epithelium (usually very small tumours centred exquisitely on the ampulla of Vater), then ampullary carcinomas can be expected to be essentially uniformly CDX2 positive and MUC 1 negative. That is, the CDX2 positive, MUC 1 negative profile would be highly sensitive for ampullary carcinoma in this subgroup which, are not difficult to classify as ampullary by a conventional anatomic approach. However, if a more liberal interpretation is applied so that larger tumours which probably, possibly or potentially originally arose from the ampullary epithelium are considered ampullary, then the CDX2 positive, MUC 1 negative profile becomes much less specific for ampullary carcinoma both because larger tumours may lose differentiation and because this expanded subgroup must include at least some tumours which originally arose from the pancreas and merely mimic ampullary carcinoma. This is problematic because it is exactly these anatomically difficult to classify tumours in which ancillary markers would be most useful clinically. Therefore a more sensible approach to the investigation of ancillary markers of ampullary status is to not compare their expression to the older anatomical classification (which is known to be flawed) but to compare their expression to outcome or response to therapy.

For example, Chang *et al*<sup>[12]</sup> subdivided anatomical

periampullary cancers based on protein expression and immunohistochemistry to distinct cancer subtypes. In their study of 208 ampullary cancers, 74% were intestinal subtype (CDX2 +ve, MUC1-ve), and 22% were pancreatobiliary subtype (CDX2 -ve, MUC 1 +ve).

The Chang study demonstrated that patients with pancreatobiliary subtype cancers have poorer survival compared with those with intestinal subtype cancers consistent with historical studies<sup>[39-41]</sup>. The Schueneman *et al*<sup>[42]</sup> study of 163 ampullary cancers validated the prognostic role of the histomolecular results of Chang *et al*<sup>[12]</sup>, using MUC 1 and CDX2. In their study, 25% of their patients had pancreatobiliary subtype tumours. These patients had significantly poorer median overall survival of 21.1 mo compared to patients with intestinal subtype tumours, 108.3 mo ( $P < 0.0001$ )<sup>[42]</sup>.

In the Schiergens retrospective study of their prospective database, patients with pancreatobiliary subtype cancers receiving adjuvant gemcitabine had improved overall survival (32 mo vs 13 mo,  $P = 0.013$ ) unlike patients with intestinal subtype cancers who tended to have poorer survival with gemcitabine (35 mo vs 112 mo,  $P = 0.193$ )<sup>[39]</sup>. This suggests patients with pancreatobiliary subtype cancers may benefit from gemcitabine.

Similarly Leo *et al*<sup>[3]</sup> demonstrated significantly higher pathological stage and worse overall survival in pancreatic compared to intestinal phenotype ampullary carcinomas. In a more recent study of 510 patients undergoing PD, histopathologic phenotype was superior to tumour anatomic location in prognosticating survival. There was no difference in survival between pancreatobiliary subtype cancers and pancreatic cancer (33.3 mo vs 31.4 mo,  $P = 0.66$ )<sup>[43]</sup>.

Whilst these studies emphasize the clinical outcome differences between pancreatobiliary phenotype and intestinal phenotype ampullary carcinomas, at the genomic level these tumours show both similarities and differences. Yachida *et al*<sup>[44]</sup> reported whole exome sequencing in a cohort of Japanese and American patients with ampullary cancers. While ampullary cancers were found to be similar to colorectal cancers, and pancreatobiliary subtype cancers

similar to pancreatic cancer, the two subtypes also share similar mutational patterns and signatures differentiating them from colorectal and pancreatic cancers. The authors found tumour suppressor gene *ELF3*, to be a significant driver of ampullary cancers present in both histological subtypes<sup>[44]</sup>.

Gingras *et al*<sup>[45]</sup> evaluated 98 ampullary adenocarcinomas, comparing these to 44 distal bile duct and 18 duodenal adenocarcinomas. Mutations in the WNT signaling pathway occurred in approximately half and *ELF3* approximately 10% of patients across all three tumour types<sup>[45]</sup>.

## A LOGICAL SUBGROUP: AMPULLARY CANCERS OF INTESTINAL SUBTYPE AND DUODENAL CANCERS

Ampullary cancers of intestinal subtype and duodenal cancers are similar in their intestinal origin and form a logical clinical and therapeutic subgroup of periampullary cancers. While *KRAS* mutation occurs in over 90% of pancreatic cancers, both these cancers have a much lower incidence of *KRAS* mutation<sup>[7,46]</sup>.

Valsangkar *et al*<sup>[11]</sup> reported the incidence of *KRAS* mutation in 75 patients with ampullary cancer was 33%. This was supported by the Kwon *et al*<sup>[47]</sup> study of 62 ampullary cancers revealing a similar 31% incidence of *KRAS* mutation.

Mikhitarian *et al*<sup>[48]</sup> analysed the incidence of *KRAS* mutation by ampullary cancer subtype. They reported that 52% of 25 intestinal subtype cancers and 42% of 24 pancreatobiliary subtype cancers had *KRAS* mutation. In the Hechtman *et al*<sup>[49]</sup> study of 18 pancreatobiliary subtype cancers and 14 intestinal subtype cancer, there was an increased frequency of *KRAS* mutation in the pancreatobiliary subtype cancers (61% vs 29%).

While small bowel cancers are rare, the duodenum represents the most common site (56%) for adenocarcinoma of the small bowel, followed by the jejunum (16%) and ileum (13%)<sup>[50,51]</sup>.

As with ampullary cancers, the incidence of *KRAS* mutation is much lower in duodenal cancers compared to pancreatic cancer. Fu *et al*<sup>[52]</sup> reported the incidence of *KRAS* mutations to be 35% in 78 duodenal cancers.

Given ampullary and duodenal cancers have a much lower incidence of *KRAS* mutation compared to pancreatic cancer, the addition of anti-epidermal growth factor receptor (EGFR) treatment in the metastatic and advanced disease may well be a fruitful area of study on the basis of the morphological and biological similarity to *KRAS* wild type colorectal adenocarcinoma where the benefits of this treatment are well proven<sup>[53,54]</sup>.

## ADJUVANT STUDIES IN PERIAMPULLARY CANCERS

Historically, non-pancreatic periampullary cancers have

been included in trials of pancreatic cancer<sup>[55]</sup>. In a summary of eleven of the most important randomized controlled trials of adjuvant trials in pancreatic cancer, 4 studies deliberately included non-pancreatic cancers. In most studies, shortcomings in pathological assessment and the lack of standardized pathology to determine the tissue of origin of these cancers may have led to the unintentional inclusion of non-pancreatic cancers<sup>[56]</sup>.

In the ESPAC-3 periampullary cancer trial, 428 patients with periampullary cancer; 297 with ampullary cancers, 96 with bile duct cancers, and 35 with other cancers were randomized to either observation ( $n = 144$ ) or 6 mo of 5-FU and Folinic acid ( $n = 143$ ) or gemcitabine ( $n = 141$ ). There was no survival benefit from adjuvant treatment. However, after adjusting for age, bile duct cancer, poor tumour differentiation and lymph node involvement, on multiple regression analysis there was a survival benefit for chemotherapy compared to observation with a HR of 0.75 (95%CI: 0.57-0.98,  $P = 0.03$ )<sup>[57]</sup>.

A recent meta-analysis of 1671 patients reported no survival benefit for adjuvant chemotherapy or chemoradiotherapy in the management of periampullary cancer<sup>[58]</sup>. The median 5-year survival was 40.0% with adjuvant treatment vs 37.5% in the control group with a HR of 1.08 (95%CI: 0.91-1.28;  $P = 0.067$ ).

Interestingly, the recent UK BILCAP study has shown a benefit for adjuvant capecitabine in bile duct cancers. Of the 447 patients in the study, 156 (35%) had extrahepatic bile duct cancers which would include distal bile duct cancers resected with a PD. In the per-protocol analysis, median survival with capecitabine was 53 mo (95%CI 40-not reached) compared to 36 mo with observation (95%CI: 30-44), HR = 0.75 (95%CI: 0.58-0.97,  $P = 0.028$ )<sup>[59]</sup>.

Duodenal cancer studies are often reported with other small bowel cancers, including those arising from the jejunum and ileum. Halfdanarson *et al*<sup>[60]</sup> in a retrospective review of 491 small bowel adenocarcinomas (57% duodenum; 29% jejunum, 10% ileum) reported a median overall survival of 20.1 mo. Adjuvant therapy did not improve survival in their study. In the Khan *et al*<sup>[61]</sup> study of 48 resected small bowel adenocarcinomas (63% duodenum, 21% jejunum, 15% ileum), 56% received adjuvant chemotherapy. Adjuvant therapy again did not improve survival in their study.

In the study by Overman *et al*<sup>[62]</sup> of 54 resected small bowel adenocarcinomas (67% duodenum, 20% jejunum, ileum 13%) although there was no improvement in overall survival with adjuvant chemotherapy, on multivariate analysis, adjuvant therapy improved disease-free survival (HR = 0.27; 95%CI: 0.07-0.98,  $P = 0.05$ )<sup>[62]</sup>.

In a more recent National Cancer Database study (NCDB), patients with resected small bowel adenocarcinoma who received adjuvant chemotherapy ( $n = 1674$ ) were compared to those undergoing surgery alone ( $n = 3072$ ). This study found that adjuvant chemotherapy improved survival in patients with AJCC stage III disease (Median OS 42.4 mo vs 26.1 mo;  $P <$



0.001)<sup>[63]</sup>. The addition of radiotherapy did not improve survival in another adjuvant NCDB study of duodenal adenocarcinoma patients<sup>[64]</sup>.

The role of adjuvant chemotherapy in small bowel adenocarcinomas will be investigated in the international phase III study (the BALLAD study) promoted by the International Rare Cancer Initiative<sup>[65]</sup>.

## SYSTEMIC CHEMOTHERAPY IN ADVANCED AND METASTATIC AMPULLARY AND DUODENAL CANCER

Several studies have investigated the role of chemotherapy in the advanced or metastatic setting<sup>[66-68]</sup>. Response rates in ampullary and small intestinal cancers with chemotherapy alone vary between 10%-50%.

A retrospective study of 905 resected periampullary cancers, reported fluoropyrimidine-based chemotherapy was superior to gemcitabine-based chemotherapy in prolonging time to progression in metastatic ampullary cancer suggesting it is a more appropriate first-line approach for ampullary cancers<sup>[69]</sup>.

Overman *et al*<sup>[70]</sup> achieved an overall response rate (complete response and partial response) of 50% (95%CI: 31%-69%) in their phase II study of capecitabine and Oxaliplatin (CAPOX) for advanced or metastatic ampullary and small intestinal adenocarcinoma<sup>[70]</sup>. Patients with intestinal adenocarcinoma ( $n = 18$ ) had a response rate of 61% (95%CI: 36-83%) and those with ampullary adenocarcinoma ( $n = 12$ ) a response rate of 33% (95%CI: 10%-65%). The poorer response rates in the ampullary compared to the intestinal cancers in this study was thought to be due to the inclusion of ampullary adenocarcinomas of pancreatobiliary origin which may be less responsive to CAPOX.

In the study by Khan *et al*<sup>[61]</sup>, 46/59 (78%) patients received systemic chemotherapy for relapsed, unresectable or metastatic small bowel adenocarcinoma (68% duodenum, 19% jejunum, 14% ileum). Of these, 40 were evaluable for response with a response rate of 50% (1 Complete response, 19 Partial response). The overall 1 year survival was better with chemotherapy 60.9% (95%CI: 45.8-76.0) vs 27.3% ( $P = 0.042$ ). Of the 23 patients who received triplet chemotherapy, 13 received EOX (Epirubicin, Oxaliplatin and Capecitabine) and 4 received ECF (Epirubicin, Cisplatin and 5-FU). Of the 18 patients on doublet chemotherapy, 6 received CAPOX, 4 received FOLFOX (5-FU and oxaliplatin), 3 received FOLFIRI (5-FU and irinotecan) and 3 received capecitabine with Mitomycin C<sup>[61]</sup>.

In a large multicentre retrospective series of different chemotherapy regimens in small bowel cancers, 38 patients received FOLFOX with a tumour response rate of 34% and 11 patients received FOLFIRI with a response rate of 9%. The authors concluded that FOLFOX is the most effective platinum-based chemotherapy for small bowel cancers<sup>[71]</sup>.

From these studies, the combination of a fluoro-

pyrimidine-regimen and oxaliplatin such as FOLFOX or CAPOX appears to be an active regimen in both ampullary and small bowel cancer (*i.e.*, duodenal cancer) suggesting this is a logical treatment regimen in this subgroup of periampullary cancers.

## ANTI-EGFR TREATMENT

The lower incidence of *KRAS* mutation in both ampullary and duodenal cancer suggest a potential role for anti-EGFR therapy trials in this subgroup<sup>[72]</sup>. In the phase II study of panitumumab in *KRAS* wild-type metastatic adenocarcinoma of the small bowel and ampulla, 9 patients (1 ampullary - pancreatobiliary subtype, 3 duodenal, 5 jejunal/ileal) received panitumumab with minimal clinical activity. This was thought to relate to these tumours being of foregut origin, given the recent findings of less benefit with anti-EGFR therapy in right sided colon cancers compared to left sided cancers<sup>[73]</sup>.

Santini *et al*<sup>[74]</sup> reported the use of anti-EGFR treatment with Cetuximab in advanced duodenal ( $n = 2$ ) and jejunal ( $n = 2$ ) cancers. Cetuximab was associated with CPT-11-based chemotherapy in first-line (2 patients) or second-line (2 patients) therapy for metastatic disease. The patients previously treated had progressed on Folfiri. One patient had a complete response, 2 patients had a partial response and one had stable disease.

While targeted therapy against anti-EGFR pathway is not established in advanced small intestinal cancers, studies are currently evaluating the safety and efficacy of these targeted therapies in this group<sup>[75,76]</sup>.

## CONCLUSION

Ampullary and duodenal cancer form a significant proportion of cancers resected with a PD. A strong argument can be made that future clinical trials should group ampullary cancers of intestinal origin and duodenal cancers together given their similarities and their response to fluoropyrimidine therapy in combination with oxaliplatin. Furthermore, treatment response should be compared to both established (CDX2 and MUC1) and more investigational biomarkers. The addition of anti-EGFR therapy in this group warrants further study.

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## Retrospective Study

# Clinical outcomes of Clutch Cutter endoscopic submucosal dissection for older patients with early gastric cancer

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**Informed consent statement:** The ethics committee of Aso Iizuka Hospital approved this study (registration No. 12120).

Written informed consent was obtained from all patients.

**Conflict-of-interest statement:** Kazuya Akahoshi and Hidenobu Akahane (Fujifilm) have applied for a patent in Europe for the CC described in this article. Japan, China, and the United States have already granted patents.

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## Abstract

### AIM

To evaluate the clinical outcome of endoscopic sub-

mucosal dissection using the Clutch Cutter (ESDCC) in older patients.

## METHODS

We reviewed 232 consecutive patients with early gastric cancer who underwent ESDCC between June 2010 and February 2014 at Aso Iizuka Hospital. We divided patients into two groups according to age: Older patients ( $> 80$  years,  $n = 64$ ) and non-older patients ( $\leq 80$  years,  $n = 168$ ). We retrospectively compared the prevalence rates of pre-existing comorbidities, anticoagulant therapy, *en bloc* resection, mean duration of hospitalization, incidence of ESDCC-related complications, change in performance status (PS) before and after ESDCC, and financial cost of admission.

## RESULTS

The older group comprised 64 patients with a mean age of 84.1 years, and the non-older group comprised 168 patients with a mean age of 69.5 years. Older patients had significantly more pre-existing comorbidities than did non-older patients, specifically heart disease ( $P < 0.05$ ). The *en bloc* resection rate in non-older patients was significantly higher than that in older patients (100% *vs* 95.3%,  $P = 0.02$ ). There were no significant differences between the older and non-older groups in the incidence of ESDCC-related complications (*i.e.*, postoperative bleeding and perforation) and the post-ESDCC change in PS. There were also no significant differences between the older and non-older groups in the mean duration of hospitalization (11.4 and 10.7 d, respectively) and financial cost of admission (657040 JPY and 574890 JPY, respectively).

## CONCLUSION

ESDCC has a good clinical outcome in older patients.

**Key words:** Older patients; Clutch Cutter; Endoscopic submucosal dissection; Early gastric cancer; Financial cost; Duration of hospitalization

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**Core tip:** No previous reports have described the clinical outcomes of endoscopic submucosal dissection using the Clutch Cutter (ESDCC) for older patients with early gastric cancer (EGC). The present study evaluated the clinical outcomes, including medical economics, associated with ESDCC for older patients. There was no significant difference between older patients and non-older patients in the rate of ESDCC-related complications. There was also no significant difference between older and non-older patients in the mean duration of hospitalization and medical economics. We conclude that ESDCC is safe and effective for older and non-older patients with EGC.

Otsuka Y, Akahoshi K, Yasunaga K, Kubokawa M, Gibo J, Osada S, Tokumaru K, Miyamoto K, Sato T, Shiratsuchi Y, Oya M, Koga H, Ihara E, Nakamura K. Clinical outcomes of Clutch Cutter



Figure 1 The distal tip of the Clutch Cutter (long type: Blade length of 5 mm).

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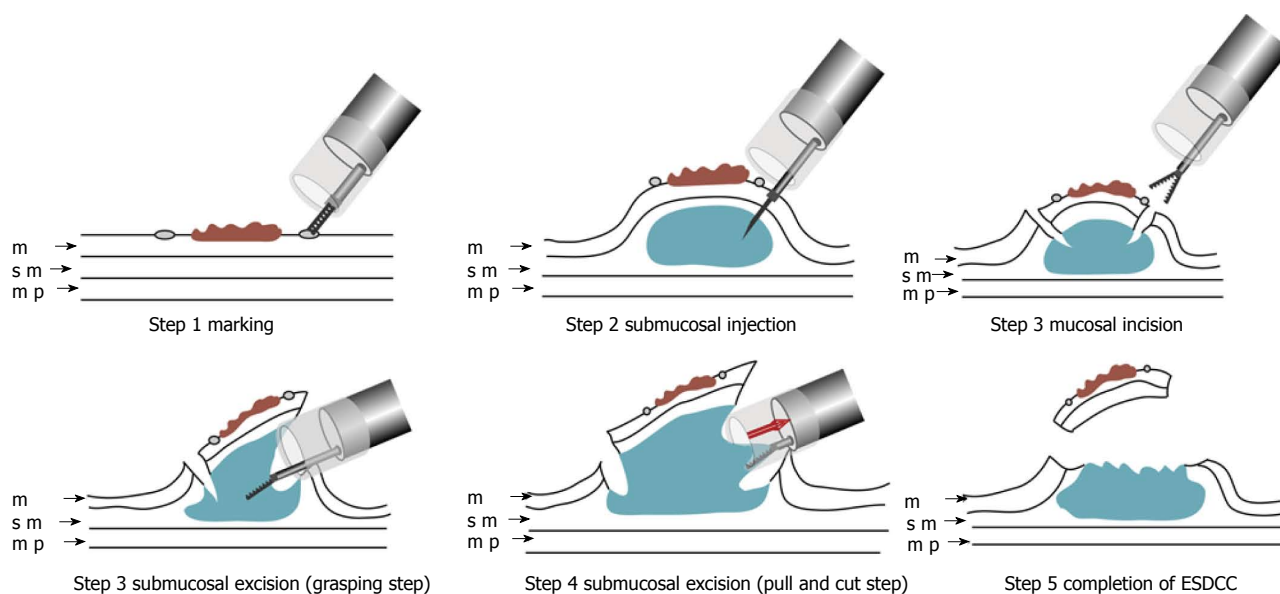
## INTRODUCTION

In an increasingly older society, a growing number of endoscopic treatments are being performed in patients with age-associated comorbidities<sup>[1]</sup>. Endoscopic submucosal dissection (ESD) for early gastric cancer (EGC) has become widely accepted, as it provides *en bloc* resection and histologically complete resection, and it is less invasive than surgical resection<sup>[2-5]</sup>. ESD recently been reported to be a safe and reliable procedure for treating early gastrointestinal tract cancer in older patients<sup>[6-10]</sup>. However, there is little information on the medical costs of ESD, the mean duration of hospitalization, and the change in performance status (PS) before and after ESD. Akahoshi and Fujifilm<sup>[11]</sup> developed a grasping-type scissors forceps [*i.e.*, the "Clutch Cutter" (CC), Fujifilm, Tokyo, Japan, Figure 1] for safe ESD. We previously showed that ESD using the CC (ESDCC) is a safe and effective method for treating patients with early cancer in the esophagus, stomach, duodenum, or colorectum<sup>[11-17]</sup>. However, no reports have described the clinical outcomes of ESDCC for older patients with EGC. The present study evaluated the clinical outcomes of ESDCC for older patients with EGC, including the medical economics associated with ESDCC.

## MATERIALS AND METHODS

### Patients

ESDCC was performed in 269 consecutive patients with EGC between June 2010 and February 2014 at Aso Iizuka Hospital. A total of 37 patients were excluded because their post-ESD histological analysis did not meet the clinical indication criteria for ESD proposed by Gotoda *et al.*<sup>[18]</sup> and the Japanese Gastric Cancer Association<sup>[19]</sup> (*i.e.*, listed in the exclusion criteria group).



**Figure 2** Schema showing endoscopic submucosal dissection using the Clutch Cutter technique. m: Mucosa; sm: Submucosa; mp: Muscularis propria; ESDCC: Endoscopic submucosal dissection using the Clutch Cutter.

A total of 232 consecutive patients with EGC were enrolled in this retrospective study.

We divided the patients into two groups according to age: Older patients (> 80 years, mean age: 84.1 SD  $\pm$  3.2 years old) and non-older patients ( $\leq$  80 years, mean age: 69.5 SD  $\pm$  7.3 years old). The following factors were retrospectively compared between the two groups: Pre-existing comorbidities, anticoagulant therapy, *en bloc* resection rate, mean duration of hospitalization, incidence of ESDCC-related complications, change in PS before and after ESD, and financial cost of admission. We used a prospectively maintained ESDCC database for the analyses of anticoagulant therapy, *en bloc* resection rate, and incidence of ESDCC-related complications; our institutional medical and accounting records for each patient were used to analyze pre-existing comorbidities, mean duration of hospitalization, change in PS after ESD, and financial cost of admission. PS was classified using the Eastern Cooperative Oncology Group scale. The indication for ESD was a PS score of 0, 1, or 2.

### ESD with the Clutch Cutter procedure

Detailed technical procedures of ESDCC have been reported previously<sup>[11-17]</sup> (Figure 2). ESDCC was conducted using a single-channel therapeutic endoscope (EG-450RD5; Fujifilm) or a two-channel multi-bending endoscope (GIF-2T240M; Olympus, Tokyo, Japan). A long, transparent hood (F-01; Top Co. Ltd., Tokyo, Japan) was attached to the tip of the endoscope to facilitate submucosal dissection by elevating the lesion. Circumferential markings were made using the CC in closed mode. A hyaluronic acid solution (MucoUp; Johnson and Johnson, Tokyo, Japan) with diluted epinephrine (0.0002%) and indigo carmine (0.0002%) was injected into the submucosal layer to lift up the lesion. The target mucosal and submucosal layer tissues

were grasped, lifted up, and cut using the CC. Finally, the lesion was completely resected using the CC (Figure 3). When bleeding occurred during the procedure, it was treated *via* coagulation with the CC. The forced coagulation mode (VIO 300D; Erbe, Tübingen, Germany) 30 W (effect 3) was used for marking, the endo cut Q mode (effect 2, duration 3, interval 1) was used for cutting, and the soft coagulation mode 100 W (effect 5) was used for hemostatic treatment.

### Statistical analysis

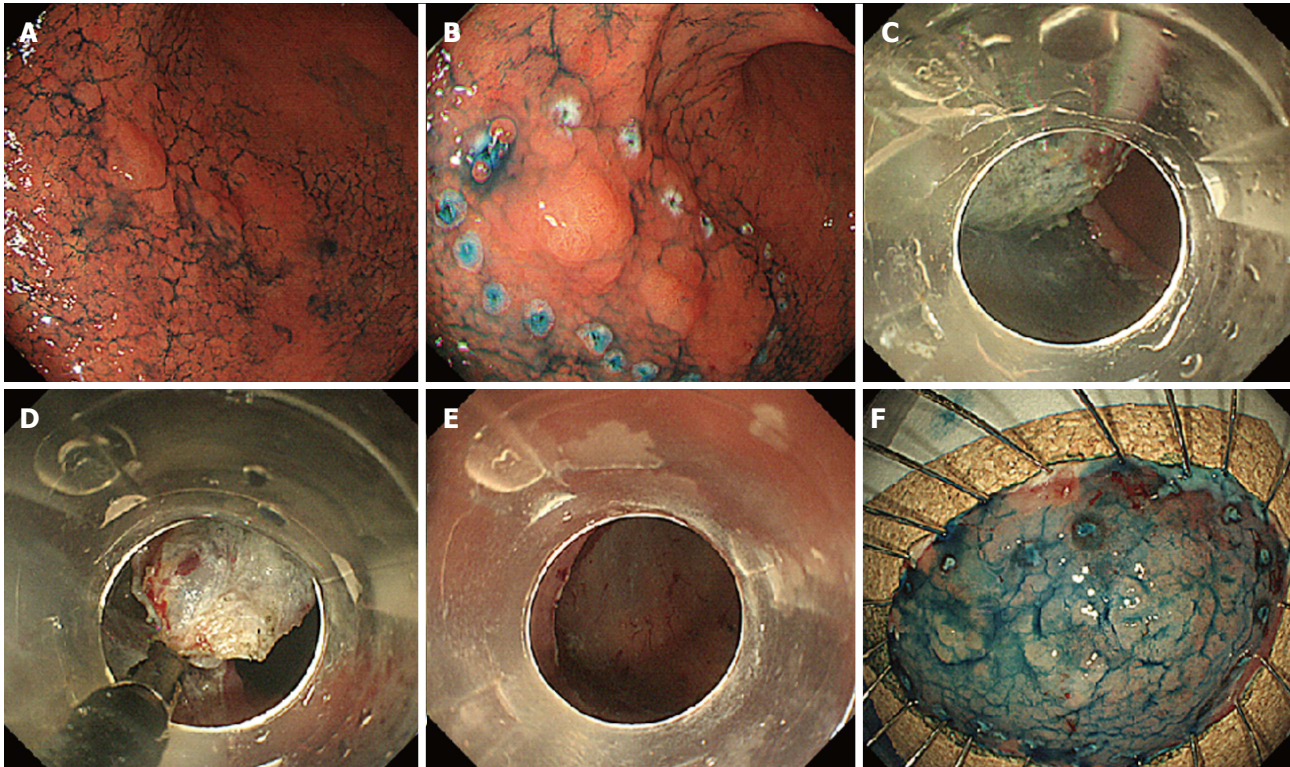
Statistical analyses for comparison between the older and non-older groups was performed using the  $\chi^2$ -test, the Wilcoxon/Kruskal-Wallis test, and Fisher's exact probability test. The  $\chi^2$ -test was used to evaluate intergroup differences in anticoagulant therapy and *en bloc* resection, incidence of ESDCC-related complications, and change in PS before and after ESD. The Wilcoxon/Kruskal-Wallis test was used to evaluate intergroup differences in the mean duration of hospitalization and financial cost of admission. Fisher's exact probability test was used to evaluate intergroup differences in pre-existing comorbidities. Differences were considered significant at  $P < 0.05$ .

## RESULTS

### Patients' characteristics

The older group comprised 64 patients with a mean age of 84.1 years, and the non-older group comprised 168 patients with a mean age of 69.5 years (Table 1). The two groups significantly differed in terms of age, but not sex. Older patients had a significantly higher rate of pre-existing comorbidities than did non-older patients ( $P < 0.05$ ), especially heart disease. The proportion of the older group receiving anticoagulant therapy was not





**Figure 3** Endoscopic submucosal dissection using the Clutch Cutter in an 82-year-old Japanese male. A: Indigo carmine was sprayed to demarcate the lesion; B: Markings outside the lesion; C and D: The submucosal tissue under the lesion was gradually grasped and dissected from the muscle layer; E: The lesion was completely cut from the muscle layer; F: Fixation of the specimen.

**Table 1** Patient characteristics *n* (%)

	Older group ( <i>n</i> = 64)	Non-older group ( <i>n</i> = 168)	<i>P</i> value
Mean age	84.2	69.5	
Gender ratio (M/F)	44/20	118/50	NS
No. of performance state 3 or 4	5 (7.8)	4 (2.4)	
Pre-existing comorbidity			
Total prevalence rates	58 (90.6)	123 (73.2)	0.0042
Cerebral infarction	11 (17.2)	17 (10.1)	NS
Cardiovascular disease	23 (35.9)	25 (14.9)	0.0004
Respiratory disease	8 (12.5)	12 (7.1)	NS
Chronic renal dysfunction	8 (12.5)	21 (12.5)	NS
Liver cirrhosis	1 (1.6)	12 (7.1)	NS
Diabetes	20 (31.2)	47 (28.0)	NS
Hypertension	44 (68.8)	98 (58.3)	NS
Senile dementia	7 (10.9)	14 (8.3)	NS
Anti-coagulant therapy	4 (6.3)	12 (7.1)	NS

NS: Not significant.

significantly different to that of the non-older group.

#### Tumor characteristics

No significant between-group differences were found regarding the macroscopic type, tumor size, histological type, and ESD indication categories (Table 2). However, the proportion of upper lesions was significantly higher in the older group (43.8%) than in the non-older group (23.2%) ( $P = 0.0042$ ).

**Table 2** Tumors characteristics

	Older group ( <i>n</i> = 64)	Non-older group ( <i>n</i> = 168)	<i>P</i> value
Location			
Upper	28	39	0.019
Middle	16	55	NS
Lower	20	72	NS
Residual stomach	0	2	
Mean tumor size	17.5	15.6	NS
Histological type			
Well differentiated	58	149	NS
Moderately differentiated	5	10	NS
Poorly differentiated	0	8	NS
Papillary differentiated	1	1	NS
Category of indication			
Guideline lesion	47	118	NS
Lesion included in the expanded indications	17	50	NS

NS: Not significant.

#### Technical outcomes

The R0 resection rate in the non-older group was significantly higher than that in the older group (100% vs 95.3%,  $P = 0.02$ , Table 3). However, the R0 resection rate was greater than 95% in both groups. The postoperative bleeding rates of the older and non-older groups were 1.6% (1/64) and 4.8% (8/168), respectively. Perforation occurred in only one (1.6%) patient in the older group; endoscopic clipping was performed in this patient and the



**Table 3** Technical outcome *n* (%)

	Older group ( <i>n</i> = 64)	Non-older group ( <i>n</i> = 168)	<i>P</i> value
<i>En bloc</i> resection	63 (98.4)	168 (100)	NS
R0 resection	61 (95.3)	168 (100)	0.03
Complications			
Intraoperative hemorrhage	0 (0)	0 (0)	NS
Intraoperative perforation	1 (1.6)	0 (0)	NS
Postoperative hemorrhage	1 (1.6)	8 (4.8)	NS
Postoperative perforation	0 (0)	0 (0)	NS

NS: Not significant.

perforation was cured. There was no significant difference between the two groups in the rate of ESDCC-related complications.

### Social and economic outcomes

Three patients in the older group and one patient in the non-older group showed a worse PS after ESD, but there was no significant difference between groups in the prevalence of a worse PS after ESD. The mean duration of hospitalization in the older and non-older groups was 11.4 and 10.7 d, respectively. The mean financial costs of admission for the older and non-older groups were 657040 JPY and 574890 JPY, respectively. There were no significant differences between the two groups in duration of hospitalization or admission costs (Table 4).

## DISCUSSION

According to the 2014 fiscal statistics published by the Ministry of Health, Labour and Welfare, the life expectancies of men and women are 80.5 years old and 86.8 years old, respectively<sup>[20]</sup>. The natural history of EGC is unclear. However, a long life expectancy and an aging population will inevitably lead to an increased number of older patients with EGC in Japan. Long-term outcomes suggest that implementation of ESD for older patients with a satisfactory PS will increase life expectancy<sup>[21]</sup>. Therefore, we investigated the clinical outcomes associated with older patients who received ESDCC for EGC, including economic and social aspects.

As expected, older patients in our study had significantly more pre-existing comorbidities than did non-older patients. In our study, the rate of pre-existing comorbidities was higher than that in previous reports because the mean age of our patients was older than that in previous reports<sup>[8,22]</sup>. Tokioka *et al.*<sup>[8]</sup> and Chinda *et al.*<sup>[22]</sup> reported that older patients were more likely to receive anticoagulant therapy than non-older patients. However, the proportions of older and non-older patients in our study who received anticoagulant therapy were almost equal. The current study included five (7.8%) and four (2.4%) patients who had PSs of 3 and 4 in the older group and the non-older group, respectively,

**Table 4** Social and economic outcomes *n* (%)

Parameter	Older group ( <i>n</i> = 64)	Non-older group ( <i>n</i> = 168)	<i>P</i> value
Worsening of the performance status	3/64 (4.7)	1/168 (0.6)	NS
Mean duration of hospitalization (d)	11.4	10.7	NS
Mean financial cost of admission (JPY)	657040	574890	NS

NS: Not significant.

as they strongly desired treatment. The PSs of these patients did not change after the procedure. However, three (4.7%) patients in the older group and one (0.6%) patient with senile dementia in the non-older group showed a worse PS after ESD. Three patients in the older group had several pre-existing comorbidities. Although ESD is less invasive than an operation, care should be taken regarding patients with several pre-existing comorbidities.

No significant between-group differences were observed regarding macroscopic type, tumor size, histological type, and ESD indication categories. ESD was performed on the lesions of these patients, similar to a previous report<sup>[8]</sup>. In our study, the proportion of upper lesions was significantly higher in the older group than in the non-older group. Furthermore, the R0 resection rate in the non-older group was significantly higher than that in the older group. The tumor location likely affects the difficulty of the ESD procedure, and so the greater number of upper lesions in older patients might have affected the technical outcomes.

The current study did not show a significant difference between older and non-older patients in the rate of ESDCC-related complications (*i.e.*, postoperative bleeding and perforation). The reported perforation and bleeding rates of ESD using a knife device range from 1.2% to 8.2% and from 5.3% to 15.6%, respectively<sup>[23–30]</sup>. Our complication rate was low compared with that reported in previous studies that used conventional knives<sup>[23–30]</sup>. Inevitable risk factors associated with knife devices for ESD-related complications include defects of fixation (inaccurate targeting) and compression (hemostatic effect), as well as pushing the knife into the target tissue (where the pushing force is in the direction of the muscle layer) with an electric discharge<sup>[14]</sup>. The CC can accurately grasp target tissue and can be energized or incised while separated from the muscular layer, thus greatly reducing the risks. There was no uncontrollable intraoperative bleeding in our previous reports on ESD<sup>[11–17]</sup>. We were able to quickly and easily stop intraoperative bleeding using the CC<sup>[15]</sup> without changing the device for the entire gastrointestinal tract. In the present study, we did not perform any unexpected incisions. Therefore, the CC has the potential to decrease the risk of ESD-related complications in older and non-older patients.

Our study found no significant difference between older and non-older patients in the mean duration of hospitalization. Tokioka *et al.*<sup>[8]</sup> also failed to find a significant difference in the mean duration of hospitalization between these two groups (13.3 d vs 10.3 d). However, these authors reported that older patients with complications due to ESD (*i.e.*, perforation) were hospitalized for significantly longer periods than non-older patients<sup>[8]</sup>. In our series, we encountered perforation in one older patient who underwent endoscopic clipping and required 16 d of hospitalization. Therefore, preventing complications, such as perforation, is important for reducing the duration of hospitalization, especially in older patients.

Few medical economic outcomes have been reported in older patients. Murata *et al.*<sup>[31]</sup> reported that mean medical costs are significantly higher for older patients undergoing ESD for EGC than for non-older patients. They also reported that chronic comorbid conditions or the use of anticoagulant drugs, as well as the occurrence of complications, might be associated with an increase in the length of stay or medical costs during hospitalization<sup>[31]</sup>. Although the older group in our study showed a significantly higher rate of comorbid cardiovascular disease than did the non-older group, the postoperative bleeding rate was low in the older group (1.6%). In our series, there were no significant between-group differences in the length of stay and medical costs during hospitalization. The reported complication rate associated with ESDCC is lower than that of ESD using conventional knives<sup>[11-17]</sup>, which might have affected our medical economic outcomes.

We conclude that ESDCC is safe and effective for older and non-older patients with EGC. This study is limited by its retrospective nature. A prospective study with a larger sample size is advised.

## ACKNOWLEDGMENTS

We thank Kelly Zammit, BVSc, for editing a draft of this manuscript. Kazuya Akahoshi and Hidefumi Akahane (Fujifilm) have applied for a patent in Europe for the CC described in this article. Japan, China, and the United States have already granted patents.

## COMMENTS

### Background

In an aging society, the opportunity to treat older patients with early gastric cancer (EGC) is increasing. However, there is little information on the medical costs of endoscopic submucosal dissection (ESD), the mean duration of hospitalization, and the change in performance status before and after ESD. It is important to assess the clinical outcomes of ESD using the Clutch Cutter (CC) (ESDCC) in older patients with EGC.

### Research frontiers

The authors previously showed that ESD using the CC (ESDCC) is a safe and effective method for treating patients with early cancer in the gastrointestinal tract. No reports have yet evaluated the efficacy and safety of ESDCC for older patients with EGC, including the economic and social aspects.

## Innovations and breakthroughs

There was no significant difference between older patients and non-older patients in mean duration of hospitalization, incidence of ESDCC-related complications, change in PS before and after ESD, and financial cost of admission.

## Application

It may be economically and socially beneficial to reduce the complication rate by performing ESDCC for older patients with EGC.

## Terminology

The CC (DP2618DT, Fujifilm Corporation, Tokyo, Japan) was developed by Akahoshi. The CC is a grasping type of scissors/forceps that can grasp tissue pieces and cut or coagulate with an electrosurgical current. To facilitate tissue grasping, the CC has a serrated cutting edge with a width of 0.4 mm, and a length of 3.5 mm or 5 mm. The forceps diameter is 2.7 mm. The outside of the forceps is insulated so that electrosurgical current energy concentrates on the closure blade to avoid unintentional incision. Furthermore, the forceps can be rotated in any desired direction. The CC is disposable and cannot be reused. The CC can be used in all steps of ESD.

## Peer-review

The authors present an interesting study on the efficacy and safety of endoscopic submucosal dissection for older patients with early gastric cancer. The study is well designed, the results are accurately noted and the discussion is concise.

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# Low-dose computed tomography with 4<sup>th</sup>-generation iterative reconstruction algorithm in assessment of oncologic patients

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## Abstract

### AIM

To compare radiation dose and image quality of low-dose computed tomography (CT) protocol combined with hybrid-iterative reconstruction algorithm with standard-dose CT examinations for follow-up of oncologic patients.

### METHODS

Fifty-one patients with known malignant diseases which underwent, during clinical follow-up, both standard-dose and low-dose whole-body CT scans were enrolled. Low-dose CT was performed on 256-row scanner, with 120 kV and automated mA modulation, and iterative reconstruction algorithm. Standard-dose CT was performed on 16-rows scanner, with 120 kV, 200-400 mAs (depending on patient weight). We evaluated density values and signal-to-noise ratio, along with image noise (SD), sharpness and diagnostic quality with 4-point scale.



## RESULTS

Density values in liver, spleen and aorta were higher in low-dose images (liver 112.55 HU *vs* 103.90 HU,  $P < 0.001$ ), as SD values in liver and spleen (liver 16.81 *vs* 14.41). Volumetric-Computed-Tomographic-Dose-Index (CTDIvol) and Dose-Length-Product (DLP) were significantly lower in low-dose CT as compared to standard-dose (DLP 1025.6 mGy\*cm *vs* 1429.2 mGy\*cm,  $P < 0.001$ ) with overall dose reduction of 28.9%. Qualitative analysis did not reveal significant differences in image noise and diagnostic quality.

## CONCLUSION

Automatic tube-current modulation combined with hybrid-iterative algorithm allows radiation dose reduction of 28.9% without loss of diagnostic quality, being useful in reducing dose exposure in oncologic patients.

**Key words:** Computed tomography; Low-dose computed tomography; Tube current modulation; Oncologic imaging; Radiation dose

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**Core tip:** Introduction of new generation of multidetector computed tomography (MDCT) scanner allowed thin-collimation scanning and high spatial resolution, and reducing at same time the delivered radiation dose to patients by using new iterative reconstruction algorithm. This new mathematical model approach permits to reduce the radiation dose, especially in patients who undergo serial follow-up study for oncologic (staging and restaging) purpose. On these basis in our study we evaluated radiation dose and image quality of CT examinations in a population of oncologic patients undergoing follow-up examinations with a new generation MDCT scanner (256-rows) using automatic modulation of tube current and iterative reconstruction algorithm (DoseRight system).

Ippolito D, Casiraghi AS, Franzesi CT, Fior D, Meloni F, Sironi S. Low-dose computed tomography with 4<sup>th</sup>-generation iterative reconstruction algorithm in assessment of oncologic patients. *World J Gastrointest Oncol* 2017; 9(10): 423-430 Available from: URL: <http://www.wjgnet.com/1948-5204/full/v9/i10/423.htm> DOI: <http://dx.doi.org/10.4251/wjgo.v9.i10.423>

## INTRODUCTION

Since the advent of computed tomography (CT) and, more recently, the development of multidetector computed tomography (MDCT) technique, the number of CT scans have increased significantly<sup>[1,2]</sup>; according to National Council on Radiation Protection and Measurements (NCRP) report 160, in 2006 United States population was exposed to more than seven times ionizing radiation from medical procedures than in the early 1980's, and CT contributes to nearly one-half of all

this radiation imaging exposure<sup>[1,2]</sup>. This high number of CT examinations have raised concern because of the potential risk of radiation-induced malignancy<sup>[3]</sup>.

In the clinical management of patients with malignant diseases CT, with other cross-sectional imaging techniques such as MRI and FDG-PET/CT, has a major role for initial diagnosis of the disease, for staging, and during follow-up to monitor response to treatment and evaluate disease remission or relapse<sup>[4]</sup>. CT of the chest, abdomen and pelvis has the ability to obtain a global evaluation of the patient and to depict both primary tumor and metastasis in most cases, more quickly, safely and accurately than other more invasive or less sensitive imaging techniques. On these bases, oncologic patients typically undergo multiple CT investigations during their clinical follow-up, and risks derived from repeated scans and exposure to ionizing radiation should be balanced against the benefits of diagnostic imaging: CT examinations must have a correct clinical justification, and every CT scans must be technically optimized to keep radiation doses as low as possible while providing requested diagnostic information<sup>[5,6]</sup>.

To optimize radiation dose in CT various technological strategies can be applied: These include active management of individual acquisition parameters (number of phases, section thickness, peak voltage, tube current-time product, pitch) or using an automated exposure control system<sup>[7]</sup>. Recently, iterative reconstruction algorithms have been introduced to reduce image noise, allowing further reduction in radiation dose beyond levels previously achievable with filtered back projection reconstruction (FBP)<sup>[7]</sup>. iDose<sup>4</sup> is a hybrid iterative algorithm that is able to reduce noise on both the raw and image data<sup>[8,9]</sup>. The purpose of our study was to evaluate the radiation dose and image quality of CT examinations in a population of oncologic patients undergoing follow-up examinations comparing a new generation MDCT scanner (256-rows), using automatic modulation of tube current and iterative reconstruction algorithm (DoseRight system and iDose<sup>4</sup>), with a 16-MDCT scanner using standard dose protocol and FBP.

## MATERIALS AND METHODS

### Patients population

Institutional research board approval was obtained for this retrospective study with waiver of the requirement for written consent.

Between August 2013 and April 2015, 259 adult patients (> 18 years old) with known malignant diseases (32 lung, 9 colorectal, 3 ovarian, 3 stomach, 2 uterine, 1 non hodgkin lymphoma and 1 testicular) underwent contrast enhanced chest-abdomen-pelvis CT examination in a single venous phase during their clinical follow-up on a 256-MDCT scanner with a protocol implemented in our institution from August 2013 (with automatic modulation of current tube and iDose<sup>4</sup> reconstruction algorithm). These examinations were identified with a retrospective

**Table 1** Descriptive table of weight groups in the patients population (*n* = 51)

Weight groups	No. of patients	M/F	Mean $\pm$ SD (yr)
Group A (41-60 kg)	8	3/5	70.5 $\pm$ 8.6
Group B (61-80 kg)	25	9/16	67.6 $\pm$ 11.5
Group C (81-90 kg)	13	11/2	71.8 $\pm$ 7.4
Group D (> 90 kg)	5	4/1	59.2 $\pm$ 10.1
All patients	51	34/18	68.3 $\pm$ 10.4

M: Male; F: Female.

review of CT studies archived with PACS system (AGFA Diagnostic Software, Impax, version 6.4.0.3125; Agfa, Mortsel; Belgium). In this group we selected patients which had undergone also standard-dose contrast enhanced CT scan on a 16-MDCT in a different time. Fifty-one patients were finally selected as our study group and were categorized into four groups according to their weight (kg): 41-60 kg (group A), 61-80 kg (group B), 81-90 kg (group C) and > 90 kg (group D) (Table 1). Total mean weight was not statistically different between the two scans ( $74.1 \pm 14.9$  kg for lower dose scans and  $73.9 \pm 15.0$  kg for standard dose scans,  $P = 0.705$ ). The mean time interval between CT acquisitions was  $4.8 \pm 2.9$  mo. Patients' mean age, calculated at the time of the most recent CT scan, was  $68.3 \pm 10.4$  years old.

### MDCT technique and image reconstruction

All 51 patients had undergone thorax-abdomen-pelvis CT examinations both on a 16-rows MDCT scanner (Brilliance, Philips Medical Systems, Eindhoven, The Netherlands) and on a 256-rows MDCT (iCT, Philips).

For both examinations, CT data were acquired after the intravenous bolus injection of non-ionic iodinated contrast material (Xenetix 350; Guerbet, Aulnay, France), injected using a 18-gauge catheter positioned into the antecubital vein at a rate of 3.5 mL/s, with image acquisition during portal venous phase (55-70 s after the initiation of the contrast bolus), in accordance with an institutionally defined protocol. The volume of contrast agent was calculated on the basis of the patient's body weight, with total dose ranging from 80 to 130 mL, and it was followed by a saline flush of 50 mL of NaCl at 3.5 mL/s. The patients were instructed to hold their breath during scanning. All studies were started from the lung apices and proceeded in a cephalocaudal direction until ischial tuberosity, to include chest, abdomen and pelvis of the patients.

The technical parameters for scanning included: (1) for 16-rows CT scanner: 120 kVp, mAs depending on patients' weight (41-60 kg: 200 mAs; 61-80 kg: 300 mAs; 81-90 kg: 350 mAs; > 90 kg: 400 mAs), section thickness 2 mm, pitch 0.813, 0.75 s rotation time, display field of view (FOV) depending on the patient's physique (median values of 350), beam collimation  $16 \times 1.5$ ; (2) for 256-rows CT scanner: 120 kVp, automated mAs with X-ray tube current automatic modulation system

(range of mean mAs: 103-468 mAs), section thickness 2 mm, pitch 0.984, 0.75 s rotation time, display field of view (FOV) depending on the patient's physique (median values of 350), beam collimation  $64 \text{ mm} \times 0.625 \text{ mm}$  (to reduce the overranging and to improve the Z-DOM modulation performance). The Brilliance iCT scanner can control radiation exposure with advanced dose reduction tools, such as X-ray tube current automatic modulation system (the automatic current selection ACS, which automatically suggests tube current settings according to estimated patient diameter in the scan region, and the Z-axis dose modulation system Z-DOM, that modulates mA along the patient longitudinal axis using the attenuation profile estimated from the Surview), SmartShape and IntelliBeam shaping filters, the Eclipse asymmetric collimator for over-ranging reduction, NanoPanel<sup>3D</sup> detectors and ClearRay 2D anti-scatter grid<sup>[10]</sup>.

The 16-MDCT images were reconstructed using a standard FBP algorithm with a standard soft-tissue kernel, while the 256-MDCT images were reconstructed with hybrid iterative reconstruction algorithm (iDose<sup>4</sup>): iDose<sup>4</sup> Level 3 was chosen<sup>[8]</sup>.

At the end of every examinations, the volumetric computed tomographic dose index (CTDIvol) and the dose-length product (DLP) were provided by the scanners in the dosimetric report.

CT values (HU) and standard deviation of CT values (SD) were also measured by placing one ROI ( $2 \text{ cm}^2$ ) within the subcutaneous fat of the anterior abdominal wall. SNR was calculated for the liver and the spleen as:  $\text{SNR} = \text{HU}_{\text{ROI}} / \text{SD}_{\text{ROI}}$ , where  $\text{HU}_{\text{ROI}}$  is the mean CT value in Hounsfield units of the tissue and  $\text{SD}_{\text{ROI}}$  the standard deviation of CT values in the same ROI.

### Statistical analysis

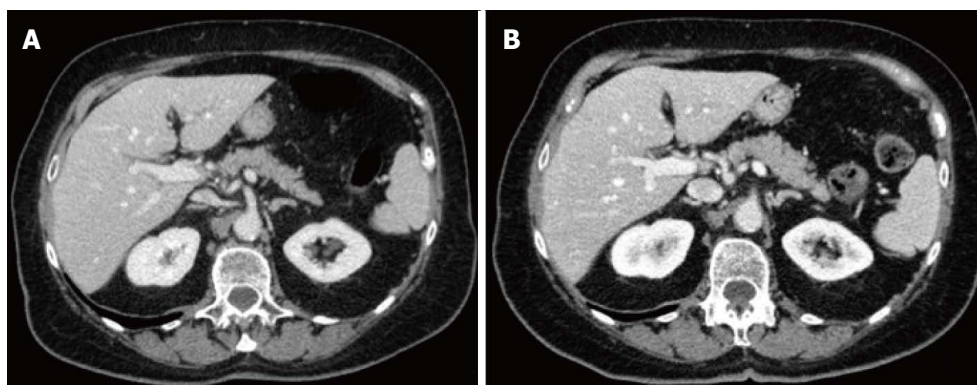
Continuous variables (HU, SD, SNR, DLP, CTDIvol, patients weight) were presented as means  $\pm$  SD. The Wilcoxon signed-rank test for paired samples or paired Student *t*-test were used, where appropriate, to compare values of continuous variables between standard dose protocol images and lower dose protocol images. To evaluate differences in qualitative analysis between the two protocols the Wilcoxon signed-rank test for paired samples was applied. A *P*-value < 0.05 was considered statistically significant.

Cohen's kappa was used to evaluate agreement between the two readers<sup>[11]</sup>. Analysis was performed with commercially available statistical software (SPSS Statistics 17.0, Chicago, IL).

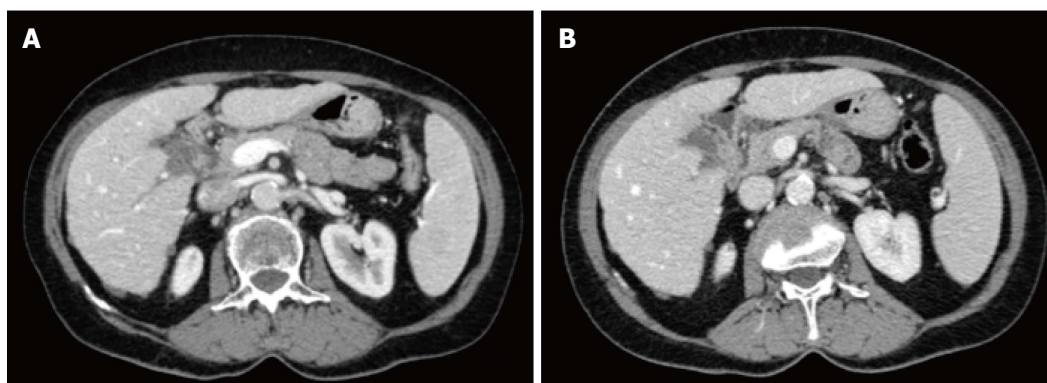
## RESULTS

### Radiation dose

Mean values of obtained DLP and CTDIvol were significantly lower with low-dose protocol in comparison with standard dose protocol ( $P < 0.001$ ): For lower-dose CT, DLP and CTDIvol were respectively  $1025.6 \pm$



**Figure 1** Axial contrast-enhanced computed tomography images at the level of upper abdomen obtained in a 81 years old female patient with lung cancer (height 160 cm, weight 61 kg). A: Standard dose protocol (120 kV, 300 mAs, DLP 1317.4 mGy\*cm, CDTI 21.1 mGy); B: Lower dose protocol (120 kV, 142-222 mAs, DLP 846.0 mGy\*cm, CDTI 13.6 mGy): Lower dose image shows increased sharpness and enhancement in comparison with standard dose image in spite of mild increase of noise, and similar diagnostic quality with a 35.8% Dose-Length-Product reduction.



**Figure 2** Axial contrast-enhanced computed tomography images at the level of upper abdomen obtained in a 70 years old female patient with ovarian cancer and some small hypoattenuating hepatic subcapsular implants with well-defined margins (height 160 cm, weight 68 kg). A: Standard dose protocol (120 kV, 300 mAs, Dose-Length-Product 1304.6 mGy\*cm, CDTI 21.0 mGy); B: Lower dose protocol (120 kV, 123-231 mAs, Dose-Length-Product 840.9 mGy\*cm, CDTI 13.1 mGy).

370.9 mGy\*cm and  $15.4 \pm 5.2$  mGy, compared with  $1429.2 \pm 297.7$  mGy\*cm and  $21.4 \pm 4.0$  mGy for standard-dose protocol. The low-dose protocol provided a mean DLP reduction of 28.9% compared to standard-dose protocol (Figure 1).

The average weight was  $74.1 \pm 14.9$  kg for low dose scans (range 41-114 kg) and  $73.9 \pm 15.0$  kg for standard dose scans (range 43-114 kg) ( $P = 0.705$ ). By dividing patients into four groups of weight, we obtained a higher rate of DLP reduction in patients in groups A, B and C with a statistically significant difference between the two protocols; in patients with high weight ( $> 90$  kg, group D), for values of DLP and CTDIvol only few differences between the two protocols were recorded, not statistically significant, with a radiation dose reduction of 2.5% (Table 2).

### Qualitative analysis

Qualitative analysis results are shown in Table 3. There was a good inter-reader agreement, as shown by  $k$  Cohen values. There weren't significant differences in the qualitative evaluation of image noise and diagnostic quality for both readers (Figure 2), and of image

sharpness for one reader. The other reader assigned to low-dose images superior grades for sharpness, with a significant difference ( $P = 0.012$ ).

### Quantitative analysis

CT values of density (HU) measured within abdominal aortic lumen and liver and spleen parenchyma were significantly higher using low-dose CT with iDose<sup>4</sup> ( $P < 0.001$ ) (Table 4). The mean liver and spleen parenchymal noise (SD) was higher with low-dose protocol as well, while SD of abdominal subcutaneous fat was higher but without reaching statistical significance. The measured noise varied according to the weight of the patients, with higher values of SD in patients with higher weight; values of SD in the liver parenchyma with low-dose protocol vs standard protocol were respectively  $14.62 \pm 1.80$  vs  $11.62 \pm 2.03$  in group A,  $16.66 \pm 1.45$  vs  $13.10 \pm 2.18$  in group B,  $18.03 \pm 1.98$  vs  $16.26 \pm 1.54$  in group C and  $17.91 \pm 2.24$  vs  $20.59 \pm 3.28$  in group D.

SNR values, calculated as  $SNR = HU_{ROI}/SD_{ROI}$ , were lower in low-dose images, reaching a significant difference within the liver parenchyma ( $6.94 \pm 1.32$  vs  $7.80 \pm 2.30$ ,  $P = 0.002$ ) and without statistical

**Table 2** Comparison of Dose-Length Product and Volumetric Computed Tomographic Dose Index obtained with standard-dose and low-dose protocols in all patients ( $n = 51$ ) and according to weight (kg)

BMI groups	No. of patients	CTDIvol (mGy)			DLP (mGy*cm)			% DLP reduction
		Standard-dose	Low-dose	P-value	Standard-dose	Low-dose	P-value	
Group A (41-60 kg)	8	14.1 ± 0.0	9.8 ± 1.5	0.012	891.9 ± 36.3	627.5 ± 92.9	0.012	29.6
Group B (61-80 kg)	25	21.1 ± 0.0	14.0 ± 2.8	< 0.001	1386.6 ± 65.9	920.0 ± 175.0	< 0.001	33.5
Group C (81-90 kg)	13	24.6 ± 0.0	17.2 ± 2.9	0.001	1656.8 ± 61.2	1162.6 ± 204.2	0.001	29.9
Group D (> 90 kg)	5	27.4 ± 1.5	26.5 ± 5.4	0.5	1910.4 ± 147.6	1835.1 ± 359.5	0.5	2.5
All patients	51	21.4 ± 4.0	15.4 ± 5.2	< 0.001	1429.2 ± 297.7	1025.6 ± 370.9	< 0.001	28.9

DLP: Dose-Length-Product; CTDIvol: Volumetric Computed Tomographic Dose Index.

**Table 3** Qualitative scoring of image noise, image sharpness and diagnostic quality of computed tomography images from Reader 1 and Reader 2

	Image noise		Image sharpness		Diagnostic quality	
	Reader 1	Reader 2	Reader 1	Reader 2	Reader 1	Reader 2
Low-dosea	3.4 ± 0.6	3.6 ± 0.6	3.7 ± 0.5	3.7 ± 0.5	3.7 ± 0.5	3.8 ± 0.4
Group A ( $n = 8$ )	3.6 ± 0.7	3.8 ± 0.5	3.6 ± 0.5	3.5 ± 0.5	3.9 ± 0.4	3.8 ± 0.5
Group B ( $n = 25$ )	3.4 ± 0.6	3.6 ± 0.6	3.8 ± 0.5	3.8 ± 0.4	3.7 ± 0.6	3.8 ± 0.4
Group C ( $n = 13$ )	3.1 ± 0.4	3.6 ± 0.5	3.6 ± 0.5	3.9 ± 0.4	3.6 ± 0.5	4.0 ± 0.0
Group D ( $n = 5$ )	3.3 ± 0.6	3.0 ± 1.0	4.0 ± 0.0	3.3 ± 0.6	4.0 ± 0.0	3.7 ± 0.6
Standard-doseb	3.6 ± 0.7	3.5 ± 0.6	3.3 ± 0.7	3.5 ± 0.6	3.7 ± 0.5	3.8 ± 0.4
Group A ( $n = 8$ )	3.9 ± 0.4	3.5 ± 0.5	3.1 ± 0.6	3.4 ± 0.5	3.9 ± 0.4	3.8 ± 0.5
Group B ( $n = 25$ )	3.7 ± 0.5	3.7 ± 0.5	3.5 ± 0.6	3.5 ± 0.6	3.8 ± 0.4	3.8 ± 0.4
Group C ( $n = 13$ )	3.3 ± 0.5	3.3 ± 0.5	2.9 ± 0.7	3.9 ± 0.4	3.6 ± 0.5	4.0 ± 0.0
Group D ( $n = 5$ )	2.3 ± 1.5	3.0 ± 1.0	3.0 ± 1.0	3.3 ± 0.6	2.7 ± 1.2	3.7 ± 0.6
P-valuea-b	0.292	0.655	0.012	0.088	0.437	0.206
k Cohen Reader 1-Reader 2	0.694a-0.756b		0.783a-0.672b		0.704a-0.786b	

**Table 4** Computed tomography values (HU), standard deviation of computed tomography values (SD) and signal-to-noise ratio obtained with standard-dose and low-dose protocols

	Low-dose	Standard-dose	P-value
HU <sub>aorta</sub> ( $n = 51$ )	166.20 ± 18.83	154.17 ± 24.82	< 0.001
HU <sub>liver</sub> ( $n = 51$ )	112.55 ± 16.49	103.90 ± 17.49	< 0.001
HU <sub>spleen</sub> ( $n = 51$ )	126.24 ± 13.60	112.77 ± 15.66	< 0.001
SD <sub>liver</sub> ( $n = 51$ )	16.81 ± 2.02	14.41 ± 3.33	< 0.001
SD <sub>spleen</sub> ( $n = 51$ )	16.78 ± 2.04	14.72 ± 3.26	< 0.001
SD <sub>fat</sub> ( $n = 51$ )	12.17 ± 2.77	12.07 ± 2.98	0.307
SNR <sub>liver</sub> ( $n = 51$ )	6.94 ± 1.32	7.80 ± 2.30	0.002
SNR <sub>liver</sub> Group A ( $n = 8$ )	8.09 ± 1.63	9.36 ± 2.27	0.05
SNR <sub>liver</sub> Group B ( $n = 25$ )	6.95 ± 1.22	8.53 ± 1.97	< 0.001
SNR <sub>liver</sub> Group C ( $n = 13$ )	6.47 ± 1.00	6.66 ± 1.34	0.972
SNR <sub>liver</sub> Group D ( $n = 5$ )	6.29 ± 1.19	4.60 ± 1.77	0.08
SNR <sub>spleen</sub> ( $n = 51$ )	7.73 ± 1.46	8.10 ± 2.04	0.153
SNR <sub>spleen</sub> Group A ( $n = 8$ )	9.25 ± 2.31	10.53 ± 1.69	0.092
SNR <sub>spleen</sub> Group B ( $n = 25$ )	7.60 ± 1.10	8.04 ± 1.86	0.177
SNR <sub>spleen</sub> Group C ( $n = 13$ )	7.26 ± 1.11	7.20 ± 1.45	0.65
SNR <sub>spleen</sub> Group D ( $n = 5$ )	7.21 ± 0.82	6.86 ± 1.89	0.893

SNR: Signal-to-noise ratio.

significance within spleen parenchyma ( $7.73 \pm 1.46$  vs  $8.10 \pm 2.04$ ,  $P = 0.153$ ). When SNR data were reviewed according to the weight of patients, SNR values decreased as patients' weight increased. SNR values were lower in low-dose protocol in comparison with standard-dose protocol in groups A and B, comparable

between two protocols in group C and higher in group D (Table 3).

## DISCUSSION

In the latest years many studies investigating the potential of radiation dose reduction by applying different iterative reconstruction algorithms have been published for abdomen, chest, head, coronary and chest angiography, and they showed significant dose reduction while maintaining, or sometimes improving, image quality<sup>[12-26]</sup>.

Arapakis *et al.*<sup>[12]</sup> addressed the effect of iterative algorithm on radiation dose and image quality of chest-abdomen-pelvis (CAP) CT scans. They applied iDose<sup>4</sup> hybrid iterative reconstruction algorithm in a group of 84 patients and compared images to those obtained with "old standard" protocol with filtered back projection reconstruction algorithm in a group of 99 patients, obtaining an overall 46.5% decrease in effective dose with lower image noise and higher values of SNR and CNR; in their study, the greatest dose reduction was recorded in patients with lower weight<sup>[12]</sup>.

Karpitschka *et al.*<sup>[13]</sup> retrospectively evaluated 40 patients which underwent CT scans for staging of malignancy with both a standard-dose (tube current time product 250 mAs and FBP reconstruction) and a reduced-dose CT scan (150 mAs and with Iterative Reconstruction



in Image Space IRIS), obtaining a greater than 45% dose reduction at maintained image quality; the authors recommend the use of IR in oncological patients in order to reduce radiation dose to patients.

Moreover, for abdominal CT scans, Prakash *et al.*<sup>[14]</sup> showed a reduction of radiation dose by 25% using weight-based adjustment of automatic exposure control technique and Adaptive Statistical Iterative Reconstruction (ASIR) in comparison with FBP reconstructed scans, while May *et al.*<sup>[15]</sup> obtained a 50% reduction in abdominal CT by using IRIS.

In our study, values of DLP obtained using a low-dose protocol with iDose<sup>4</sup> iterative algorithm were, on average, 28.9% lower compared to our standard dose with FBP reconstruction. Despite higher levels of quantitative noise (as demonstrated by SD values) within liver and spleen parenchyma, however, qualitative analysis didn't reveal significant differences in overall image noise and diagnostic quality when compared with standard-dose images in the same patients (Table 2). These results and lower rate of dose reduction in comparison with other CAP studies may be correlated to a different level of strength of the iterative reconstruction of iDose<sup>4</sup> applied in our institution (L3) which determines less noise reduction and, with a fixed noise index, can be associated with higher levels of tube current and radiation dose.

The measured noise and SNR in low-dose images varied according to the weight of the patients, with higher values of SD and lower values of SNR in patients with higher weight (Table 3). These data were confirmed by qualitative analysis, which showed increasing levels of subjective noise by increasing patients weight, but without significant differences in image sharpness and diagnostic quality between the four groups (Table 2). In our patient group with weight greater than 90 kg (group D), values of SD within liver and spleen parenchyma and abdominal fat were the highest, with worst values of SNR; however, compared to standard-dose images, these values of SNR were higher indicating a better image contrast, although the difference was not statistically significant.

In group D the DLP reduction rate was substantially lower than those in the other patient groups (2.5% vs 29.6%, 33.5% and 29.9%). With automatic tube current modulation, tube current is automatically adjusted to the X-ray attenuation of the patient to keep the radiation dose as low as possible while maintaining a constant image noise level and specified image quality as in a previously defined reference image. In patients with large body habitus, to maintain a constant image quality, there is a risk of high radiation doses when this technique is applied in abdominal MDCT<sup>[23]</sup>. Our results could be explained then by the higher tube current needed and used in this group of patients to fulfill the fixed noise level and desired final image quality. In heavy patients, the optimal noise indexes and image quality should be adjusted to patient habitus, considering that subjective image quality in abdomen CT of these patients is usually higher because of the

amount of fat deposition around the abdominal organ that improve tissue contrast<sup>[23]</sup>.

There were several limitations in our study. First of all, it was a retrospective study and acquisition of CT paired studies in our patients were not realized on the same scanner; despite many technical parameters were identical between the two protocols, some of them and the scanners were different and it may have introduced some bias. Because of the interval time between studies, which was remarkable in a small number of patients, and the retrospective nature of the study, we couldn't compare conspicuity or detection rate of the primary tumor and secondary lesions between the two protocols, which should be addressed in further prospective studies.

In conclusion, in our sample of oncologic patients, automatic modulation of tube current and iDose<sup>4</sup> reconstruction algorithm allowed a mean radiation dose reduction of 28.9%, without significant loss of subjective diagnostic quality, and this protocol could be useful in reducing dose exposure in patients with malignancy which undergo a high number of chest-abdomen-pelvis CT scan during their clinical follow-up.

## COMMENTS

### Background

In the latest years, the number of computed tomography (CT) scans have increased significantly and this high number of examinations has raised concern because of the potential risk of radiation-induced malignancy. CT has a major role in the clinical management of patients with malignant diseases, which typically undergo multiple CT investigations during their follow-up, and risks derived from repeated scans and exposure to ionizing radiation should be balanced against the benefits of diagnostic imaging. On this basis in the study authors compared the diagnostic quality and the radiation dose of whole body CT scan examination obtained with a low-dose setting protocol combined with the new state of art iterative reconstruction algorithm with those obtained with a standard-dose protocol.

### Research frontiers

New generation of high row number multidetector computed tomography (MDCT) scans allow thin-collimation, high spatial resolution and better multiplanar reconstructions (MPRs), and are increasingly used in clinical practice in oncologic field, because MDCT can assess in a single examination, the entire abdomen, pelvis and chest, allowing for local tumour staging and distant metastases evaluation. Several dose reduction tools are actually integrated in these new CT-scanners, including hardware components as dynamic helical collimator, adaptive axial collimator and tube-current modulation, and software post-acquisition improvements as iterative reconstruction algorithms. These technical solutions permit to reduce the dose delivered to the patients, maintaining high diagnostic quality of images.

### Innovations and breakthroughs

CT protocols should be properly designed and carefully applied in order to obtain the highest amount of information by using the lowest radiation dose achievable, since the theoretical risk of radiation-induced cancer from CT examinations has been reported as not negligible. New CT scanners are equipped with several iterative reconstruction (IR) algorithms that allow a reduction of the radiation dose without theoretically affecting the image quality, especially if used in association with a low kV scanning protocol. The study was designed in order to obtain the best image quality with the lowest effective dose, using dose reduction strategies available with our scanner. iDose<sup>4</sup> is a fourth-generation hybrid IR algorithm introduced by Philips Healthcare, and the major component of this algorithm deals with subtraction of the image noise while

preserving the underlying edges associated with true anatomy or pathology.

## Applications

In this study the authors compared radiation dose and image quality of CT examinations in a population of oncologic patients undergoing follow-up examinations with a new generation MDCT scanner (256-rows) using automatic modulation of current tube and iterative reconstruction algorithm (DoseRight system and iDose<sup>4</sup>) and with a 16-MDCT scanner with standard dose protocol and FBP. The importance of this work relies on the fact that the results confirm the high diagnostic quality and the important radiation dose sparing of whole body CT scan examination obtained with a low-dose setting protocol combined with the new state of art iterative reconstruction algorithm in comparison with a standard-dose protocol. Moreover in this manuscript the authors compared and commented the results with those of previous literature on this field by using different vendor approach.

## Terminology

MDCT: Multidetector row-CT are new generation of CT scanner with high number of detector, which allow to obtain high spatial resolution images with thinner collimation; FBP: Filtered back projection (FBP) has been the industry standard for CT image reconstruction for decades, representing a very fast and fairly robust method to reconstruct the raw data obtained from routinely CT scan acquisition; Hybrid Iterative Reconstruction Algorithms: In the literature, the term hybrid IR usually refers to algorithms that mainly decrease image noise by iterative methods. IR approaches are not new and were, in fact, the initially proposed method for data reconstruction in the early days of CT technology during the 1970s. However, due to its mathematically demanding properties and the large amount of data in CT imaging, until recently IR has not been practical for clinical purposes. The current evidence on the clinical implementation of IR into CT protocols shows substantial promise for major improvements in image quality, chiefly noise reduction-with subsequent radiation dose reduction-and artifact suppression; iDose<sup>4</sup>: iDose<sup>4</sup> is a fourth-generation hybrid IR algorithm introduced by Philips Healthcare. With this algorithm the noise can be controlled for high spatial resolution reconstructions, hence providing high-quality, low-contrast, and spatial resolution within the same image, when radiologist work with low dose approach; through an iterative mathematical process, the noisy data are penalized and edges are preserved.

## Peer-review

This is a very interesting attempt to achieve lower radiation dose in follow-up CT of oncologic patients with parallel comparison of thorax-abdomen-pelvis CT with 4<sup>th</sup> generation hybrid iterative reconstruction algorithm and standard dose examination.

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## Primary esophageal diffuse large B cell lymphoma presenting with tracheoesophageal fistula: A rare case and review

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**Informed consent statement:** Patient's legal guardian provided verbal informed consent authorizing discloses and use his information.

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### Abstract

Primary non-Hodgkin lymphomas in the esophagus are rare. Tracheoesophageal fistulas mainly arise from solid esophageal carcinoma or mediastinal malignancies. Our patient presented with cough, dysphagia and weight loss, and upon initial computed tomography imaging and esophagogastroduodenoscopy, a malignant mass in the middle third of esophagus with tracheoesophageal fistula was found. The location of the mass and presence of malignant tracheoesophageal fistula were strongly suggestive of squamous cell carcinoma. However, tumor biopsy revealed diffuse large B-cell lymphoma. This case report details a rare incident of a primary diffuse large B-cell lymphoma presented as tracheoesophageal fistula and reviews previous literature.

**Key words:** Non-Hodgkin lymphoma; Tracheoesophageal fistula; Esophageal cancer; Esophageal lymphoma

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**Core tip:** Primary non-Hodgkin lymphoma of esophagus is a rare disease, and tracheoesophageal fistula secondary to this condition prior to treatment is extremely rare and fatal. However, it has better prognosis than fistulas secondary to solid tumor if patients receive timely treatment.

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mary esophageal diffuse large B cell lymphoma presenting with tracheoesophageal fistula: A rare case and review. *World J Gastrointest Oncol* 2017; 9(10): 431-435 Available from: URL: <http://www.wjgnet.com/1948-5204/full/v9/i10/431.htm> DOI: <http://dx.doi.org/10.4251/wjgo.v9.i10.431>

## INTRODUCTION

Lymphomas, the most common blood cancers, are characterized by proliferation of lymphocytes in the lymph nodes and of lymphoid tissue<sup>[1,2]</sup>. Lymphomas are categorized into two groups: Hodgkin and non-Hodgkin lymphomas (NHLs). Among NHLs, diffuse large B-cell lymphomas (DLBCLs) account for 40% of all lymphoma cases worldwide<sup>[3]</sup>. Primary gastrointestinal (GI) lymphoma is the most common extranodal presentation NHL; however, most cases involve the stomach, small intestine and colon. Esophageal involvement is the rarest. Malignant tracheoesophageal fistula (TEF) from NHL is uncommon and presents mostly as complication of radiation therapy or chemotherapy.

Here, we present a rare case of a primary esophageal NHL presented with malignant TEF. To the best of our knowledge, this is the first case of primary DLBCL with malignant TEF prior to cancer treatment.

## CASE REPORT

A 60-year-old male with past medical history of diabetes mellitus type 2, hypothyroidism and chronic tobacco smoking presented with gradually worsening 3-wk dry cough, dysphagia and cough provoked with all oral intake. On review of systems, patient had unintentional 30-pound weight loss in the past 3 mo. On physical exam vital signs were unremarkable except for oxygen saturation of 91% on room air with respiratory rate of 18 breaths per minute. Moreover, the patient was not in acute distress; his breathing was non-labored; liver and spleen were not palpable; superficial lymphadenopathy was not found. The initial CBC revealed a white blood cell count of 21900/ $\mu$ L, 5% bands, 81% segmented neutrophils, 5% lymphocyte, and 8% monocytes. Lactate dehydrogenase was 223 units/L (normal value; 135-225 units/L), liver functions and renal functions were unremarkable and human immunodeficiency virus (HIV) was negative. A chest computed tomography (CT) imaging revealed a mid-esophageal wall thickening and enhancement, a fistulous connection between the membranous portion of the trachea and the anterior portion of the mediastinum, nonspecific mediastinal lymph nodes enlargement and some of ground glass opacity in posterior segment of the upper lobes and superior segments of the lower lobes bilaterally (Figure 1). Abdominal and pelvic CT imaging revealed multiple lytic lesions in pelvic bone, mild hepatic steatosis, normal spleen and no intraabdominal or pelvic lymphadenopathy. Our patient was started on levofloxacin for concern of aspiration pneumonia.

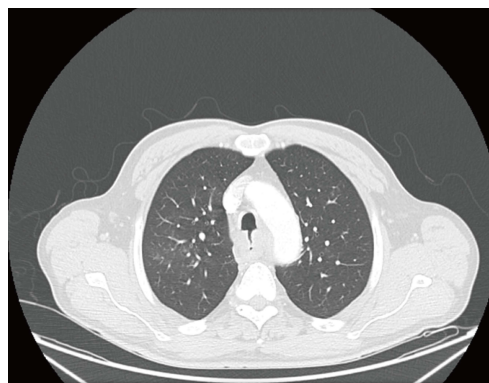


Figure 1 Contrasted chest computed tomography imaging showing tracheoesophageal fistula in a 60-year-old male patient.

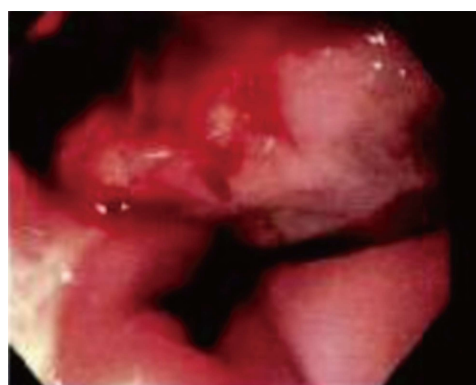
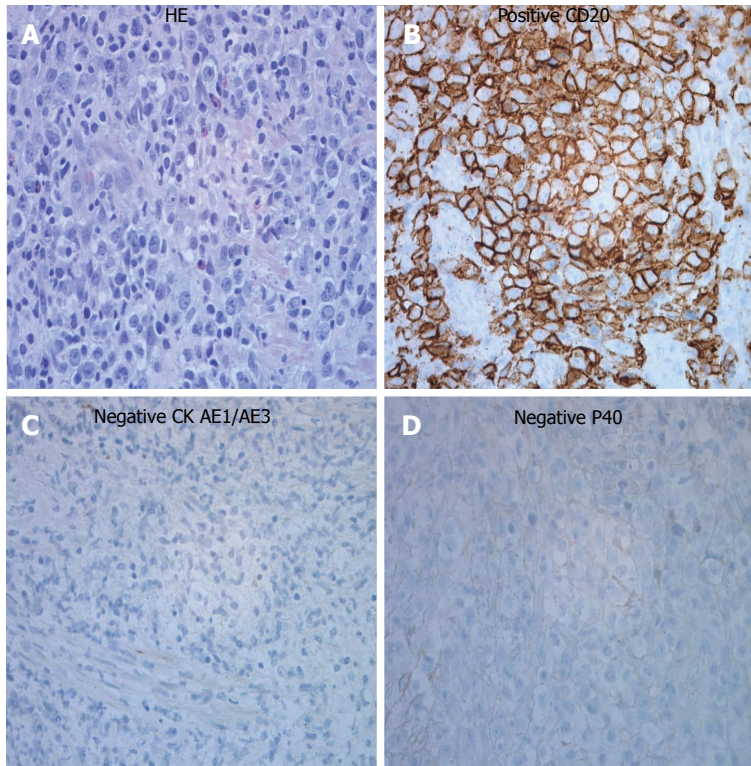


Figure 2 Esophagogastroduodenoscopy showing a partially obstructing mid-esophageal tumor and tracheoesophageal fistula in a 60-year-old male patient.

Esophagogastroduodenoscopy (EGD) found a large fungating and ulcerating mass in the middle third of the esophagus with partial obstruction, and TEF was found in the middle third of the esophagus with tracheal rings (Figure 2). Bronchoscopy revealed 3 cm TEF in the trachea and 1.5 cm bronchoesophageal fistula in left mainstem. The esophageal mass biopsy showed large, highly pleomorphic cells with diffuse growth pattern (Figure 3A). Various immunohistochemical staining were performed. Tumor cells had strong and diffuse expression for CD20 (Figure 3B), CD10, CD45, CD79a and bcl2. CD 3 and CD5 were negative. Cytokeratin (CK) AE1/AE3 was negative for the cells of tumor infiltrate (Figure 3C). Tumor cells did not show any expression for P40, a marker for squamous cell carcinoma (Figure 3D). These findings were consistent with diffuse large B-cell lymphoma diagnosis. Bone marrow biopsy was not performed because CT imaging suggested bone marrow involvement. Lumbar puncture was not done as well. Patient underwent for percutaneous endoscopic gastrostomy tube placement, esophageal stent placement and tracheobronchial stent placement. He received rituximab 375 mg/m<sup>2</sup> for 1 dose, and a week later he subsequently received complete first cycle of rituximab, cyclophosphamide, doxorubicin, vincristine and prednisone (R-CHOP regimen). Patient completed 6



**Figure 3** Histological features of primary diffuse large B-cell lymphoma in a 60-year-old male patient. A: HE staining shows highly pleomorphic large cell proliferation on sections of neoplasm; B: Immunohistochemistry shows tumor cells with a strongly diffused positive expression for CD20; C: Cytokeratin (CK) AE1/AE3 was negative for the cells of tumor infiltrate; D: P40 was negative for squamous carcinoma.

cycles of R-CHOP with good response in tumor but still has persistent TEF with intermittent aspiration. He lost 30 pounds during the course of treatment due to poor feeding intolerance.

## DISCUSSION

GI lymphomas comprise 5%-20% of extranodal lymphomas<sup>[4]</sup> and only 1%-4% of all GI cancers<sup>[5]</sup>. Primary esophageal lymphoma accounts for less than 1% of GI lymphomas. The earliest reported primary esophageal NHL was in 1979<sup>[6]</sup>. Heretofore, there have been less than 25 reported cases of primary esophageal cancer from NHL<sup>[7-16]</sup>. Sometimes, it can be difficult to differentiate between primary GI lymphomas and lymphomas that disseminated to GI tract. Dawson *et al.*<sup>[17]</sup> described the diagnostic criteria of primary GI lymphomas: (1) absence of peripheral lymphadenopathy; (2) absence of mediastinal adenopathy; (3) no involvement of liver and spleen; and (4) normal peripheral blood count. Majority of primary GI lymphomas are DLBCL<sup>[18]</sup>. A major risk factor for primary esophageal lymphoma is immunosuppression, such as HIV infection<sup>[19]</sup>. Radiologic features of primary esophageal lymphoma are ulceration, stenosis, polypoid masses, aneurysmal dilatation and TEF, which are non-specific<sup>[19,20]</sup>.

Malignant TEF is a serious late complication of cancers. Most malignant TEF cases are related to esophageal and lung cancers<sup>[21]</sup>. TEF from primary esophageal lymphoma is an uncommon complication. TEF in lymphoma frequently develops during or after treatment

with radiation or chemotherapy, but it can occur due to the disease itself. Most of the reports were of Hodgkin lymphomas<sup>[22-25]</sup>. Even though literature review reveals case reports of NHL with esophageal-tracheobronchial connection, the reported NHLs are not primary esophageal NHLs<sup>[26-28]</sup>. Malignant TEF usually has very poor prognosis; however, if lymphomas are recognized and treated early, TEF repair and chemotherapy treatment will result in good prognosis<sup>[29]</sup>. Standard treatment of DLBCL is R-CHOP regimen. Management of TEF is predominantly a non-surgical intervention because of the difficulty of and risk from surgery. Esophageal stent and/or airway stent is effective to prevent aspiration of GI contents and risk of pneumonia. In addition, general treatments, such as gastrostomy/jejunostomy tube, antibiotics and airway secretion prevention help reduce further risk of aspiration<sup>[22,30]</sup>.

Novelty of this case report is the co-presence of malignant TEF with primary DLBCL in the esophagus. Primary esophageal lymphoma-related TEF is extremely rare but fatal. Physicians should suspect it for timely diagnosis since NHL with TEF has better prognosis with interventions and chemotherapy alone than TEFs caused by esophageal cancer or lung cancer.

## COMMENTS

### Case characteristics

A 60-year-old man presented with worsening 3-wk dry cough, dysphagia and cough provoked with all oral intake.

## Clinical diagnosis

Clinical examination was unremarkable.

## Differential diagnosis

Stroke, esophageal spasm, esophageal tumor, tracheoesophageal fistula-related or pulmonary infection.

## Laboratory diagnosis

Blood count showed leukocytosis suggested of infection or inflammation, but lactate dehydrogenase and liver function were unremarkable.

## Imaging diagnosis

Chest, abdominal and pelvic computed tomography imaging revealed fungating and ulcerating mass in the middle third of the esophagus with partial obstruction and tracheoesophageal fistula (TEF) without significant lymphadenopathy.

## Pathological diagnosis

Esophageal mass biopsy revealed diffuse large B-cell lymphoma.

## Treatment

Patient received chemotherapy R-CHOP regimen and underwent to have PEG tube placement, tracheal and esophageal stents.

## Related reports

Most primary esophageal lymphoma cases are the rarest among primary gastrointestinal lymphoma, and TEF is seldom found as a presenting symptom.

## Term explanation

Tracheoesophageal fistula is an abnormal connection between the esophagus and trachea. Diffuse large B cell lymphoma is a subtype of non-Hodgkin lymphoma.

## Experiences and lessons

Primary esophageal lymphoma is extremely rare, and malignant TEF is fatal. However, patients with this condition have better prognosis if they receive a proper management.

## Peer-review

This case report is very interesting and rare. It is helpful to know if the patient has been immunologically investigated. The manuscript is well written and illustrations are informative.

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*WJGO* covers topics concerning carcinogenesis, tumorigenesis, metastasis, diagnosis, prevention, prognosis, clinical manifestations, nutritional support, molecular mechanisms, and therapy of benign and malignant tumors of the digestive tract. The current columns of *WJGO* include editorial, frontier, diagnostic advances, therapeutics advances, field of vision, mini-reviews, review, topic highlight, medical ethics, original articles, case report, clinical case conference (Clinicopathological conference), and autobiography. Priority publication will be given to articles concerning diagnosis and treatment of gastrointestinal oncology diseases. The following aspects are covered: Clinical diagnosis, laboratory diagnosis, differential diagnosis, imaging tests, pathological diagnosis, molecular biological diagnosis, immunological diagnosis, genetic diagnosis, functional diagnostics, and physical diagnosis; and comprehensive therapy, drug therapy, surgical therapy, interventional treatment, minimally invasive therapy, and robot-assisted therapy.

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Retrospective Cohort Study

# Paradoxical expression pattern of the epithelial mesenchymal transition-related biomarkers CD44, SLUG, N-cadherin and VSIG1/Glycoprotein A34 in gastrointestinal stromal tumors

Attila Kövecsi, Simona Gurzu, Zoltan Szentirmay, Zsolt Kovacs, Tivadar Jr Bara, Ioan Jung

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## Abstract

### AIM

To evaluate the immunohistochemical (IHC) expression of five biomarkers, commonly involved in epithelial mesenchymal/mesenchymal epithelial transition (EMT/MET), in gastrointestinal stromal tumors (GISTs).

### METHODS

In 80 consecutive GISTs the IHC examinations were performed using the EMT-related antibodies E-cadherin,

N-cadherin, SLUG, V-set and immunoglobulin domain containing 1 (VSIG1) and CD44.

## RESULTS

The positivity rate was 88.75% for SLUG, 83.75% for VSIG1, 36.25% for CD44 and 10% for N-cadherin. No correlation was noted between the examined markers and clinicopathological parameters. Nuclear positivity for SLUG and VSIG1 was observed in all cases with distant metastasis. The extra-gastrointestinal stromal tumors (e-GISTs) expressed nuclear positivity for VSIG1 and SLUG, with infrequent positivity for N-cadherin and CD44. The low overall survival was mainly dependent on VSIG1 negativity ( $P = 0.01$ ) and nuclear positivity for SLUG and/or CD44.

## CONCLUSION

GIST aggressivity may be induced by nuclear up-regulation of SLUG and loss or cytoplasm-to-nuclear translocation of VSIG1. SLUG and VSIG1 may act as activated nuclear transcription factors. The CD44, but not N-cadherin, might also have an independent prognostic value in these tumors. The role of the EMT/MET-related transcription factors in the evolution of GISTs, should be revisited with a larger dataset. This is the first study exploring the IHC pattern of VSIG1 in GISTs.

**Key words:** SLUG; Glycoprotein A34; N-cadherin; V-set and immunoglobulin domain containing gastrointestinal stromal tumors

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**Core tip:** In this paper we proved for the first time in the current literature the possible role of V-set and immunoglobulin domain containing 1 (VSIG1) in gastrointestinal stromal tumors (GISTs) in correlation with the expression of the other markers involved in the epithelial mesenchymal/mesenchymal epithelial transition. Based on the obtained results, we hypothesized that the GIST aggressivity may be induced by nuclear upregulation of SLUG and the loss or cytoplasm-to-nuclear translocation of VSIG1.

Kövecsi A, Gurzu S, Szentirmay Z, Kovacs Z, Bara T Jr, Jung I. Paradoxical expression pattern of the epithelial mesenchymal transition-related biomarkers CD44, SLUG, N-cadherin and VSIG1/Glycoprotein A34 in gastrointestinal stromal tumors. *World J Gastrointest Oncol* 2017; 9(11): 436-443 Available from: URL: <http://www.wjgnet.com/1948-5204/full/v9/i11/436.htm> DOI: <http://dx.doi.org/10.4251/wjgo.v9.i11.436>

## INTRODUCTION

Despite the existence of several molecular pathways described as being involved in the genesis and

evolution of gastrointestinal stromal tumors (GISTs), the invasive and metastatic behavior of these tumors is not completely understood. The aim of this immunohistochemistry (IHC) study was to evaluate the possible role of five of the biomarkers commonly involved in the epithelial mesenchymal transition/mesenchymal epithelial transition (EMT/MET) and also in maintaining the stem cell capacity of tumor cells, in the GIST histogenesis. The inspiration for this examination comes from the findings of some recent studies that proved a negative prognostic role of the EMT/MET-related markers in malignant tumors including GISTs<sup>[1-4]</sup>.

In carcinomas, the EMT is defined as the loss of the expression of the transmembrane protein E-cadherin and gain in the positivity of tumor cells for mesenchymal markers such as N-cadherin. Another EMT-related biomarker is known as SLUG (SNAIL2), which is a member of the SNAIL family. SLUG is a zinc-finger nuclear transcription protein that can suppress the E-cadherin expression of epithelial cells and favor carcinoma progression<sup>[1,2]</sup>. There is little known about the clinical significance of E-cadherin, N-cadherin or SLUG in GISTs<sup>[3,4]</sup>. The first report concerning the clinical significance of SLUG expression in GIST was published in 2017<sup>[3]</sup>. This study is the second.

CD44 is a transmembrane glycoprotein that plays role in cell-cell adhesion, migration and cell differentiation; during pathological processes, it is involved in tumor cell proliferation, invasion and metastasis<sup>[5,6]</sup>. CD44 expression is correlated with the phenotype of cancer stem cells but its role in GIST is unclear<sup>[7]</sup>.

V-set and immunoglobulin domain containing 1 (VSIG1) or membrane glycoprotein A34, is a member of the junctional adhesion molecules family expressed in normal gastric mucosa and tumors of the upper, but not lower, gastrointestinal tract. Testicular germ cells and ovarian cancers can also display VSIG1 positivity<sup>[2,8,9]</sup>. The clinical significance and the function of VSIG1 expression in GISTs or other mesenchymal tumors has not yet been explored in the studies published to date.

## MATERIALS AND METHODS

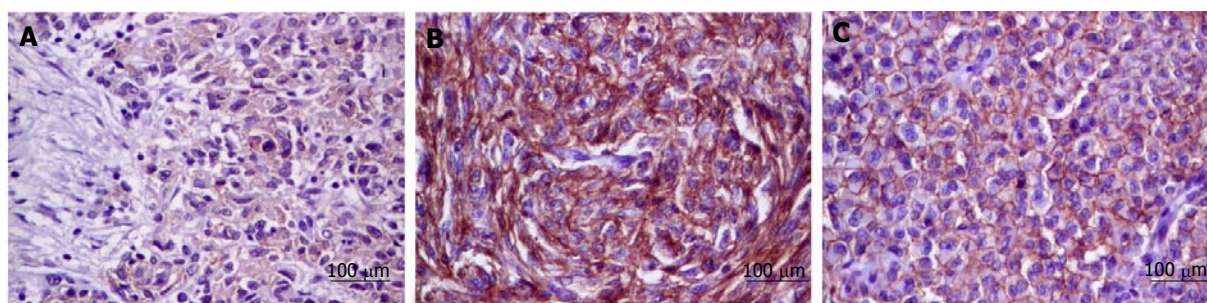
In the present study we retrospectively evaluated the paraffin-embedded specimens provided from 80 consecutive cases of GISTs diagnosed in our department from 2003 to 2015 in our clinic. The Ethical Committee approval was obtained from the University of Medicine and Pharmacy of Targu-Mures, Romania, and the research was performed according to the Helsinki criteria.

The diagnosis of GISTs was performed according to the modified National Institute of Health consensus classification<sup>[10]</sup>. The IHC diagnosis was based on the the c-KIT/DOG-1/PKCθ panel<sup>[11]</sup>. The aggressivity was assessed based on the mitotic count associated with the Ki67 index<sup>[10]</sup>.

**Table 1** Immunohistochemical antibodies used in the study

Antibody (company)	Clone	Dilution
C-KIT (Dako)	Rabbit polyclonal	1:500
DOG1 (Novocastra)	NCL-L-DOG1	1:50
PKCθ (ABCAM)	Polyclonal	1:200
SLUG (Santa Cruz Biotech)	Rabbit polyclonal	1:100
E-cadherin (Dako)	Monoclonal mouse NCH-38	1:50
N-cadherin (Dako)	Monoclonal mouse 6G11	1:100
Ki67 (LabVision)	SP6	1:200
CD44 (Dako)	Monoclonal mouse DF1485	1:50
VSIG1 (SIGMA)	Rabbit polyclonal HPA036311	1:200

VSIG1: V-set and immunoglobulin domain containing 1.



**Figure 1** Immunohistochemical profile of gastrointestinal stromal tumors ( $\times 20$ ). A: Cytoplasmic expression of N-cadherin; B: Cytoplasmic expression of CD44; C: Membrane positivity of CD44.

Tissue microarray (TMA) blocks were constructed for this study. From each case, three representative areas of each GIST tissue (3 mm diameter core) were used. The following IHC markers have been assessed: E-cadherin, N-cadherin, SLUG, VSIG1 and CD44 (Table 1). For each antibody, a cut-off value of 5% was used. The E-cadherin and N-cadherin were quantified in the cell cytoplasm. For CD44, the cytoplasmic and/or membrane positivity was taken into account (Figure 1). Regarding SLUG and VSIG1, the cases were considered positive based on the nuclear and/or cytoplasmic staining (Figure 2). Two pathologists independently performed the IHC assessment.

Statistical analysis was done with the GraphPad InStat 3 software and two-sided tests with a  $P$ -value  $< 0.05$  and a 95%CI were considered as statistically significant. Kaplan-Meier curves and long-rank test were used to evaluate the independent prognostic value of the examined biomarkers. The median follow-up was  $74 \pm 44.87$  mo (range: 9-163 mo) and the overall survival (OS) was considered to be the time (in months) from operation to death or last follow-up.

## RESULTS

### Clinicopathological characteristics

Overall, 80 patients were included in the study, 45 women and 35 men, with a median age of  $61.58 \pm 11.84$  years (range from 19 to 80 years). The most common location of GISTs was the stomach ( $n = 35$ ), followed by the small intestine ( $n = 25$ ), colorectum

( $n = 6$ ) and extra-gastrointestinal area ( $n = 14$ ). The median tumor size was of  $6.47 \pm 1.34$  cm (range: 0.4-21 cm). The spindle cell morphology predominated ( $n = 64$ ), followed by the epithelioid ( $n = 2$ ) and mixed architecture ( $n = 14$ ). There was no lymph node metastases observed in the examined cases. Distant metastases ( $n = 11$ ) were localized in peritoneum ( $n = 6$ ) and liver ( $n = 5$ ) (Table 2).

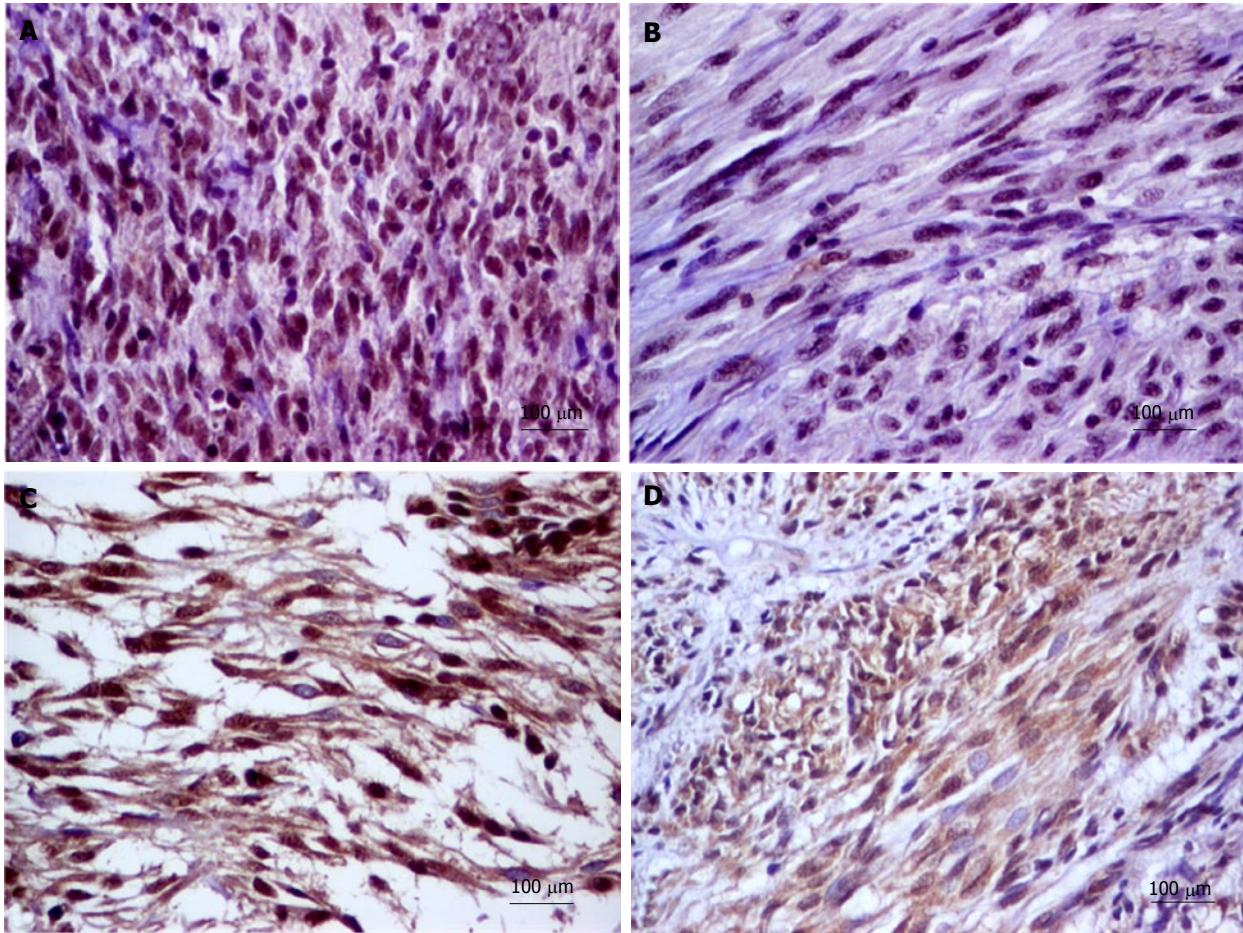
### Immunohistochemical features

E-cadherin positivity was not noted in the examined cases. Most of the cases ( $n = 71$ ; 88.75%) showed SLUG positivity and VSIG1 positivity was seen in 67 of the 80 cases (83.75%). CD44 and N-cadherin showed positivity in 29 out of 80 (36.25%) and 8 out of 80 cases (10%) respectively.

Not one of the four positive markers (SLUG, CD44, N-cadherin and VSIG1) was statistically correlated with the clinicopathological factors, which included gender, age, tumor size, mitotic rate, tumor location, histological type, intratumoral necrosis, risk degree, Ki67 proliferation index, local invasion, presence or absence of distant metastasis. Most of the extra-gastrointestinal stromal tumors (e-GISTs) displayed SLUG and VSIG1 expression without N-cadherin and CD44 positivity (Table 2).

All of the cases with distant metastasis showed the immunophenotype SLUG nuclear positivity/VSIG1 nuclear positivity/N-cadherin $\pm$ /CD44 $\pm$ . All of the 13 cases, which were negative for VSIG1, displayed nuclear SLUG positivity and were negative for





**Figure 2** Subcellular localization of the immunohistochemical markers (nuclear and/or cytoplasmic) in gastrointestinal stromal tumors ( $\times 20$ ). A, B: SLUG; C, D: V set and immunoglobulin domain containing 1 (VSIG1).

N-cadherin. They were included in the cases with a high mitotic rate, high Ki67 index and the high-risk group.

The nine SLUG negative cases that displayed positivity for VSIG1 (predominantly in the cytoplasm) but not for N-cadherin, did not present necrosis and were included in the cases with a low mitotic rate, Ki67 negative and low-risk group.

All of the six c-KIT negative cases expressed SLUG positivity and were negative for N-cadherin. These cases were positive or negative for CD44 or VSIG1. The expression of SLUG was not correlated with N-cadherin expression ( $P = 0.58$ ). A reverse correlation was seen between PKC $\theta$  and N-cadherin ( $P = 0.029$ ) and also between N-cadherin and VSIG1 ( $P = 0.021$ ). The VSIG1 expression was directly correlated with the PKC $\theta$  pattern ( $P = 0.012$ ) (Table 3).

### Clinical outcome

The patients with VSIG1-negative GISTs showed a shorter OS than those with tumors that display VSIG1 positivity ( $P = 0.01$ ). A univariate Cox regression analysis showed that OS also decreased with CD44 positivity ( $P = 0.06$ ) and slightly decreased in patients with SLUG or N-cadherin positive GISTs (Figure 3). The VSIG1 expression was the most significant independent prognostic factor.

Based on the above-mentioned aspects, we presume that the loss of VSIG1 is an independent predictor of low OS whereas nuclear positivity for VSIG1 might indicate risk for distant metastasis. The cytoplasmic expression of a GIST is not an indicator of high risk. SLUG positivity indicates an increased risk of metastatic behavior whereas the loss of SLUG positivity is associated with longer OS. Double nuclear positivity for SLUG and VSIG1 indicates aggressive behavior especially for e-GISTs. The GISTs might be classified as tumors with high (SLUG nuclear positivity/VSIG1 negative or nuclear positivity/N-cadherin $\pm$ /CD44 $\pm$ ) or low risk for MET-induced aggressivity (SLUG negative/VSIG1 negative or cytoplasmic positivity/N-cadherin $\pm$ /CD44 $\pm$ ).

### DISCUSSION

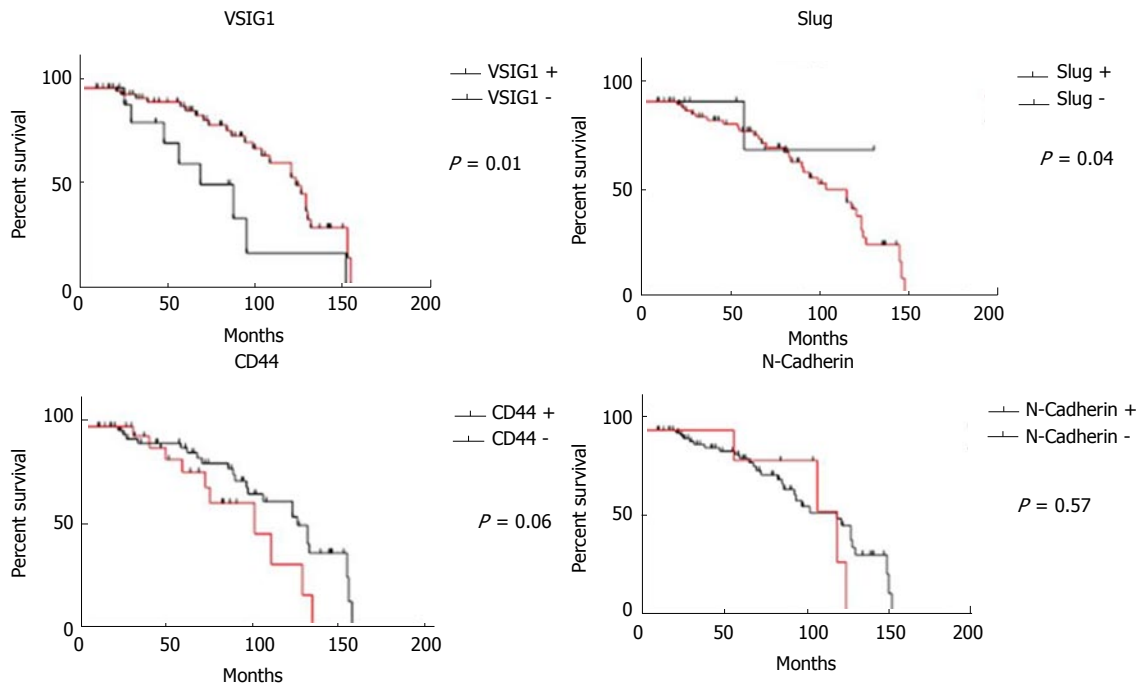
The EMT/MET-related biomarkers examined in the present study may have induced aggressivity as result of their role as nuclear transcription factors but CD44. It is important to note that CD44 is also known as a stemness-related biomarker.

About 20%-50% of GISTs can display SLUG expression<sup>[3,12-15]</sup>. Due to the cut-off value of 5% used here, compared to the 20% used in other studies<sup>[3]</sup>,



Table 2 Correlation of SLUG, N-Cadherin, CD44 and V-set and immunoglobulin domain containing 1 expression with the clinicopathological parameters in gastrointestinal stromal tumors																	
n	SLUG			CD44			N-Cadherin			VSG1			P				
	-	+	OR (95%CI)	P <sub>vaule</sub>	-	+	OR (95%CI)	P	-	+	OR (95%CI)	P					
Gender																	
Male	35	2	33	0.32 (0.06-1.69)	0.28	22	13	0.93 (0.37-2.33)	0.88	33	2	2.53 (0.47-13.4)	0.45	5	30	0.77 (0.22-2.60)	0.76
Female	45	7	38			29	16			39	6			8	37		
Age																	
≤ 45	8	0	8	0.39 (0.02-7.38)	0.58	6	2	1.8 (0.33-9.56)	0.7	7	1	0.75 (0.08-7.05)	0.58	0	8	0.25 (0.01-4.77)	0.34
> 45	72	9	63			45	27			65	7			13	59		
Tumor size																	
≥ 5 cm	45	6	39	1.64 (0.38-7.08)	0.72	29	16	1.07 (0.42-2.68)	1	40	5	0.75 (0.16-3.37)	0.95	9	36	1.93 (0.54-6.91)	0.37
< 5 cm	35	3	32			22	13			32	3			4	31		
Mitotic rate (50HPF)																	
High (≥ 5)	29	1	28	0.19 (0.02-1.62)	0.14	18	11	0.89 (0.34-2.29)	0.81	24	5	0.30 (0.06-1.36)	0.13	5	24	1.11 (0.32-3.80)	1
Low (< 5)	51	8	43			33	18			48	3			8	43		
Tumor location																	
Stomach	35	4	31	NA	0.47	26	9	NA		32	3	NA	0.26	8	27	NA	0.21
Small intestine	25	2	23			10	15		0.93	23	2			2	23		
Colorectum	6	0	6			5	1			4	2				4		
E-GIST	14	3	11			10	4			13	1			1	13		
Histological pattern																	
Spindle cell type	64	7	57	NA	0.82	40	24	NA		58	6	NA	0.62	11	53	NA	0.72
Epithelioid cell type	2	0	2			1	1		0.75	0	2			0	2		
Mixed type	14	2	12			10	4			14	0			2	12		
Risk group																	
Very low	10	2	8	NA	0.59	5	5	NA		10	0	NA	0.5	1	9	NA	0.77
Low	21	3	18			14	7			19	2			3	18		
Intermediate	16	2	14			13	3		0.19	15	1			2	14		
High	33	2	31			19	14			28	5			7	26		
Local invasion																	
Positive	14	1	13	0.55 (0.06-4.85)	0.96	6	8	0.35 (0.10-1.13)	0.12	11	3	0.30 (0.06-1.44)	0.14	1	13	0.34 (0.04-2.90)	0.44
Negative	66	8	58			45	21			61	5			12	54		
Distant metastasis																	
Present	11	0	11	0.27 (0.01-5.09)	0.38	7	4	0.99 (0.26-3.73)	1	8	3	0.20 (0.04-1.04)	0.07	0	11	0.18 (0.01-3.28)	0.19
Absent	69	9	60			44	25			64	5			13	56		
Necrosis																	
Present	32	1	31	0.16 (0.09-1.36)	0.07	18	14	0.58 (0.23-1.47)	0.23	27	5	0.36 (0.07-1.62)	0.25	3	29	0.39 (0.09-1.55)	0.22
Absent	48	8	40			33	15			45	3			10	38		

VSIG1: V-set and immunoglobulin domain containing 1; GIST: Gastrointestinal stromal tumors.



**Figure 3** Kaplan Meier survival analysis in gastrointestinal stromal tumors. Immunoexpression of some epithelial mesenchymal/mesenchymal epithelial transition-related markers influences the overall survival.

the positivity rate was found to be higher (88.75%) in our study. Although a possible link between the *KIT* signaling pathway and the *SLUG* transcription factor has been proven in experimental studies, it was not proven in our material<sup>[3]</sup>. *SLUG* is also proposed to have stemness properties<sup>[3]</sup> but we did not find it to correlate with *CD44*. In GISTs, *SLUG* positivity is considered to be an indicator of a high cell proliferation rate but not for cancer progression<sup>[3,12,13]</sup> especially in e-GISTs<sup>[12-14]</sup>.

In line with the literature, we confirm the role of *SLUG* in GISTs aggressivity, especially for e-GISTs. *SLUG* acts as a nuclear transcription factor, being more frequently expressed by large GISTs with pleomorphic nuclei and high mitotic index<sup>[3]</sup>, and as an indicator of risk for systemic metastases and/or local invasion<sup>[3,15]</sup>.

In the present material, double nuclear positivity for *SLUG* and *VSIG1* has been identified in the metastatic cases and the loss of *VSIG1* is associated with a lower OS. Although no data regarding the role of *VSIG1* in GIST have been published, its nuclear positivity indicates its possible role as a nuclear transcription factor. In normal gastric epithelium, *VSIG1* plays the role of the junctional adhesion molecule that can be lost in carcinomas, as an indicator for a worse clinical outcome<sup>[8,9]</sup>. In mesenchymal tumors such as GISTs, its loss may indicate a lower survival rate whereas membrane/cytoplasm to nuclear transcription may stimulate tumor cells proliferation and their migration in the blood vessels. As *VSIG1* is considered to be a novel target for antibody-based cancer immunotherapy<sup>[8]</sup>, this therapy may benefit patients with *VSIG1*-positive metastatic GISTs. We found a

direct correlation between *VSIG1* and the expression of *PKCθ* and a reverse correlation with *N-cadherin* expression.

The potential role of *N-cadherin* in increasing the metastatic potential of GISTs was previously proposed<sup>[16]</sup> but not confirmed<sup>[4]</sup>.

The cell-cell adhesion molecule *E-cadherin* and *AE1/AE3* keratin might be expressed by one third of GISTs<sup>[12,13]</sup> as an indicator of low invasion properties and low risk for recurrence<sup>[17,18]</sup>. In leiomyosarcomas the increased expression of *E-cadherin* and decreased *SLUG* expression was associated with decreased cell proliferation, invasion, and migration<sup>[19]</sup>. In this study, lower levels of aggressive behavior were shown by *SLUG* negative GISTs.

The *CD44* stemness marker was expressed in one quarter of the cases but its positivity can be shown by more than 70% of the GISTs<sup>[20,21]</sup>. The role of *CD44* in tumor progression and metastatic capacity of GISTs has been analyzed in a few studies, however the results are controversial. *CD44* positivity might be an indicator of better prognosis<sup>[20]</sup>. The high-risk group GISTs displayed a significant loss of *CD44* expression<sup>[21]</sup>. Being universally expressed in GISTs, *CD44* and *CD133* may represent a linkage rather than cancer stem cell markers<sup>[22,23]</sup>. We did not prove a statistical correlation between *CD44* and *SLUG*. A slightly lower OS was proven for *CD44* positive cases compared with *CD44* negative ones.

In conclusion, we hypothesized that the EMT/MET of GISTs involves the upregulation of the nuclear transcription factors *SLUG* and *VSIG1*. The main shortfall of this paper is the small number of examined

**Table 3** Correlation of the diagnostic biomarkers with the epithelial mesenchymal/mesenchymal epithelial transition -related factors SLUG, N-Cadherin, CD44 and V-set and immunoglobulin domain containing 1 in gastrointestinal stromal tumors

	<i>n</i>	SLUG				CD44				N-Cadherin				VSIG1			
		-	+	OR (95%CI)	<i>P</i>	-	+	OR (95%CI)	<i>P</i>	-	+	OR (95%CI)	<i>P</i>	-	+	OR (95%CI)	<i>P</i>
Ki67 index																	
Low	60	9	51	7.56 (0.42-136.02)	0.16	39	21	1.23 (0.43-3.50)	0.68	55	5	1.94 (0.42-8.97)	0.4	9	51	0.70 (0.19-2.60)	0.6
High	20	0	20			12	8			17	3			4	16		
C-KIT																	
Positive	74	9	65	1.88 (0.09-36.23)	0.67	47	27	0.87 (0.14-5.06)	0.87	66	8	0.60 (0.03-11.65)	0.73	11	63	0.34 (0.05-2.14)	0.25
Negative	6	0	6			4	2			6	0			2	4		
DOG-1																	
Positive	61	7	54	1.10 (0.20-5.81)	0.11	37	24	0.55 (0.17-1.72)	0.3	54	7	0.42 (0.04-3.72)	0.44	6	55	0.18 (0.05-0.65)	0.01
Negative	19	2	17			14	5			18	1			7	12		
C-theta																	
Positive	72	7	65	0.321 (0.05-1.91)	0.21	45	27	0.55 (0.10-2.95)	0.49	67	5	8.04 (1.47-43.81)	0.02	9	63	0.14 (0.03-0.67)	0.02
Negative	8	2	6			6	2			5	3			4	4		

VSIG1: V-set and immunoglobulin domain containing 1.

cases. The role of the adhesion molecule N-cadherin and stemness factor CD44 in GISTs should be further explored in studies which include a higher number of GISTs. The possible predictive role of VSIG1 expression for immunotherapy and the prognostic significance of its subcellular localization also deserve further exploration.

## COMMENTS

### Background

There are no data in literature regarding the role of V-set and immunoglobulin domain containing 1 (VSIG1) in the gastrointestinal stromal tumors (GISTs) aggressivity even about its interaction with other biomarkers involved in the epithelial mesenchymal transition/mesenchymal epithelial transition. This is the first immunohistochemistry study exploring the VSIG-related aggressivity of GISTs.

### Research frontiers

The subcellular location of the mesenchymal epithelial transition-related biomarkers might influence the GIST evolution.

### Innovations and breakthroughs

In this paper, the authors hypothesized for the first time in the current literature that the GIST aggressivity may be induced by upregulation of the nuclear transcription factor SLUG and the loss or cytoplasm-to-nuclear translocation of VSIG1.

### Applications

The possible predictive role of VSIG1 expression for immunotherapy and the prognostic significance of its subcellular localization also deserve further exploration.

### Terminology

Epithelial mesenchymal transition represents loss of the epithelial phenotype with reverse gain of a mesenchymal immunoprofile. Mesenchymal epithelial transition is the reverse phenomenon. These processes are mediated through several signalling pathways that are incompletely understood in GISTs.

### Peer-review

This paper reported possible role of VSIG1 in GISTs for the first time, which is related with expression of the other markers involved in the epithelial mesenchymal/mesenchymal epithelial transition.

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Clinical Trials Study

# Value of histomorphometric tumour thickness and smoothelin for conventional m-classification in early oesophageal adenocarcinoma

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**Data sharing statement:** The data set and search algorithms are available from the corresponding author at [bruno.maerkl@klinikum-augsburg.de](mailto:bruno.maerkl@klinikum-augsburg.de).

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## Abstract

### AIM

To test the validity of tumour thickness measurement in distinguishing between the different infiltration depths, especially when the duplication of muscularis mucosae cannot be demarcated clearly.

### METHODS

We re-evaluated 100 completely embedded Barrett's adenocarcinomas regarding m-classification, maximum tumour thickness, and muscularis mucosae duplication. For validation, smoothelin staining was performed on a subset of cases.

### RESULTS

The m1-, m2- and m3-classified adenocarcinomas

showed a significant lower tumour thickness compared to the m4- and sm1-classified lesions ( $P < 0.001$ ). Smoothelin staining determined a clear muscularis mucosae duplication in 64% of the tested samples and enabled the differentiation of the two layers in diffuse and merged splits.

### CONCLUSION

Tumour thickness in early oesophageal adenocarcinoma significantly correlates with the depth of infiltration and demonstrates its worth as an accurate pT classification in non-polypoid lesions. We created a new algorithm, which combines histomorphology with morphometric analyses. It is noteworthy that it facilitates the assessment of mucosal *vs* submucosal infiltration depth. The smoothelin staining strengthened our results of the tumour thickness evaluation and can be used in cases of doubt.

**Key words:** Smoothelin; Endoscopic submucosal dissection; Muscularis mucosae; Barrett's carcinoma

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**Core tip:** The aim of this study was to determine whether histomorphometric measurement of tumor thickness and immunohistochemical staining for smoothelin facilitate the exact pT substaging in early oesophageal adenocarcinoma. Our data showed that there is clear cut-off of 1000  $\mu\text{m}$  to distinguish advanced early lesions (M4/sm1) from such lesions that do not reach the deep muscularis mucosae or the submucosa. Moreover, smoothelin staining is of help to distinguish the superficial from the deep muscularis mucosa by different staining intensities. Therefore, both methods could be shown to be of help for the often challenging task to T-classify early oesophageal adenocarcinomas.

Endhardt K, Märkl B, Probst A, Schaller T, Aust D. Value of histomorphometric tumour thickness and smoothelin for conventional m-classification in early oesophageal adenocarcinoma. *World J Gastrointest Oncol* 2017; 9(11): 444-451 Available from: URL: <http://www.wjgnet.com/1948-5204/full/v9/i11/444.htm> DOI: <http://dx.doi.org/10.4251/wjgo.v9.i11.444>

## INTRODUCTION

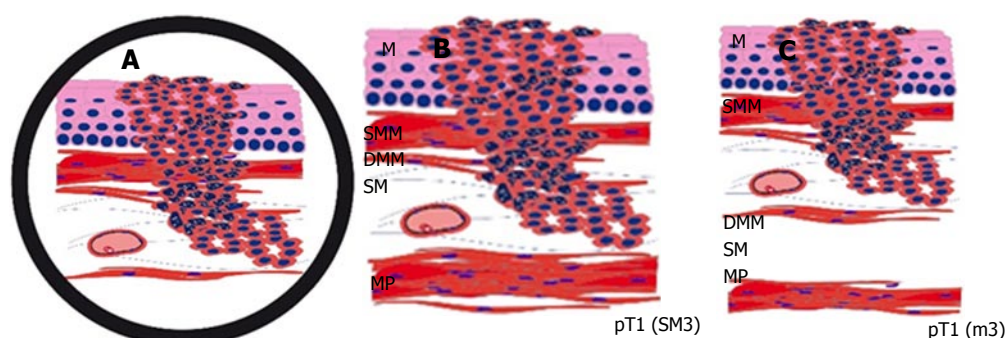
Early oesophageal adenocarcinoma, arising from Barrett Oesophagus, most frequently occurs in Caucasians and shows a rising incidence in recent decades<sup>[1-5]</sup>. The latest studies confirm that endoscopic submucosal dissection (ESD) is a safe and curative endoscopic method, yielding better R0 resection rates than endoscopic mucosal resections (EMR)<sup>[1,3,5,6]</sup>. Since the current endoscopic techniques allow for minimally invasive curative en-bloc resection of early carcinoma,

it is essential to determine the accurate depth of infiltration. In particular, it is of crucial importance to distinguish between mucosal and submucosal invasion. While this is simple in most areas of gastrointestinal tumours, it can be a challenging problem in Barrett's carcinoma. This is mainly caused by muscularis mucosae duplication, which was first described in 1990<sup>[6,7]</sup>. Based on Vieth and Stolte, the intramucosal carcinoma is divided into 4 subgroups, depending on the infiltration depth as follows: m1, limited to the Lamina propria mucosae; m2, the superficial muscularis mucosae (SMM); m3, the layer in between the superficial and deep muscularis mucosae (DMM) and m4, the DMM<sup>[7,8]</sup>. Submucosal invasion sm1 ( $< 500 \mu\text{m}$ ), in association with poor differentiation (G3) or diameters  $> 20 \text{ mm}$ , as well as sm2-invasion ( $> 500 \mu\text{m}$ ) or deeper, are indications for a subsequent surgical resection<sup>[8,9]</sup>. Furthermore, significantly higher rates of lymphatic invasion and lymph node metastasis identify sm1-invasion and are important prognostic factors<sup>[9,10]</sup>. Due to the diffuse and confusing splits of the muscularis mucosae, poor material quality or piecemeal resections (EMR), the subclassification cannot be properly defined in each sample. When evaluating the slides, it is always important to keep in mind that the tumour beneath a layer of smooth muscle is not necessarily submucosal (sm) but could just as well be located within a splintered muscularis mucosae (m3) (Figure 1). Penetration of the SMM can be mistaken for an initial submucosal invasion and may have severe consequences to the further treatment plan, including mismanagement, such as oesophagectomy without adequate indication<sup>[10,11]</sup>. We, therefore, examined our ESD collection with early oesophageal adenocarcinoma and searched for a new parameter facilitating the exact subclassification as an adjunct to the conventional histomorphological method. With the help of a histomorphometric tumour thickness measurement, we intended to establish a cut-off value for distinguishing between the penetration of the SMM and the DMM, where the latter is equivalent to submucosal infiltration. The use of smoothelin immunohistochemistry for distinguishing between the muscularis mucosae and the muscularis propria is well established in bladder carcinoma<sup>[12]</sup>. In staging Barrett's adenocarcinoma, it is not yet fully evaluated, but recent studies emphasized the different staining intensities in the superficial compared to DMM as an important discriminatory marker<sup>[13,14]</sup>. To confirm the reported findings and evaluate the additional discriminatory power, the smoothelin staining was used in a subset of cases in combination with the histomorphometric analysis.

## MATERIALS AND METHODS

### Specimens

The study was performed according to the national rules and was approved by the institutional ethical



**Figure 1** The difficulty in distinguishing between the smooth muscle cells of the muscularis mucosae, which can eventually be splintered in the superficial muscularis mucosae and the deep muscularis mucosae, and those of the muscularis propria. This schematic drawing shows a section of a Barrett's adenocarcinoma that may, at first glance appear, relatively straightforward, but determining the type of muscle layer can be challenging. Identifying large vessels (A) may suggest the diagnosis of submucosal invasion (B) and is, therefore, a well-known pitfall, as large vessels can also be found in between the superficial and DMM (C). An intramucosal carcinoma pT1 (m3) can therefore easily be mistaken as a submucosal carcinoma pT1 (sm). M: Mucosa; SMM: Superficial muscularis mucosae; DMM: Deep muscularis mucosae; MP: Muscularis propria.

review board of the Klinikum Augsburg. All the ESDs of Barrett Oesophagus with adenocarcinoma between 2008 and the end of 2014 were retrieved from the archives of the Institute of Pathology, Klinikum Augsburg (Augsburg, Germany), and all the sections (the lesions were oriented and completely paraffin-embedded) were reviewed by at least two independent experienced pathologists without knowledge of the initial report (BM, DA, and HA). In discrepant cases, a consensus diagnosis was established after a re-evaluation on a multi-headed microscope. Haematoxylin and eosin-stained sections were reviewed regarding the pT (m/sm)-subclassification, grading, and resection margins. Neoplastic superficial lesions with a polypoid morphology (Paris endoscopic classification type I) were excluded from the study to avoid a bias of the measuring method. Flat, slightly elevated or slightly depressed tumours (Paris endoscopic classification type II) were included. A validation set of 25 cases (beginning of 2015 to mid-2015) was selected from our archives and was analysed as described.

### Morphometric analysis

After detecting the area with the deepest infiltration, the tumour thickness was measured in millimetres using a digital camera with calibrated software (ProgRes C10, Jenoptik, Jena). It is important to mention that the invasion depth was not of interest, but we were interested in the complete thickness of the tumour throughout all the mucosal layers, from the surface to the invasion front. In case of undermining growth beneath the intact epithelium, tumour thickness was measured from the most superficial neoplastic cell layer to the point of deepest invasion (Figure 2). Size values were assigned to the subgroups depending on the infiltration level.

### Immunohistochemistry and evaluation of the muscularis mucosae

Slides showing the deepest infiltration level from 33 randomly selected cases were sectioned and stained for smoothelin (Cell Marque Clone R4 A, monoclonal

mouse, dilution 1:100) and desmin (Dako, Clone D33, monoclonal mouse, dilution 1:200). The Ventana Ultraview detection system (Roche Diagnostics, Mannheim, Germany) was used for the development of the reactions. The slides were evaluated for the following parameters: Length of muscularis mucosae duplication within the Barrett's lesion in percent ( $\leq 5\%$ ,  $> 5$  and  $\leq 50\%$ ,  $> 50$  and  $\leq 95\%$ ,  $> 95\%$ ); scattered split and notable difference in staining intensity of the SMM vs the DMM. Discrepant cases were reviewed together for a consensual diagnosis.

### Statistical analysis

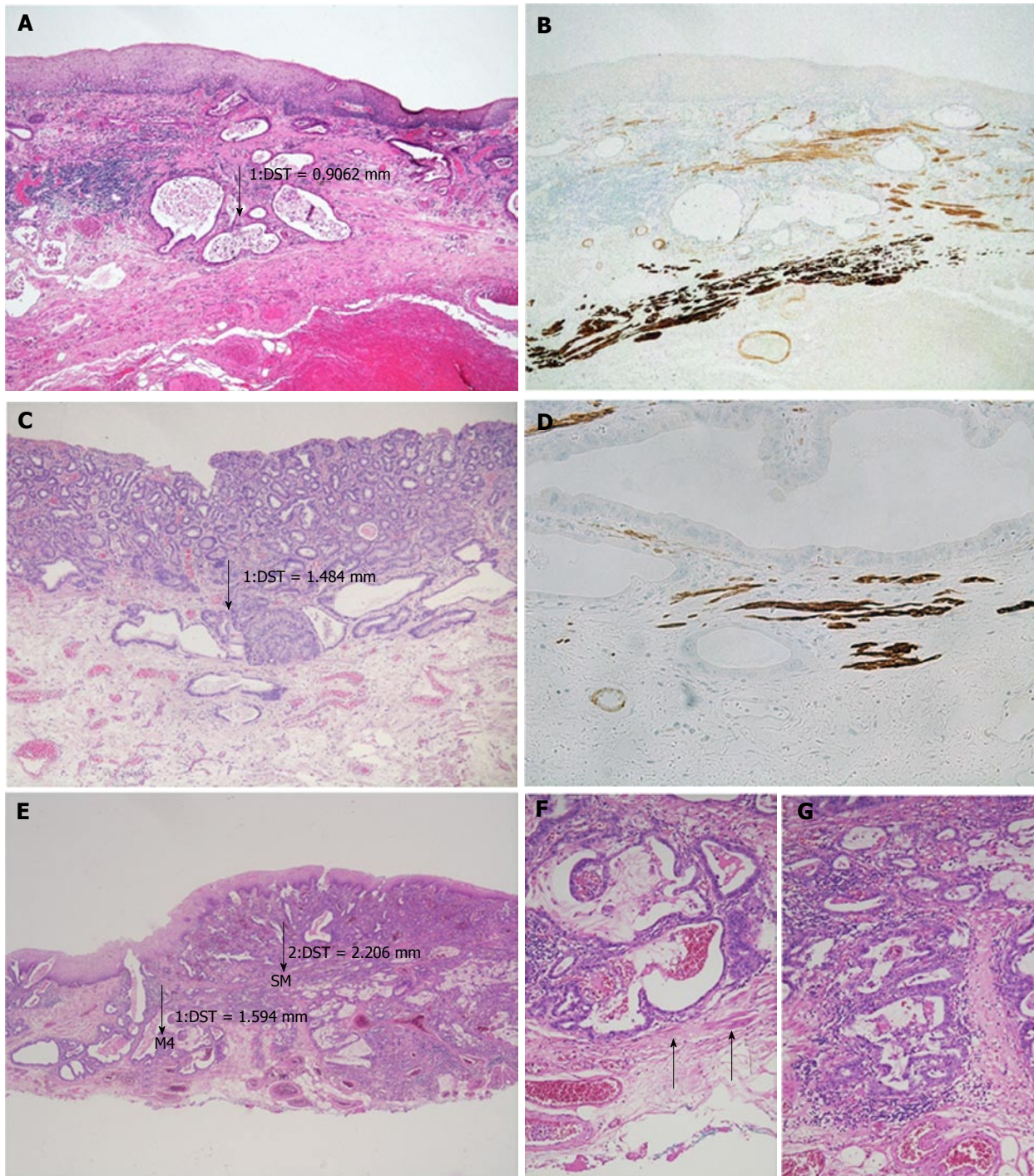
The Spearman Rank Order Correlation Test was used to calculate the correlation between tumour thickness and pT stage. A One-Way Repeated Measures Analysis of Variance was used to compare the numeric values of the pT subgroups. To isolate the groups that differed from the others, the Holm-Sidak method was applied. The mean values are given with  $\pm 1$  SD. A *P*-value of  $< 0.05$  was considered significant. The sensitivity, specificity, and the positive and negative predictive values were calculated to measure the diagnostic accuracy of the morphometric evaluation. All the calculations were performed using the Sigma Plot 13.0 software package (Systat, Richmond, VA, United States).

## RESULTS

### Morphometry

Cases were primarily included for analysis based on the diagnosis in the original pathology report. A total of 100/100 cases (100%) were confirmed as harbouring an early adenocarcinoma arising in Barrett's mucosa. However, 12/100 cases (12%) had to be excluded due to a polypoid growth pattern. Thanks to the good quality of the ESD specimens, the overall orientation was excellent, and representative cross sections were available throughout most specimens. Only 5/100 cases (5%) had to be excluded due to technical issues, where rotated and tilted positions caused imprecise





**Figure 2** In case of undermining growth beneath the intact epithelium, tumour thickness was measured from the most superficial neoplastic cell layer to the point of deepest invasion. A: HE 50 ×; ESD specimen with early Barrett's adenocarcinoma pT1 (m3) with infiltration between the SMM and DMM and a tumour thickness of 900 µm. Tumour thickness was measured from the most superficial tumour cell layer to the deepest point of the invasion; B: Smoothelin IHC 50 ×; Immunohistochemical staining discriminates between the SMM (light brown) and DMM (dark brown). A tumour gland can be seen in-between the two muscle layers; C: HE 16 ×; Neoplastic glands reach the DMM. Smooth muscle fibres are found in the neighbourhood of the glands. The tumour thickness is approximately 1500 µm (m4); D: Smoothelin IHC 200 ×; Immunohistochemical staining confirms the m4 stage. Dark brown fibres of the DMM are found on the same level as the tumour glands; E: HE 16 ×; ESD specimen with an adenocarcinoma of the oesophagus that reaches the submucosa. The left-sided measurement was performed in an area where the tumour was restricted to a m4-stage (tumour thickness approximately 1600 µm). The right-sided measurement was in an area where the tumour already showed the beginning of an infiltration of the submucosa (tumour thickness approximately 2200 µm); F: HE 100 ×; Higher magnification of the m4-area of (E). Smooth muscle fibres (arrows) discriminate from sm-stage; G: HE 100 ×; Higher magnification of the sm-area of (E). The lack of muscle fibres indicates the sm-stage. ESD: Endoscopic submucosa dissection; SMM: Superficial muscularis mucosae; DMM: Deep muscularis mucosae; IHC: Immunohistochemistry; HE: Haematoxylin and eosin.

measurements and subclassifications. In 83/100 eligible cases (83%) with proper evaluation, the

infiltration depth was divided as follows: m1: 13/83 (16%); m2: 36/83 (43%); m3: 4/83 (5%); m4:

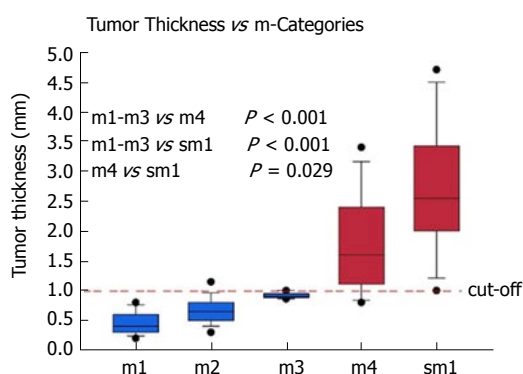


**Table 1** Comparison of the different subgroups

pT1 subgroup	Total	m1	m2	m3	m4	sm
No. of cases ( <i>n</i> )	83	13	36	5	13	16
Tumour thickness (mm)	0.2-4.7	0.2-0.8	0.3-1.4	0.9-1.0	0.8-3.4	1.0-4.7
Mean $\pm$ SD (mm)	-	0.46 $\pm$ 0.19	0.64 $\pm$ 0.24	0.91 $\pm$ 0.05	1.78 $\pm$ 0.79	2.69 $\pm$ 1.03

**Table 2** Muscularis mucosae duplication, *n* (%)

Total cases	33 (100)
Duplication	21 (64)
Percent of duplication in the lesion	
≤ 5%	3 (9)
> 5% and ≤ 50%	5 (15)
> 50% and ≤ 95%	3 (9)
> 95%	10 (30)

**Figure 3** Box plot of the median tumour thickness in the different m-categories. Circles = 5/95 percentiles. The dashed line indicates the proposed cut-off to discriminate between m1-3 and m4/sm1.

14/83 (7%) and sm: 16/83 (19%). Table 1 shows the tumour thickness in the different pT subgroups. It ranged from 0.2 to 4.7 mm.

pT1a Barrett's carcinomas that were restricted to the mucosa and did not show any infiltration of the DMM (m1-m3), showed significantly ( $P < 0.001$ ) lower levels of tumour thickness when compared to the pT1a (m4) and pT1b (sm1 or deeper) tumours (Figure 3). With increasing infiltration depth, the mean values of the tumour thickness steadily rose. Overall, there was a very strong correlation between the pT (m/sm) substages and tumour thickness ( $P < 0.0001$ ). The values were summarized by two groups, either m1/m2/m3 or m4/sm. After separation, we detected six cases (7%) with a slightly overlapping result, where the tumour thickness did not fit the diagnosed pT stage. The mean tumour thickness of the subgroup m1-m3 was significantly different compared to group m4 ( $P < 0.001$ ) and group sm1 and deeper ( $P < 0.001$ ). The overlaps were observed at approximately 1000  $\mu$ m. The statistical analysis, with a cut-off value of 1000  $\mu$ m, showed high sensitivity (94%) and specificity (90%) for the distinction between m1/m2/m3 vs m4/sm. The negative predictive value was 94%, and the positive predictive value 91%, showing

a strong correlation between the tumour thickness and infiltration depth. Only one case of this collection with a clear submucosal infiltration and tumour thickness of 3600  $\mu$ m showed lymphatic invasion. Venous invasions were not identified. Therefore, correlations between tumour thickness and vascular invasion could not be calculated.

To confirm our findings, a validation set of 25 cases from the beginning of 2015 to mid-2015 was reviewed and analysed as described. In this smaller data set, we confirmed the findings of the evaluation set with a specificity of 100%, whereas the sensitivity was 83.3%.

### Immunohistochemical evaluation of the muscularis mucosae

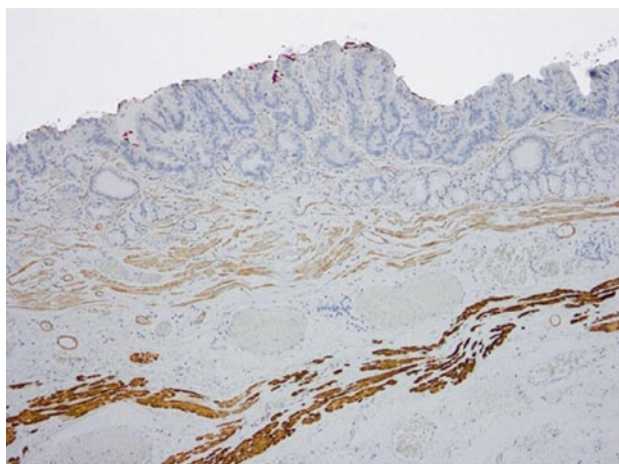
Immunohistochemistry for desmin showed a similar staining intensity in the superficial and DMM, even though the SMM usually appeared finer and more delicate. In 21/33 cases (64%), desmin and smoothelin showed a clearly identifiable duplication, with at least a focal dissociation of the muscularis mucosae. Table 2 shows the details of the percentages of the detected duplication within these 21 specimens.

A total of 9/33 cases (27%) did show a broadened muscular layer, where the superficial and DMM could not be demarcated. In 3/33 cases (9%), a single thin muscular layer appeared not to show any split at all.

The staining pattern with smoothelin varies considerably from desmin, with a weak-to-moderate staining of the SMM, while the DMM resembles the strong staining with desmin (Figure 4). In the 21 cases described with duplication, this remarkable difference in staining intensity between the two muscular layers was determined. Cases without any definable muscularis mucosae duplication showed a uniform smoothelin staining intensity. It is noteworthy, that all the duplicated specimens, even if duplication was only detected in a minor fraction of the lesion, showed the different intensity with the smoothelin staining.

## DISCUSSION

A significant observer variability is described for early oesophageal adenocarcinoma, leading to overdiagnosis followed by overtreatment<sup>[6,11,15]</sup>. In our experience, it is often challenging to determine the exact pT classification in early Barrett's adenocarcinoma. Most difficulties are caused by duplication or diffuse branchlike splintering of the muscularis mucosae (Figure 1). The appearance of duplication was first



**Figure 4** Expression of smoothelin immunohistochemistry demonstrating the different staining intensity in the duplicated muscularis mucosae layers.

described in the early 1990s<sup>[6,10,13-15]</sup>, and since then, it has been analysed in several studies, showing a strict limitation to Barrett's adenocarcinoma. It is reported in up to approximately 90% of Barrett-related neoplasia<sup>[10,13,14]</sup>. The origin and significance of the duplication still needs to be defined. Diffuse, irregular, and merged splits remain problematic in the histological assessment, leading to confusion and misinterpretation. In cases of inadequate orientation, an invasion of the space between the duplicated muscular layers (m3) could easily be mistaken as a submucosal invasion (sm). Since muscularis mucosae duplication is an inconsistent event and is usually not captured in all slides, the establishment of further standard evaluation criteria would be of great help.

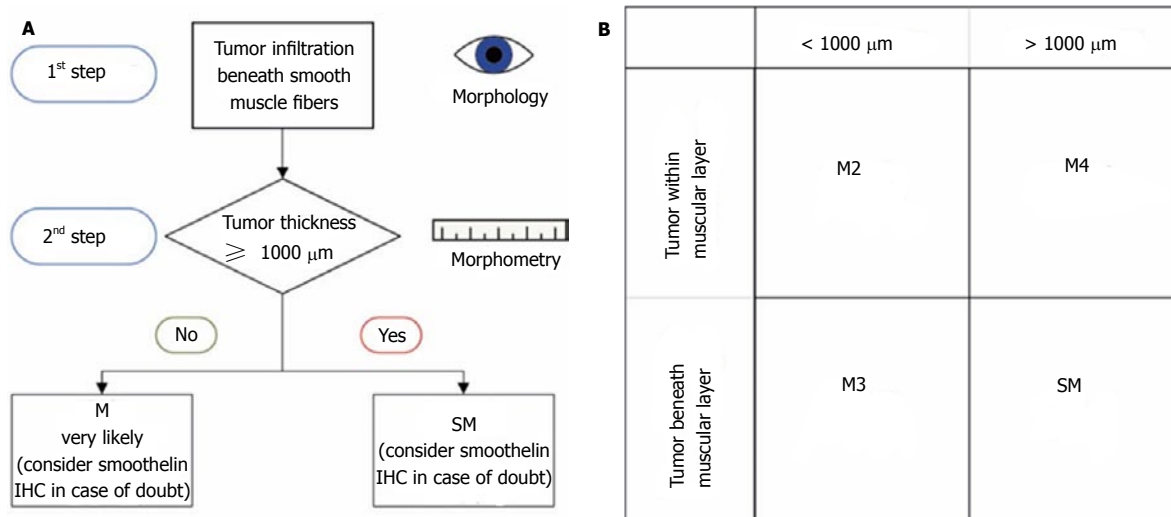
This study aimed, therefore, to find a complementary tool to support the histomorphologic assessment. The superficial and DMM are the histological hallmark structures to determine pT subclassification. However, this is complicated by the morphological variability of these structures. The ESD is almost exclusively performed in specialized gastroenterological centres and therefore, most studies, to date, are limited to the more common piecemeal specimens gained from EMR. The basis for this study was a unique dataset of 100 ESD specimens of early oesophageal adenocarcinoma. Due to *en-bloc* resection, an outstanding benefit in the histological examination is given compared to the EMR specimens. The lesions can be oriented accurately regarding the horizontal diameter, invasion depth and basal as well as circumferential resection margins. Moreover, orthogonal cutting provides an excellent overview through the different tissue layers. The majority of slides in this study depicted a complete cross section through the mucosa and in parts the submucosa, whereas the muscularis propria was demonstrably not captured in any of the samples.

Our hypothesis was that tumour thickness, which is easy to measure, might facilitate the pT assessment.

Exact pT staging is of crucial importance in early adenocarcinoma of the oesophagus as the risk of lymph node metastasis increases with infiltration depth<sup>[16,17]</sup>. In gastric and colorectal cancers, a morphometric measurement is already accepted in a different context for the evaluation of submucosal infiltration. An infiltration beyond the cut-off value indicates a higher risk for lymph node metastases and is defined as a limit for endoscopic treatment. Endoscopic mucosectomy is justified for a submucosal invasion of 500  $\mu\text{m}$  or less (stomach) or 1000  $\mu\text{m}$  or less (large bowel)<sup>[18-20]</sup>. In view of the morphometric measurement, it is important to mention that excessive stretching of the resected unfixed tissue can thin out the mucosal and submucosal layer and, therefore, lead to biased values<sup>[21]</sup>. To prevent this bias, all the specimens are pinned on a cork immediately after ablation, and no further tractions or tensions during the processing can influence the tumour thickness. For establishing our morphometric method, we investigated the tumour thickness from the surface to the invasion front by measuring the maximum infiltration depth. Since the initial point of invasion cannot always be defined and might lead to inter-observer variability, it appeared reasonable to not restrict our measurement to the invasive part of the tumour.

From a therapeutic point of view, the distinction between the mucosal and submucosal involvement is essential and usually the main issue at stake. In this study, we showed that the tumour thickness measurement, in combination with the histomorphology, is a robust method for the distinction of the infiltration depth. A Barrett adenocarcinoma with a muscular penetration and a tumour thickness < 1000  $\mu\text{m}$  is most likely a mucosal lesion pT1a (m3), whereas a muscular penetration with a tumour thickness  $\geq$  1000  $\mu\text{m}$  is most likely a submucosal lesion pT1b (sm). In certain cases, the immunohistochemical smoothelin staining is additionally helpful because it reveals the different muscle layers in the duplicated but irregularly merged or branchlike arranged muscle fibres. The detected cut-off value of 1000  $\mu\text{m}$  will help, especially in small and fragmented specimens, such as EMR, or specimens where the duplication cannot be properly classified. It is noteworthy that our method is restricted to non-polypoid lesions, which account for more than 85% of the cases.

Approximately 60%-70% of the analysed Barrett's adenocarcinomas harbour a distinctive muscularis mucosae split, which is close to the reported ranges of 92% ( $n = 50$ ) and 66% ( $n = 110$ )<sup>[13,14]</sup>. Faragalla *et al.*<sup>[10]</sup> did not report the frequency of duplication since their study is based merely on the cases showing duplication. Many cases in our study showed a clear identification of the two muscularis mucosae layers by smoothelin immunohistochemistry. The staining was, first of all, useful for determining the borders of the individual layer itself and was even more helpful in distinguishing the superficial from DMM, especially in the described



**Figure 5 Suggested algorithm for the pT staging in early adenocarcinoma of the oesophagus.** A: In the first step, the relationship between the tumour and the smooth muscle fibres is determined. The second step includes the measurement of the tumour thickness; B: This table illustrates the combinations of the tumour relationships to the smooth muscle fibres/tumour thickness and the corresponding T-sub-stages. M: Mucosa; SM: Superficial muscularis.

critical cases with irregular splits. In those cases, the differentiation often seemed virtually impossible to assess in the haematoxylin and eosin-stained sections and with desmin staining but was quite obvious with the smoothelin staining. Some diffuse splits can only be demarcated with the help of immunohistochemical analyses. Faragalla *et al.*<sup>[10]</sup> suggested the development of a modified smoothelin antibody that only stains the DMM, since, in his opinion, staining of the SMM could cause confusion. In our eyes, it was helpful to identify both layers with different staining intensities<sup>[10]</sup>. Solely staining with the smoothelin antibody can be insufficient for precise staging, but it offers an important additional benefit in complex and confusing areas of duplicated lesions. All three observers independently confirmed the diagnostic advantage of the smoothelin staining, in comparison with haematoxylin and eosin, as well as desmin.

In conclusion, our results show a very strong correlation between pT stage and tumour thickness. However, a clear cut-off was identified only for the discrimination of m1/m2/ m3 vs m4/sm stages. At first glance, this seems of minor value because the clinically most relevant discrimination is the separation between the m- and sm-cases. Nevertheless, we developed an algorithm to overcome this problem. The histomorphological evaluation of the tumour invasion, in relation to the smooth muscle fibres in combination with the tumour thickness measurement, allows us to translate our results into a mucosal vs submucosal discrimination (Figure 5). This, therefore, considerably facilitates accurate pT staging. In cases of doubt, further immunohistochemical staining with smoothelin is advocated. Our data consequently indicate that a morphometric measurement of tumour thickness and smoothelin staining supplement the histomorphological discrimination of mucosal vs submucosal invasion in

non-polypoid oesophageal adenocarcinoma. It has to be emphasized that the morphometric analysis is an adjunct to the conventional histomorphological evaluation and not its replacement.

## COMMENTS

### Background

The main problem of determine the infiltration depth in early oesophageal adenocarcinoma is caused by the duplication of the muscularis mucosae in early oesophageal adenocarcinoma. Based on Vieth and Stolte, the intramucosal carcinoma is divided into 4 subgroups, depending on the infiltration depth as follows: m1, limited to the Lamina propria mucosae; m2, the superficial muscularis mucosae (SMM); m3, the layer in between the superficial and deep muscularis mucosae (DMM) and m4, the DMM.

### Research frontiers

There are still controversies concerning the criteria for the histological diagnosis of barrett mucosa. Further research topics deal with the techniques to treat early oesophageal lesions.

### Innovations and breakthroughs

This work provides helpful adjuncts to evaluate the pT stage in early oesophageal adenocarcinoma. Because of its high clinical relevance this can be seen as a major advance.

### Applications

Histomorphometric measurement of the tumor thickness and smoothelin staining can be used as an adjunct to the conventional pT evaluation which is solely based on morphology.

### Terminology

EMR: Endoscopic mucosa resection; ESD: Endoscopic submucosa dissection; M: Mucosa; SMM: Superficial muscularis mucosae; DMM: Deep muscularis mucosae.

### Peer-review

This is a well-conducted and well-written study.

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# Gastric metastasis from ovarian adenocarcinoma presenting as a subepithelial tumor and diagnosed by endoscopic ultrasound-guided tissue acquisition

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**Institutional review board statement:** Since this is not a clinical study, this case report was not reviewed by the institutional review board.

**Informed consent statement:** Written informed consent for endoscopic examinations was obtained from the patient.

**Conflict-of-interest statement:** The authors state that they have no conflicts of interest regarding this case report.

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## Abstract

We describe an uncommon case of a patient with a metastatic adenocarcinoma of ovarian origin presented as a gastric subepithelial tumor (SET) and that was diagnosed by endoscopic ultrasound fine-needle biopsy (EUS-FNB). Malignant gastric lesions are rarely metastatic and the primary tumor is mainly breast, lung, esophageal cancer or cutaneous melanoma. Gastric metastasis from ovarian cancer is unusual, presenting synchronously with the primary tumor but also several years later than the initial diagnosis. From an endoscopic point of view, gastric metastasis does not present specific features. They may mimic both a primary gastric tumor or, less frequently, an SET.

This case demonstrates the importance of EUS-FNB in distinguishing SETs and how this may alter treatment and prognosis.

**Key words:** Metastasis; Subepithelial lesion; Gastric cancer; Ovarian; Endoscopic ultrasonography

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**Core tip:** Gastric metastasis from ovarian cancer is unusual, either as synchronous with the primary tumor or appearing several years after its initial diagnosis. The diagnosis is challenging because of the low incidence, especially when gastric metastases present as a subepithelial tumor. This case emphasizes the crucial role of endoscopic ultrasound fine-needle biopsy in the differential diagnosis of this rare condition.

Antonini F, Laterza L, Fuccio L, Marcellini M, Angelelli L, Calcina S, Rubini C, Macarri G. Gastric metastasis from ovarian adenocarcinoma presenting as a subepithelial tumor and diagnosed by endoscopic ultrasound-guided tissue acquisition. *World J Gastrointest Oncol* 2017; 9(11): 452-456 Available from: URL: <http://www.wjgnet.com/1948-5204/full/v9/i11/452.htm> DOI: <http://dx.doi.org/10.4251/wjgo.v9.i11.452>

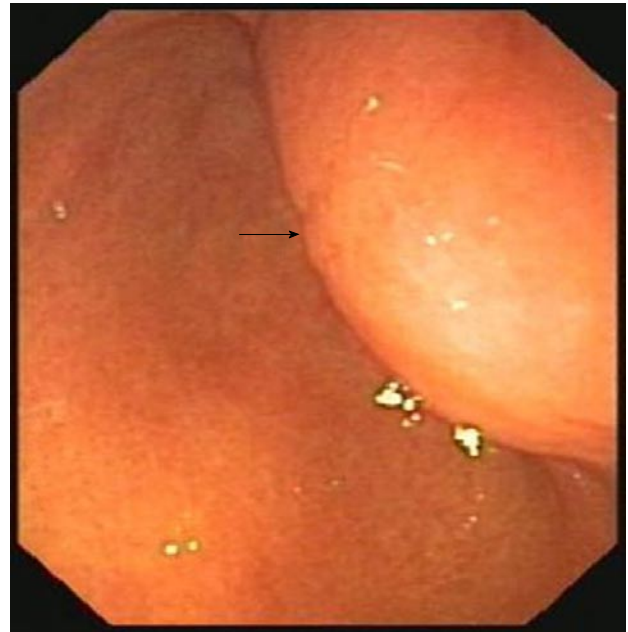
## INTRODUCTION

Gastrointestinal (GI) subepithelial tumors (SETs) are lesions located under a normal-appearing mucosa that include several neoplastic and non-neoplastic conditions. Most of the GI-SETs are asymptomatic, therefore their real incidence is unknown. Stomach is the GI tract where the highest incidence is documented<sup>[1]</sup>. The diagnosis of SETs can be challenging because conventional endoscopic biopsies are frequently inconclusive. Endoscopic ultrasound (EUS) is currently recommended as the preferred investigation modality to establish the exact nature of SETs because of its accuracy in differentiating them from extrinsic compression and providing information about morphology and layer of origin<sup>[2]</sup>. Lesions arising from the muscularis propria usually represent mesenchymal tumors, such as gastrointestinal stromal tumors (GIST), leiomyomas and schwannomas<sup>[1,2]</sup>. Metastasis to the GI tract generally involves the fourth and fifth layers and can be misleading as a GIST<sup>[1,2]</sup>.

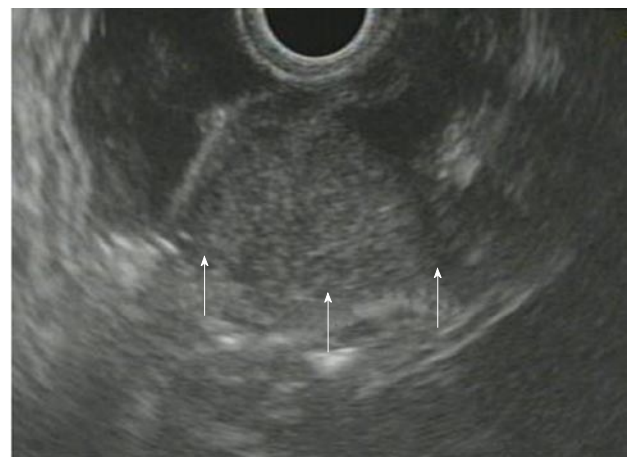
Herein, we describe an uncommon case of a patient with a metastatic adenocarcinoma of ovarian origin presented as a gastric SET mimicking a GIST and diagnosed by EUS-guided tissue acquisition.

## CASE REPORT

The patient was a 61-year-old woman diagnosed in November 2013 with stage IV high-grade serous carcinoma of the ovary treated with cycles of carboplatin



**Figure 1** Upper gastrointestinal endoscopy revealing a subepithelial tumor with intact overlying mucosa (black arrow) on the posterior wall of the gastric antrum.



**Figure 2** Endoscopic ultrasonography showing a homogenous, hypoechoic mass within the muscularis propria (white arrows). Its echogenicity appears to be more hyperechoic than that of the muscle layer.

and taxol chemotherapy with partial clinical response. After 1-year of therapy, due to the persistence of abdominal and pelvic disease, cytoreductive surgery had been performed. In June 2015, her CA125 levels had increased to 138 U/mL (normal value < 35 U/mL) and a computed tomography (CT) of the abdomen showed progression of the disease.

She was referred at that time to our endoscopic center for evaluation of dyspepsia. Upper GI endoscopy revealed an SET covered by normal mucosa on the posterior wall of the gastric antrum (Figure 1). Biopsies of the overlying mucosa proved inconclusive. EUS showed a 23-mm mass within the muscularis propria, hypoechoic but more hyperechoic than the muscular tissue (Figure 2). EUS-guided fine needle biopsy (FNB)

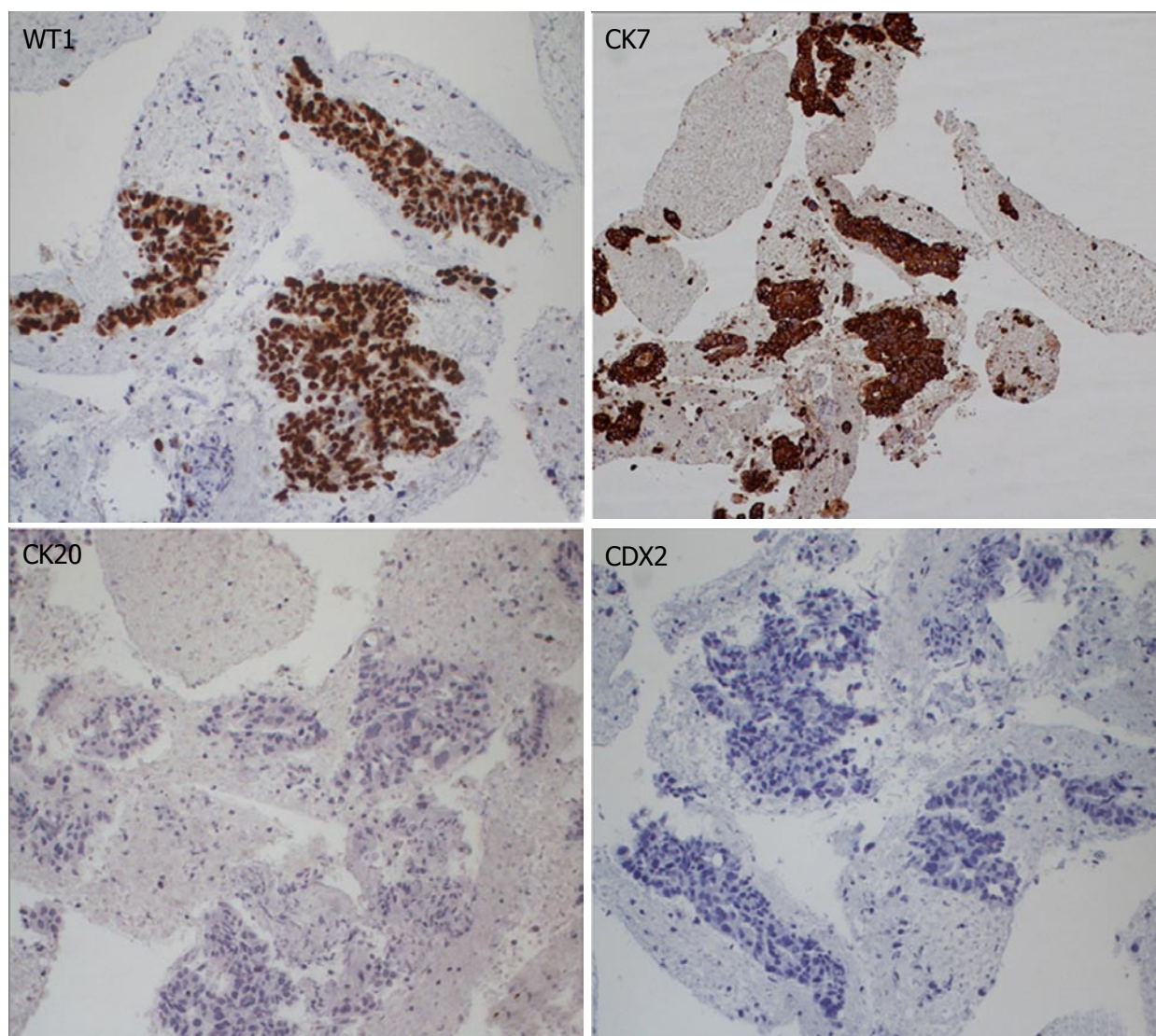


Figure 3 Histopathological images showing adenocarcinoma with immunohistochemistry positive for WT1 ( $\times 20$ ) and CK7 ( $\times 16$ ), and negative for CK20 ( $\times 20$ ) and CDX2 ( $\times 20$ ).

was performed for tissue diagnosis. Histology showed adenocarcinoma with immunohistochemistry positive for WT1 and CK7, and negative for CK20 and CDX2 (Figure 3). These findings supported the final diagnosis of a metastatic adenocarcinoma of ovarian origin. The patient started paclitaxel chemotherapy and was alive at the 18-mo follow-up visit.

## DISCUSSION

Gastric metastasis is rare and has been reported mainly from breast, lung, esophageal cancer or cutaneous melanoma<sup>[3]</sup>. Ovarian carcinoma regularly metastasizes to the peritoneal surface<sup>[4]</sup>. The acquisition of invasiveness in ovarian carcinoma is accompanied by the process of epithelial to mesenchymal transition. Cancer-associated fibroblasts originating from stromal fibroblastic cells are a component of the tumor microenvironment and promote tumor angiogenesis and lymphangiogenesis<sup>[5]</sup>. Also, the MUC4 mucin has a role

in the invasiveness of ovarian cancer cells because it is overexpressed in ovarian tumors. The overexpression of MUC4 in ovarian cancer is a morphological alteration, along with a decreased expression of epithelial markers (E-cadherin and cytokeratin-18) and an increased expression of mesenchymal markers (N-cadherin and vimentin)<sup>[6]</sup>.

GI involvement from ovarian cancer is limited to the seromuscular layer of the small and large bowels but it also metastasizes through the lymphatic and hematogenous route, with a frequency ranging from 0.7% to 1.8%<sup>[7]</sup>. The stomach is highly vascularized, therefore the dissemination of ovarian carcinoma is possible but rare. Gastric metastasis from ovarian cancer is unusual, either as synchronous with the primary tumor or appearing several years after its initial diagnosis<sup>[8]</sup>. The diagnosis is challenging because of its low incidence. Clinical manifestations include epigastric pain, nausea, vomiting, anemia, melena or occult GI blood loss. Obstructive symptoms may



**Table 1 Cases of gastric metastases from ovarian adenocarcinoma presenting as subepithelial tumor and diagnosed by endoscopic ultrasound-guided tissue acquisition**

Ref.	Age	Clinical presentation	Diagnosis of ovarian cancer	Endoscopic aspect of gastric SET	EUS morphology
Shanga <i>et al</i> <sup>[13]</sup> , 2003	62	Epigastric discomfort	7 yr before	Body, 4 cm	Irregular border, hypoechoic lesion, fourth layer
Jung <i>et al</i> <sup>[10]</sup> , 2009	49	Asymptomatic	52 mo from surgery	Antrum, 2.5 cm × 2.5 cm	Hypoechoic lesion, fourth layer
Carrara <i>et al</i> <sup>[14]</sup> , 2011	70	Mild anemia, dyspepsia	NR	Body, 3.8 cm × 4.8 cm, ulcerated	Third layer
Akce <i>et al</i> <sup>[15]</sup> , 2012	55	Anemia, melena	5 yr before	Antrum, 3.4 cm × 3.7 cm and body, 1.2 cm × 0.8 cm	Hypoechoic lesions, fourth layer
Yamao <i>et al</i> <sup>[16]</sup> , 2015	51	NR	25 mo from surgery	Antrum, 3 cm	Hypoechoic lesion with marginal rim, fourth layer
Current case	61	Dyspepsia	2 yr before	Antrum, 2.3 cm	Hypoechoic lesion (more hyperechoic than the muscular tissue), fourth layer

EUS: Endoscopic ultrasound; NR: Not reported; SET: Subepithelial tumor.

be present in case of involvement of the cardia or pylorus<sup>[9]</sup>. In asymptomatic patients, CA-125 levels beyond normal range may be the only warning sign<sup>[7,9]</sup>. The prognosis of gastric metastases of ovarian carcinoma is still unknown, a 1-year survival rate can be optimistically expected<sup>[10,11]</sup>. From an endoscopic point of view, gastric metastases do not present specific features. They may mimic both a primary gastric tumor or, less frequently, an SET<sup>[8,12]</sup> and can be solitary or more rarely multiple<sup>[13]</sup>.

Several cases of metastatic ovarian cancer presenting as gastric SET have been reported in the literature<sup>[10,13-17]</sup> but only few have been diagnosed by EUS-guided tissue acquisition, as in our case (Table 1). In other cases, surgical excision or endoscopic submucosal dissection with enucleation has been performed<sup>[8,11]</sup>.

In the present case, the lesion was mimicking a GIST, even if a metastasis from ovarian cancer had been considered. For these reasons, a tissue diagnosis was considered necessary. After a diagnosis of metastatic ovarian cancer to the stomach had been achieved, surgical intervention or more aggressive options would become unnecessary considering the progression of the disease.

In conclusion, although rare, gastric metastasis from primary ovarian cancer should be considered in any patient with a history of ovarian adenocarcinoma who presents with gastric tumor. This case emphasizes the crucial role of EUS-FNB in the differential diagnosis.

## COMMENTS

### Case characteristics

A 61-year-old woman diagnosed 3 years previously with stage IV high-grade serous carcinoma of the ovary and treated with debulking surgery and chemotherapy presented for evaluation of dyspepsia.

### Clinical diagnosis

General physical examination was unremarkable.

### Differential diagnosis

Gastrointestinal stromal tumor, gastric tumor, metastasis.

### Laboratory diagnosis

CA125 levels had increased to 138 U/mL (normal value < 35 U/mL).

### Imaging diagnosis

Endoscopic ultrasonography (EU) showed a 23-mm mass within the muscularis propria, hypoechoic but more hyperechoic than the muscular tissue.

### Pathological diagnosis

Histology obtained via EUS-guided fine needle biopsy showed adenocarcinoma with immunohistochemistry positive for WT1 and CK7, and negative for CK20 and CDX2, supporting the final diagnosis of a metastatic adenocarcinoma of ovarian origin.

### Treatment

Palliative chemotherapy.

### Related reports

Several cases of metastatic ovarian cancer presenting as gastric subepithelial tumor have been reported in the literature, but only few of them have been diagnosed by EUS-guided tissue acquisition, as in our case. In other cases, surgical excision or endoscopic submucosal dissection with enucleation has been performed.

### Experiences and lessons

Although rare, metastatic ovarian cancer to the stomach should be considered in any patient with a history of ovarian adenocarcinoma who presents with gastric tumor.

### Peer-review

This is an interesting case report.

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*WJGO* covers topics concerning carcinogenesis, tumorigenesis, metastasis, diagnosis, prevention, prognosis, clinical manifestations, nutritional support, molecular mechanisms, and therapy of benign and malignant tumors of the digestive tract. The current columns of *WJGO* include editorial, frontier, diagnostic advances, therapeutics advances, field of vision, mini-reviews, review, topic highlight, medical ethics, original articles, case report, clinical case conference (Clinicopathological conference), and autobiography. Priority publication will be given to articles concerning diagnosis and treatment of gastrointestinal oncology diseases. The following aspects are covered: Clinical diagnosis, laboratory diagnosis, differential diagnosis, imaging tests, pathological diagnosis, molecular biological diagnosis, immunological diagnosis, genetic diagnosis, functional diagnostics, and physical diagnosis; and comprehensive therapy, drug therapy, surgical therapy, interventional treatment, minimally invasive therapy, and robot-assisted therapy.

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## Neoadjuvant therapy for resectable pancreatic cancer

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### Abstract

The use of neoadjuvant therapies has played a major role for borderline resectable and locally advanced pancreatic cancers (PCs). For this group of patients, preoperative chemotherapy or chemoradiation has increased the likelihood of surgery with negative resection margins and overall survival. On the other hand, for patients with resectable PC, the main rationale for neoadjuvant therapy is that the overall survival with current strategies is unsatisfactory. There is a consensus that we need new treatments to improve the overall survival and quality of life of patients with PC. However, without strong scientific evidence supporting the theoretical advantages of neoadjuvant therapies, these potential benefits might turn out not to be worth the risk of tumors progression while waiting for surgery. The focus of this paper is to provide the readers an overview of the most recent evidence on this subject.

**Key words:** Pancreatic adenocarcinoma; Neoadjuvant chemotherapy; Neoadjuvant chemoradiation therapy; Meta-analysis; Decision analysis; Borderline resectable; Locally advanced; Randomized controlled trial; Phase I trial; Phase II trial; Phase III trial

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**Core tip:** The use of neoadjuvant therapy for patients with resectable pancreatic cancer (PC) has been used by an increasing number of cancer centers around

the world. The main rationale of using neoadjuvant therapies in resectable PC is the hope that patients' likelihood of long-term overall survival will benefit from the chemo or chemoradiation therapy administered when their overall conditions allow them to tolerate the treatment. At this time, there is no phase III trial to support the use of neoadjuvant therapies in resectable PC. Without strong scientific evidence supporting the theoretical advantages of neoadjuvant therapies, these potential benefits might turn out not to be worth the risk of tumors progression while waiting for surgery.

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## INTRODUCTION

The most common form of pancreatic cancers (PCs) originates from the ductal cells of the exocrine gland<sup>[1,2]</sup>. In the United States, it represents the fourth leading cause of cancer-related deaths with 44000 new cases per year<sup>[2,3]</sup>. The prognosis of patients with PC remains poor with only 5%-10% of patients alive after five years<sup>[4]</sup>. Their outcome is significantly improved if they undergo surgery; however, even in this case, 5-year survival is only 25%-40%<sup>[1,4]</sup>. PC is a difficult tumor to cure as it behaves as a systemic disease even in its early stages. Although surgery remains the only potential cure, it is still inadequate for most of the patients who will develop recurrent disease within five years. The use of multimodality therapy (surgery, chemotherapy and radiation therapy) provides the best chance for long-term survival<sup>[5]</sup>, but the ideal sequence and duration of these treatments remain unknown due to the lack of scientific evidence.

Despite these limitations, there is a consensus that, because of the poor outcomes observed with old treatment modalities, new strategies are necessary<sup>[6]</sup>. Among them, the use of neoadjuvant chemotherapy has gained traction and, in recent years, an increasing number of oncologists and surgeons are recommending it<sup>[7,8]</sup>.

For borderline resectable and locally advanced PC, there is evidence that neoadjuvant therapy increases the probability of negative resection margins and the number of patients who can undergo surgery<sup>[8,9]</sup>. On the other hand, for resectable PC, neoadjuvant chemotherapy or chemoradiation remains debatable because of the conflicting data on its effectiveness, and because there is no phase III trial to support their use<sup>[10-12]</sup>. The focus of this publication is to provide an overview of the most recent evidence on this topic, appraise the potential benefits and disadvantages of neoadjuvant vs surgery first approach, and finally, to

review the ongoing phase III trials that might address some of the questions that are still unanswered.

## RESECTABILITY

Surgery remains the only potential cure for patients with PC. Determining if the disease is resectable or not at the time of diagnosis is crucial, but often subjective to the interpretation of preoperative imaging tests. Resectability is usually determined using a combination of imaging tests and laparoscopic assessment of the peritoneal cavity to rule out small hepatic or peritoneal metastases that might be missed even with high-quality contrast enhanced computerized tomography (CT scans) or magnetic resonance imaging (MRI) studies<sup>[2,13]</sup>. There are several definitions of tumor resectability that are summarized in Table 1<sup>[13-16]</sup>. All criteria currently used to identify patients with resectable disease are based on the degree of contact between the tumor and blood vessels adjacent to the pancreas in the absence of distant disease.

## TREATMENT STRATEGIES

Until recently, the most accepted treatment paradigm for resectable PC was surgery followed by postoperative systemic chemotherapy or chemoradiation. In recent years, the use of systemic pre-operative chemotherapy alone or in combination with radiation therapy has been offered to an increasing number of patients with the main intent of reducing the size of the tumor, increase the likelihood of negative resection margins, and test the effects of cytotoxic medications *in vivo*<sup>[9]</sup>. Most patients who are treated with neoadjuvant chemotherapy or chemoradiation receive oral or intravenous medications for the duration of three to six months before undergoing surgery<sup>[17]</sup>.

## ADVANTAGES AND DISADVANTAGES OF NEOADJUVANT THERAPY

Neoadjuvant therapy has several theoretical benefits but also drawbacks (Table 2). It is usually well tolerated, does not increase the perioperative morbidity, reduces the interval between diagnosis and the initiation of systemic treatment<sup>[17]</sup> and has the potential benefit of facilitating radical resections by lessening the size of the tumors before surgery. Despite these advantages, postponing surgery for neoadjuvant treatment might give enough time for the tumor to progress and become unresectable<sup>[17,18]</sup>.

## RECENT STUDIES

Table 3 summarizes details of the latest phase I and II trials reporting the outcomes of patients treated with neoadjuvant chemotherapy or chemoradiation for radiologically resectable PC. In all these studies, tumor

**Table 1 Operational definitions of resectability of pancreatic cancer**

Classification of resectability of pancreatic cancer	Definition by AHPBA/SSO/SSAT	Definition by MD Anderson Cancer Centre
Resectable	The tumor does not abut or encase any of the following vascular structures: the superior mesenteric vein or portal vein, superior mesenteric artery or common hepatic artery or celiac trunk	The tumor abuts or encases the superior mesenteric vein or portal vein without occluding the lumen. Absence of abutment or encasement of the superior mesenteric artery, common hepatic artery or celiac trunk
Borderline resectable	Abutment, encasement or occlusion of the superior mesenteric vein or portal vein. Abutment of the superior mesenteric artery. Abutment or short segment encasement of the common hepatic artery. Absence or abutment or encasement of the celiac trunk	Tumor causing a short-segment occlusion of the superior mesenteric vein or portal vein. Presence of abutment of the superior mesenteric artery, abutment or encasement of a short segment of the common hepatic artery, absence of abutment or encasement of the celiac trunk
Locally advanced	Tumor located in the proximity of the superior mesenteric vein or portal vein and the superior mesenteric vein or portal vein are unable to be resected and reconstructed. Tumor encasing the superior mesenteric artery, or long-segment encasement of the common hepatic artery, or abutment of the celiac trunk	Tumor located in the proximity of the superior mesenteric vein or portal vein that are not reconstructible. Presence of tumor encasement of the superior mesenteric artery, long-segment encasement of the common hepatic artery and encasement of the celiac trunk

AHPBA: Americas Hepato-Pancreato-Biliary Association; SSO: Society of Surgical Oncology; SSAT: Society for Surgery of the Alimentary Tract.

**Table 2 Summary of the benefits and drawbacks of neo-adjuvant and adjuvant therapies for the treatment of patients with resectable pancreatic cancer**

Neo-adjuvant therapy		Adjuvant therapy	
Advantages	Disadvantages	Advantages	Disadvantages
In comparison to the strategy of adjuvant chemotherapy or chemoradiation therapy where up to 50% of patients who undergo surgery cannot complete their therapy due to complications or decline of their function, neoadjuvant strategy has been shown to be well tolerated by the majority of patients and therefore a greater proportion receive systemic therapy The use of neo-adjuvant therapy might sterilize the presence of small metastatic disease and reduce the size of the primary tumor. Downsizing the primary tumor might increase the likelihood of negative resection margins	Neoadjuvant therapy requires the placement of biliary stents to decompress the biliary obstruction prior to surgery of patients with jaundice. The placement of biliary stents before surgery increases the risk of infections in the perioperative period Pre-operative therapy delays surgery and increases the risk of progression of the disease to the point of becoming unresectable	One of the advantages of surgery first approach is that patients have a short period of time between when they are diagnosed and when they undergo resections of their tumor. This might have some benefits on patients' and their families' anxiety Since patients undergo surgery as soon as possible after their diagnosis, their risk of tumor progression is smaller than patients who wait a longer time before being operated on	About 20%-50% of patients will not be able to complete their postoperative therapy due to surgical complications or overall decline of their performance status One of the risk of undergoing surgery first for pancreatic cancer is that, some patients will undergo a major operation without the benefit of being cured as they might already have micrometastases
Treating patients before surgery, gives physicians some time to identify the tumors with poor prognosis that do not respond to the therapy. The identification of those patients who are likely to experience early metastases is very important because prevents them to undergo unnecessary surgery One of the advantages of using chemotherapy or chemoradiation therapy before surgery is that the blood supply to the pancreatic tumor is not compromised by the ligation of vessels. Therefore, chemotherapy agents can be delivered to the pancreatic tumor in higher concentrations	The use of neoadjuvant therapies might increase the risk of perioperative morbidity and mortality due to the side effects of chemotherapy or chemoradiation	Patients who undergo surgery first do not routinely need the placement of biliary stents to release their jaundice before undergoing resection	Patients who undergo surgery first have a higher risk of positive resection margins

response was evaluated differently as some investigators reported radiographic or clinical response before surgical exploration and others the histopathological response observed in the surgical specimen.

Gillen *et al*<sup>[17]</sup> published the first systematic meta-analysis on the effects of preoperative therapy in PC.

The authors reviewed 515 studies, but only 111 trials were included with a total of 4394 patients. Among these studies, 15 were a phase I, 13 were a phase I / II , 28 were phase II , 14 were cohort studies, and 41 were case series. Most the studies were prospective (No. 78). Chemotherapy was used as neoadjuvant therapy in



**Table 3 Phase I and Phase II studies assessing the outcomes of patients with resectable pancreatic cancer treated with neoadjuvant therapies**

Author (yr)/ journal/trial/ institution	No. of patients	Clinical stage/ duration of neoadjuvant therapy	Study design	Chemotherapy/ chemoradiation	Radiological response	Resection rate (%)	Negative resection margins (%)	Median overall survival (mo)
Hoffman (1998)/ <i>J Clin Oncol</i> /ECOG	53	Resectable PC/2.8 mo	Phase II, prospective study, November 1991 to September 1993	5-FU (1000 mg/m <sup>2</sup> per day + Mitomycin C (10 mg/m <sup>2</sup> ) + RT (50 Gy)	Partial response 8%; Stable disease 78%; Progression 16%	45	67	15 with surgery; without surgery 8; 10.9 for the entire cohort
PistersPister (2002)/ <i>J Clin Oncol</i> /MD Anderson Cancer Centre	35	Resectable PC/1.8 mo	Phase II, prospective study, timeframe not specified	Paclitaxel (60 mg/m <sup>2</sup> ) weekly, RT (30 Gy)	Partial response 4%; Stable disease 23%; Progression 20%	57	68	12 for the entire cohort; 19 with surgery; 10 without surgery
Joensuu (2004)/ <i>Int J Radiat Oncol Biol Phys</i> /Helsinki University	28	Resectable PC/3.5 mo	Phase I - II prospective study, November 1999 to December 2001	Gemcitabine (20 mg/m <sup>2</sup> vs 50 mg/m <sup>2</sup> vs 100 mg/m <sup>2</sup> ) twice a week + RT (50 GY)	NA	71	NA	13.6 for the entire cohort
Talamonti (2006)/ <i>Ann Surg Oncol</i> / Northwestern University	20	Resectable PC/3.8 mo	Phase II prospective, multi- institutional study, April 2002 to October 2003	Gemcitabine (1000 mg/m <sup>2</sup> weekly) + RT (36 Gy)	Partial response 15%; Stable disease 80%; Progression 5%	85	94	26 mo with surgery
Palmer (2007)/ <i>Ann Surg Oncol</i> / University of Birmingham	24	Resectable PC/4 mo	Phase II, prospective study, November 1999 to May 2003	Gemcitabine (1000 mg/m <sup>2</sup> weekly)	Partial Response 0%; Stable Disease 29%; Progression 4%; Unable to measure 4%	38	75	28.4 with surgery; 9.9 for the entire cohort
Palmer (2007)/ <i>Ann Surg Oncol</i> / University of Birmingham	26	Resectable PC/4 mo	Phase II, prospective study, November 1999 to May 2003	Gemcitabine (1000 mg/m <sup>2</sup> weekly) + Cisplatin (25 mg/m <sup>2</sup> )	Partial Response 0%; Stable Disease 66%; Progression 21%; Unable to measure 4%	70	75	28.4 with surgery; 9.9 for the entire cohort
Le Scodan (2009)/ <i>Ann Oncol</i> /SFRO- FFCD	41	Resectable PC/3 mo	Phase II, prospective study, January 1998 to March 2003	RT (50 Gy) + 5-FU (300 mg/m <sup>2</sup> daily) + Cisplatin (20 mg/m <sup>2</sup> )	Partial response 10%; Stable Disease 65%; Progression 25%	63	81	11.7 with surgery; 9.4 for the entire cohort
Heinrich (2008)/ <i>Ann Surg</i> /University Hospital of Zurich	28	Resectable PC/2 mo	Phase II, prospective study, August 2001 to April 2007	Gemcitabine (1000 mg/m <sup>2</sup> twice weekly) + Cisplatin (50 mg/m <sup>2</sup> )	Partial response 4%; Stable Disease 61%; Progression 13%	89	80	19.1 mo with surgery
Evans (2008)/ <i>J Clin Oncol</i> /MD Anderson Cancer Centre	80	Resectable PC/3 mo	Phase II, prospective study, July 1998 to October 2001	Gemcitabine (400 mg/m <sup>2</sup> weekly) + RT (30 Gy)	NA	85	82	34 mo with surgery; 22.7 mo for the entire cohort; 7 mo without surgery
Varadhachari (2008)/ <i>J Clin Oncol</i> /MD Anderson Cancer Centre	90	Resectable PC/4.3 mo	Phase II, prospective study, October 2002 to February 2006	Gemcitabine (750 mg/m <sup>2</sup> weekly) + Cisplatin (30 mg/m <sup>2</sup> ) every 2 wk + RT (30 Gy)	NA	58	96	31.0 mo with surgery; 17.4 mo for the entire cohort; 10.5 mo without surgery
Turrini (2009)/ <i>Oncology /University Mediterranean</i>	34	Resectable PC/2.1 mo	Phase II, prospective study, May 2003 to July 2005	Docetaxel (30 mg/m <sup>2</sup> ) weekly + RT (45 GY)	Partial response 9%; Stable disease 59%; Progression 32%	68	100	32 mo with surgery; 15.5 mo for entire cohort; 11 mo without surgery

Landry (2010)/ <i>J Surg Oncol</i> /Emory University/Multicenter ECOG	21	Resectable PC/3 mo	Phase II, prospective two-arm study, October 2013 to June 2015	Arm A: Gemcitabine (500 mg/m <sup>2</sup> ) weekly + RT (50 Gy) Arm B: Gemcitabine (175 mg/m <sup>2</sup> ) + Cisplatin (20 mg/m <sup>2</sup> ) + 5-FU (600 mg/m <sup>2</sup> ) + RT (50 Gy)	Arm A: Partial response 10%, Arm B: Partial response 18.2%	NA	NA	Arm A: Entire cohort 19.4 mo. Arm B: entire cohort 13.4 mo. 26.3 mo with surgery
Wo (2014)/ <i>Radiother Oncol</i> /Multicentric	10	Resectable PC	Phase I, prospective study	Capecitabine (1650 mg/m <sup>2</sup> ) over 10 d + RT (30 Gy)	NA	80	NA	NA
Shinoto (2013)/ <i>Cancer</i> /Japan	26	Resectable PC	Phase I, prospective study, April 2003 to December 2010	RT (30 Gy)	Partial response 3.8%; Stable disease 96.1%	81	90	18.6 mo for entire cohort; NA for patients who underwent surgery
O'Reilly (2014)/ <i>Ann Surg</i> /Memorial Sloan Kettering Cancer Centre	38	Resectable PC	Phase II, prospective study, July 2007 to December 2011	Gemcitabine (1000 mg/m <sup>2</sup> ) + Oxaliplatin (80 mg/m <sup>2</sup> ) every 2 wk	Partial response 10.5%; Stable disease 73.7%; Progression 7.9%; NA 7.9%	77	74	27.2 mo for the entire cohort; 22 mo progression free survival with surgery
Golcher (2015)/ <i>Strahlenther Oncol</i> /Germany	66 (33 patients allocated to surgery + 33 patients allocated to chemoradiation followed by surgery)	Resectable PC	Phase II, prospective randomized trial with two arms: Primary surgery vs preoperative chemoradiation followed by surgery. June 2003 to December 2009	Gemcitabine (300 mg/m <sup>2</sup> ) + Cisplatin (30 mg/m <sup>2</sup> ) + RT (50.4 Gy) (Preoperative for patients enrolled in Arm A)	NA	Preoperative chemoradiation: 69% Surgery first: 57%	Arm A (preoperative chemoradiation): 48. Arm B (surgery first): 51	Arm A (preoperative chemoradiation): 18.9 mo. Arm B (surgery first): 25.0 mo
Van Buren (2013)/ <i>Ann Surg Oncol</i> /Multicenter/United States	59	Resectable PC	Phase II, prospective study, February 2007 to February 2011	Gemcitabine (1500 mg/m <sup>2</sup> ) ever 2 wk + Bevacizumab (10 mg/kg) + RT (30 Gy)	Partial response 8.4%; Stable disease 73.7%; Progression 7.9%	74	88	19.7 mo with surgery; 16.8 mo for the entire cohort

NA: Not available.

107 (96%) and radiotherapy in 104 (94%) with doses ranging from 24 to 63 Gy. In 13 trials, patients received intraoperative radiation therapy with doses between 10 and 30 Gy.

Six studies stated that the RECIST criteria were used to assess the preoperative radiological response to neoadjuvant therapy. The criteria used to evaluate tumor response were clearly stated in 44 studies, while in 61 studies the criteria used were not adequately reported. Pooled results of patients with resectable cancers at the time of diagnosis showed a complete response in 3.6%, partial response in 30.6%, progression in 20.9% and stable disease in 42.1%. Resections were performed in 73.6% (95%CI: 65.9%-80.6%) of patients. Perioperative morbidity occurred in 26.7% (95%CI: 20.7%-33.3%) and mortality in 3.9% (95%CI: 2.2%-6.0%) which were comparable to the outcomes

of patients undergoing surgery first. Negative resection margins (R0) were observed in 82.1% of patients (95%CI: 73.1%-89.6%) with a median survival of 23.3 mo (range 12-54). Analysis of trials with monotherapy vs poly-chemotherapy revealed higher rates of complete or partial response when multiple chemotherapy agents were used. Higher response rates, however, did not translate into higher resection rates.

One year later, Assifi *et al*<sup>[19]</sup>, published a second systematic review and meta-analysis of only phase II neoadjuvant therapy trials. Out of 397 studies published from 1993 to 2010, 14 trials were included with a total of 536 patients. All studies were prospective, with 12 out of 14 (86%) being a single arm. Patients who had resectable tumors were 402 (75% of the sample). Gemcitabine was used in 8 trials, while the remaining 6 used 5-FU. Radiotherapy was given in 12 of 14 studies

(85%) with doses ranging between 30 and 50.4 Gy. In patients with resectable disease at diagnosis, complete radiological response was observed in 0.8% (95%CI: 0.0%-2.6%), partial response in 9.5% (95%CI: 2.9%-19.4%), stable disease in 73.9% (95%CI: 63.2%-83.3%) and progression in 17.0% (95%CI: 11.9%-22.7%). After neoadjuvant therapy, the resection rate was 65.8% (95%CI: 55.4%-75.6%) and negative resection margins were observed in 85.1% (95%CI: 76.8%-91.9%). Median survival was 23.0 mo (range 11.7-34.0). The most significant finding of these two meta-analyses was that even if safe, neoadjuvant therapy did not seem to add any substantial survival advantage<sup>[18]</sup>.

Due to the heterogeneity of these studies, no conclusion can be drawn regarding the overall impact on survival and what are the most effective chemotherapy agents or the best combination of chemotherapy agents for resectable PC.

More recently, D'Angelo *et al.*<sup>[20]</sup> completed another systematic review of randomized controlled trials on adjuvant and neoadjuvant therapies for resectable PC. Fifteen studies were included covering a period of 30 years (1985 to 2015). Their analysis suggested that despite all the best efforts, the question whether neoadjuvant therapy provides a better overall survival than adjuvant therapy remains unanswered.

## DECISION ANALYSES

VanHouten *et al.*<sup>[21]</sup> used a decision analysis model to assess what is the best treatment strategy for resectable PC. A survival advantage of 7 mo was found in patients who underwent neoadjuvant therapy in comparison to surgery first (27.2 mo vs 19.9 mo).

Another Markov decision analysis by de Geus *et al.*<sup>[22]</sup> supported the use of neoadjuvant chemotherapy that provided longer overall survival (32 mo vs 27 mo) and quality-adjusted life expectancy (25 mo vs 21 mo) in comparison to surgery followed by adjuvant chemotherapy. Sensitivity analysis of the model showed that if the probability of surgical resection after neoadjuvant therapy was lower than 57%, upfront surgery was the best treatment option.

Another group led by Sharma *et al.*<sup>[23]</sup> compared the efficacy of neoadjuvant-based chemotherapy with adjuvant treatment with an intention-to-treat analysis using a two-arms Markov model. In the neoadjuvant group, patients were treated with an average of 3 mo of neoadjuvant therapy followed by surgery. After surgery, patients who received preoperative chemotherapy did not receive any adjuvant treatment. On the other hand, patients who underwent surgery first, underwent chemotherapy after they recovered from their operations. In this model, the median overall survival was longer for the neoadjuvant cohort (22 mo) in comparison to the adjuvant group (20 mo), and the cumulative quality-adjusted survival for patients who underwent the neoadjuvant strategy was

19.8 mo compared to 18.4 mo for patients who had adjuvant therapy. One-way sensitivity analysis showed that surgery first provided higher quality-adjusted survival rates if more than 44% of patients treated with neoadjuvant therapy experienced progression of their disease and failed to undergo surgical resection.

All these models provided evidence that neoadjuvant therapies have better overall survival and quality of life in comparison to surgery first, although the differences were clinically quite small.

## PERSISTENT CONTROVERSY

For borderline or locally advanced PC, the use of neoadjuvant therapy makes sense, and it is desirable for both patients and physicians. For patients' perspective, neoadjuvant treatments might decrease the tumor burden and give them the chance of becoming resectable. Similarly, for the surgeons' perspective, any reduction of the tumor size is welcome as it facilitates the technical aspect of the resection around critical vascular structures such as the superior mesenteric-portal vein junction or superior mesenteric artery.

However, this is not the case for resectable PC. Neoadjuvant therapy does not facilitate surgery, as the tumor is resectable at the time of diagnosis. Preoperative therapy might increase the rate of negative margins; however, this needs to be proven in randomized controlled trials, as the current evidence is not sufficient. Furthermore, for patients' perspective, there is a considerable risk of missing out the only opportunity of being cured with surgery as the tumor might progress to become unresectable while neoadjuvant therapies are delivered.

Because the current evidence is inadequate, there are no unequivocal criteria able to assist health-care providers to select the strategy with the best long-term survival for resectable PC. Physicians are left to decide whether to use neoadjuvant therapy and whether to use of one or multiple pre-operative chemotherapeutic agents or chemoradiation is worth the risk of toxicities and the possibility of disease progression. In theory, neoadjuvant treatments would be unanimously recommended for patients at high risk of positive resection margins, as their surgery would not be curative. The selection of these patients is not easy. To overcome this concerns, Bao *et al.*<sup>[24]</sup> developed a predictive module to maximize the probability of identifying patients with true resectable tumors by using commonly available preoperative imaging modalities. With this model, the authors could classify patients with low-risk and high-risk for non-curative resections and concluded that until better evidence is available, patients who are unlikely to have R0 margins should be treated with neoadjuvant therapy.

## FUTURE DIRECTIONS

D'Angelo *et al.*<sup>[20]</sup> pointed out that the current literature is biased because the likelihood that radiologically

**Table 4** List of ongoing phase II and phase III trials comparing neoadjuvant therapies *vs* adjuvant strategies for resectable pancreatic adenocarcinoma

Study	Design	No. of patients needed	Therapy	Primary outcome
NEOPAC (NCT01314027)	Phase III Enrollment 2009-2014	350	Neoadjuvant gemcitabineoxaliplatin + adjuvant gemcitabine <i>vs</i> Adjuvant gemcitabine	Progression free survival
NEOPAC (NCT01521702)	Phase III Initiated in 2011	310	Preoperative FOLFIRINOX, followed by adjuvant gemcitabine after surgery <i>vs</i> adjuvant gemcitabine after resection	Five-year progression free survival
NCT01900327	Phase III	410	Neoadjuvant gemcitabine-based chemoradiation therapy followed by adjuvant gemcitabine <i>vs</i> adjuvant gemcitabine	Three-year overall survival
NCT01771146	Phase II	100	Neoadjuvant FOLFIRINOX	Progression free survival
NEONAX (NCT02047513)	Randomized phase II	166	Neoadjuvant gemcitabine + nab-paclitaxel followed by adjuvant gemcitabine + nab-paclitaxel <i>vs</i> adjuvant gemcitabine + nab-paclitaxel	Disease-free survival at 18 mo
NCT01150630	Randomized phase II / III	370	Adjuvant PEXG <i>vs</i> adjuvant gemcitabine <i>vs</i> neoadjuvant PEXG - followed by surgery and then adjuvant PEXG	One year event-free survival
ACOSOG-Z5041 (NCT00733746)	Phase II	123	Neoadjuvant gemcitabine + erlotinib (completed; results pending)	Two-year overall survival
NCT00727441	Phase II	87	Neoadjuvant GVAX +/- IV or oral cyclophosphamide followed by adjuvant gemcitabine + CRT	Safety, feasibility, and immune response
NCT02178709	Phase II	48	Neoadjuvant FOLFIRINOX	Pathologic complete response
GEMCAD1003 (NCT01389440)	Phase II	24	Neoadjuvant gemcitabine + erlotinib	R0 resection rate
NCT02562716	Phase II Enrollment 2015-2019	112	Neoadjuvant and adjuvant mFOLFIRINOX <i>vs</i> neoadjuvant and adjuvant Nab-paclitaxel and gemcitabine	Overall survival
NCT02243007	Randomized phase II	112	Neoadjuvant FOLFIRINOX <i>vs</i> gemcitabine + nab-paclitaxel	18-mo overall survival
NCT02030860	Pilot	15	Neoadjuvant gemcitabine + nab-paclitaxel ± paricalcitol	Number of adverse events
NCT02305186	Randomized phase I b/ II	56	Neoadjuvant capecitabine-based CRT ± pembrolizumab (MK-3745)	Safety and immune response

CRT: Chemoradiation therapy; GVAX: Granulocyte-macrophage colony-stimulating factor gene-transfected tumor cell vaccine; PEXG: Cisplatin, epirubicin, capecitabine, gemcitabine; R0: Margin-negative surgical resection.

resectable PCs is indeed unresectable at the time of surgery is only about 40%<sup>[25]</sup>. Therefore, the only way to find out if there is any benefit from neoadjuvant therapy is to complete an intention to treat randomized controlled trial where one arm entails surgery followed by adjuvant therapy (current standard of care) and the second arm involves neoadjuvant therapy followed by surgery followed by adjuvant therapy (experimental group).

Recent chemotherapy regimens, such as FOLFIRINOX [folinic acid (leucovorin)/5-FU/Irinotecan/Oxaliplatin], have already demonstrated promising results in a small group of patients with borderline resectable tumors<sup>[26,27]</sup>. Given these findings, several ongoing prospective studies are examining the role of FOLFIRINOX in a neoadjuvant setting for resectable disease (Table 4). Other studies include NEOPAC, NEONAX, NCT01660711, and NCT02172976. NEOPAC (Adjuvant *vs* Neoadjuvant Plus Adjuvant Chemotherapy in Resectable Pancreatic Cancer) will compare neoadjuvant gemcitabine and oxaliplatin plus adjuvant gemcitabine *vs* adjuvant gemcitabine alone. NEONAX, (Neoadjuvant Plus Adjuvant or Only Adjuvant Nab-Paclitaxel Plus Gemcitabine for Resectable Pancreatic Cancer) will assess the effects of neoadjuvant plus

adjuvant Nab-Paclitaxel plus gemcitabine *vs* adjuvant only Nab-Paclitaxel plus gemcitabine. Other ongoing trials are a single-arm nonrandomized trial evaluating preoperative and postoperative FOLFIRINOX in patients with resectable disease (NCT01660711) and the multicenter German randomized trial investigating adjuvant gemcitabine compared with neoadjuvant and adjuvant FOLFIRINOX (NCT02172976).

## CONCLUSION

Based on the current literature, there is still insufficient evidence to fully support the use of neoadjuvant therapy for all patients with radiologically resectable PC. Randomized controlled trials are urgently needed to address many of the questions that are still unanswered. Until then, clinicians need to weigh the pros and cons of the two treatment strategies and guide their patients. Ideally, patients should be educated on the advantages, and detrimental effects associated with each of the two possible therapies and their preferences should be elicited. Since each patient is unique, proposing neoadjuvant therapy with one-size-fits-all approach should be discouraged, and patients should become active participants and share with their



physicians the responsibility of selecting the treatment strategy that fits best with their goals and values.

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Retrospective Cohort Study

# Tumor-stroma ratio as prognostic factor for survival in rectal adenocarcinoma: A retrospective cohort study

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**Author contributions:** Scheer R and Klaase JM designed the research; Scheer R and Zoidze S performed the research; Baidoshvili A supervised the histological scoring and took final decisions in case of discrepancy in scores between Scheer R and Zoidze S; Elferink MAG collected data from the population-based The Netherlands Cancer Registry and analyzed the data; Berkel AEM, Klaase JM and van Diest PJ supervised and interpreted the results; Scheer R and Zoidze S wrote the paper; all authors critically reviewed and accepted the final version of the manuscript.

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presented data are anonymized and risk of identification is low.

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## Abstract

### AIM

To evaluate the prognostic value of the tumor-stroma ratio (TSR) in rectal cancer.

### METHODS

TSR was determined on hematoxylin and eosin stained histological sections of 154 patients treated for rectal adenocarcinoma without prior neoadjuvant treatment in the period 1996-2006 by two observers to assess

reproducibility. Patients were categorized into three categories: TSR-high [carcinoma percentage (CP)  $\geq$  70%], TSR-intermediate (CP 40%, 50% and 60%) and TSR-low (CP  $\leq$  30%). The relation between categorized TSR and survival was analyzed using Cox proportional hazards model.

### RESULTS

Thirty-six (23.4%) patients were scored as TSR-low, 70 (45.4%) as TSR-intermediate and 48 (31.2%) as TSR-high. TSR had a good interobserver agreement ( $\kappa = 0.724$ , concordance 82.5%). Overall survival (OS) and disease free survival (DFS) were significantly better for patients with a high TSR ( $P = 0.01$  and  $P = 0.02$ , respectively). A similar association existed for disease specific survival ( $P = 0.06$ ). In multivariate analysis, patients without lymph node metastasis and an intermediate TSR had a higher risk of dying from rectal cancer (HR = 5.27, 95%CI: 1.54-18.10), compared to lymph node metastasis negative patients with a high TSR. This group also had a worse DFS (HR = 6.41, 95%CI: 1.84-22.28). An identical association was seen for OS. These relations were not seen in lymph node metastasis positive patients.

### CONCLUSION

The TSR has potential as a prognostic factor for survival in surgically treated rectal cancer patients, especially in lymph node negative cases.

**Key words:** Rectal cancer; Adenocarcinoma; Prognosis; Recurrence; Pathology; Tumor-stroma ratio

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**Core tip:** The tumor-stroma ratio (TSR) can be determined accurately on routine histopathological sections by different observers. The TSR has potential as a prognostic factor for survival in surgically treated rectal cancer patients, especially in lymph node negative cases. It could therefore be useful in decision making regarding adjuvant treatment in individual patients.

Scheer R, Baidoshvili A, Zoidze S, Elferink MAG, Berkel AEM, Klaase JM, van Diest PJ. Tumor-stroma ratio as prognostic factor for survival in rectal adenocarcinoma: A retrospective cohort study. *World J Gastrointest Oncol* 2017; 9(12): 466-474 Available from: URL: <http://www.wjgnet.com/1948-5204/full/v9/i12/466.htm> DOI: <http://dx.doi.org/10.4251/wjgo.v9.i12.466>

## INTRODUCTION

Colorectal cancer (CRC) is a common form of cancer in both men and women. More than 15000 new patients with a colorectal carcinoma were diagnosed in The Netherlands in 2016<sup>[1]</sup>. The common form to stage this type of cancer is the TNM staging system of the Union Internationale Contre le Cancer/American

Joint Cancer Committee (UICC/AJCC)<sup>[2]</sup>. This system is also used in decision making about the appliance of (neo)adjuvant (chemo)radiotherapy. Although the TNM staging system is still regarded as the most important prognostic factor<sup>[3]</sup>, it seems insufficiently able to predict the prognosis of the individual patient. This applies in particular to patients with stage II rectal cancer<sup>[4]</sup>. A part of the patients is overtreated and consequently exposed to a higher risk on therapy related complications, indicating a need for additional prognostic factors.

More recently, some studies have focused on the tumor-host interaction in relation to metastatic invasion. This interaction is enacted in an environment including cancer cells, the stromal tissue, consisting of different cell types like fibroblasts, myofibroblasts, endothelial cells and immune cells, and the extracellular matrix<sup>[5]</sup>. Mesker *et al*<sup>[6]</sup> showed that a high tumor-stroma ratio (TSR), the proportion of carcinoma relative to the proportion of tumor stroma in the histopathological section through the tumor, is an indicator of a better outcome of disease in colon cancer. This is more outspoken for right sided tumors<sup>[6]</sup>. Similar results were seen in breast cancer, oral squamous cell carcinoma and prostate cancer<sup>[7-9]</sup>. A high TSR is possibly related to both a longer disease free and overall survival (OS) according to a study on a small number of rectal cancer patients<sup>[10]</sup>. In this respect, it is meaningful to explore the relevance of the TSR in a larger cohort of patients with rectal adenocarcinoma.

## MATERIALS AND METHODS

### Patients

Patients with rectal adenocarcinomas under the peritoneal reflection were identified out of all patients, who underwent surgery for left sided colorectal malignancies at our hospital between 1996 and 2006, by analyzing the histopathological reports. Only patients treated with curative intent were included, *i.e.*, patients without known distant metastases at surgery and radically resected tumors (M0, R0 resections). Patients who received neoadjuvant therapy, with malignancies in the past, other than radically excised basal cell carcinoma of the skin, and cases where no tumor was found in the resected specimen, despite preoperative adenocarcinoma in the biopsy of a suspected abnormality, were excluded. Other exclusion criteria were the presence of synchronous colorectal tumors, Lynch syndrome, familial adenomatous polyposis, and inflammatory bowel diseases. Patients who died within thirty days after surgery, with incomplete follow-up or unavailable histopathological material were also excluded (Figure 1).

Data concerning local recurrences, distant metastases, death, and cause of death were collected from the patient records and by consultation of general practitioners. Furthermore, dates of death were retrieved from the population-based Netherlands Cancer Registry. All data were handled in a coded anonymous



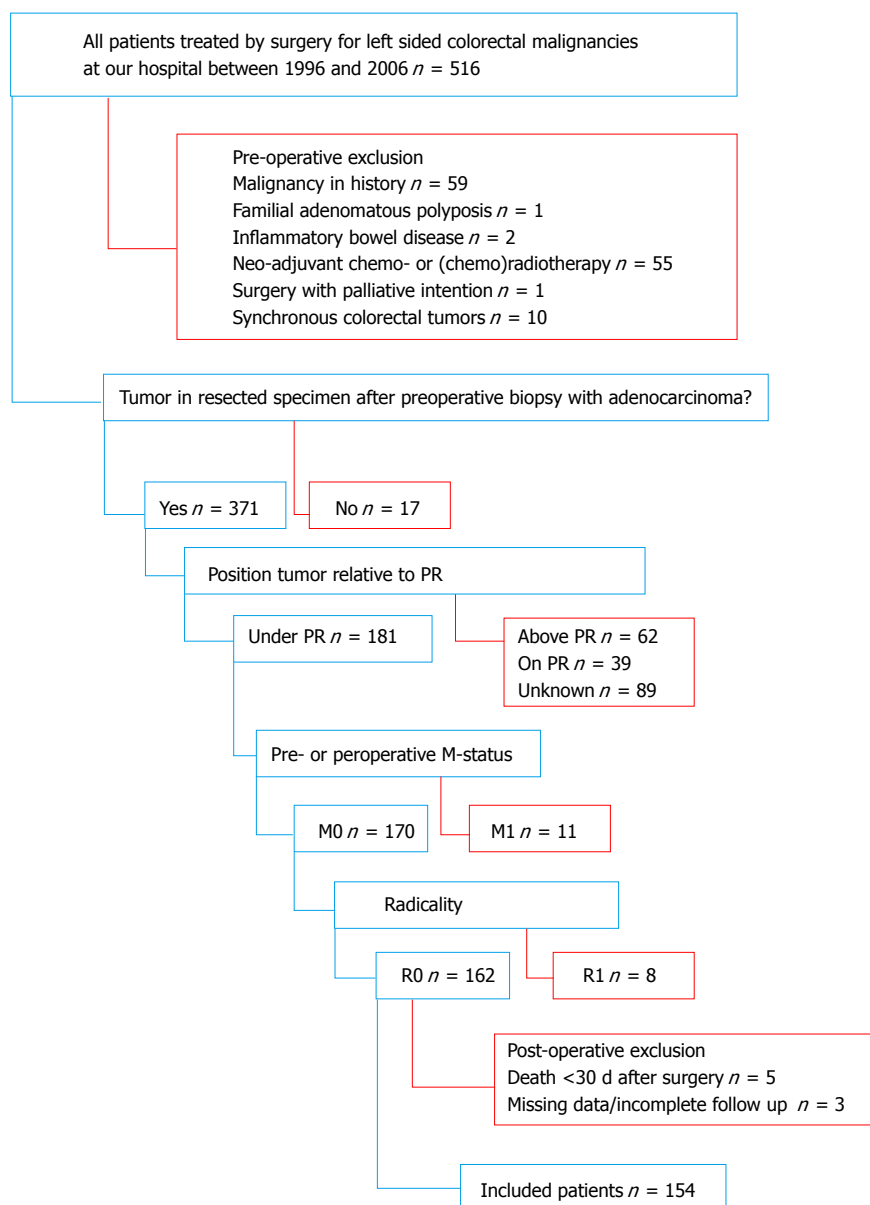


Figure 1 Flowchart of exclusion criteria applied to the dataset of all patients. PR: Peritoneal reflection.

fashion according to the Code for proper secondary use of human tissue from the Dutch Federation of Medical Scientific Societies and with respect to the Helsinki Declaration.

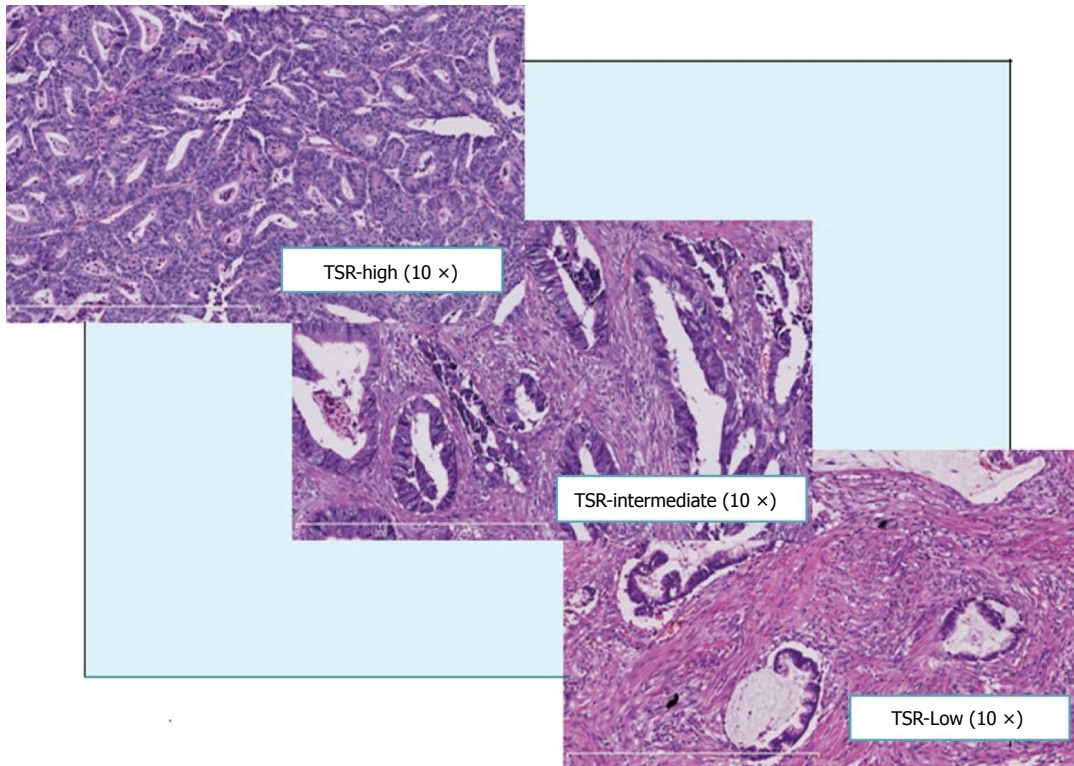
### TSR assessment

TSR was determined on hematoxylin and eosin (H and E) stained histological sections. The section with the most invasive part of the tumor was identified to semiquantitatively assess the carcinoma percentage (CP) in 10% steps. The CP is a derivative of the TSR and is complementary to the percentage of stroma and other components, like mucus. For example, a CP of 20% corresponds to a stroma percentage of 80%, which coincides with a low TSR. The section was viewed with a  $5 \times$  objective (50 times magnification). The CPs were determined on all image fields of the entire section with tumor cells in all sides of it (North-

East-South-West). Areas with the lowest CP were given more weight in rating the mean CP of the total assessed area, as is common practice in routine pathology in determining tumor differentiation. All sections were assessed separately by two observers (René Scheer and Shorena Zoidze) to allow assessment of reproducibility.

The absolute CPs were categorized for a good clinical reproducibility and clarity reasons into three categories, finally. TSR-low including the CP-values  $\leq 30\%$ , TSR-intermediate including the CP-values 40%, 50% and 60%, and TSR-high including the CP-values  $\geq 70\%$  (Figure 2). In the results only the categorized TSR are shown for clarity.

In case of a difference of 10% in determined CPs, which lead to a different TSR category, the lowest CP was used for the determination of the final TSR. The sections were reviewed by a third observer (AB) in case of  $> 10\%$  difference in determined CPs leading



**Figure 2** Examples of different categories of the tumor-stroma ratio. H and E stained 2 µm paraffin sections of primary rectal adenocarcinoma. TSR-high (carcinoma percentage  $\geq 70\%$ ), TSR-intermediate (carcinoma percentage 40%, 50% and 60%), and TSR-low (carcinoma percentage  $\leq 30\%$ ). TSR: Tumor-stroma ratio.

to different TSR categories. This third opinion was considered as decisive.

### Statistical analysis

Data were analyzed using SPSS, version 19.0 (SPSS Inc., Chicago, IL, United States) and Stata, version 12.0 (StataCorp LP, College Station, Texas, United States). The statistical methods of this study were reviewed by Elferink MA, from the Netherlands Comprehensive Cancer Organization.

Patient characteristics were compared using Pearson  $\chi^2$  tests and one-way ANOVA. Interobserver reproducibility for the absolute and categorized CPs was analyzed by using Cohen's Kappa ( $\kappa$ ) coefficient. A  $\kappa$ -value of 0.0 or less was considered to represent poor agreement, 0.01-0.20 slight agreement, 0.21-0.40 fair, 0.41-0.60 moderate, 0.61-0.80 sufficient to good, and 0.81-1.00 near-perfect agreement<sup>[11]</sup>. Survival analyses based on categorized CPs included comparison of OS, disease free survival (DFS), and disease specific survival (DSS) by Kaplan-Meier survival analysis and log-rank statistics. Follow-up time in OS analyses was defined as the period between the date of primary surgery and the date of death from any cause, or the date of last follow-up. The DSS was restricted to death from rectal cancer only. Follow-up time in DFS analyses was defined as the time from the date of primary surgery until the date of a local recurrence or distant metastasis (irrespective of site). In DFS analyses, cases were censored in case of

a second primary tumor (colorectal or other types) or death. The date of last follow-up was used as endpoint to calculate follow-up time, if none of these events occurred.

The relation between categorized TSR and survival (OS, DFS, and DSS) was analyzed, and adjusted for confounders (age, gender, grading, pathological T- and N-stage, and adjuvant treatment), using Cox proportional hazards model. Probability values  $< 0.05$  (2-sided) were considered statistically significant.

## RESULTS

### Patient characteristics

A total of 154 patients met the inclusion criteria for this study. Three types of resections were used: Abdominoperineal resection in 67 (43.5%), low anterior resection in 63 (40.9%), and Hartmann resection, a modulated low anterior resection without construction of an anastomosis, in 24 patients (15.6%). The median follow-up of all patients was 5.3 years. Out of the analyzed samples, 36 (23.4%) were scored as TSR-low, 70 (45.4%) as TSR-intermediate, and 48 (31.2%) as TSR-high. There were more lymph node metastasis positive patients with a low TSR in comparison with patients with a higher TSR ( $P = 0.029$ ), who consequently received adjuvant treatment. Radiotherapy was the most common form of adjuvant therapy. Detailed patient characteristics are shown by categorized TSR in Table 1.

**Table 1 Patient characteristics by categorized tumor-stroma ratio**

	TSR-low ( <i>n</i> = 36)		TSR-intermediate ( <i>n</i> = 70)		TSR-high ( <i>n</i> = 48)		<i>P</i> -value <sup>1</sup>
	<i>n</i>	%	<i>n</i>	%	<i>n</i>	%	
Gender							NS
Male	24	66.7	44	62.9	32	66.7	
Female	12	33.3	26	37.1	16	33.3	
Age (yr)	M 68.0 (range 49.0-82.0)	SD 8.0	M 67.3 (range 40.0-87.0)	SD 10.3	M 65.7 (range 43.0-91.0)	SD 10.3	NS <sup>2</sup>
Treatment							NS
APR	19	52.6	31	44.3	17	35.4	
LAR	11	30.6	28	40.0	24	50.0	
Hartmann	6	16.7	11	15.7	7	14.6	
T-status							NS
pT1	1	2.8	1	1.4	4	8.3	
pT2	9	25.0	23	32.9	18	37.5	
pT3	24	66.7	43	61.4	25	52.1	
pT4	2	5.6	3	4.3	1	2.1	
N-status							0.029
pN0	16	44.4	44	62.9	34	70.8	
N1	15	41.7	13	18.6	11	22.9	
N2	5	13.9	13	18.6	3	6.3	
Stage							NS
I	7	19.4	21	30.0	17	35.4	
II	9	25.0	23	32.9	17	35.4	
III	20	55.6	26	37.1	14	29.2	
Grading							NS
Well	0	0	1	1.4	3	6.3	
Moderate	31	86.1	58	82.9	40	83.3	
Poor	5	13.9	11	15.7	5	10.4	
Adjuvant treatment	19	52.8	21	30.0	11	22.9	0.012 <sup>3</sup>
Radiotherapy	17		18		9		
Chemoradiotherapy	1		3		2		
Chemotherapy	1		-		-		

<sup>1</sup>Pearson  $\chi^2$  test; <sup>2</sup>One-Way ANOVA; <sup>3</sup>*P*-value for adjuvant treatment in general. Significant *P*-values are shown bold. *n*: Number of patients; %: Percentage; Age defined as period from birth until date of primary surgery; LAR: Low anterior resection; APR: Abdominoperineal resection; pT: Pathological tumor status; pN: Pathological nodal status; Stage according to UICC/AJCC TNM staging system, 5<sup>th</sup> edition; TSR: Tumor-stroma ratio; UICC/AJCC: Union Internationale Contre le Cancer/American Joint Cancer Committee.

### Interobserver reproducibility

A third opinion about a final TSR in case of inter-observer disagreement about CPs with > 10% difference in determined CPs leading to different TSR categories was needed in 12 patients (7.8%). Mainly strong heterogeneity of the tumor complicated the determination of the CP for the total section. CPs were scored in a range between 10 and 90 percent. Lower CPs were found in mucinous adenocarcinomas.

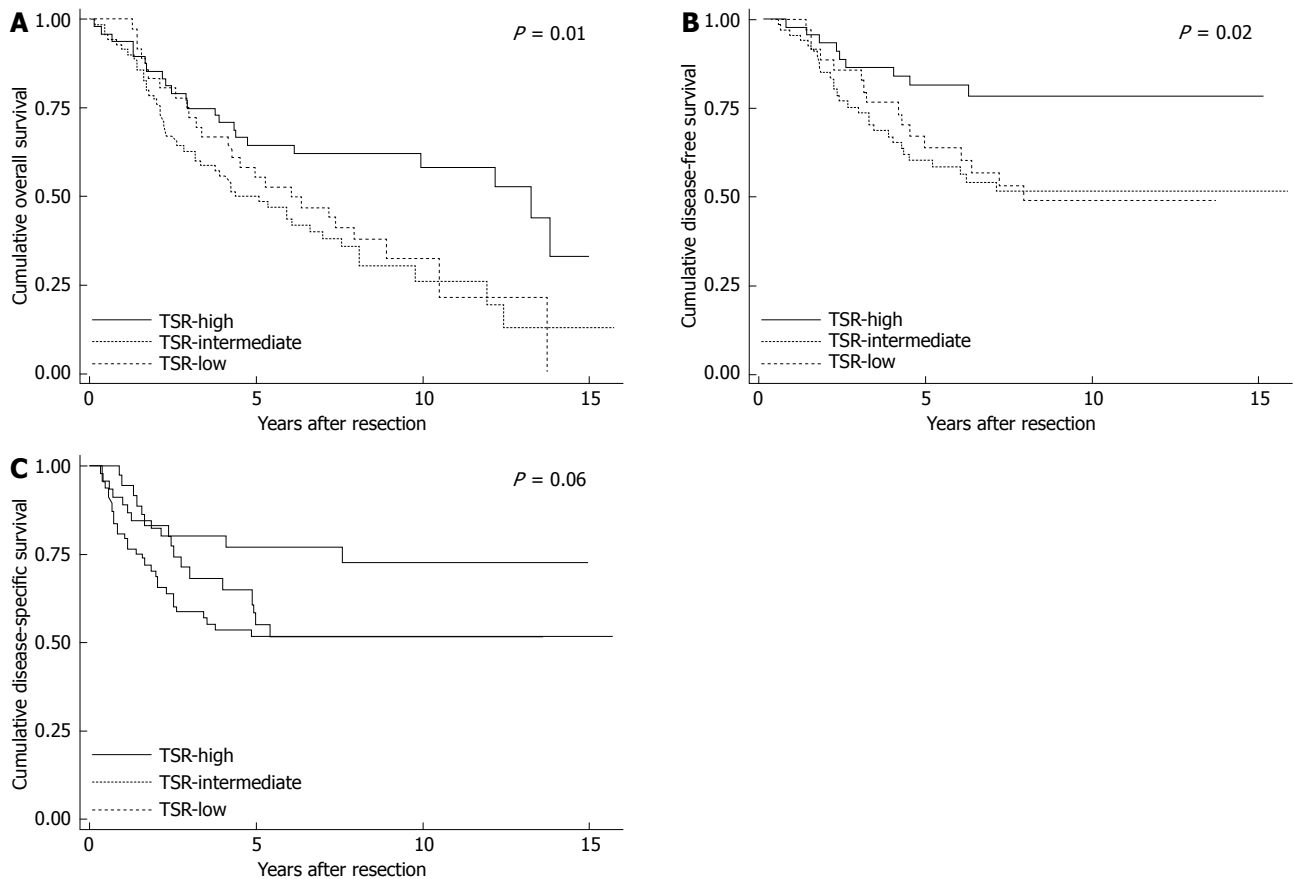
Cohen's Kappa ( $\kappa$ ) coefficient for interobserver agreement of the absolute CP showed a moderate agreement ( $\kappa$  = 0.522, concordance 59.1%). By categorizing the CP into three categories TSR (TSR-low, TSR-intermediate, and TSR-high) the  $\kappa$ -value improved and showed a good agreement ( $\kappa$  = 0.724, concordance 82.5%).

### Prognostic impact on outcome

The 5-year survival rate for OS was 64.6% in the TSR-high population, vs 50.0% and 55.6% in the TSR-intermediate and TSR-low population, respectively. For the DFS, the 5-year survival rates for TSR-high, TSR-intermediate, and TSR-low were 77.2%, 51.8%, and

55.2%, respectively. The OS and DFS were significant different between the three TSR categories (*P* = 0.01 and *P* = 0.02, respectively). The 5-year survival rates for DSS were 81.6% for TSR-high, 60.3% for TSR-intermediate, and 63.9% for TSR-low. Although a higher DSS for the TSR-high population was thereby seen, the differences between the three TSR categories were just not significant (*P* = 0.06). The Kaplan-Meier survival curves are shown in Figure 3.

After adjusting for known prognostic factors (age, grading, and the use of adjuvant therapy), an intermediate TSR in lymph node metastasis negative patients showed a trend to a lower OS rate (HR = 2.04, 95%CI: 0.99-4.21) in comparison with a high TSR. There were no statistical differences between the TSR categories in OS among lymph node metastasis positive patients (Table 2). A statistically significant worse DFS was seen among the lymph node metastasis negative patients with an intermediate TSR (HR = 6.41, 95%CI: 1.84-22.28) compared to patients with a high TSR. Among lymph node metastasis positive patients, no statistically significant differences were seen between TSR categories for DFS (Table 3). Lymph node meta-



**Figure 3** Kaplan-Meier survival curves of overall survival, disease free survival and disease specific survival by categorized tumor-stroma ratio. A: Overall survival; B: Disease free survival; C: Disease specific survival. *P*-values of Log-rank statistics. TSR-high (carcinoma percentage  $\geq 70\%$ ), TSR-intermediate (carcinoma percentage 40%, 50% and 60%), and TSR-low (carcinoma percentage  $\leq 30\%$ ). TSR: Tumor-stroma ratio.

stasis negative patients with an intermediate TSR had a higher risk of dying from rectal cancer (HR 5.27, 95%CI: 1.54-18.10) in comparison with patients with a high TSR. These differences were not seen in lymph node metastasis positive patients (Table 4).

## DISCUSSION

This study, analyzing data of 154 patients with rectal adenocarcinoma diagnosed in the period 1996-2006, showed that the TSR is a prognostic factor for patients without lymph node metastasis. In such cases, a high TSR had a longer local recurrence and distant metastasis free period, and a lower risk of death from rectal adenocarcinoma. Besides, a high TSR was associated with a lower risk of death from any cause. The determination of the TSR may therefore contribute to stratify patients for prognosis. Determination of the TSR turned out to be feasible and reproducible among observers on routinely made sections of rectal cancers. The TSR therefore has the potential to contribute to decision making regarding the individual treatment policy in rectal cancer.

The relation between the prognosis and the TSR may be explained pathophysiologically. A dual effect of the tumor stroma in the tumor-host interaction

has been described. The tumor stroma is able to exert inhibitory effects on the malignant cells at first. With ongoing tumor growth, the tumor can exploit its stroma, for example by changing its composition (*e.g.*, vasculature), to promote tumor growth and metastasis. A process called stromagenesis, which occurs parallel with tumor progression. Stromagenesis is characterized by bidirectional communication between the tumor and its stroma. The interactional pathways are multiple and complex<sup>[12-14]</sup>. Despite this complexity it is justifiable to conclude that the stromal tissue is not a passive component surrounding the tumor. A sufficient amount of stroma contributes to a more aggressive phenotype of tumor, as is shown in this study as well.

Indeed, the poor prognosis for lymph node negative patients with an intermediate TSR is remarkable. The survival rates for death from all causes, death from rectal cancer, and the occurrence of local recurrences and distant metastasis are the lowest for this group of patients. This may be explained by a favorable balance between the tumor and its stroma. In this way, the tumor may be able to exploit the surrounding tumor stroma very efficiently. The concept of a balance between pro- and antitumor factors had been hypothesized earlier. For example, there is a relation between the degree of the peritumoral inflammatory



**Table 2** Cox multivariate analysis for overall survival

	N0		N+	
	HR	95%CI	HR	95%CI
Age				
< 70 yr	1	Ref.	1	Ref.
> 70 yr	3.32 <sup>a</sup>	1.75-6.28	2.26 <sup>a</sup>	1.10-4.65
Grading				
Poor	1	Ref.	1	Ref.
Moderate	1.03	0.43-2.49	0.52	0.25-1.10
Well	0.36	0.04-2.99	0.56	0.06-5.31
Adjuvant treatment				
No	1	Ref.	1	Ref.
Yes	0.47	0.06-3.50	0.66	0.27-1.60
TSR				
TSR-high	1	Ref.	1	Ref.
TSR-intermediate	2.04	0.99-4.21	1.19	0.50-2.84
TSR-low	1.43	0.57-3.60	1.04	0.40-2.69

<sup>a</sup>P < 0.05. Age defined as period from birth until date of primary surgery. N0: Lymph node metastasis negative patients; N+: Lymph node metastasis positive patients; TSR: Tumor-stroma ratio.

**Table 3** Cox multivariate analysis for disease free survival

	N0		N+	
	HR	95%CI	HR	95%CI
Age				
< 70 yr	1.00	Ref.	1.00	Ref.
> 70 yr	0.36	0.13-1.01	0.89	0.36-2.22
pT-status				
T1	1.00	Ref.	1.00	Ref.
T2	0.11	0.01-1.21	0.66	0.10-4.44
T3	0.85	0.09-7.58	1.51	0.31-7.30
T4	1.61	0.07-39.27	<sup>1</sup>	
Grading				
Poor	1.00	Ref.	1.00	Ref.
Moderate	1.43	0.29-6.99	0.58	0.25-1.35
Well	<sup>1</sup>		<sup>1</sup>	
Adjuvant treatment				
No	1.00	Ref.	1.00	Ref.
Yes	0.2	0.01-2.57	1.04	0.32-3.41
TSR				
TSR-high	1.00	Ref.	1.00	Ref.
TSR-intermediate	6.41 <sup>a</sup>	1.84-22.28	1.31	0.49-3.51
TSR-low	3.7	0.84-16.42	0.93	0.31-2.74

<sup>a</sup>P < 0.05; <sup>1</sup>Too small numbers to analyze. Age defined as period from birth until date of primary surgery. N0: Lymph node metastasis negative patients; N+: Lymph node metastasis positive patients; pT: Pathological tumor status; TSR: Tumor-stroma ratio.

reaction and its ability to destroy invading colorectal malignant cells<sup>[15]</sup>. Lymph node metastasis can be seen as an expression of a developed tumor that has exploited its environment successfully. When lymph node metastasis has occurred, the effect of the tumor micro-environment may be negligible. This statement may explain why we did not find differences in survival in lymph node metastasis positive patients survival based on the TSR.

The effect of a high TSR on survival demonstrated in this study is in line with other studies on the prognostic impact of the TSR in colorectal carcinomas (CRCs) and

**Table 4** Cox multivariate analysis for disease specific survival

	N0		N+	
	HR	95%CI	HR	95%CI
Age				
< 70 yr	1.00	Ref.	1.00	Ref.
> 70 yr	0.47	0.16-1.40	1.19	0.53-2.67
pT-status				
T1	1.00	Ref.	1.00	Ref.
T2	0.12	0.01-1.29	0.48	0.06-3.50
T3	0.69	0.08-6.25	1.29	0.27-6.07
T4	0.46	0.02-8.70	<sup>1</sup>	
Grading				
Poor	1.00	Ref.	1.00	Ref.
Moderate	1.03	0.20-5.30	0.46	0.21-1.04
Well	<sup>1</sup>		<sup>1</sup>	
Adjuvant treatment				
No	1.00	Ref.	1.00	Ref.
Yes	0.2	0.01-2.57	1.04	0.32-3.41
TSR				
TSR-high	1.00	Ref.	1.00	Ref.
TSR-intermediate	5.27 <sup>a</sup>	1.54-18.1	1.60	0.54-4.70
TSR-low	3.48	0.78-15.55	1.22	0.41-3.66

<sup>a</sup>P < 0.05; <sup>1</sup>Too small numbers to analyze. Age defined as period from birth until date of primary surgery. N0: Lymph node metastasis negative patients; N+: Lymph node metastasis positive patients; pT: Pathological tumor status; TSR: Tumor-stroma ratio.

other malignancies<sup>[6-10,16]</sup>. The present study is however the first that has identified a subgroup of patients with rectal cancer, namely lymph node metastasis negative patients with an intermediate TSR, whereby the TSR is a strong prognostic factor.

The interobserver agreement for absolute scores was moderate. The correlation coefficient improved to good, when grouping as TSR-low, TSR-intermediate, and TSR-high. The categorization into these categories was performed with the aim of generating enhanced prognostic information based on the TSR, which had been executed earlier in a previous study on the TSR in oesophageal adenocarcinomas<sup>[17]</sup>. Other studies concerning the TSR used an arbitrary cut-off value of 50%. No differences in the given survival rates were found at this and other cut-off values in our population (Appendix 1). The rate of agreement of the present study is slightly lower compared to these studies<sup>[7,8]</sup>, which may be attributed to the determination of absolute CPs before the categorization and the addition of an extra CP-category.

This study has some shortcomings to be noted. Neo-adjuvant radiotherapy for rectal malignancies was applied more frequently at our hospital relatively late in the study period and consequentially patients received adjuvant therapy frequently. Neoadjuvant treated patients were excluded, while in most cases a neoadjuvant (chemo)radiotherapy regimen is given nowadays. Though, it remains valuable to investigate tissue based prognostic factors in non-pretreated patients. There is a tendency to treat elderly, for whom there is an increasing incidence of rectal cancer, without neoadjuvant radiotherapy due to postoperative

wound complications in The Netherlands. According to the Dutch Surgical Colorectal Audit, no neoadjuvant therapy was used in 26% of cT2 patients aged > 75 years with rectal carcinoma in comparison with 14% in younger patients<sup>[18]</sup>. Furthermore, there is still a debate about adjuvant chemotherapy for rectal cancer. Future research about the balance between the oncological benefit, *i.e.*, relative risk reduction of 50% in local recurrences and the side effects, *i.e.*, relative risk increase of 50% in acute treatment-related toxicity, and long-term anorectal and sexual dysfunction<sup>[19-21]</sup> of neoadjuvant radiotherapy will help to determine the position of pretreatment dependent tissue-based markers like the TSR in predicting an individual prognosis.

It would be of interest to analyze the TSR and its prognostic value in biopsy specimens of well described areas of a rectal tumor. Prognostic information could then be provided before the use of neoadjuvant therapy. The visual estimation of the TSR could be made more accurate by the use of tumor or stroma specific stainings. Besides, it would be desirable to develop a more objective instrument to determine the TSR than the visual estimation in this study. This could be provided by the use of tumor specific staining and the development of computer software in the growing field of digital pathology.

Determination of the TSR has the potential to identify patients without lymph node metastasis with a good and a poor clinical outcome and can thereby help in decision making on (neo)adjuvant treatment policy in individual cases. Determination of the TSR in routine sections is feasible and can be done with a good concordance by different observers.

## COMMENTS

### Background

Colorectal cancer is one of the most common form of cancer in both men and women. The TNM staging system, the most common system to stage colorectal tumors, is used to discriminate between patients with a better and a poor prognosis, but it seems insufficiently able to predict the prognosis of the individual patient. Additional prognostic factors are desirable, because a part of the patients is overtreated and consequently exposed to a higher risk on therapy related complications. The tumor-stroma ratio (TSR), the proportion of carcinoma relative to the proportion of tumor stroma in the histopathological section through the tumor, has proven to be of prognostic value in several malignancies.

### Research frontiers

A previous study, on a small number of rectal cancer patients, showed that a high TSR is possibly related to both a longer disease free and overall survival. In this respect, it is meaningful to explore the relevance of the TSR in a larger cohort of patients with rectal adenocarcinoma, as the authors did.

### Innovations and breakthroughs

This paper showed that the TSR has potential as a prognostic factor for survival in surgically treated rectal cancer patients, especially in lymph node negative cases. The effect of a high TSR on survival demonstrated in this study is in line with other studies on the prognostic impact of the TSR in colorectal carcinomas and other malignancies. The present study is however the first that has identified a subgroup of patients with rectal cancer, namely lymph node

metastasis negative patients with an intermediate TSR, whereby the TSR is a strong prognostic factor.

### Applications

The determination of the TSR may contribute to stratify patients for prognosis and has the potential to contribute to decision making regarding the individual treatment policy in rectal cancer.

### Terminology

The TSR is the proportion of carcinoma relative to the proportion of tumor stroma in the histopathological section through the tumor. The carcinoma percentage (CP) is a derivative of the TSR and is complementary to the percentage of stroma and other components, like mucus. TSR-low including the CP-values  $\leq 30\%$ , TSR-intermediate including the CP-values 40%, 50% and 60%, and TSR-high including the CP-values  $\geq 70\%$ .

### Peer-review

The study is well designed and clearly presented and the topic of high interest for oncologists that should decide after surgery what patients will benefit more from an adjuvant treatment.

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# Laparoscopic vs open complete mesocolic excision with central vascular ligation for colon cancer: A systematic review and meta-analysis

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## Abstract

### AIM

To compare the effectiveness of laparoscopic complete mesocolic excision (CME) with central vascular ligation (L-CME) with its open (O-CME) counterpart.

### METHODS

We conducted an electronic search of the PubMed/MEDLINE, Excerpta Medica Database, Web of Science Core Collection, Cochrane Center Register of Controlled Trials, Cochrane Database of Systematic Reviews, SciELO, and Korean Journal databases from their inception until May 2017. We considered randomized controlled trials (RCTs) and controlled clinical trials (CCTs) that included patients with colonic cancer comparing L-CME and O-CME. Primary outcomes included the quality of the resected specimen (lymph nodes retrieved, complete mesocolic plane excision, tumor to arterial high tie, resected mesocolon surface). Secondary outcomes included the three-year and five-year overall and disease-free survival rates, recurrence of the disease, surgical data, and postoperative morbidity and mortality. Two authors of the review screened the methodological quality of the eligible trials and independently extracted data from individual



studies.

## RESULTS

A total of one RCT and eleven CCTs (four from Europe and seven from Asia) met the inclusion criteria for the current meta-analysis. These studies involved 1619 patients in L-CME and 1477 patients in O-CME. The L-CME was associated with the same quality of the resected specimen, with no differences regarding the retrieved lymphnodes (MD = -1.06, 95%CI: -3.65 to 1.53,  $P = 0.42$ ), and tumor to high tie distance (MD = 14.26 cm, 95%CI: -4.30 to 32.82,  $P = 0.13$ ); the surface of the resected mesocolon was higher in the L-CME group (MD = 11.75 cm<sup>2</sup>, 95%CI: 9.50 to 13.99,  $P < 0.001$ ). The L-CME was associated with a lower rate of blood transfusions (OR = 0.45, 95%CI: 0.27 to 0.75,  $P = 0.002$ ), faster recovery of gastrointestinal function, and less postoperative overall complication rate. The L-CME approach was associated with a statistical significant better three-year overall (OR = 2.02, 95%CI: 1.31 to 3.12,  $P = 0.001$ ,  $I^2 = 28\%$ ) and disease-free (OR = 1.45, 95% CI: 1.00 to 2.10,  $P = 0.05$ ,  $I^2 = 0\%$ ) survival.

## CONCLUSION

The laparoscopic approach offers the same quality of the resected specimen as the open approach in complete mesocolic excision with central vascular ligation for colon cancer. The laparoscopic complete mesocolic excision with central vascular ligation is superior in all perioperative results and at least non-inferior in long-term oncological outcomes.

**Key words:** Colon cancer; Complete mesocolic excision; D3 lymphadenectomy; Central vascular ligation

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**Core tip:** The laparoscopic complete mesocolic excision with central vascular ligation was associated with the same quality of the resected specimen, with no differences regarding the retrieved lymphnodes, and tumor to high tie distance; the surface of the resected mesocolon was higher in the laparoscopic group. Laparoscopy was associated with a lower rate of blood transfusions, faster recovery of gastrointestinal function, and less post-operative overall complication rate. The laparoscopic approach was associated with a statistical significant better three-year overall and disease-free survival.

Negoi I, Hostiuc S, Negoi RI, Beuran M. Laparoscopic vs open complete mesocolic excision with central vascular ligation for colon cancer: A systematic review and meta-analysis. *World J Gastrointest Oncol* 2017; 9(12): 475-491 Available from: URL: <http://www.wjgnet.com/1948-5204/full/v9/i12/475.htm> DOI: <http://dx.doi.org/10.4251/wjgo.v9.i12.475>

## INTRODUCTION

Complete mesocolic excision (CME) with central

vascular ligation (CVL) represents an extension to the colonic cancer of the already standardized resection for rectal cancer. It adheres to the same guiding principle that sharp surgical dissection, following embryological planes, with central vascular ligation, should improve oncological outcomes<sup>[1]</sup>.

Hohenberger *et al*<sup>[2]</sup> (2007) published the technical details of a new concept termed CME and central ligation for colonic cancer. During CME with CVL for right-sided tumors, the ileocolic and right colic vessels should be ligated at their origin from the superior mesenteric artery. Transverse colon tumors require transection of the middle colic artery at its origin. Left-sided tumors require transection of the inferior mesenteric artery (IMA) at its origin from the aorta<sup>[3]</sup>. Using CME and CVL, Hohenberger *et al*<sup>[4]</sup> reported a reduction of the local five-year recurrence rate from 6.5% to 3.6% and an increase in the cancer-related five-year survival rate from 82.1% to 89.1%. This specimen-oriented technique is associated with the removal of more tissue compared with standard surgery, a wider distance from the tumor to the high vascular tie (131 mm vs 90 mm,  $P < 0.0001$ ), a longer length of large bowel (314 mm vs 206 mm,  $P < 0.0001$ ), a wider area of removed mesentery (19657 mm<sup>2</sup> vs 11829 mm<sup>2</sup>,  $P < 0.0001$ ) and a greater lymph node yield (30 vs 18,  $P < 0.0001$ )<sup>[5]</sup>. These differences may partially explain the higher reported survival rates with CME and CVL.

One should note the similarities between D3 lymphadenectomies, recommended as a standard of care for stage II and III colon cancer in Eastern countries, and Western CME<sup>[3,6]</sup>. The Japanese nomenclature includes D1 as pericolic (close to the bowel wall), D2 as intermediate (along the feeding artery), and D3 as main (at the origin of the feeding artery) lymph nodes. For right-sided tumors, a D3 lymphadenectomy requires the transection of the feeding arteries next to their origin from the superior mesenteric artery. In left-sided cancers, a D3 lymphadenectomy requires transection of the IMA close to its aortic origin<sup>[7]</sup>.

Current evidence is consistent with a faster postoperative recovery for laparoscopic colectomies compared with the open approach; the former is not associated with any negative impact regarding local recurrence and survival rates. Therefore, according to the latest National Comprehensive Cancer Network guidelines, the laparoscopic approach is preferred given access to a surgeon with experience in advanced minimally invasive procedures<sup>[8]</sup>.

The objective of this systematic review and meta-analysis is to summarize the current evidence regarding laparoscopic CME (L-CME) and to compare its effectiveness with its open (O-CME) counterpart.

## MATERIALS AND METHODS

This systematic review and meta-analysis was conducted according to the Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) statement<sup>[9]</sup>.

Electronic search, study selection, data extraction, and quality assessment was performed independently by two reviewers.

### Data sources and search strategy

We conducted an electronic search to identify all published randomized controlled trials (RCTs) and controlled clinical trials (CCTs) using the following databases: United States National Library of Medicine - National Institutes of Health PubMed/MEDLINE, EMBASE, Web of Science Core Collection, Cochrane Center Register of Controlled Trials (CENTRAL), Cochrane Database of Systematic Reviews, SciELO, and Korean Journal databases from their inception until May 2017. We did not use any language restrictions. The most recent search in PubMed was performed in May 2017.

We constructed the search strategy using various combinations of terms related to CME or D3 lymphadenectomy using an open or laparoscopic approach to colon cancer. We used in different combinations the following key words: colon, cancer, complete mesocolic excision, central vascular ligation, D3 lymphadenectomy, minimally invasive, laparoscopy, open, surgery, colectomy and resection. These words were identified as truncated words in the title, abstract, or in the medical subject headings (MeSH). We additionally used electronic and manual cross-referencing to find other relevant sources. The search strategy used in PubMed/Medline was: [colon (MeSH Terms)] OR colonic (Title/Abstract) OR lower intestinal (Title/Abstract) OR large bowel (Title/Abstract) AND cancer (MeSH Terms) OR neoplasia (Title/Abstract) OR neoplasm (Title/Abstract) OR tumor (Title/Abstract) AND laparoscopy (MeSH Terms) OR minimally invasive (Title/Abstract) OR laparoscopic (Title/Abstract) AND complete mesocolic excision (Title/Abstract) OR central vascular ligation (Title/Abstract) OR D3 lymphadenectomy (Title/Abstract).

### Trial selection

**Study eligibility criteria:** We considered RCTs and CCTs comparing open with laparoscopic CME or D3 lymphadenectomy as eligible for inclusion if they included patients with colonic cancer.

### Outcome measures

**Primary outcome:** Quality of the resected specimen (lymph nodes retrieved, complete mesocolic plane excision, tumor to arterial high tie, resected mesocolon surface).

**Secondary outcomes:** Three-year and five-year overall and disease-free survival rates, recurrence of the disease, surgical data (operation time, length of the abdominal incision, conversion rate), intraoperative complications, blood loss, postoperative complications (anastomotic leakage, wound infections, overall complications), length of hospital stay, thirty-day mortality, immunologic response, quality of life, and cost.

### Data extraction

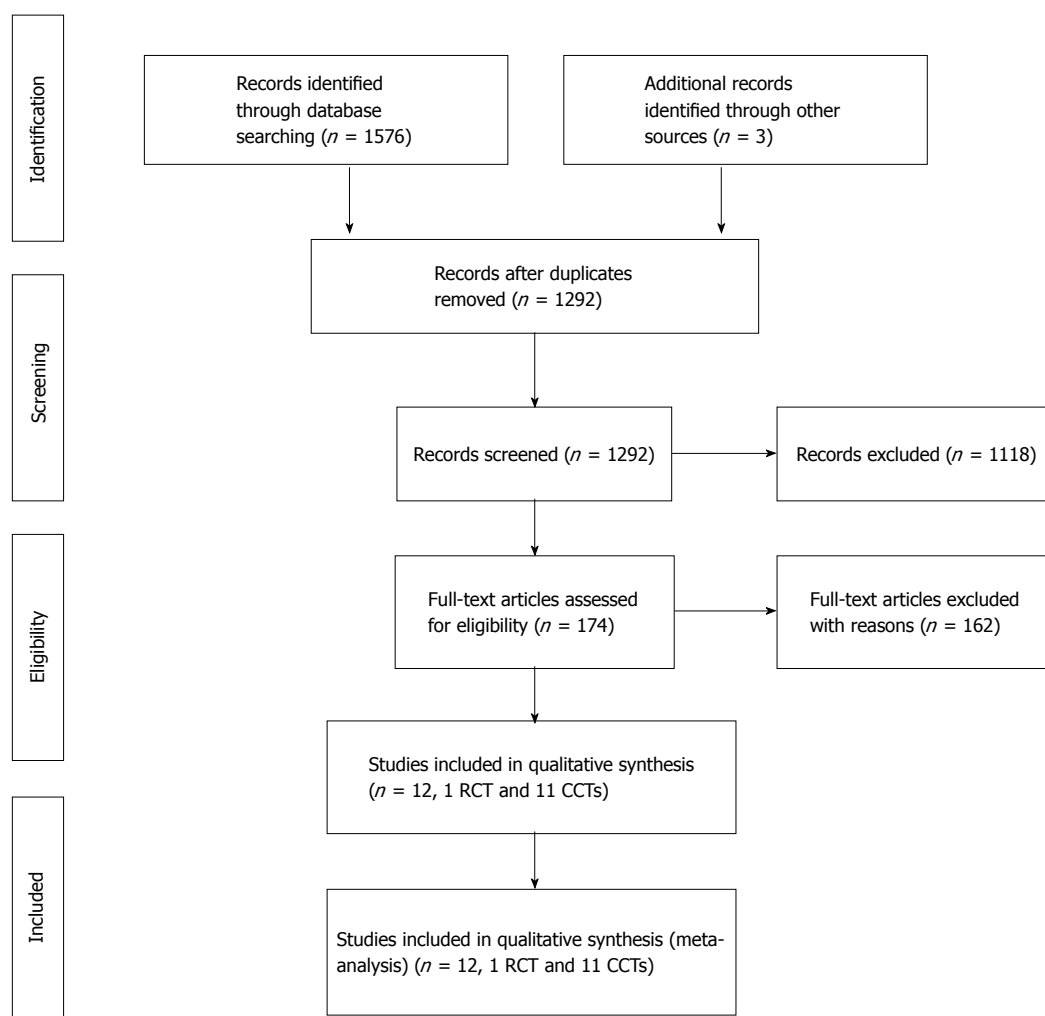
Two authors<sup>[10]</sup> (Negoi and Hostiuc) assessed the methodological quality of the eligible trials and independently extracted data from individual studies using a data-extraction form. We extracted the following data: Year of publication, source, title, first author, contact address, criteria for patient inclusion and exclusion, sample size, baseline characteristics, and patient characteristics including mean age, sex ratio, location of the tumor, number of patients assigned to each treatment group, and details of the intervention regimens. We registered the following outcomes: One-, three- and five-year overall and disease-free survival rates, number of removed lymph nodes, length of the resected colon, resection of the mesocolic plane, operation time, length of hospital stay, number and frequency of postoperative complications, and quality of life.

### Assessment of risk of bias

To assess the risk of bias, we used the Cochrane Collaboration tool for RCTs. This tool grades the random sequence generation, allocation concealment, blinding of participant and personnel, blinding of outcome assessment, incomplete outcome data, selective reporting, and other biases<sup>[11]</sup>. To evaluate the non-randomized trials, we used the methodological index of non-randomized studies (MINORS)<sup>[12]</sup>. We scored all of the 12 methodological items for non-randomized comparative studies as follows: 0 - not reported; 1 - reported but inadequate; or 2 - reported and adequate. The global ideal score for comparative (non-comparative) studies was 24.

### Statistical analysis

For statistical analysis, we used Review Manager Software 5.3.5 (The Nordic Cochrane Centre, Copenhagen, Denmark)<sup>[11]</sup> provided by the Cochrane Collaboration and OpenMetaAnalyst<sup>[13]</sup> with metaphor package<sup>[14]</sup> as statistical softwares. We selected the mean difference (MD) as an effect measure for continuous data and the odds ratio (OR) for dichotomous data; we also reported the 95%CI. In cases of continuous data presented as median and range, we estimated the mean and standard deviation according to the methods described by Hozo *et al.*<sup>[15]</sup>. We used Chi-square and  $I^2$  statistics to assess the studies' heterogeneity and explain the total variation observed between the studies that be generated by the differences between the trials rather than the sampling error (chance). An  $I^2$  value  $\leq 25\%$  indicates less heterogeneity, an  $I^2$  value  $> 25\%$  but  $\leq 75\%$  indicates a moderate heterogeneity, and  $I^2$  values  $> 75\%$  indicate higher heterogeneity<sup>[16]</sup>. We explored the reasons behind the statistical heterogeneity using sensitivity analyses and the exclusion of specific studies. We used fixed-effect model analysis for outcomes with low heterogeneity. If we found clinical heterogeneity between included studies due to differences with respect to eligibility criteria (study population), the type of surgical technique, and lacking or differing definitions of outcomes, we performed meta-analysis by applying a random-



**Figure 1** Flow diagram of the systematic literature search and study selection according to prisma statement. RCT: Randomized control trial; CCT: Controlled clinical trial.

effect model (the DerSimonian-Laird method)<sup>[17]</sup>. We used Begg's funnel plot and Egger's test for assessing publication bias<sup>[18]</sup>. The statistical significance was defined as  $P < 0.1$  in Egger's test and  $P < 0.05$  for the other statistical tests. To correct possible publication bias, we performed trim and fill analysis<sup>[19]</sup>. The statistical methods of this study were reviewed by Sorin Hostiuc from the Department of Legal Medicine and Bioethics, Carol Davila University of Medicine and Pharmacy, Bucharest, Romania.

## RESULTS

### Description of studies

**Results of the search:** The initial electronic and manual literature searches revealed 174 full-text articles. A total of one RCT (from Japan)<sup>[20,21]</sup> and eleven CCTs (four from Europe and seven from Asia)<sup>[22-32]</sup> met the inclusion criteria for the qualitative and quantitative (meta-analysis) synthesis; these studies involved 1619 patients in L-CME and 1477 patients in O-CME. Eleven studies were published in English and one in Chinese. The reasons for exclusion in each stage of the process are shown in Figure 1.

**Included studies:** The characteristics of the included studies are summarized in Table 1. All of the studies were published between 2012 and 2016, the RCT being published in 2014. The sample size of the studies ranged from 23 to 533 patients. The CME or D3 lymphadenectomy was defined as dissection along the Told's fascia space and a high (apical or central) ligation of the feeding vessel. Colonic mobilization was conducted using a medial-to-lateral or a lateral-to-medial approach according to the surgeon's preference. For right-sided tumors, the vascular pedicles were divided at their origin together with removal of the draining lymph nodes along the border of the superior mesenteric vein. For left-sided tumors, removal of the central lymph nodes from the origin of the inferior mesenteric artery was performed with high ligation or with preservation of the left colic artery. In the JCOG 0404 study, the accredited surgeons had completed more than 30 laparoscopic and 30 open colorectal resections<sup>[20]</sup>. In all of the other studies, the procedures were performed or supervised by colorectal surgeons. Conversion to laparotomy was defined as the extension of the abdominal incision more than eight cm or as the inability to complete the dissection fully laparoscopically. The reported rate

Table 1 Characteristics of the included studies

Ref.	Country of origin	Study type	Study period	Female (number, L/O)	Mean age (yr, L/O)	Intervention (L-CME) right/transverse/left location of the tumor	Control (O-CME) right/transverse/left location of the tumor	Adjuvant chemotherapy
Kim <i>et al</i> <sup>[23]</sup> , 2016	South Korea	Case control, unicentre, prospective database	2008-2013	62/44	69/67	116/0/0	99/0/0	L-CME = 68 pts (58.62%), O-CME = 78 pts (78.78%), recommended to all stage II and III
Storli <i>et al</i> <sup>[22]</sup> , 2016	Norway	Prospective non RT, unicentre	2007-2014	22/13	73/23	0/33/0	0/23/0	L-CME = 8 (61.5%), O-CME = 5 (62.5%), all stage III below 75 yr
Huang <i>et al</i> <sup>[24]</sup> , 2015	China	Case control, unicentre	2012-2013	20/21	56/55	53/0/0	49/0/0	NR
Yamamoto <i>et al</i> <sup>[20]</sup> , 2014	Japan	RCT, multicentre	2004-2009	248/215	64/64	144/0/389	156/0/368	NR, recommended for all stage III
Munkedal <i>et al</i> <sup>[25]</sup> , 2014	Denmark	Prospective nonRT, unicentre	2008-2011	30/38	69.1/72.9	30/0/53	41/0/38	NR
Bae <i>et al</i> <sup>[27]</sup> , 2014	South Korea	Case control, unicentre	2006-2008	40/38	64/65	73/12/0	76/9/0	All stage III and II with poor prognosis
Han <i>et al</i> <sup>[26]</sup> , 2014	China	Case control, unicentre	2003-2010	94/67	67/65	177/0/0	147/0/0	NR, recommended for high risk stage II and stage III
Zhao <i>et al</i> <sup>[28]</sup> , 2014	China	Case control, multicentre	2000-2009	53/44	61.3/64.5	89/30/0	65/36/0	NR, recommended for high risk stage II and stage III
Cong <i>et al</i> <sup>[29]</sup> , 2014	China	Case control, unicentre	2008-2011	53/45	61.5/62.3	96/0/0	82/0/0	NR
Storli <i>et al</i> <sup>[30]</sup> , 2013	Norway	Prospective nonRT, unicentre	2007-2010	49/60	71.9/73.1	50/18/60 2 pts - multiple	35/44/42	All stage III below 75 yr
Gouvas <i>et al</i> <sup>[31]</sup> , 2012	Greece	Prospective nonRT, multicentric	2006-2010	19/17	62.1/66.3	7/9/33	9/9/23	NR
Sun <i>et al</i> <sup>[32]</sup> , 2012	China	Case control, unicentre	2000-2008	58/45	60.1/61.9	49/7/91	43/9/74	NR, according to stage

L-CME: Laparoscopic complete mesocolic excision; O-CME: Open complete mesocolic excision; RT: Randomized control trial; Non RT: Non randomized control trial; L/O: Laparoscopy/open groups; NR: Not reported.

of conversion to laparotomy was between 2.82% and 7.6%<sup>[20,22-32]</sup>. Transverse colon cancers were excluded from the JCOG 0404 study<sup>[20]</sup>. Storli *et al*<sup>[30]</sup> performed 9 (7.3%) transverse colectomies in the open approach but none in the laparoscopic group. In a second paper, Storli *et al*<sup>[22]</sup> published their experience regarding CME only in transverse colon cancer. Gouvas *et al*<sup>[31]</sup> managed all of the transverse colon cancers using an extended right hemicolectomy. Munkedal *et al*<sup>[25]</sup> excluded all cancers in the transverse colon or flexures from their analysis. Bae *et al*<sup>[26]</sup>, Han *et al*<sup>[27]</sup>, and Zhao *et al*<sup>[28]</sup> managed all cases by a right or extended right hemicolectomy. All studies exhibited remarkable similar exclusion criteria: Stage IV disease and emergency surgery. All of the studies described the technique of laparoscopic CME. Perioperative care was not described in most trials.

The patient demographics and baseline clinical data were similar between the treatment groups; the L-CME group exhibited a mean age of 69.91 years, and the O-CME group exhibited a mean age of 65.41 years.

Women comprised 46.20% and 41.23% of the L-CME and O-CME patients, respectively. None of the studies were blinded, and all of the studies were powered to demonstrate the non-inferiority of the laparoscopic approach.

**Excluded studies:** We excluded all studies in which the surgical technique did not comply with CME or D3 lymphadenectomy principles<sup>[33-42]</sup>. We also excluded studies based on the hand-assisted laparoscopic technique<sup>[43,44]</sup>. Due to the probability of overlapping patients, we have excluded first report of Kim *et al*<sup>[45]</sup> which includes only T4 patients.

#### Risk of bias in the included studies

The risk of bias in the one Japanese RCT was low in all domains<sup>[20]</sup>. Although blinding of patients and medical personnel was not performed in either trial, the endpoints were considered to be objective, particularly when they were supported by photos. The prospective and



**Table 2** Quality assessment of included non-randomized controlled trials

Quality evaluation criteria	Kim <i>et al.</i> <sup>[23]</sup> , 2016	Storli <i>et al.</i> <sup>[22]</sup> , 2016	Storli <i>et al.</i> <sup>[30]</sup> , 2016	Huang <i>et al.</i> <sup>[24]</sup> , 2015	Munkedal <i>et al.</i> <sup>[25]</sup> , 2014	Bae <i>et al.</i> <sup>[27]</sup> , 2014	Han <i>et al.</i> <sup>[26]</sup> , 2014	Zhao <i>et al.</i> <sup>[28]</sup> , 2014	Cong <i>et al.</i> <sup>[29]</sup> , 2014	Gouvas <i>et al.</i> <sup>[31]</sup> , 2012	Sun <i>et al.</i> <sup>[32]</sup> , 2012
Clear stated aim	2	2	2	2	2	2	2	2	2	2	2
Inclusion of consecutive patients	2	2	2	2	2	2	2	2	1	2	2
Prospective data collection	2	2	2	0	2	0	0	0	0	2	0
Endpoints appropriate to the study aim	2	2	2	2	2	2	2	2	2	2	2
Unbiased assessment of study end-point	2	2	2	2	2	2	2	1	1	2	1
Appropriate follow-up period	2	2	2	1	2	2	2	2	2	2	2
Loss to follow-up less than 5%	1	2	2	1	2	2	2	1	1	2	1
Prospective calculation of the study size	0	0	0	0	0	0	0	0	0	0	0
Adequate group control	2	2	2	2	2	2	2	2	0	2	2
Contemporary groups	2	2	2	2	2	2	2	2	2	2	2
Baseline equivalence	2	2	2	2	2	2	2	2	2	2	2
Adequate statistical analysis	2	2	2	2	2	2	2	2	2	2	1
Total	21	22	22	18	22	20	20	18	15	22	17

0: Non-reported; 1: Reported but inadequate; 2: Reported and adequate.

retrospective non-randomized studies had good MINORS scores, although the risk of selection, performance, and detection bias was high (Table 2). As expected, the prospective observational studies<sup>[22-23,25,30,31]</sup> had a higher methodological quality comparing with the retrospective studies<sup>[24,26-29]</sup>.

### Effects of intervention

**Overall survival:** Three-year overall survival was reported by four studies, including 1010 patients (Table 3). The laparoscopic approach was associated with a statistical significant better three-year overall survival, with an OR of 2.02 (95%CI: 1.31 to 3.12,  $P = 0.001$ ,  $I^2 = 28\%$ ). The five-year overall survival was reported by three studies, with a high heterogeneity between them ( $I^2 = 63\%$ ). The combined data revealed no statistical significant differences between the L-CME and O-CME (OR = 0.77,  $P = 0.38$ , 95%CI: 0.44 to 1.37) (Figure 2). Meta-regression of studies on three-year overall survival according to the number of included patients revealed a trend, although not statistical significant (omnibus  $P = 0.127$ ), for decreasing of the size of the effect with increasing the number of patients (Figure 3A). The subgroup analysis of studies that include or not only right sided colon cancers, revealed statistical significant results irrespective of that ( $P = 0.003$  and  $P = 0.018$ , respectively) (Figure 3B). On the other hand, the cumulative meta-analysis showed a progressively increasing of the size effect while experience is accumulating (Figure 3C).

### Disease-free survival

Three studies, with a total of 686 patients, reported the three-year DFS with a low heterogeneity between them ( $I^2 = 0\%$ ). However, to adjust for possible methodological differences we used the random-effects model, which revealed that the laparoscopic approach is associated with a statistical significant better three-

year DFS (OR = 1.45, 95%CI: 1.00 to 2.10,  $P = 0.05$ ) (Figure 4). Meta-regression of studies on three-year overall survival according to the number of the included patients revealed a trend, although not statistical significant (Omnibus  $P = 0.718$ ), for decreasing of the size of the effect with increasing the number of patients (Figure 5).

### Local and distant recurrences

The local recurrence rate was presented by five studies, including 1233 patients. In the fixed-effect meta-analysis there were no statistical significant differences between L-CME and O-CME (OR = 0.67, 95%CI: 0.38 to 1.17,  $P = 0.16$ ,  $I^2 = 0\%$ ) (Figure 6).

The distant recurrence rate was presented by four studies, with a moderate heterogeneity between them ( $I^2 = 40\%$ ). In the random-effects meta-analysis there were no statistical significant differences between the two groups (OR = 0.98, 95%CI: 0.61 to 1.58,  $P = 0.94$ ). Using Egger's test, no publication bias was found for local ( $t = 0.22$ ,  $P = 0.42$ ) or distant recurrences ( $t = 0.38$ ,  $P = 0.36$ ).

The port size metastasis rate was reported by two studies including 494 patients, with a low heterogeneity between them ( $I^2 = 0\%$ ). In the fixed-effect analysis model there was no difference regarding the port size metastasis rate between laparoscopic and open CME (OR = 1.52, 95%CI: 0.20 to 11.42,  $P = 0.69$ ).

### Quality of the resected specimen

Standardized evaluation of the resected specimen and grading its quality are objective measures that predict recurrence rate and survival. These data are correlated with the accuracy of the surgical technique.

### Lymphnodes retrieved

Ten studies reported the number of retrieved lymph nodes for 1376 L-CME patients and 1271 O-CME pa-

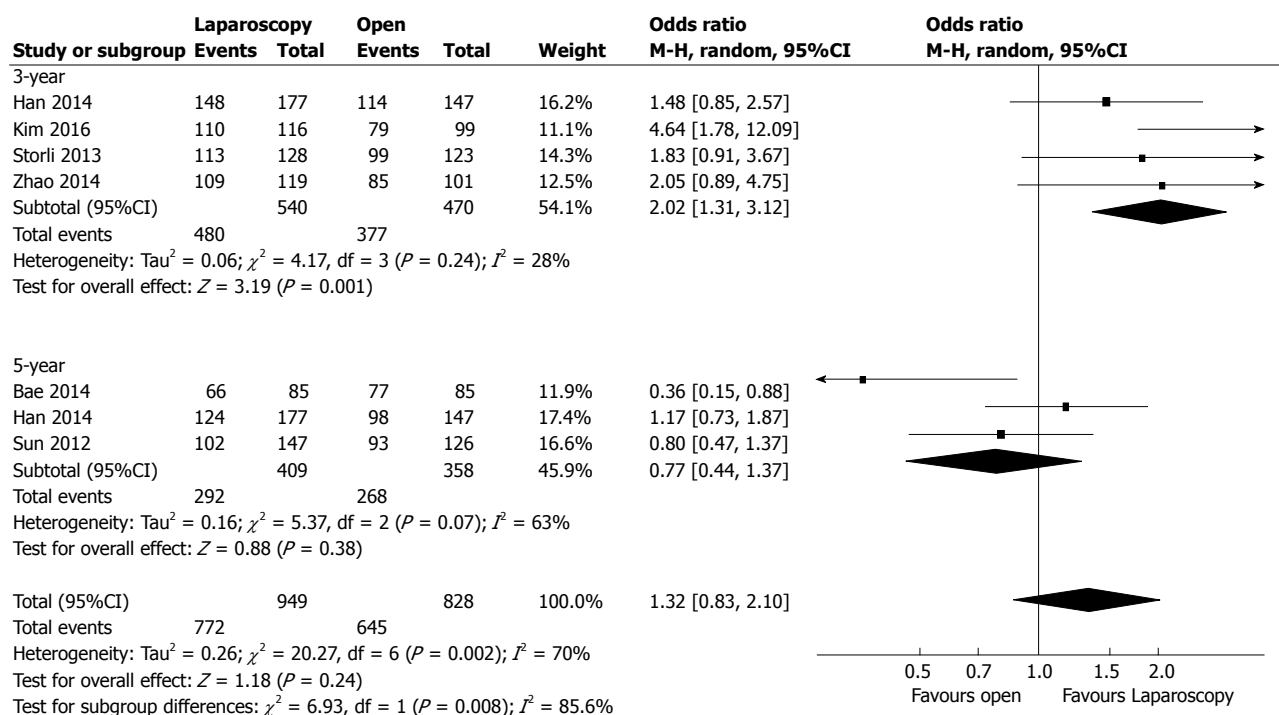
**Table 3** Results of meta-analysis comparing laparoscopic with open complete mesocolic excision for colon cancer

Outcome or subgroups	No. of Studies	Participants	Statistical method (95%CI)	Effect estimate (95%CI)	P value	Heterogeneity <i>P</i> , <i>I</i> <sup>2</sup> (%)
Survival and recurrences						
Overall survival	6	1777	OR (M-H, random)	1.32 (0.83, 2.10)	0.24	0.002, 70
Three-year	4	1010	OR (M-H, random)	2.02 (1.31, 3.12)	0.001	0.24, 28
Five-year	3	767	OR (M-H, random)	0.77 (0.44, 1.37)	0.38	0.07, 63
Disease free survival	4	856	OR (M-H, random)	1.15 (0.70, 1.87)	0.58	0.09, 54
Three-year	3	686	OR (M-H, random)	1.45 (1.00, 2.10)	0.05	0.89, 0
Five-year	1	170	OR (M-H, random)	0.50 (0.24, 1.05)	0.07	NA
Local recurrences	5	1233	OR (M-H, fixed)	0.67 (0.38, 1.17)	0.16	0.60, 0
One-year	2	466	OR (M-H, fixed)	0.52 (0.20, 1.35)	0.18	0.30, 7
Five-year	3	767	OR (M-H, fixed)	0.77 (0.38, 1.54)	0.46	0.52, 0
Distant recurrences	4	1018	OR (M-H, random)	0.98 (0.61, 1.58)	0.94	0.17, 40
Three-year	1	251	OR (M-H, random)	1.28 (0.54, 3.03)	0.58	NA
Five-year	3	767	OR (M-H, random)	0.90 (0.48, 1.69)	0.75	0.10, 57
Port site metastasis	2	494	OR (M-H, fixed)	1.52 (0.20, 11.42)	0.69	0.55, 0
Quality of the resected specimen						
Lymphnodes retrieved	10	2647	MD (IV, random)	-1.06 (-3.65, 1.53)	0.42	< 0.001, 92
RCTs	1	1057	MD (IV, random)	1.00 (-0.34, 2.34)	0.14	NA
NRCTs	9	1590	MD (IV, random)	-1.32 (-4.42, 1.78)	0.40	< 0.001, 92
Lymphnodes retrieved	10	2647	MD (IV, random)	-1.06 (-3.65, 1.53)	0.42	< 0.001, 92
< 100 patients	4	478	MD (IV, random)	-3.18 (-8.69, 2.33)	0.26	< 0.001, 85
> 100 patients	6	2169	MD (IV, random)	0.29 (-1.64, 2.21)	0.77	< 0.001, 83
Lymphnodes retrieved	10	2647	MD (IV, random)	-1.06 (-3.65, 1.53)	0.42	< 0.001, 92
Europe	4	559	MD (IV, random)	-3.33 (-8.31, 1.64)	0.19	< 0.001, 90
Asia	6	2088	MD (IV, random)	0.56 (-1.33, 2.46)	0.56	< 0.001, 77
Tumor to arterial high tie (mm)	2	252	MD (IV, random)	14.26 (-4.30, 32.82)	0.13	< 0.001, 92
Resected mesocolon surface (cm <sup>2</sup> )	2	252	MD (IV, fixed)	11.75 (9.50, 13.99)	< 0.001	0.55, 0
Complete mesocolic plane excision	1	90	OR (M-H, fixed)	0.77 (0.20, 2.96)	0.71	NA
Operative data						
Duration of surgery	7	2266	MD (IV, random)	26.26 (5.06, 47.46)	0.02	< 0.001, 94
Incision length (cm)	2	1159	MD (IV, random)	-14.01 (-14.35, -13.66)	< 0.001	0.89, 0
Blood loss (mL)	5	1868	MD (IV, random)	-52.11 (-78.57, -25.65)	< 0.001	< 0.001, 89
Transfusion requirement	2	1272	OR (M-H, random)	0.45 (0.27, 0.75)	0.002	0.54, 0
Intraoperative morbidity	1	1057	OR (M-H, fixed)	2.12 (0.95, 4.72)	0.07	NA
Postoperative course						
Time to first flatus (d)	4	1771	MD (IV, random)	-0.90 (-1.46, -0.34)	0.002	< 0.001, 97
Time to liquid diet (d)	5	1031	MD (IV, random)	-1.84 (-2.93, -0.74)	0.001	< 0.001, 98
Short-term morbidity and mortality						
Thirty-day overall morbidity	7	2144	OR (M-H, fixed)	0.57 (0.46, 0.71)	< 0.001	0.76, 0
RCTs	1	1057	OR (M-H, fixed)	0.66 (0.49, 0.89)	0.006	NA
NRCTs	6	1087	OR (M-H, fixed)	0.49 (0.36, 0.68)	< 0.001	0.89, 0
Wound complications	8	2322	OR (M-H, fixed)	0.43 (0.30, 0.61)	< 0.001	0.80, 0
Postoperative bleeding	4	1662	OR (M-H, fixed)	1.20 (0.46, 3.12)	0.71	0.75, 0
Pneumonia	5	867	OR (M-H, random)	0.61 (0.20, 1.84)	0.38	0.21, 32
Anastomotic leakage	8	2471	OR (M-H, fixed)	0.82 (0.54, 1.25)	0.36	0.77, 0
Need for reoperation	2	1113	OR (M-H, fixed)	0.59 (0.28, 1.23)	0.16	0.79, 0
Thirty-day mortality	6	2237	OR (M-H, fixed)	0.42 (0.16, 1.12)	0.07	0.98, 0
Hospital stay (d)	9	2573	MD (IV, random)	-4.07 (-5.87, -2.28)	< 0.001	< 0.001, 91

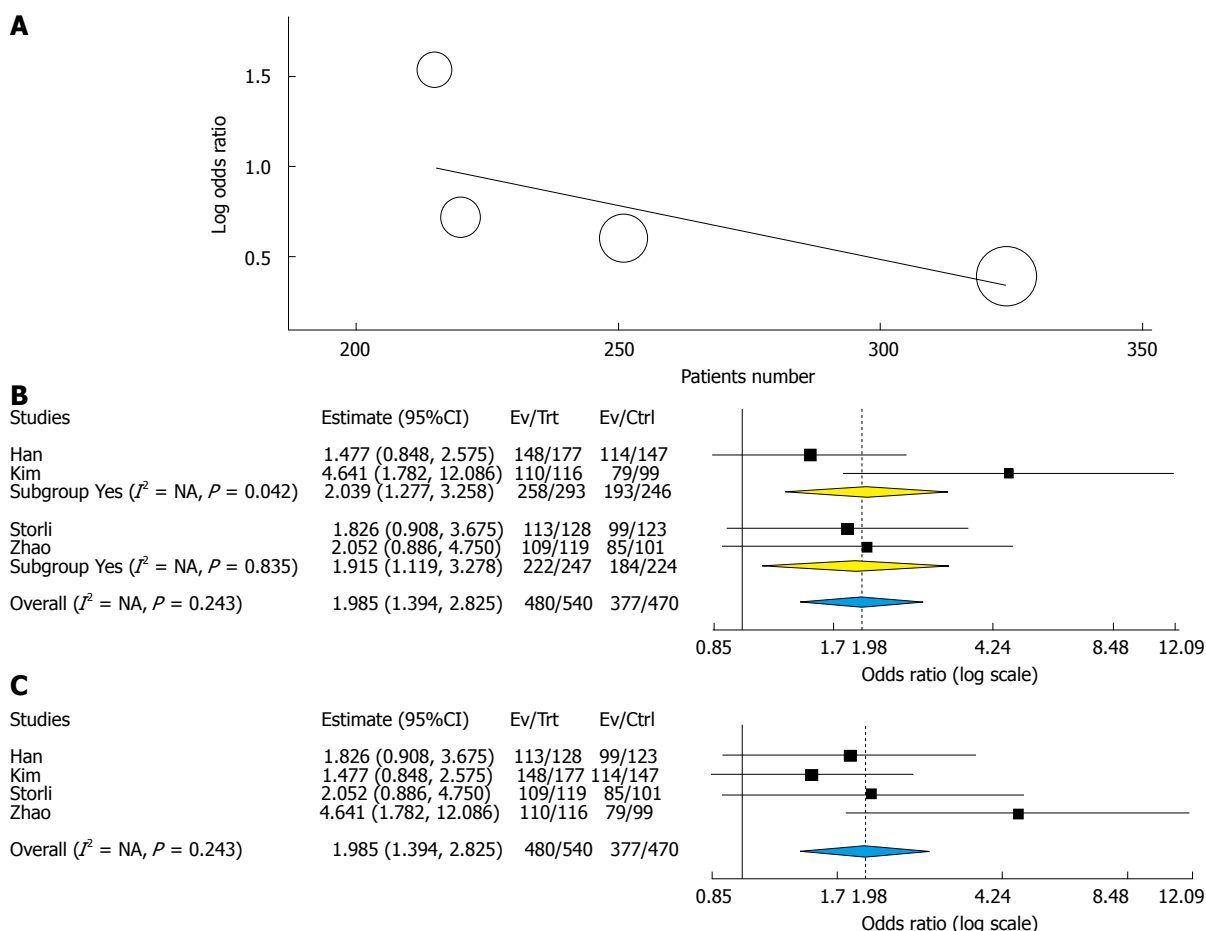
M-H: Mantel-haenszel analysis method; IV: Inverse variance analysis method; RCT: Randomized control trial; NRCTs: Non-randomized control trials; MD: Mean difference; OR: Odds ratio; NA: Not applicable.

tients. There was a high heterogeneity between the studies ( $I^2 = 92\%$ ). In the random-effects model, we found no statistically significant mean difference between L-CME and O-CME (MD = -1.06, 95%CI: -3.65 to 1.53,  $P = 0.42$ ) (Figure 7). In order to address the observed heterogeneity, we performed subgroup analysis according to the number of included patients (less or more than 100 patients in each group) and the geographical location of the study (Europe and Asia). The subgroup analysis revealed a high heterogeneity between studies with less than ( $I^2 = 85\%$ ) or more ( $I^2 = 83\%$ ) than 100 patients into laparoscopy or open group.

The results remained with no statistical significance into the two subgroups. Studies coming from Europe showed a high heterogeneity ( $I^2 = 90\%$ ) and with no differences regarding the number of retrieved lymphnodes ( $P = 0.19$ ). Studies published in Asia had also a high heterogeneity ( $I^2 = 77\%$ ), and no statistical significant difference between L-CME and O-CME ( $P = 0.56$ ). Meta-regression of retrieved lymphnodes according to the number of patients revealed that the equivalence between laparoscopic and open approach is stronger with the increased experience in laparoscopic approach (number of the included patients - omnibus  $P$



**Figure 2** Meta-analysis of studies on overall survival of patients undergoing laparoscopic vs open complete mesocolic excision with central vascular ligation excision for colon cancer.



**Figure 3** Results of statistical analysis. A: Meta-regression on three-year overall survival according with the number of included patients in each study, of patients undergoing laparoscopic vs open complete mesocolic excision with central vascular ligation excision for colon cancer; B: Subgroup meta-analysis according with the selection of patients with only right colon cancers (Yes group) or all-localizations colon cancer (No group); C: Cumulative meta-analysis according to the year of publishing for each study.

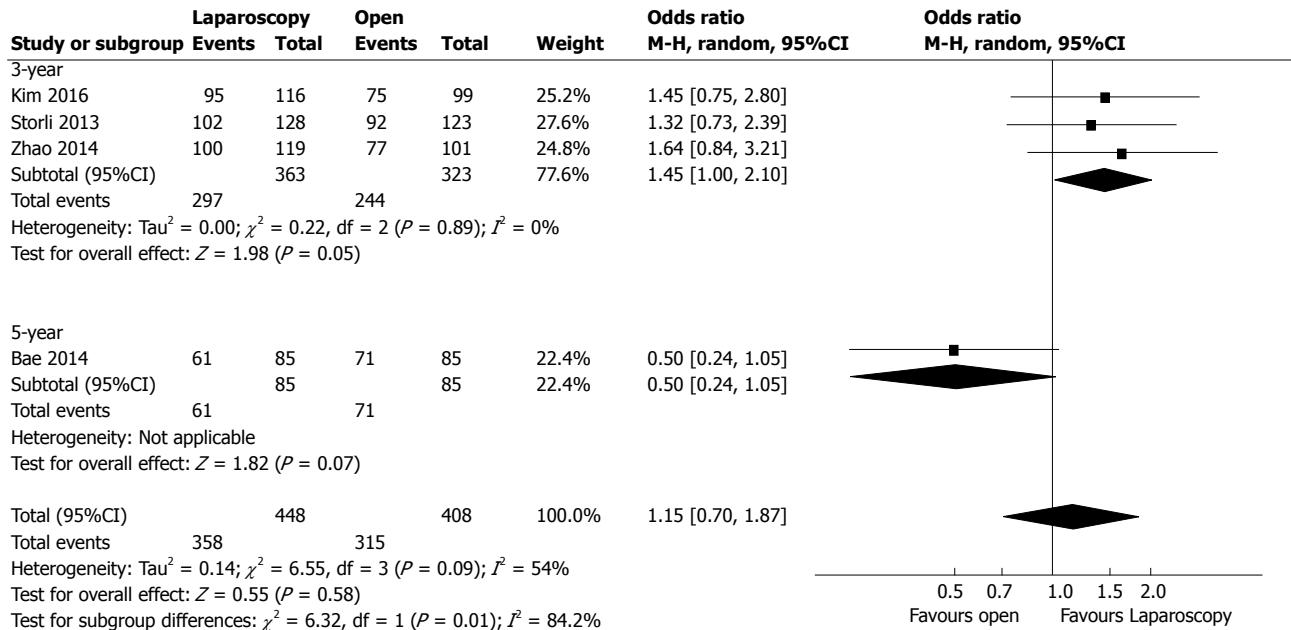


Figure 4 Meta-analysis of studies on disease-free survival of patients undergoing laparoscopic vs open complete mesocolic excision with central vascular ligation excision for colon cancer.

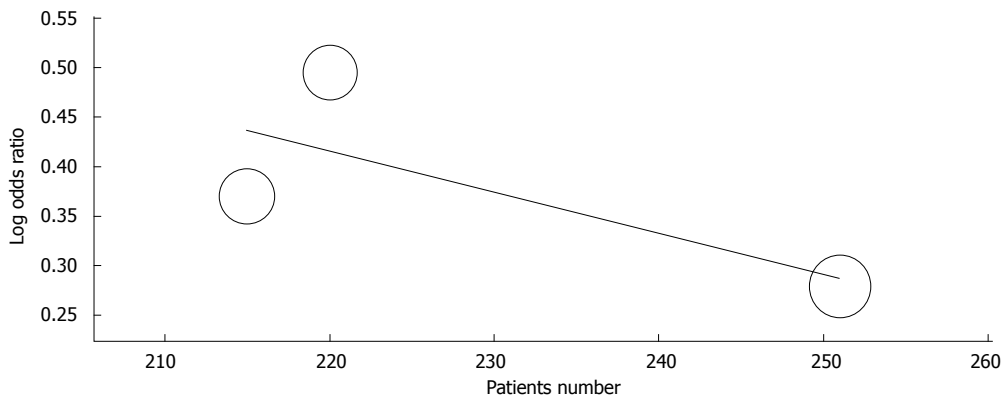


Figure 5 Meta-regression of studies on three-year disease-free survival of patients undergoing laparoscopic vs open complete mesocolic excision with central vascular ligation excision for colon cancer.

= 0.314, Figure 8A; and year of publishing of the study, Figure 8B).

#### Tumor to high tie distance

The mean distance from the tumor to the arterial high tie was reported by two studies that included 132 patients in the L-CME group and 120 patients in the O-CME group; we noted high heterogeneity among the studies ( $I^2 = 92\%$ ). Using the random-effects model, we did not find any statistically significant difference between the L-CME and O-CME groups (MD = 14.26 cm, 95%CI: -4.30 to 32.82,  $P = 0.13$ ) (Figure 9).

#### Surface of the resected mesocolon

The surface of the resected mesocolon was reported by two studies with 132 patients in the L-CME group and 120 patients in the O-CME group. The surface of the resected mesocolon was larger in the L-CME group (MD = 11.75 cm<sup>2</sup>, 95%CI: 9.50 to 13.99,  $P < 0.001$ ) (Figure

10).

#### Complete mesocolic plane excision rate

One study reported the rate of complete mesocolic plane excision, with no statistically significant difference between the laparoscopic and open approach (OR = 0.77, 95%CI: 0.20 to 2.96).

#### Duration of surgery

The duration of surgery was reported by seven studies, with a high heterogeneity between data ( $I^2 = 94\%$ ). The L-CME group had a longer duration of surgery with a mean difference of 26.26 min (95%CI: 5.06 to 47.46,  $P = 0.02$ ). Using Egger's test, no publication bias was found ( $t = 0.71$ ,  $P = 0.26$ ).

#### Incision length

The incision length was reported by two studies, including 586 patients in the L-CME group and 573 patients in the



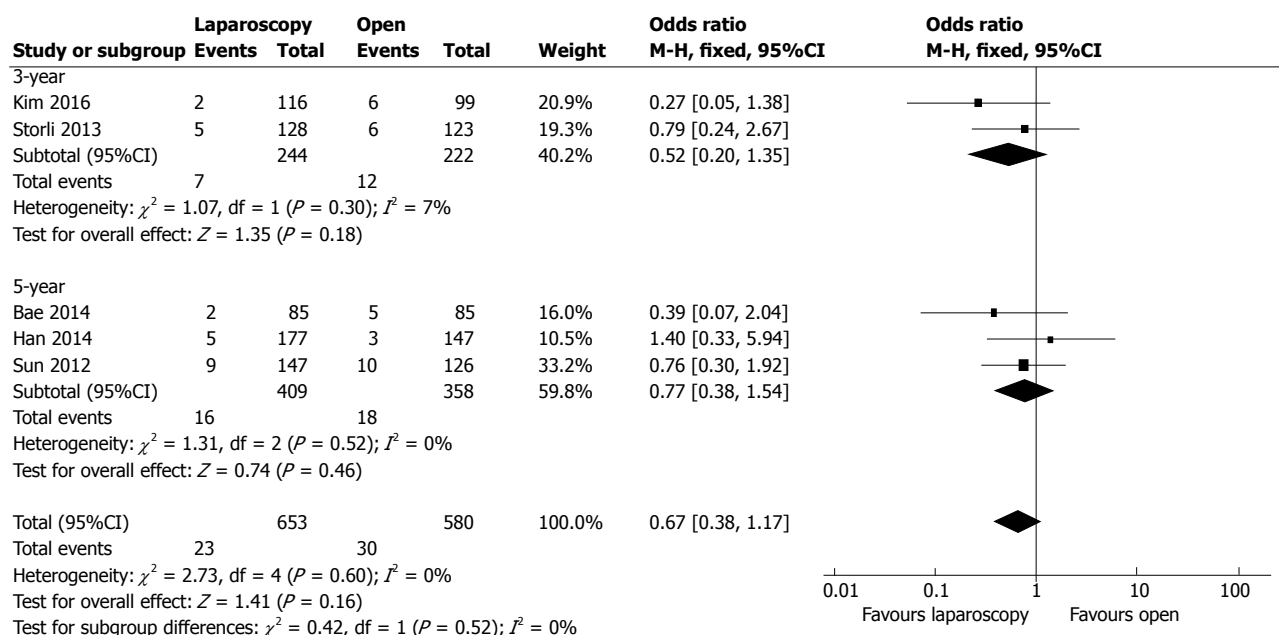


Figure 6 Meta-analysis of studies local recurrence rate of patients undergoing laparoscopic vs open complete mesocolic excision with central vascular ligation excision for colon cancer.

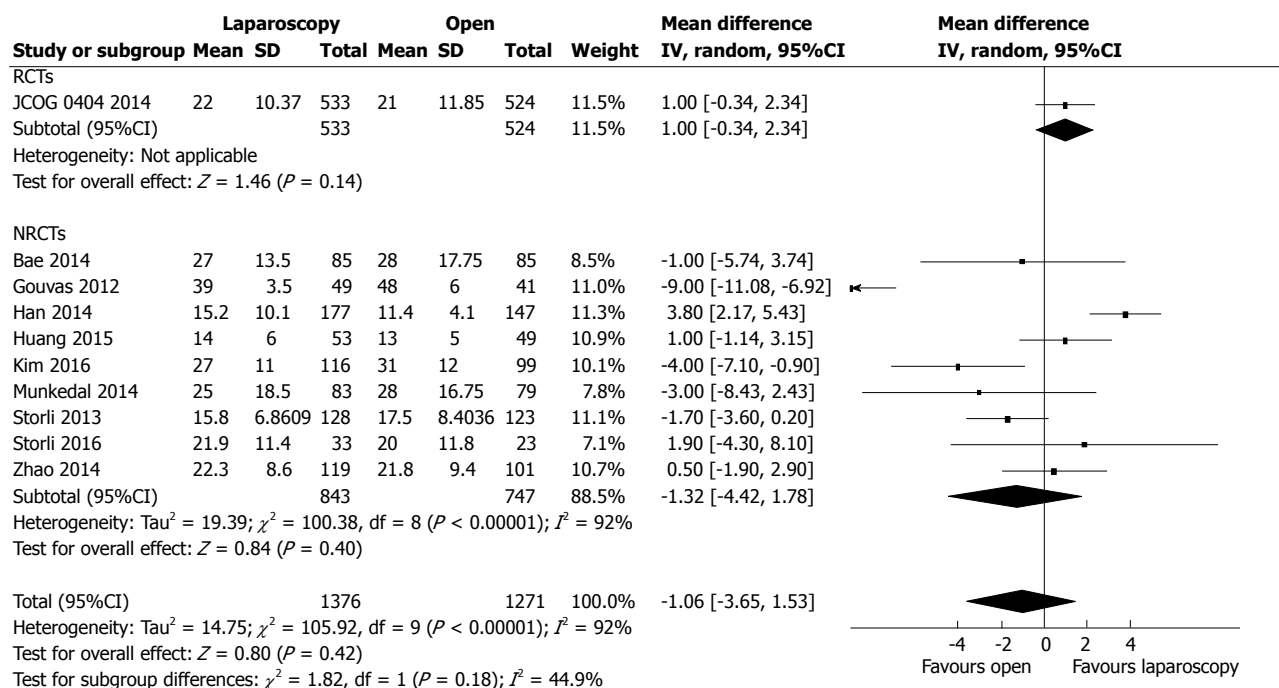


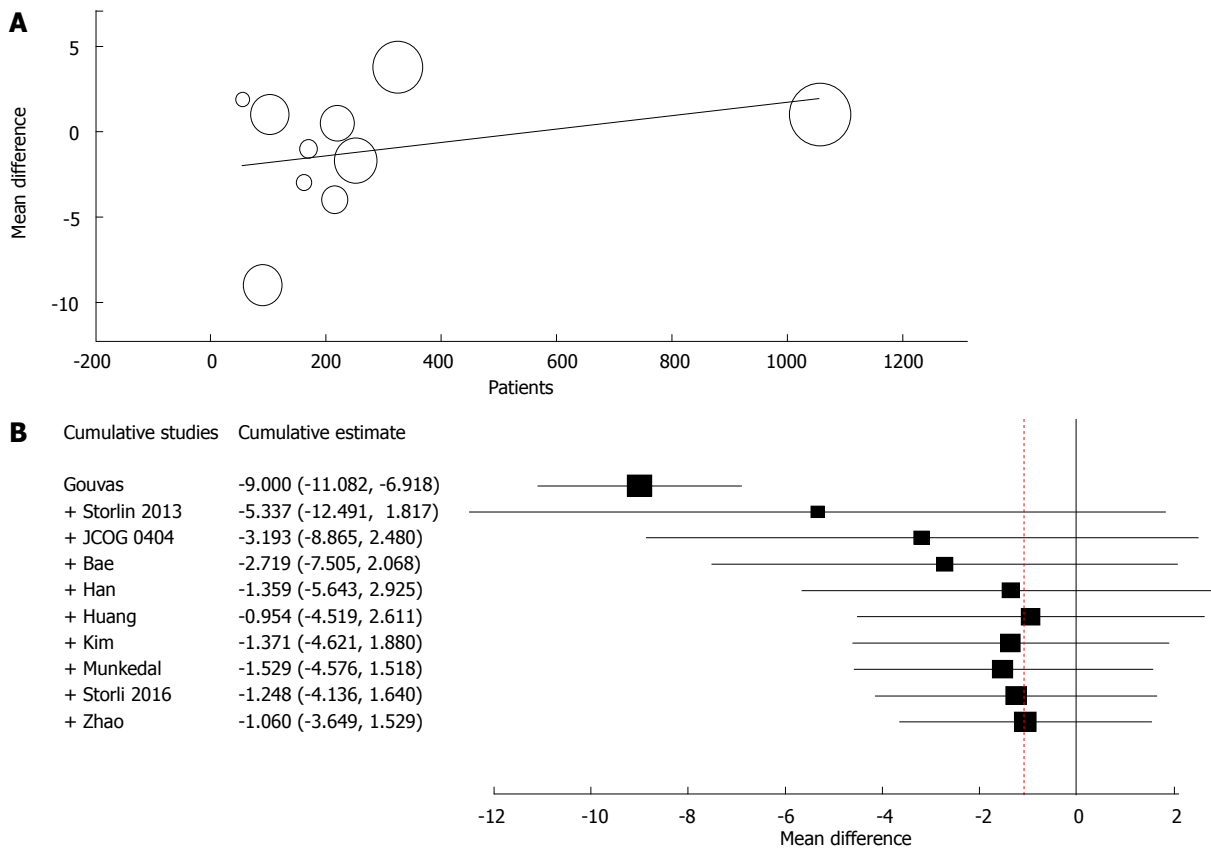
Figure 7 Meta-analysis of studies on lymphnodes retrieved of the specimen of patients undergoing laparoscopic vs open complete mesocolic excision with central vascular ligation excision for colon cancer.

O-CME group. Patients from the laparoscopic group had a shorter incision, with a mean difference of 14.01 cm (95%CI: -14.35 to -13.66,  $P < 0.001$ ).

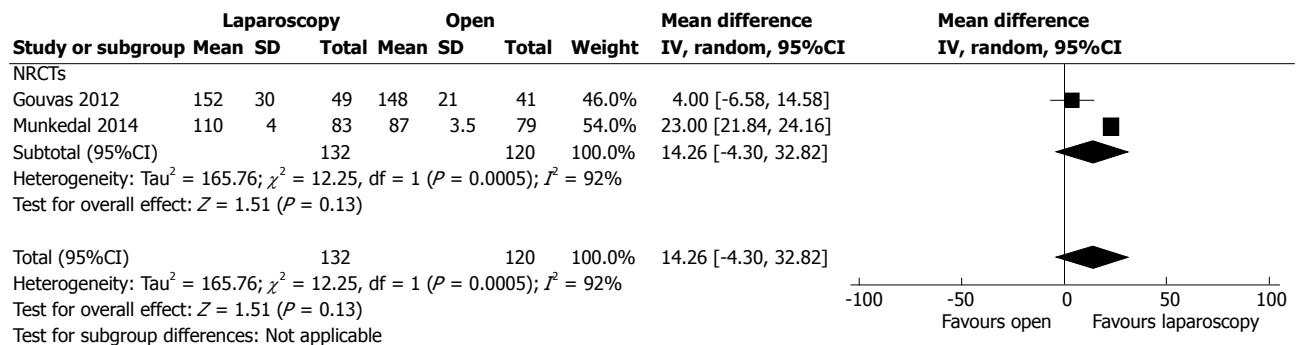
### Blood loss

Intraoperative blood-loss data were presented by five studies, with 964 and 904 patients in the L-CME and O-CME, respectively. Due to the high heterogeneity of the data ( $I^2 = 89\%$ ) we have used the random-effect

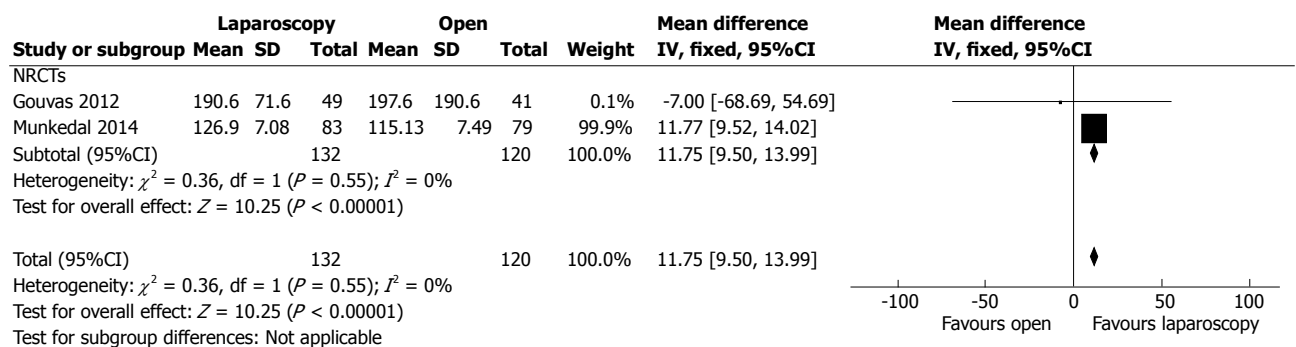
analysis. The laparoscopic approach was associated with statistical significant lower intraoperative bleeding, with a mean difference of 52.11 mL (95%CI: -78.57 to -25.65,  $P < 0.001$ ). Using Egger's test, no publication bias was found ( $t = 0.17$ ,  $P = 0.44$ ). Should be noted the clinical significance of lower intraoperative blood loss associated with laparoscopic approach, which was translated in a lower need for transfusion rate (OR = 0.45, 95%CI: 0.27 to 0.75,  $P = 0.002$ ). Two studies,



**Figure 8 Results of statistical analysis.** A: Meta-regression of studies on lymphnodes retrieved of the specimen according to the number of the included patients in each study; B: Cumulative meta-analysis according to the year of publishing of the article of patients undergoing laparoscopic vs open complete mesocolic excision with central vascular ligation excision for colon cancer.



**Figure 9 Meta-analysis of studies on tumor to arterial high tie (mm) distance of the specimen of patients undergoing laparoscopic vs open complete mesocolic excision with central vascular ligation excision for colon cancer.**



**Figure 10 Meta-analysis of studies on resected mesocolon surface (cm<sup>2</sup>) of the specimen of patients undergoing laparoscopic vs open complete mesocolic excision with central vascular ligation excision for colon cancer.**

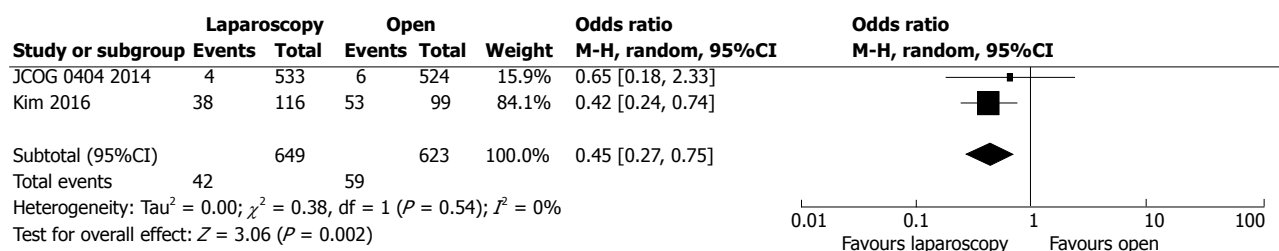


Figure 11 Meta-analysis of studies on transfusion requirements of patients undergoing laparoscopic vs open complete mesocolic excision with central vascular ligation excision for colon cancer.

including a total of 1272 patients, reported the need for blood transfusions, with a low heterogeneity between them ( $I^2 = 0\%$ ) (Figure 11).

### Recovery of gastrointestinal function

The time to first flatus was reported by four studies, including 914 and 857 patients in the L-CME and O-CME, respectively. In the random-effects meta-analysis the laparoscopic approach was associated with a shorter time interval to first flatus, with a mean difference of 0.90 d (95%CI: -1.46 to -0.34,  $P = 0.002$ ,  $I^2 = 97\%$ ).

The time to liquid diet was reported by five studies, with a high heterogeneity between them ( $I^2 = 98\%$ ). The time to liquid diet was shorter for the L-CME patients, with a mean difference of 1.84 d (95%CI: -2.93 to -0.74,  $P = 0.001$ ).

### Short-term morbidity and mortality

Seven studies presented the postoperative overall morbidity, and these studies included 1116 patients in the L-CME group and 1028 patients in the O-CME group. There was low statistical heterogeneity among the studies ( $I^2 = 0\%$ ). The L-CME procedure was associated with a lower postoperative morbidity (OR = 0.57, 95%CI: 0.46 to 0.71,  $P < 0.001$ ) (Figure 12).

Wound complications, reported by eight studies, were significantly less frequent in the L-CME group (OR = 0.43, 95%CI: 0.30 to 0.61,  $P < 0.001$ ). There was no statistical heterogeneity among the studies ( $I^2 = 0\%$ ).

There was no difference between the two groups regarding postoperative bleeding (OR = 1.20, 95%CI: 0.46 to 3.12,  $P = 0.71$ ), anastomotic leakage (OR = 0.82, 95%CI: 0.054 to 1.25,  $P = 0.36$ ), need for reoperation (OR = 0.59, 95%CI: 0.28 to 1.23,  $P = 0.16$ ), and pulmonary complications (OR = 0.61, 95%CI: 0.20 to 1.84,  $P = 0.38$ ).

The 30-d mortality was reported by six studies with 1158 patients in the L-CME group and 1079 patients in the O-CME group. There was low heterogeneity among the studies ( $I^2 = 0\%$ ). In the fixed-effects meta-analysis we observed no statistically significant difference between the L-CME and O-CME groups (OR = 0.42, 95%CI: 0.16 to 1.12).

Nine studies, with 1340 and 1233 patients in the L-CME and O-CME, respectively reported the hospital stay. There was a high heterogeneity between the studies ( $I^2 = 91\%$ ). In the random-effects meta-

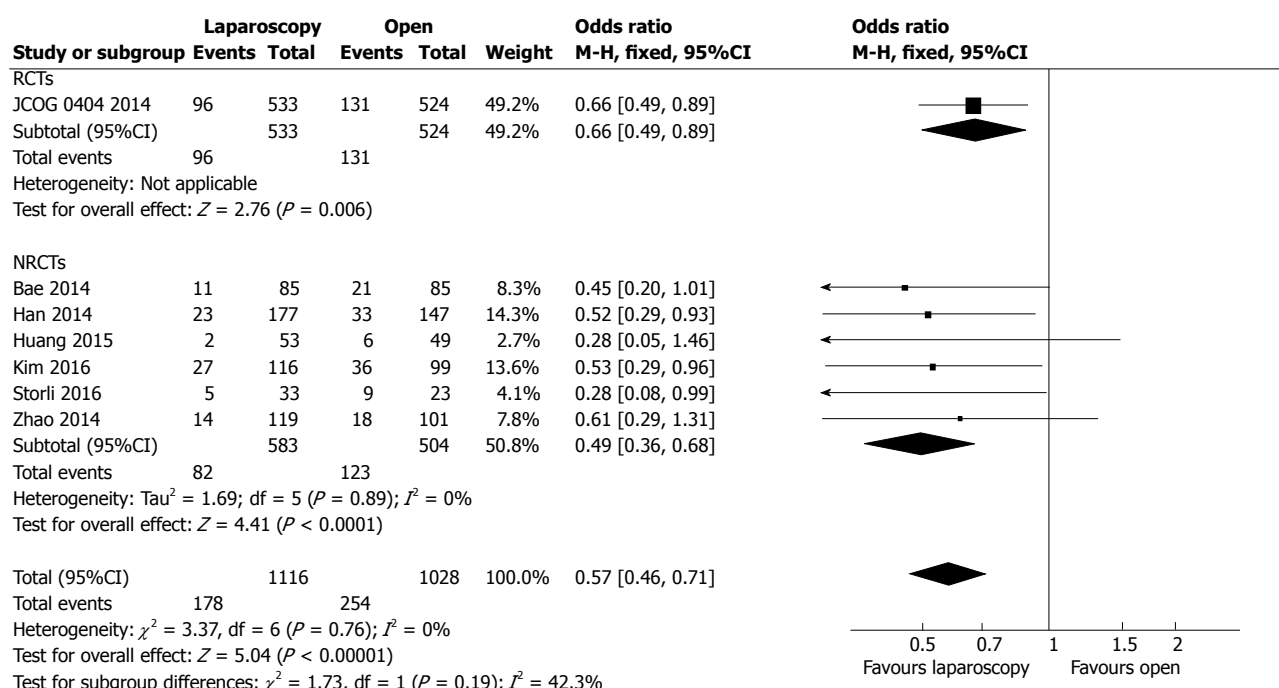
analysis we found a statistical significant lower hospital stay for laparoscopic group, with a mean difference of 4.07 d (95%CI: -5.87 to -2.28,  $P < 0.001$ ).

### Risk of bias across studies

We conducted sensitivity analysis to assess statistical heterogeneity based on excluding specific studies with a high risk of bias (Figure 13). There were no relevant changes in the overall effects of the quantitative synthesis. Our analysis of the funnel plots reveals no significant asymmetries for the studied outcomes (Figures 14 and 15).

## DISCUSSION

Our meta-analysis revealed that laparoscopic CME with CVL for colon cancer offers the same quality of the resected specimen as the open approach, being superior in all perioperative results and at least non-inferior in long-term oncological outcomes. Although not addressed the complete mesocolic excision or D3 lymphadenectomy technique, the equivalence of laparoscopy in terms of resected lymphnodes was showed in four large, multi-center, studies-Clinical Outcomes of Surgical Therapy (COST), Conventional vs Laparoscopic-Assisted Surgery in Colorectal Cancer (CLASSIC), Colon Cancer Laparoscopic or Open Resection I (COLOR I), and the Australasian Randomized Clinic Study Comparing Laparoscopic and Conventional Open Surgical Treatments for Colon Cancer (ALCCaS); the mean number of resected lymph nodes was 10.13 in the laparoscopic group and 10.14 in the open group<sup>[40-42]</sup>. An RCT from Taiwan comparing open with laparoscopic left-sided D2 resections for stage II or III colon cancer reported  $16 \pm 3$  dissected lymph nodes in its laparoscopic group and  $16 \pm 6$  in its open group<sup>[33]</sup>. The long-term oncological outcomes between the L-CME and O-CME groups were also comparable; there were no differences regarding the local and distant recurrence rate, the three- and five-year overall rates and the disease-free survival rates. In our study, the three-year overall and disease-free survival were superior in the laparoscopic group; however, should be noted the extensive experience in laparoscopy of the reporting centers. In Barcelona study, the laparoscopic approach was associated with a slight increase in survival rate, a faster postoperative recovery, and a shorter in-hospital stay duration<sup>[38]</sup>. In the COLOR



**Figure 12** Meta-analysis of studies on postoperative overall morbidity of patients undergoing laparoscopic vs open complete mesocolic excision with central vascular ligation excision for colon cancer.

Leave-one-out summary  
Continuous random-effects model  
Metric: Mean difference  
Model results

Studies	Estimate	Lower bound	Upper bound	Std. error	P-value
Overall	-1.749	-4.442	0.944	1.374	0.203
Han 2014	-2.462	-5.120	0.196	1.356	0.069
Kim 2016	-1.502	-4.379	1.375	1.468	0.306
Storlin 2013	-1.792	-4.871	1.287	1.571	0.254
Zhao 2014	-2.043	-5.046	0.960	1.532	0.182
JCOG 0404 2014	-2.148	-5.368	1.073	1.643	0.191
Bae 2014	-1.828	-4.694	1.037	1.462	0.211
Gouvas 2012	-0.524	-2.433	1.385	0.974	0.590
Huang 2015	-2.111	-5.139	0.916	1.545	0.172
Munkedal 2014	-1.646	-4.483	1.191	1.447	0.255
Storli 2016	-1.253	-4.011	1.506	1.408	0.373

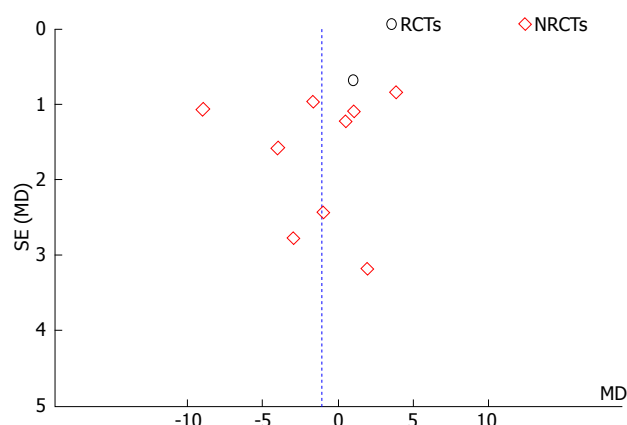
**Figure 13** Leave-one-out meta-analysis for the endpoint number of retrieved lymphnodes of patients undergoing laparoscopic vs open complete mesocolic excision with central vascular ligation excision for colon cancer.

study, 1248 patients were randomized for open or laparoscopic colon resection<sup>[46]</sup>. After a median follow-up of 53 mo, the combined three-year, disease-free survival rate was 74.2% in the laparoscopic group and 76.2% in the open group ( $P = 0.70$ ). The combined three-year overall survival rate was 81.8% in the laparoscopic group and 84.2% in the open group ( $P = 0.45$ ). The authors concluded that a difference in the three-year, disease-free survival rate could not be ruled out due to limitations of the study<sup>[46]</sup>. In the CLASSIC trial, 794 patients with colorectal cancer were randomized for open or laparoscopic resection<sup>[47]</sup>. An analysis of the subgroup of patients with colon cancer, 140 in the open group and 273 in the laparoscopic group, did not reveal any differences in terms of three-year overall survival rates ( $P = 0.51$ ). After a median follow-up of 62.9 mo, there

were no statistically significant differences in overall survival and disease-free survival rates<sup>[48]</sup>. In the COST study, 872 patients were randomized to receive an open or laparoscopic colectomy<sup>[49]</sup>. The 3- and 5-year follow-ups revealed no differences regarding recurrence rate and overall survival rates<sup>[49,50]</sup>.

We found a longer duration of surgery in the laparoscopic group. However, all the perioperative outcomes, such as blood loss, need for transfusion, incision length, wound complications, and thirty-day overall morbidity were less frequent in the laparoscopic group. In the COST, CLASSIC, COLOR I, and ALCCaS trials, the mean duration of surgery was 145-180 compared to 95-135 min, the hospital stay was 5-10 vs 6-11 d, the 30-d morbidity was 21%-38% vs 20%-45%, and the 30-d mortality was 0.5%-4.0% vs 0.7%-5.0% in the



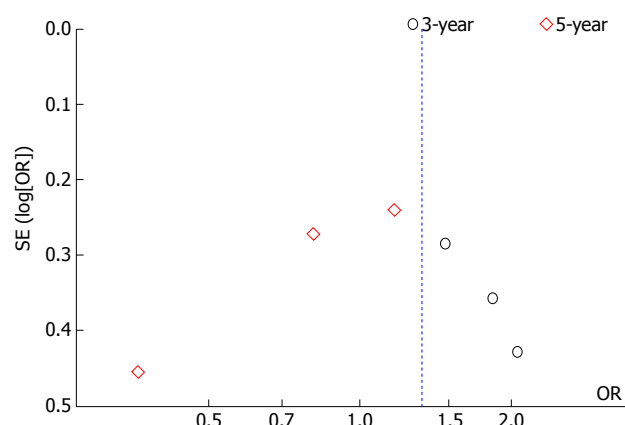


**Figure 14** Begg's funnel plot for the endpoint number of retrieved lymphnodes of patients undergoing laparoscopic vs open complete mesocolic excision with central vascular ligation excision for colon cancer. RCTs: Randomized control trial; NRCTs: Non-randomized clinical studies.

laparoscopic and open groups, respectively<sup>[40-42,49]</sup>. Liang *et al*<sup>[33]</sup> found a longer operative time for left-sided resections ( $224.4 \pm 44.8$  min vs  $184.0 \pm 30.6$  min), less blood loss ( $54 \pm 12$  mL vs  $240 \pm 34$  mL), a shorter wound incision ( $10.6 \pm 1.6$  cm vs  $18 \pm 3.1$  cm) for the laparoscopic approach, but there were no statistically significant differences regarding total postoperative complications (20 vs 29,  $P = 0.15$ ).

Our meta-analysis showed that patients from the laparoscopic group had a shorter hospital stay and a shorter recovery time to regain gastrointestinal function. This result is consistent with the current evidence that supported earlier recovery of bowel functioning and oral diet with an in-hospital stay duration 1.7 d shorter in the laparoscopic group<sup>[51]</sup>. The studies included in the current meta-analysis did not evaluate how surgery affected immune functioning. According to Liang *et al*<sup>[33]</sup>, the postoperative proinflammatory response, evaluated by C-reactive protein and the erythrocyte sedimentation rate and postoperative immunosuppression and assessed by alteration of lymphocyte counts and the  $CD4^+/CD8^+$  ratio, was significantly less in the laparoscopic group ( $P < 0.001$ ).

An important concern regarding laparoscopic colon surgery is the reproducibility of results given the nature of multicenter, specialized centers and the heterogeneity of general surgeons. All surgical procedures from the studies included in this meta-analysis were performed by highly experienced or accredited surgeons. An analysis of the short-term outcomes of colon and rectal laparoscopic resections in Sydney South West Area Health Service revealed a lower morbidity (28.8% vs 54.4%,  $P < 0.001$ ), fewer transfusions (0.4 units vs 0.7 units,  $P = 0.0028$ ), a longer operative time (24.1 min,  $P < 0.0001$ ) and a shorter length of stay (7 vs 10 d,  $P = 0.0011$ ) for laparoscopic procedures<sup>[52]</sup>. Dobbins *et al*<sup>[53]</sup> published the results of laparoscopic resections for colon and rectal cancer from all of the public and private hospitals in New South Wales, Australia. The laparoscopic colon resections were associated with a



**Figure 15** Begg's funnel plot for the endpoint overall survival of patients undergoing laparoscopic vs open complete mesocolic excision with central vascular ligation excision for colon cancer.

reduced rate of extended stay (OR = 0.60, 95%CI: 0.49-0.72) and 28-d readmissions (OR = 0.86, 95%CI: 0.74-0.99). Survival benefits for laparoscopy, regarding cancer-specific survival, were observed in higher-caseload hospitals but not in lower-caseload hospitals<sup>[53]</sup>.

The current meta-analysis has as a main limitation the clinical heterogeneity of the included studies, and caution should be exercised when interpreting its results. This meta-analysis involves several types of study designs, including retrospective, prospective, and RCT. There is an increased heterogeneity of the tumor localization on the colon, with the transverse colon cancers being excluded from the analysis in two studies, while the others included them into the right/extended right hemicolectomy group. Excepting the one randomized controlled trial, the experience in minimally invasive surgery of the surgeons from the laparoscopic group is not quantified, although all procedures were performed or supervised by trained colorectal surgeons. However, using random-effects meta-analysis, with subgroup analysis and meta-regression, we limited the variance of the included outcomes.

In summary, the current data suggest that the laparoscopic approach offers the same quality of resected specimens as the open approach in CME with CVL for colon cancer while maintaining all of the short-term benefits of a minimally invasive approach. Although a specimen-oriented surgical dissection in colon cancer *via* a laparoscopic approach is challenging, the magnification and predisposition to details of a minimally invasive technique are associated with a lower postoperative morbidity.

## COMMENTS

### Background

Complete mesocolic excision with central vascular ligation represents an extension to the colonic cancer of the already standardized resection for rectal cancer. It adheres to the same guiding principle that sharp surgical dissection, following embryological planes, with central vascular ligation, should improve oncological outcomes. The technical details of this new concept were published in 2007.

## Research frontiers

A high-level evidence that laparoscopic approach offers the same quality of the resected specimen as open surgery for complete mesocolic excision with central vascular ligation for colon cancer is lacking.

## Innovations and breakthroughs

Current evidence is consistent with a faster postoperative recovery for laparoscopic colectomies compared with the open approach; the former is not associated with any negative impact regarding local recurrence and survival rates. This study reveals that laparoscopy offers the same quality of the resected specimen as the open approach in complete mesocolic excision with central vascular ligation for colon cancer. The laparoscopic complete mesocolic excision with central vascular ligation is superior in all perioperative results and at least non-inferior in long-term oncological outcomes.

## Applications

Due to all advantages of laparoscopy, the teaching and mentoring of minimally invasive techniques for colon resections should be accentuated, in order to increase the proportion of laparoscopic over open procedures.

## Terminology

During complete mesocolic excision with central vascular ligation for right-sided tumors, the ileocolic and right colic vessels should be ligated at their origin from the superior mesenteric artery, medial (patient left-hand side) to the superior mesenteric vein. Transverse colon tumors require transection of the middle colic artery at its origin. Left-sided tumors require transection of the inferior mesenteric artery at its origin from the aorta.

## Peer-review

This is an interesting meta-analysis and review of a highly debatable topic in surgery, the consensus about laparoscopic vs open surgery in high ligation.

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## Cystic metastasis from a mucinous adenocarcinoma of duodenum mimicking type II choledochal cyst: A case report

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### Abstract

A 51-year-old male patient was referred to our hospital because of an incidentally detected cystic mass near the common bile duct (CBD). Imaging studies demonstrated a cystic mass that was suspected to communicate with the CBD. Gastroscopy showed irregular mucosal thickening with hyperemic change in the second portion of the duodenum. A type II choledochal cyst combined with duodenal malignancy was suspected. The patient underwent surgical resection and the histological diagnosis was mucinous adenocarcinoma of the duodenum with cystic metastasis. Although its incidence is extremely rare, care should be taken to check for other sites of malignancy when a pericholedochal cystic mass is detected.

**Key words:** Duodenal cancer; Choledochal cyst; Cystic metastasis; Mucinous adenocarcinoma

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**Core tip:** Mucinous adenocarcinoma is very rare in the

duodenum, and a cystic metastasis from mucinous adenocarcinoma of duodenum has never been reported. This is the first report of primary mucinous adenocarcinoma of duodenum with cystic metastasis. Although rare, careful evaluation with a high suspicion for other sites of malignancy is needed when a pericholedochal cystic mass is detected.

Kim YN, Song JS. Cystic metastasis from a mucinous adenocarcinoma of duodenum mimicking type II choledochal cyst: A case report. *World J Gastrointest Oncol* 2017; 9(12): 492-496 Available from: URL: <http://www.wjgnet.com/1948-5204/full/v9/i12/492.htm> DOI: <http://dx.doi.org/10.4251/wjgo.v9.i12.492>

## INTRODUCTION

The small intestine is the longest gastrointestinal (GI) tract organ, reaching six to seven meters in average length. Despite its length and the large mucosal surface area of the small intestine, only 5% of malignant neoplasms of the GI tract occur in the small intestine<sup>[1]</sup>. Among them, primary adenocarcinoma of the duodenum represents approximately 25%-52% of malignant neoplasms of the small intestine and 4.6% were mucinous adenocarcinoma<sup>[2]</sup>. Choledochal cysts are rare, congenital dilatation of the extrahepatic or intrahepatic biliary tree. Among them, type II choledochal cyst, a diverticulum of the common bile duct (CBD), is the rarest type. Here, we present a case of mucinous adenocarcinoma of the duodenum with cystic metastasis, which is extremely rare and was initially misinterpreted as a type II choledochal cyst.

## CASE REPORT

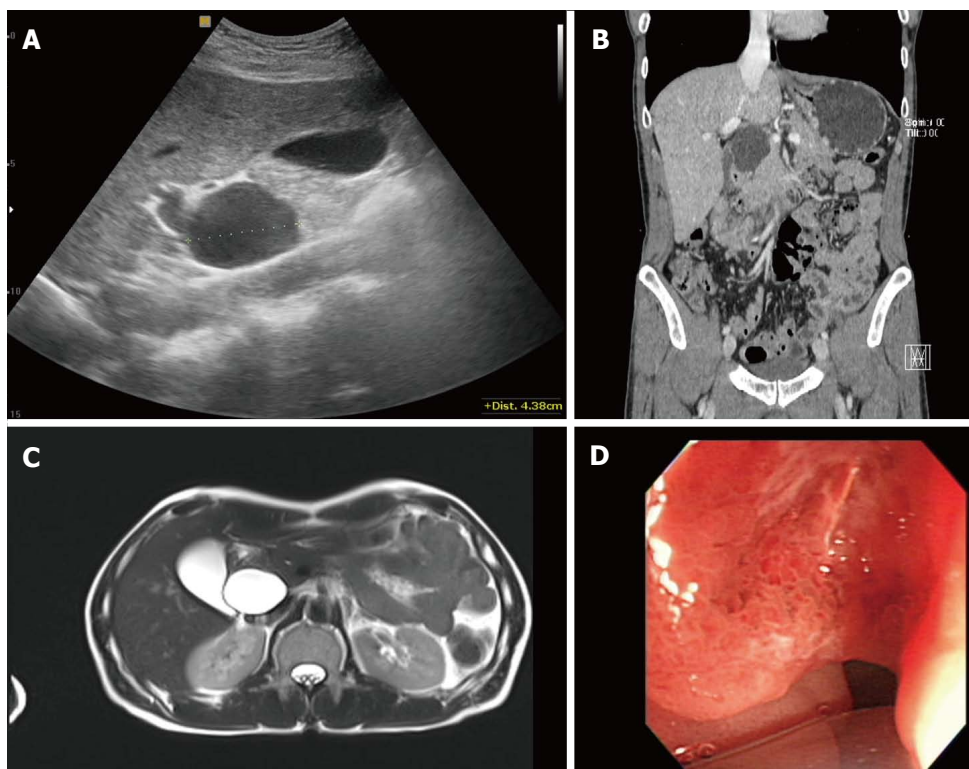
A 51-year-old male patient visited a local hospital because of dyspepsia and epigastric pain. Ultrasonography revealed a 4.5 cm sized cystic mass near the CBD and pancreatic head (Figure 1A). He was transferred to our hospital for further evaluation of the cystic mass. His medical history and laboratory findings were unremarkable. Tumor markers such as alpha-fetoprotein, carcinoembryonic antigen, and carbohydrate antigen 19-9 were within normal limits. Contrast-enhanced abdominal computed tomography (CT) showed a homogeneous low-density cystic mass with thin, smooth walls next to the CBD, and there were suspicions of a communication between the two structures (Figure 1B). Under the impression that the lesion was a type II choledochal cyst, which is a discrete diverticulum of the extrahepatic bile duct, magnetic resonance (MR) imaging and endoscopic ultrasound (EUS) were done. The cystic mass showed low signal intensity on the T1-weighted MR image and high SI on the T2-weighted MR image with nearly imperceptible walls and there was no evidence of an enhancing solid portion in the cyst (Figure 1C). EUS also revealed a 4.5 cm sized cystic mass which

seemed to be connected with the CBD, and gastroscopy showed irregular mucosal thickening with hyperemic change in the second portion of the duodenum (Figure 1D). Based on these findings, the patient underwent Whipple's operation under the impression the lesion was a type II choledochal cyst with extrinsic compression of the duodenum, and the possibility of combined duodenal malignancy due to the mucosal lesion in the duodenum. An examination of the resected specimen revealed a duodenal cancer in the second portion of the duodenum 2.5 cm proximal to the ampulla of Vater, and the cystic mass did not show communication with the CBD (Figure 2A and B). The histological diagnosis was mucinous adenocarcinoma of the duodenum with cystic metastasis and subpyloric lymph node metastasis (Figure 2C and D). The postoperative course of the patient was uneventful. The patient was disease-free 12 mo after the initial diagnosis. However, the patient died 18 mo after the recurrence.

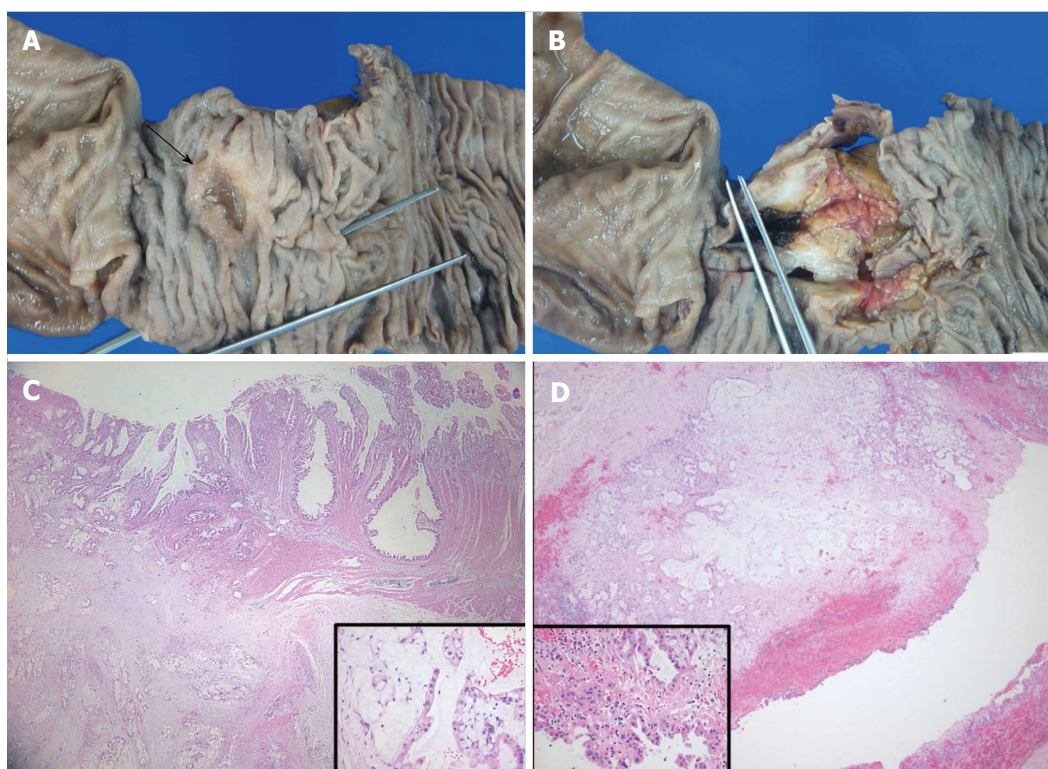
## DISCUSSION

We initially suspected a type II choledochal cyst combined with duodenal malignancy due to the mucosal lesion in the duodenum and the gastroscopic biopsy revealed a moderate degree of dysplasia. All of the imaging studies showed a well-margined, homogeneously thin-walled cyst adjacent to the CBD which is regarded as a diverticulum of the extrahepatic bile duct, and the duodenal lesion was invisible. Since surgical resection is generally considered for the treatment of choledochal cysts, the patient underwent Whipple's operation, and the patient was confirmed to have mucinous adenocarcinoma of the duodenum with cystic metastasis and subpyloric lymph node metastasis.

Mucinous adenocarcinoma is one of the histologic subtypes of carcinoma and is very rare in the duodenum. A recent study from South Korea by Chang *et al.*<sup>[3]</sup> revealed that 54.8% of small intestinal carcinomas were located in the duodenum and 4.6% were mucinous adenocarcinoma. Due to its rarity, to the best of our knowledge, this is the first case report of primary mucinous adenocarcinoma of duodenum with cystic metastasis. Although there are several studies in the literature describing the imaging findings of small bowel carcinoma including duodenal carcinoma<sup>[3-6]</sup>, there are no previous reports reporting the imaging findings of mucinous adenocarcinoma of the duodenum. According to previous studies, duodenal cancer typically appears as an irregular thickening of the duodenal wall with regional lymph node enlargement on CT. Since the duodenal lesion of our patient was flat and small (2.0 cm), the primary lesion in the second portion of the duodenum and metastatic lymph node in the subpyloric area were missed on initial imaging studies including CT and magnetic resonance imaging (MRI). In a retrospective review of CT and MRI, the metastatic lymph node in the subpyloric area was identified. However, the primary lesion was invisible.



**Figure 1 Evaluation of clinical findings.** A: Ultrasonography of upper abdomen shows a 4.5 cm sized anechoic cystic mass adjacent to the head of the pancreas and common bile duct; B: Coronal multiplanar reformatted image of contrast-enhanced abdominal computed tomograph shows a homogeneous low-density cystic mass with thin, smooth walls abutting the common bile duct, with possible communication between the two structures; C: Axial T2-weighted magnetic resonance image demonstrates the cystic mass as a homogeneously high signal intensity lesion with thin walls, and there was no evidence of mural nodularity in the cystic mass; D: Endoscopic image of the duodenum shows irregular mucosal thickening with hyperemic change in the second portion of the duodenum.



**Figure 2 Gross specimen and pathological findings.** A: The ulcerofungating mass measuring 2.4 cm × 2.0 cm (arrow), 2.5 cm distant from the ampulla of Vater, is observed in the duodenum; B: The cut surface reveals a grey-white mass that abuts the head of the pancreas; C: Histologically, the duodenal mass proved to be an infiltrative adenocarcinoma with subserosal invasion, note the abundant extracellular mucin with floating neoplastic epithelium (Inset); D: Photomicrograph of the cystic mass shows invasive tumor cells in the lining of the cyst and the surrounding soft tissue which is a definite malignant feature (Inset).



Choledochal cysts are a rare congenital anomaly of the intrahepatic or extrahepatic biliary tree and is known to occur in 1 in 100000 to 1 in 150000 live births<sup>[7]</sup>. Choledochal cysts occur more frequently in Asian populations, with more than two-thirds of all reported cases originating in Asia. Traditionally, choledochal cysts presented predominantly in young age with the triad of abdominal pain, palpable right upper quadrant mass, and intermittent jaundice. Nonetheless, recent analyses show increasing numbers of adults presenting with choledochal cysts<sup>[8]</sup>. According to Todani's classification, choledochal cyst can be divided into 5 types: Type I, a cystic or fusiform dilatation of the CBD, which is subdivided into saccular, segmental, and diffuse types; type II, a diverticulum arising from the CBD; type III, choledochoceles or a bulbous dilation of the terminal CBD within the ampulla of Vater; type IV, multiple intrahepatic and extrahepatic cysts; and type V, intrahepatic bile duct cysts or Caroli disease<sup>[9]</sup>. Among them, type II cysts are the most rare form of choledochal cysts, usually making up less than 2%-5% of cases<sup>[10]</sup>. They usually manifest as a pericholedochal cystic mass, of various shapes, some being gallbladder-like, and others being diverticulum-like. Choledochal cysts have been associated with an approximately 20 to 50-fold increase in biliary malignancies when compared with the general population<sup>[11]</sup>. The risk of malignancy in type II choledochal cysts has been estimated to range from 7%-9%, which is a slightly lower than the risk for other types of choledochal cysts (14.3% in the third decade)<sup>[12]</sup>. Current recommendations for management of choledochal cysts is surgical resection regardless of cyst type, including hepaticojejunostomy, Whipple procedure, partial liver resection, or liver transplantation<sup>[13]</sup>.

In conclusion, we have described the first case of a mucinous adenocarcinoma of the duodenum with cystic metastasis. Even though the incidence of this particular type of cancer is extremely low, careful evaluation with a high suspicion for other sites of malignancy must be done when a pericholedochal cystic mass is detected incidentally.

## ARTICLE HIGHLIGHTS

### Case characteristics

A 51-year-old male patient was admitted because of incidentally detected cystic mass near the common bile duct (CBD).

### Clinical diagnosis

About 4.5 cm sized cystic mass near the CBD, with irregular mucosal thickening in the second portion of the duodenum.

### Differential diagnosis

Type II choledochal cyst combined with duodenal malignancy.

### Laboratory diagnosis

Laboratory findings were unremarkable, including tumor markers such as alpha-fetoprotein, carcinoembryonic antigen, and carbohydrate antigen 19-9.

### Imaging diagnosis

Findings from gastroscopy, ultrasonography, computed tomograph, and magnetic resonance imaging led to a diagnosis of type II choledochal cyst with extrinsic compression of the duodenum, and the possibility of combined duodenal malignancy.

### Pathological diagnosis

Mucinous adenocarcinoma of the duodenum with cystic metastasis and subpyloric lymph node metastasis.

### Treatment

Whipple's operation.

### Related reports

Mucinous adenocarcinoma of the duodenum is very rare, and this is the first case report of primary mucinous adenocarcinoma of duodenum with cystic metastasis.

### Experiences and lessons

Although rare, careful evaluation with a high suspicion for other sites of malignancy is needed when a pericholedochal cystic mass is detected.

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## Extrapancreatic solid pseudopapillary neoplasm followed by multiple metastases: Case report

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### Abstract

Solid pseudopapillary neoplasm (SPN), also known as Gruber-Frantz tumor, is a rare form of neoplasm that almost exclusively occurs in the pancreas and in young females. While the potential of malignancy is low for SPN, these tumors can mimic other diseases and require a meticulous investigation and a standard treatment by total surgical resection. We present an unusual case of SPN arising in the mesentery of a 40-year-old man with subsequent multiple metastases. Histopathological examination showed similar properties of the mesenteric neoplasm to those of SPN in pancreas. Although the mass was surgically removed, the patient died of recurrent disease 4 years after the initial presentation. We speculate that SPN originates from pancreatic progenitor cells. Further histopathological analyses are required for the prediction of SPN recurrence after resection.

**Key words:** Solid pseudopapillary neoplasm; Mesentery; Metastasis

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**Core tip:** Solid pseudopapillary neoplasm (SPN) has been recognized by World Health Organization since 2010, and classified as a low malignant potential neoplasm. Such neoplasm is characterized by the presence of a mutation in the gene that encodes  $\beta$ -catenin.  $\beta$ -catenin is an important factor in the Wnt signaling pathway ( $\beta$ -catenin-dependent Wnt signaling). The identification of extrapancreatic SPN, especially in the mesentery, indicates a possible endoderm link between pancreatic progenitor cells and SPN cells.

Wu H, Huang YF, Liu XH, Xu MH. Extrapancreatic solid pseudopapillary neoplasm followed by multiple metastases: Case

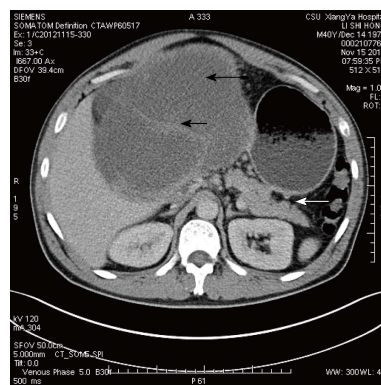
report. *World J Gastrointest Oncol* 2017; 9(12): 497-501 Available from: URL: <http://www.wjgnet.com/1948-5204/full/v9/i12/497.htm> DOI: <http://dx.doi.org/10.4251/wjgo.v9.i12.497>

## INTRODUCTION

Solid pseudopapillary neoplasm (SPN) is a rare and indolent type of neoplasm that occurs in pancreas; SPN forms 0.3% to 2.7% of all pancreatic exocrine tumors. A large body of SPN indices are found in young female patients, and well-circumscribed. A margin negative surgical resection shows curative result in majority of cases<sup>[1-3]</sup>; recurrence after surgical resection is reported in 2% to 10% of patients<sup>[4,5]</sup>. Patients with unresectable SPN may have a long-term survival (5 years), and require complex chemo- and radio-therapy treatments; the efficacy of adjuvant therapies in the SPN treatment remains largely unknown and a clinical challenge. Thus, it is important to differentiate the risk of recurrence in SPN patients. An extrapancreatic development of SPN is a rare incident; only 16 cases of extrapancreatic SPN have been reported so far worldwide (Table 1). In the present article, we report a patient, in whom SPN was found in the mesentery; no invasion or attachments to adjacent organs was observed. To the best of our knowledge, this article is the first to report a SPN case in the mesentery.

## CASE REPORT

A 40-year-old Chinese male came to hospital on November 15, 2012. His main complaint was abdominal distention that lasted over 6 mo. His physical examination revealed a 30 cm soft mass in the abdomen. An abdominal computed tomography (CT) scan exhibited solid and mixed cystic lesions, measuring > 28 cm diameter (Figure 1). Patient's blood test results were unremarkable. On November 22, 2012, the patient underwent an exploratory laparotomy, and the tumor protruding from the mesentery was completely excised. At that time, no invasion or attachments to adjacent organs was observed. In addition, the postoperative course was uneventful. The resected specimen of the mesenteric tumor was 25 cm × 15 cm × 28 cm, and showed a multilobulated structure with rich microvasculature. Microscopic characterization of the tumor showed that the tumor formation was a mix of solid and pseudopapillary areas. There was no evidence of pancreatic tissue in the analyzed sample. Further, the specimen was positive for alpha-1-antitrypsin, vimentin, CD56 and  $\beta$ -catenin immunostaining, whereas negative for S-100, neuron-specific enolase, E-cadherin, calretinin, progesterone receptor, chromogranin, and pancytokeration (Figure 2). Such results led to the diagnosis of SPN in the mesentery. Following 3.5 years, the patient continued to complain about abdominal distention and occasional polypnea. An abdominal CT scan exhibited multiple tumors in peritoneum, greater omentum, and colonic wall (Figure 3). Meanwhile, cells in the pleural effusion were



**Figure 1** An abdominal computed tomography scan exhibited solid and mixed cystic lesions, measuring > 28 cm diameter (black arrow). The tumor was apart from the pancreas (white arrow).

found positive for alpha-1-antitrypsin, vimentin, CD56 and  $\beta$ -catenin. It was clear that the patient was suffering from recurrence of the disease. Before the surgical operation to clean the recurrent tumors, the patient received the treatment of 60 mg cisplatin by hyperthermic intraperitoneal chemotherapy (HIPEC). Unfortunately, there was no response to the treatment, and the patient was transferred to the palliative care unit. Soon after the patient's physical conditions worsened, we lost the patient on November 2016, 4 years after the initial surgery.

## DISCUSSION

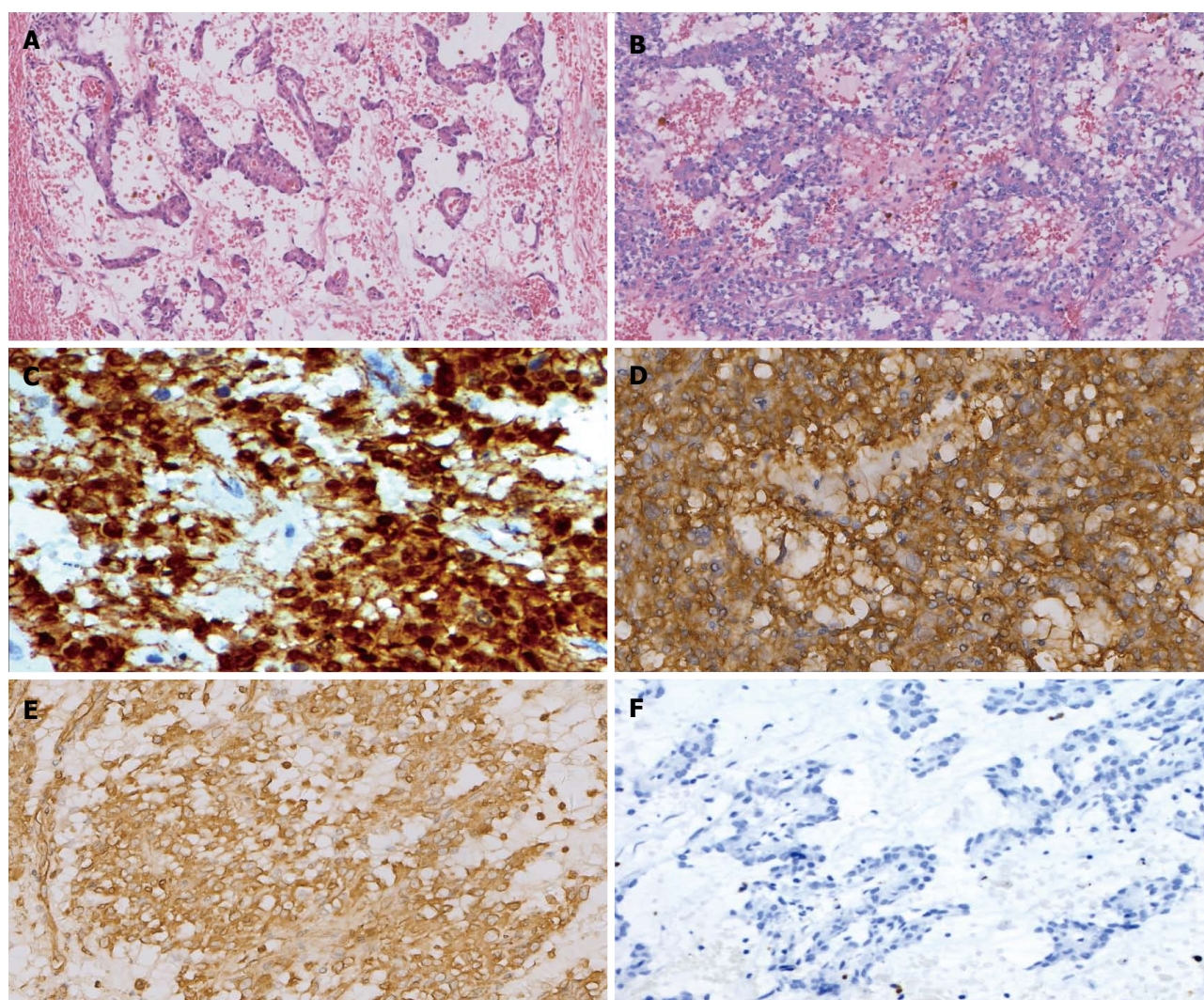
SPN has been recognized by the WHO classification as a low malignant potential neoplasm in 2010<sup>[3]</sup>. It was first named as Gruber-Frantz tumor and after that it had been called the pancreatic solid papillary epithelial neoplasm, pancreatic papillary cystic neoplasm, pancreatic solid cystic tumor and solid pseudopapillary tumor. The differential diagnosis of SPN may include: pseudocyst, pancreatic mucinous neoplasms, well-differentiated ductal adenocarcinoma, pancreatic endocrine neoplasm, and acinic cell carcinoma. The pathogenesis of SPN remains unclear. Likewise, genetic events that contribute to the development of SPN are yet to be discovered. There are two basic proposals for the SPN origin: (1) genital ridge-related cells and (2) pancreatic progenitor cells<sup>[1,6]</sup>. To note, an important proportion of SPN cases show mutations in the somatic  $\beta$ -catenin coding gene (*CTNNB1*)<sup>[7-9]</sup>. Such mutations can affect Wnt signaling pathways as well as self-renewal capability of stem cells<sup>[10]</sup>. SPN cells were reported to be positive for  $\beta$ -catenin, vimentin, alpha-1-anti-trypsin, CD10, CD56, and progesterone receptors by immunohistochemical analysis<sup>[11]</sup>; however this staining pattern fails to reveal a clear phenotypic relationship between SPN and any of the defined cell lineages of the pancreas. Thereby, it can be speculated as SPN cells show multipotential differentiation. According to the study concerned with the embryonic development of the human pancreas, dorsal and ventral pancreatic buds were reported to proliferate



**Table 1** Review of extra-pancreatic solid pseudopapillary neoplasm

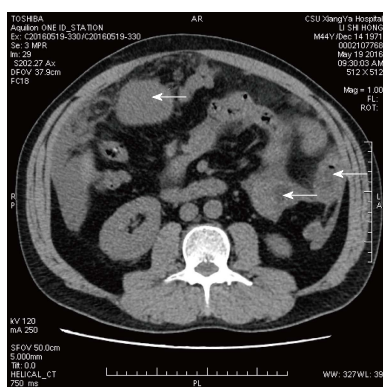
Ref.	Age	Sex	Location	Size (cm)	Procedure	Follow-up
Miyazaki <i>et al</i> <sup>[19]</sup>	22	F	Retroperitoneum	7	Laparoscopy	6 mo NED
Hibi <i>et al</i> <sup>[20]</sup>	45	M	Omentum	15	Laparoscopy	96 mo DOD
Deshpande <i>et al</i> <sup>[21]</sup>	17	F	Left ovary	25.5	Open surgery	72 mo NED
	57	F	Right ovary	3	Open surgery	NA
	21	F	Left ovary	14	Open surgery	NA
He <i>et al</i> <sup>[22]</sup>	39	F	Right ovary	6	Laparoscopy	36 mo NED
Fukunaga <i>et al</i> <sup>[23]</sup>	46	F	Omentum	5	Laparoscopy	3 mo NED
Ishikawa <i>et al</i> <sup>[24]</sup>	13	F	Mesocolon	4	Open surgery	36 mo NED
Guo <i>et al</i> <sup>[25]</sup>	47	F	Retroperitoneum	16	Open surgery	14 mo NED
Geng <i>et al</i> <sup>[26]</sup>	37	F	Retroperitoneum	8	Open surgery	NA
Zhu <i>et al</i> <sup>[27]</sup>	22	F	Retroperitoneum	6	Laparoscopy	14 mo NED
Chen <i>et al</i> <sup>[28]</sup>	47	F	Left ovary	6	Open surgery	18 mo NED
Cheuk <i>et al</i> <sup>[29]</sup>	25	F	Right ovary	16.5	Open surgery	144 mo NED
Walter <i>et al</i> <sup>[30]</sup>	32	F	Stomach	10	Open surgery	24 mo LWD
	73	M	Duodenum	14	Open surgery	3 mo DOD
Stoll <i>et al</i> <sup>[31]</sup>	48	F	Left ovary	8	Open surgery	9 mo NED
Present case	40	M	Mesentery	28	Open surgery	48 mo DOD

NED: No evidence of disease; DOD: Dead of disease; LWD: Live with disease; NA: Not available; F: Female; M: Male.



**Figure 2** Histological and immunohistochemical findings of the tumor (× 200). The tumor cells are arranged in solid sheets, pseudopapillary and microcysts (A and B: Hematoxylin-eosin stain), and are immunohistochemically positive for alpha-1-antitrypsin (C),  $\beta$ -catenin (D: Cytoplasmic and nuclear staining), CD56 (E), whereas negative for chromogranin (F).





**Figure 3** An abdominal computed tomography scan exhibited multiple tumors in peritoneum, greater omentum, and colonic wall (white arrow).

from gut epithelium of endoderm during the 4<sup>th</sup> week of gestation. Dorsal pancreas fuses with ventral pancreas at the 7<sup>th</sup> week of gestation due to the rotation of the stomach and duodenum development<sup>[12]</sup>. Identification of extrapancreatic SPN in the ovary, retroperitoneum and the omentum, as listed in Table 1, indicates a possible endoderm link, substantiated by the migration of pancreas during embryogenesis. We therefore believe that extrapancreatic SPN originates from pancreatic progenitor cells.

In SPN patients, tumor resection confers an 8 year survival rate in 85% of cases; nevertheless, local recurrence or distant metastases can occur in some patients<sup>[13]</sup>. Histological and clinical parameters for prediction of disease recurrence after the initial surgical operation remain a challenge as there is still no consensus in the medical community. Many clinicians and researchers have been working to determine such criteria. For example, Kang *et al.*<sup>[14]</sup> listed: (1) a tumor size larger than 8 cm; (2) cellular atypia; (3) vascular invasion; (4) perineural invasion; (5) systemic metastasis; and (6) peritoneal seeding as significant prognostic factors for tumor recurrence in a multicenter study. A case series study conducted by Yang *et al.*<sup>[15]</sup> showed that vascular invasion, extra-pancreatic invasion, lymph node metastasis, and Ki-67 index  $\geq 4\%$  are associated with SPN recurrence. It is important to note that a rupture of the tumor or laparoscopic biopsy may seed the tumor cells into the peritoneal cavity, and could be an etiological factor responsible for the peritoneal recurrence<sup>[16]</sup>. Nonetheless, a recurrence prediction scoring model require more investigation. Such model will help clinicians to distinguish a high-risk group from low-risk group. Likewise, there is still no consensus on the treatment strategy in patients with SPN recurrence. A previous report described a 35 years old woman relapsing 8 mo after the resection of an SPN, which ruptured preoperatively. The patient firstly underwent a complete cytoreductive surgery, but relapsed within 8 mo, and received another cytoreductive surgery combined with HIPEC (oxaliplatin and irinotecan). At 31 mo of follow-up, the patient showed no evidence of disease recurrence<sup>[17]</sup>.

Thus, a complete cytoreductive surgery combined with HIPEC stands as an important treatment solution for high-risk group of SPN. Further, another report concluded that SPN are radiosensitive, and can be successfully treated by using radiation therapy<sup>[18]</sup>. Future clinical and molecular studies are required to provide more precise tools to predict the biological behavior of SPN.

## ARTICLE HIGHLIGHTS

### Case characteristics

Abdominal distension.

### Clinical diagnosis

Abdominal mass.

### Differential diagnosis

Pancreatic mucinous neoplasms.

### Laboratory diagnosis

All labs were within normal limits.

### Imaging diagnosis

Mesenchymal neoplasm.

### Pathological diagnosis

Solid pseudopapillary neoplasm (SPN).

### Treatment

Complete cytoreductive surgery combined with hyperthermic intraperitoneal chemotherapy.

### Related reports

Grading and staging play an important role in treatment and prognosis.

### Term explanation

SPN: Solid pseudopapillary neoplasm.

### Experiences and lessons

Future clinical and molecular studies are required to provide more precise tools to predict the biological behavior of SPN.

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