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REVIEW

Advancements and challenges in treating advanced gastric cancer in the West

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Abstract

Gastric cancer is a leading cause of cancer incidence and death worldwide. Patients with advanced gastric cancer benefit from a multi-modality treatment regimen. Sound oncologic resection with negative margins and complete lymphadenectomy plays a crucial role in long-term survival for patients with resectable disease. The utilization of minimally invasive techniques for gastric cancer has been slowly increasing and is proving to be both technically and oncologically safe. Perioperative chemotherapy is the current standard of care for advanced gastric cancer. A variety of chemotherapy regimens have been used with the combination of docetaxel, oxaliplatin, 5-fluorouracil, and leucovorin being the current recommendation given its superior ability to induce a complete pathologic response and prolong survival. The use of radiation has been more controversial with its optimal place in the treatment sequence being unclear. There are current ongoing studies assessing the impact of radiation as an adjunct or in place of chemotherapy. Targeted treatments (*e.g.*, trastuzumab for human epidermal growth factor receptor 2 positive tumors and pembrolizumab for programmed death-ligand 1 positive tumors) are showing promise and are part of a continued emphasis on individualized care. Intraperitoneal chemotherapy may also play a role in preventing peritoneal recurrences for patients with high risk lesions. The treatment of patients with advanced gastric cancer in the West continues to advance and improve with a better understanding of optimal treatment sequences and the utilization of personalized treatment regimens.

Key words: Gastric cancer; D2 lymphadenectomy; Minimally invasive surgery; Neoadjuvant chemotherapy; Chemoradiation; Targeted treatments

Core tip: The treatment of advanced gastric cancer in the West continues to evolve and

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advance. Surgery-related outcomes continue to improve and have included the addition of minimally invasive surgery techniques. The use of chemotherapy to improve long-term survival outcomes has been demonstrated in randomized-controlled trials, though the best regimen to use continues to be investigated. Chemoradiation has also been shown to improve outcomes, though the timing, sequence, and patient-population for optimal benefit has yet to be determined. Targeted-therapies and intraperitoneal chemotherapy may also play a role in the treatment of patients with advanced gastric cancer.

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INTRODUCTION

Gastric cancer is the fifth most frequently diagnosed cancer and is the third leading cause of cancer death worldwide[1]. However, nearly 50% of these cases are diagnosed in Eastern Asia with over 70% of gastric cancer occurring outside of the United States[2]. Overall, the incidence of gastric cancer in the United States has been declining while overall survival (OS) rates have been steadily increasing[3]. Non-cardia gastric cancers have seen a decrease in incidence which has been attributed to changes in diet and treatment of chronic *Helicobactor pylori* infections which account for nearly 90% of new non-cardia gastric cancer cases[2,4]. Gastric cancers of the cardia, on the other hand, have seen an increase in incidence, and are associated with factors like obesity, Epstein-Barr virus and gastroesophageal reflux disease[1,3,5]. Additionally, recent studies suggest that while the incidence of gastric cancer in the United States is declining for those aged 40-84, the incidence of gastric cancer in the young is increasing, particularly in young Hispanic males[4]. Young gastric cancer patients are more likely to present with aggressive histologic factors such as poor differentiation, signet ring cells, diffuse histology, and linitis plastica, as well as more advanced nodal metastasis at presentation[6,7].

Outcomes for patients with advanced gastric cancer greatly depend on whether they have resectable disease. Patients with unresectable advanced disease have very poor outcomes with median survivals of just 10-18 mo[8,9]. Long-term survival for patients with resected advanced gastric cancer has improved as medical and surgical therapies have advanced. OS at 5 years after a curative resection was once just 19% in the 1980's but has now improved to 40%-70%[10-12]. Unfortunately, nearly half of patients with an R0 resection have a recurrence and median survival after a recurrence is just 6 mo[13]. These outcomes show that there remains room for improvement in the treatment of advanced gastric cancer. Here, we review the advancements and challenges of treating advanced gastric cancer in the West.

SURGERY

Surgery for gastric cancer is associated with significant morbidity and mortality. A study of more than 700 American College of Surgeons (ACS) approved cancer programs in the United States during the 1980s reported a 30-d mortality of 7%[12]. With improvements in surgical technique, instruments, anesthesia, and peri-operative care, the morbidity and mortality associated with gastrectomy has improved in the United States. A 2005-2010 ACS NSQIP study looking at outcomes in patients undergoing total or partial gastrectomies for gastric cancer found that 24% experienced a major morbidity and the 30-d mortality was 4%[14]. If additional procedures were required (*e.g.*, splenectomy or pancreatectomy), major morbidity increased to nearly 30%[14]. A second NSQIP study in the same time period looking at total gastrectomies alone found similar results with 36% of patients experiencing a complication and a 30-day mortality of 5%[15]. Additionally, the 30-day mortality increased to 13% in patients who underwent a pancreatectomy in addition to a total gastrectomy[15].

The benefit of multivisceral resection has been debated given the higher morbidity and mortality associated with these procedures, which is generally reported around

30%-40% and 3% respectively[16-18]. The number of patients undergoing curative-intent resection who require a multivisceral resection is anywhere from 19%-66%[16,17]. A Canadian study found that combining systemic therapy with multivisceral resections can result in a high rate of margin negative resection[19]. The 5-year survival was 34% and there were few locoregional recurrences with an acceptable morbidity and mortality[19]. A systematic review reported similar conclusions in that multivisceral resections may be helpful in achieving an R0 resection margin[20]. The United States Gastric Cancer Collaborative found that patients who underwent a multivisceral resection without a pancreatectomy had higher post-operative morbidity than those who underwent a gastrectomy alone but there was no change in mortality, while a multivisceral resection with a pancreatectomy was an independent predictor of worse OS[17]. This data would suggest that in appropriately selected patients with locally advanced gastric cancer, a multivisceral resection can be performed at high volume centers with a high rate of negative margins and an acceptable morbidity and mortality. The inclusion of pancreatectomy is an independent predictor of poor outcome and must be used selectively.

The stomach has a multidirectional and complex network of regional lymphatic vessels and nodes[21]. Gastric cancer has a high tendency to metastasize to these regional lymph nodes and their involvement is an important prognostic factor[22]. As such, lymph node retrieval is an important aspect of staging for gastric cancer and has implications in adjuvant treatment recommendations (Table 1)[22-24]. The final publication of the Dutch trial reported lower locoregional recurrence and gastric cancer-related death in patients undergoing a D2 lymphadenectomy compared to a D1 lymphadenectomy[25]. Similarly, an Italian trial demonstrated a survival benefit for patients with positive nodes treated with D2 gastrectomy without splenectomy and pancreatectomy[26]. As such, a pancreas and spleen preserving, or modified D2 lymphadenectomy, is now the standard of care at most academic institutions throughout the United States.

Similarly, the total number of lymph nodes removed during gastrectomy is an independent predictor of survival, with an increasing number of excised lymph nodes being associated with a decreased risk of death[27]. As such, the recently, published American Joint Committee on Cancer 8th edition tumor-node-metastasis-staging guidelines recommend a minimum of 16 lymph nodes be assessed in gastric cancer with 30 or more lymph nodes being desirable[28]. Despite this, most patients in the west do not receive an adequate lymphadenectomy. A recent NCDB study found that only 23% of patients had evaluation of the recommended 15 lymph nodes[29]. That same study showed that patients who were treated in National Cancer Institute-designated centers or high-volume centers were more likely to have the recommended lymph node retrieval[29]. A Surveillance, Epidemiology and End Results (SEER) analysis found that from 2004-2010, only 42% of patients had at least 15 lymph nodes evaluated after surgery, and while being treated at a cancer program increased the odds of having 15 nodes removed, improved survival was significant with removal of 15 or more lymph nodes regardless of treatment location[30]. The ability to obtain 15 lymph nodes can be difficult if a D1 resection, rather than a D2, is utilized[31]. Recently, the National Comprehensive Cancer Network (NCCN) has recommended performing a D2 lymphadenectomy with the goal of obtaining at least 15 lymph nodes while avoiding a splenectomy[32].

There has been a slow increase in minimally invasive surgery for gastric cancer over time. The first description of a laparoscopic gastrectomy was in 1994 from a group in Japan[33]. In the United States, a SEER analysis from 2008-2013 showed that only 8% of gastrectomies were performed laparoscopically and only 2% were performed robotically[34]. There was also no significant increase in the last three years of this analysis indicating that the use of laparoscopic surgery for gastric cancer has not continued to increase[34]. This is in contrast to a nearly 26% utilization by Eastern countries[35]. Data from the NCDB showed that only 13% of cases were done laparoscopically between 1998-2011 with a conversion from laparoscopic to open in nearly 24% of cases[36]. Another study from the NCDB showed that between 2010 and 2012, there was an increase in the use of minimally invasive techniques with 4% being performed robotically and 23% undergoing laparoscopic gastrectomies[37]. This study also found that open gastrectomies are decreasing overall, with a rise in both laparoscopic and robotic gastrectomies[37].

Two prospective trials, KLASS-01 and JCOG 0703, as well as several retrospective studies support the safety and oncologic feasibility of laparoscopic surgery for early gastric cancer (Table 1)[38,39]. Fewer studies have addressed laparoscopy in advanced gastric cancer. The KLASS-02 trial randomized patients with locally advanced gastric cancer to either laparoscopic or open distal gastrectomy[40]. Short term outcomes from this trial were recently published and demonstrated benefits in terms of lower complication rates, lower pain score, earlier return of bowel function, and shorter

Table 1 Surgery trials

Trial name	Country	Status	Years	Factors	Outcome
DGCT Trial	Netherlands	Complete	1989-1993	Randomization to D1 or D2 lymphadenectomy	Increased morbidity and mortality in D2 group Decreased gastric cancer-related deaths and locoregional recurrences after D2 resection
IGCSG-R01	Italy	Complete	1998-2006	Randomization to D1 or D2 lymphadenectomy	No difference in morbidity and mortality Improved 5-yr survival in subgroup analysis of patients with positive LN after D2 resection
KLASS-01[1]	Korea	Complete	2006-2010	Randomization to open distal gastrectomy or laparoscopic distal gastrectomy	Decreased wound complication rate in laparoscopic group with no difference in morbidity or mortality
KLASS-02[2]	Korea	Complete	2011-2015	Randomization to open or laparoscopic gastrectomy and D2 lymph node resection	Decreased complication rates and pain scores with shorter hospital stays in laparoscopic resections
JCOG 0703[1]	Japan	Complete	2007-2008	Prospective study with patients undergoing laparoscopic distal gastrectomy with D1 lymph node resection	Laparoscopic surgery was safe with lower than expected rates of anastomotic leaks and pancreatic fistulas

[1]Clinical stage I gastric cancers only;
[2]Locally advanced gastric cancers only. DGCT: Dutch Gastric Cancer Group Trial; IGCSG: Italian Gastric Cancer Study Group; LN: Lymph node; KLASS: Korean Laparoscopic Surgical Society; JCOG: Japan Clinical Oncology Group.

hospital length of stays compared to open surgery[41]. A primary concern regarding minimally invasive surgery for advanced gastric cancer is whether adequate lymph node retrieval could be achieved. A study from the United States Gastric Cancer Collaborative showed similar rates of lymph nodes retrieved between a minimally invasive cohort and an open cohort, both overall and after propensity matching[42]. From an outcomes standpoint, a NSQIP analysis of patients undergoing laparoscopic *vs*. open gastrectomies showed fewer complications for patients undergoing laparoscopic gastrectomies with no difference in mortality[43]. As western surgeons become more experienced and minimally invasive technology improves (*e.g.*, 3D visualization and robotic surgery), minimally invasive surgery will likely become safer and more oncologically sound for western patients with advanced gastric cancer.

CHEMOTHERAPY

The role of systemic chemotherapy for the treatment of advanced gastric cancer has evolved over time as we continue to search for optimal therapies and treatment sequencing that will positively influence outcomes (Table 2). The MAGIC trial was the first large prospective trial that observed improved outcomes in patients who received perioperative chemotherapy[44]. The agents used in this study were epirubicin, cisplatin, and 5-fluorouracil (ECF). This study showed improved outcomes even with only 42% of patients receiving the full six cycles of chemotherapy, with many patients being unable to complete their post-operative cycles. Patients who received pre-operative chemotherapy demonstrated significant down-staging as evidenced by smaller tumors and less lymph node involvement, but most importantly, had significantly better overall and progression-free survival[44]. Perioperative chemotherapy has since become standard of care for patients with non-metastatic stage II or higher gastric cancers. The use of perioperative chemotherapy in the United States has increased overtime since the MAGIC trial. In the year 2003, just 25% of patients were receiving neoadjuvant chemotherapy while in 2012 the rate

increased to over 45%[45]. A later trial, the ACCORD 07, was a phase III randomized trial that again showed a significant improvement in overall and progression free survival in patients that underwent perioperative chemotherapy and surgery compared to those that underwent surgery alone[46]. This trial used cisplatin and 5-fluorouracil without epirubicin and OS were similar between the MAGIC and the ACCORD 07 trial with 5-year survivals around 30%[44,46].

Neoadjuvant chemotherapy has several potential benefits to adjuvant chemotherapy. First, pre-operative chemotherapy is consistently better tolerated than post-operative chemotherapy in multiple trials[44,46]. Second, blood supply to the tumor is not disrupted by surgical resection and micrometastasis can be treated at the earliest possible time[47]. Down-staging or shrinkage of the tumor may lead to higher R0 resection rates, particularly in advanced gastric cancer, and it allows the assessment of response to therapy allowing postoperative therapy to be tailored to the individual response to pre-operative therapy[48]. Unfortunately, very few patients receiving pre-operative ECF are able to achieve a complete pathologic response following neoadjuvant chemotherapy[49]. Tumor regression on final surgical pathology has been reported to be an independent factor associated with improved survival in patients receiving neoadjuvant chemotherapy for a number of cancers, including gastric[50,51]. In a phase II study where patients received epirubicin, cisplatin, and capecitabine (ECX), a complete pathologic response was found in just 6%[49]. This compares to a complete response rates as high as 17%-20% in phase II studies where docetaxel is part of the treatment regimen[52,53].

The AIO-FLOT4 trial looked to compare the rates of pathological regression in patients who received neoadjuvant ECF/ECX *vs* the docetaxel-based regimen FLOT (5-fluorouracil, leuocovorin, oxaliplatin, and docetaxel)[54]. Results of this study showed a significant increase in complete regression with FLOT treatment when compared to ECF (16% *vs* 6%). Recent phase III results of the FLOT4 randomized trial showed improved median OS of 50 mo for patient receiving the FLOT regimen compared to 35 mo for those on ECF/ECX with similar toxicities between the two groups[55]. The current NCCN guidelines recommend perioperative FLOT as the preferred regimen for medically fit patients with oxaliplatin and a fluoropyrimidine without docetaxel to be used in patients with a poor performance status or significant medical comorbidities[32].

RADIATION

The benefit of chemoradiation in patients with locally advanced gastric cancer has been controversial (Table 3). In the early 2000s, the Intergroup 0116 Trial showed that patients who underwent post-operative chemoradiation radiation therapy with 5-fluorouracil and leucovorin after a complete resection had improved long-term outcomes compared to patients who underwent surgery alone[56]. These patients did not undergo perioperative chemotherapy and the median survival in the surgery plus chemoradiation group was 36 mo compared to 27 mo in the surgery alone group[56]. This trial was criticized for its low (10%) utilization of D2 lymphadenectomy suggesting that chemoradiation may compensate for inadequate surgery. In contrast, a large retrospective United States multi-institutional study in which patients had a median 18 lymph nodes removed showed that post-operative chemoradiation with 5-fluorouracil improved both overall and recurrence-free survival when compared to patients who received perioperative chemotherapy alone[57]. On subgroup analysis, patients with nodal disease (N1) and lymphovascular invasion derived the most benefit[57].

Neoadjuvant chemoradiation may improve R0 resection rate and achievement of pathological complete response (pCR) in advanced gastric cancer. A phase I multi-institutional single-arm trial in the US reported a 70% R0 resection rate and a 30% pCR rate with preoperative chemoradiation consisting of two 28-d cycles of induction fluorouracil, leucovorin, and cisplatin followed by fluorouracil-based chemoradiation therapy (CRT) to 45 Gy[58]. Pathological partial or complete response were associated with improved OS[59]. Similarly, another multi-institutional phase II trial in the United States reported a 77% R0 resection rate and 26% pCR rate following preoperative induction chemotherapy with two cycles of fluorouracil, leucovorin, and cisplatin followed by paclitaxel, fluorouracil, and concurrent 45 Gy radiotherapy[60]. A small retrospective series from Spain comparing neoadjuvant chemotherapy to neoadjuvant chemoradiation in resectable advanced gastric cancer demonstrated a significantly higher likelihood of achieving a Becker Ia-b response (58% *vs* 32%), a grade D nodal regression (30% *vs* 6%) and a favorable pathological response (23% *vs* 3%). Nodal, but not primary, response was associated with a longer 5-year progression-free and OS[61].

Table 2 Chemotherapy trials

Trial name	Country	Status	Years	Groups	Chemotherapy regimens	Outcome
MAGIC	UK	Complete	1994-2002	Surgery alone	-	Smaller tumors in chemo group (3 cm *vs* 5 cm)
				Surgery with perioperative chemo	Epirubicin, cisplatin, 5-FU (ECF)	Better PFS and OS in chemo group (5-yr OS 36% *vs* 23%)
ACCORD 07	France	Complete	1995-2003	Surgery alone	-	Improved curative resection rates with chemo (84% *vs* 73%)
				Surgery with perioperative chemo	Cisplatin and 5-FU	Better DFS and OS in chemo group (5-yr OS 38% *vs* 24%)
AIO-FLOT4 (Phase II)	Germany	Complete	2010-2012	Neoadjuvant ECF/ECX	Epirubicin and cisplatin with either 5-FU (ECF) or capecitabine (ECX)	Improved pathological complete regression in FLOT *vs* ECF/ECX
				Neoadjuvant FLOT	Docetaxel, oxaliplatin, 5-FU with leucovorin	
AIO-FLOT4 (Phase III)	Germany	Complete	2010-2015	Neoadjuvant ECF/ECX	Epirubicin and cisplatin with either 5-FU (ECF) or capecitabine (ECX)	Improved OS in FLOT group with no increase in toxicities
				Neoadjuvant FLOT	Docetaxel, oxaliplatin, 5-FU with leucovorin	

MAGIC: Medical Research Council Adjuvant Gastric Infusional Chemotherapy; UK: United Kingdom; 5-FU: 5-fluorouracil; PFS: Progression-free survival; OS: Overall survival; DFS: Disease-free survival; ECF: Epirubicin, cisplatin, and 5-fluorouracil; ECX: Epirubicin, cisplatin, and capecitabine; FLOT: 5-fluorouracil, leuocovorin, oxaliplatin, and docetaxel.

Whether chemoradiation offers a benefit in addition to or in place of chemotherapy is being actively prospectively investigated. The CRITICS trial randomized patients to perioperative chemotherapy *vs* pre-operative chemotherapy and post-operative CRT and was not able to show a survival difference between the two groups[11], however only 50% of patients received their intended post-operative treatment regimens in the CRITICS trial. The follow up CRITICS-II trial aims to optimize neoadjuvant therapy by comparing neoadjuvant chemotherapy alone, neoadjuvant chemotherapy followed by chemoradiation, and neoadjuvant chemoradiation alone[62]. Similarly, the ARTIST trial, which was designed to compare adjuvant chemotherapy to chemoradiation, failed to demonstrate a difference in disease-free and OS; however, subgroup analysis suggested that patients with intestinal type histology and lymph node metastasis, in particular, may benefit from chemoradiation[63]. A follow-up trial, ARTIST II, is currently under way addressing this question (NCT01761461). The TOPGEAR trial is another randomized prospective trial aimed at determining if pre-operative chemoradiation, in addition to perioperative chemotherapy, improves outcomes over perioperative chemotherapy alone[64].

The mode of radiation administration has traditionally been through three-dimensional conformal radiotherapy (3D-CRT). The potential advantage of using intensity-modulated radiotherapy (IMRT) over 3D-CRT is the ability to limit exposure to critical surrounding structures while delivering therapeutic doses to the organs of interest[65,66]. One study found that overall and disease-free survival were significantly improved in patients treated with adjuvant IMRT compared to similar patients treated with adjuvant 3D-CRT[67]. There has been little investigation into the use of proton beam radiation for the treatment of locally advanced gastric cancer. Reasons for this include the limited availability of proton beam radiation in the United States and that a moderate dose of radiation over a large area may be ideal for treating the resection margins and wide nodal basin[68]. A benefit of proton beam radiation is its ability to provide a very high dose of radiation to a single lesion with relative sparing of adjacent structures, limiting damage to surrounding tissue[69]. Given these properties, there has been more interest in using proton beam radiation for unresectable primary liver tumors like hepatocellular carcinoma, or even gastric

Table 3 Radiation trials

Trial name	Country	Status	Years	Trial group(s)	Regimens	Outcome
INT-0116	US	Complete	1991-1998	Surgery alone	-	Improved DSF and OS in radiation group (median survival 36 mo *vs* 27 mo)
				Surgery + adjuvant CRT	45 Gy with 5-FU and leucovorin	
Ajani *et al*[60]	US	Complete	1999-2004	CRT	45 Gy with 5-FU, leucovorin, and cisplatin	77% R0 resection rate 26% complete pathologic response rate
Martin-Romano *et al*[61]	Spain	Complete	2004-2014	Neoadjuvant chemotherapy	Variable	Improved pathologic response and nodal regression in CRT group
				Neoadjuvant CRT	Radiation: 45 Gy Chemo: Variable	
CRITICS	Netherlands, Sweden, Denmark	Complete	2007-2015	Perioperative chemo	Epirubicin, cisplatin or oxaliplatin, capecitabine	No difference in OS or DFS
				Neoadjuvant chemo and adjuvant CRT	Chemo: Epirubicin, cisplatin or oxaliplatin, capecitabine CRT: 45 Gy with capecitabine	
CRITICS-II (NCT02931890)	Netherlands, Sweden, Denmark	Ongoing	-	Neoadjuvant chemo	Docetaxel, oxaliplatin, capecitabine	-
				Neoadjuvant chemo and CRT	Chemo: Docetaxel, oxaliplatin, capecitabine CRT: 45 Gy with paclitaxel and carboplatin	
				Neoadjuvant CRT	45 Gy with paclitaxel and carboplatin	
ARTIST	Korea	Complete	2004-2008	Adjuvant chemo	Capecitabine and cisplatin	No difference in DFS overall
				Adjuvant chemo and CRT	Chemo: Capecitabine and cisplatin CRT: 45 Gy with capecitabine	Superior DFS in radiation group on subgroup analysis of patients with positive LNs
ARTIST-II (NCT01761461)	Korea	Ongoing	-	Adjuvant chemo	S-1	-
				Adjuvant chemo	S-1 and oxaliplatin	
				Adjuvant chemo and CRT	Chemo: S-1 and oxaliplatin CRT: 45Gy with S-1	
TOPGEAR (ACTRN12609000035224)	Australia	Ongoing	-	Perioperative chemo	Epirubicin, cisplatin, 5-FU (ECF)	-
				Perioperative chemo with Neoadjuvant CRT	Chemo: Epirubicin, cisplatin, 5-FU (ECF) CRT: 45 Gy with 5-FU	

INT: Intergroup; US: United States; CRT: Chemoradiation therapy; Gy: Gray; DFS: Disease-free survival; OS: Overall survival; 5-FU: 5-fluorouracil; CRITICS: Chemoradiotherapy after Induction chemotherapy In Cancer of the Stomach; chemo: Chemotherapy; LN: Lymph node; TOPGEAR: Trial Of Preoperative therapy for Gastric and Esophago-gastric junction Adenocarcinoma; ECF: Epirubicin, cisplatin, and 5-fluorouracil.

cancer hepatic metastases[69,70]. For similar reason, stereotactic body radiotherapy (SBRT) has been under investigation for the treatment of liver tumors, though there has been a description of using SBRT for the treatment of lymph node recurrences after gastric resection[71,72].

FUTURE DIRECTIONS

While outcomes for patients with advanced gastric cancer have improved with medical and surgical advancement, there are still areas for improvement and exploration. The use of targeted treatments as adjuncts to traditional chemotherapeutic agents is under continued investigation (Table 4). Epidermal growth factor receptor (EGFR) is overexpressed in most gastric cancers, however the trials that used anti-EGFR antibodies such as cetuximab (EXPAND Trial), and panitumumab (REAL3 Trial) failed to improve survival[8,73]. In contrast, in the randomized phase III AVAGAST trial, bevacizumab, a monoclonal antibody that targets vascular endothelial growth factor (VEGF), when given with cisplatin and a fluoropyrimidine was found to significantly improve progression-free survival in patients with unresectable or metastatic advanced gastric cancer though it did not improve OS (50% *vs* 42%)[74]. However, the use of ramucirumab, a VEGFR-2 inhibitor, in the REGARD trial was able to show a significant improvement in OS as a second-line option for patients with metastatic disease unresponsive to first-line therapy so there may be a role for its use in the perioperative setting in the future[75].

Another advance and future direction is using molecular analysis to better predict prognosis as well as treatment options for individual patients. One of these molecular characteristics is identification of patients with human epidermal growth factor receptor 2 (HER2) overexpression. Around 12%-24% of patients with gastric cancer have been found to have HER2 overexpression with most showing associated poorer outcomes when compared to patients with normal HER2 expression[76-78]. Intestinal type gastric cancers are much more likely to have overexpression than diffuse type (32% *vs* 6%)[79]. The randomized controlled ToGA trial found that in patients with HER2 overexpression, treatment with chemotherapy with trastuzumab was associated with improved OS when compared to patients treated with chemotherapy alone[79]. These results have led to NCCN guidelines recommending the assessment of HER2 expression and the addition of trastuzumab to all HER2 overexpressing metastatic gastric adenocarcinomas[32].

The use of pembrolizumab, a monoclonal antibody against programmed death 1 (PD-1), in patients with PD-L1-positive cancers is another example of how individualized treatment may impact specific patients based on their tumor's molecular characteristics. In a phase I study, pembrolizumab showed promising results as a single agent in patients with PD-L1-positive metastatic or recurrent gastric cancer that had failed other treatment regimens[80]. In this cohort, 22% of patients had a partial tumor response and the 1-year survival was 42%[80].

Additionally, there has been investigation into the adjuvant use of intraperitoneal chemotherapy. Peritoneal recurrence develops in about 60% of the patients with T3 and T4 tumors following curative-intent resection, and up to 40% of resected gastric cancer patients die as a direct result of peritoneal dissemination[13]. Few systemic chemotherapeutic agents penetrate the peritoneum well and intraperitoneal chemotherapy has less adverse effects with higher doses in the intraperitoneal regions than systemic chemotherapy[81]. A recent meta-analysis of 23 prospective randomized trials including 2767 advanced gastric patients from Japan, China, Korea, and Austria demonstrated that adjuvant intraperitoneal chemotherapy was associated with improved 1, 2 and 3-year survival rate, as well as a 30% reduction in the incidence of peritoneal recurrence[82]. Currently, the GASTRICHIP Trial, a randomized multicenter phase III clinical study, is evaluating the effects of hyperthermic intraperitoneal chemotherapy with oxaliplatin on patients with locally advanced gastric cancer and will hopefully provide further direction on the effectiveness of intraperitoneal chemotherapy for locally advanced gastric cancer[83].

CONCLUSION

Treatment for advanced gastric cancer continues to evolve with better understanding of optimal treatment regimens, treatment sequences, and surgical optimization with improved technique. Additionally, future efforts in providing individualized treatment recommendations based on molecular characteristics at the time of the initial diagnosis have the opportunity to improve long-term outcomes.

Table 4 Targeted treatment trials

Trial name	Country	Status	Years	Target	Groups	Chemotherapy regimens	Outcome
EXPAND	Multiple	Complete	2008-2010	EGFR	Standard chemo	Capecitabine, cisplatin	No difference in PFS
					Standard chemo with cetuximab	Capecitabine, cisplatin, cetuximab	
REAL3	Multiple	Complete	2008-2011	EGFR	Standard chemo	Epirubicin, oxaliplatin, capecitabine	No difference in OS
					Standard chemo with panitumumab	Epirubicin, oxaliplatin, capecitabine, panitumumab	
AVAGAST	Multiple	Complete	2007-2008	VEGFR	Standard chemo	Capecitabine, cisplatin	Improved PFS in the bevacizumab group (median survival 6.7 mo *vs* 5.3 mo)
					Standard chemo with bevacizumab	Capecitabine, cisplatin, bevacizumab	
REGARD	Multiple	Complete	2009-2012	VEGFR	Best supportive care	-	Improved OS in ramucirumab group (median survival 5.2 mo *vs* 3.8 mo)
					Best supportive care with ramucirumab	Ramucirumab	
ToGA Trial	Multiple	Complete	2005-2008	HER2	Standard chemo	Cisplatin with capecitabine or 5-FU	Improved OS in the trastuzumab group (median survival 13.8 mo *vs* 11.1 mo)
					Standard chemo with trastuzumab	Cisplatin, capecitabine or 5-FU, trastuzumab	
KEYNOTE-012	Multiple	Complete	2013-2014	PD-L1	Pembrolizumab	Pembrolizumab	Median OS of 11.4 mo
GASTRICHIP (NCT01882933)	Multiple	Ongoing	-	HIPEC	Curative gastrectomy with D1-D2 lymph node dissection	-	-
					Curative gastrectomy with D1-D2 lymph node dissection with HIPEC	IP Oxaliplatin with IV 5-FU and leucovorin	

EGFR: Endothelial growth factor receptor; chemo: Chemotherapy; PFS: Progression-free survival; OS: Overall survival; AVAGAST: Avastin in Gastric Cancer; VEGFR: Vascular endothelial growth factor receptor; ToGA: Trastuzumab for Gastric Cancer; HER2: Human epidermal growth factor receptor 2; 5-FU: 5-fluorouracil; PD-L1: Programmed death-ligand 1; HIPEC: Hyperthermic intraperitoneal chemotherapy; IP: Intraperitoneal; IV: Intravascular.

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REVIEW

Premalignant lesions and gastric cancer: Current understanding

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Abstract

Over the last two decades there has been a broad paradigm shift in our understanding of gastric cancer (GC) and its premalignant states from gross histological models to increasingly precise molecular descriptions. In this review we reflect upon the historic approaches to describing premalignant lesions and GC, highlight the current molecular landscape and how this could inform future risk assessment prevention strategies.

Key words: *Helicobacter pylori*; Correa cascade; Atrophic gastritis; Intestinal metaplasia; Point of no return; Dysplasia; Stem cells; Gastric cancer

Core tip: Despite recent advances in our understanding of the molecular and cellular events involved in gastric cancer, little is known about how gastric premalignant lesions actually lead to this usually lethal disease (5 years survival about 20% in most Western countries). It is still not clear whether some or all of these lesions are directly involved in the process of gastric carcinogenesis or whether they are simply bystanders. In this review, we attempt to shed some light into how our current understanding of premalignant lesions may be used to improve patient stratification and lead to better overall patient survival rates.

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INTRODUCTION

Gastric cancer (GC) is the fifth most common cancer worldwide and third highest cause of cancer-related death. In 2012, 950000 individuals were diagnosed with the disease and 723000 died. High incidence areas are Eastern Asia, particularly China, Japan and South Korea, Eastern Europe, Central and South America. Low incidence areas are Australia and New Zealand, North America, Western Europe, South Central Asia and most parts of Africa[1]. Risk factors for GC include male sex, age, high salt intake, including salt preserved foods, smoked or dried meat and fish, pickled food, low intake of fresh fruit and vegetables, smoking, radiation exposure, low levels of physical activity, obesity and low socioeconomic status[2-15].

HISTOLOGICAL AND MOLECULAR CLASSIFICATIONS OF GC

The majority of GC are adenocarcinomas and these can be subdivided by the Lauren histopathology system into intestinal and diffuse subtypes[16]. The intestinal subtype of GC (IGC) is characterised by tumour cells that form gland-like structures whereas the diffuse subtype (DGC) has single or groups of tumour cells that are poorly differentiated or undifferentiated infiltrating the gastric wall. GC with components of both DGC and IGC are referred to as mixed. Given all three subtypes are adenocarcinomas this raises questions regarding the pre-malignant pathways and aetiologies of each. Given they arise from the same gastric inflammatory milieu are they a spectrum of the same disease with overlapping molecular identities or do they represent unique entities with disparate causes and premalignant pathways?

The Cancer Genome Atlas (TCGA) Research Network published a landmark study into molecular classification of established GC in 2014. The study performed integrative genomic and epigenomic analysis of 295 gastric adenocarcinomas and reported on four major subclasses based on somatic copy number, mutation analysis, methylation and gene expression status. These were named: Epstein Barr virus positive, microsatellite unstable (MSI), genomically stable (GS) and chromosomal unstable subtypes[17]. While there was significant overlap regarding the molecular signatures between the IGC, DGC and mixed types consistent with common aspects of oncogenesis, 75% of the DGCs were of the GS subclass suggesting a divergent pathway. The TCGA analysis also demonstrates the potential limitation of histological systems such as the Lauren classification, with cellular phenotypes often not reflecting the heterogeneous nature of complex underlying molecular changes.

There are a number of inherited genetic conditions that predispose to GC such as somatic mismatch repair mutations in Lynch Syndrome and CDH1 mutations in Hereditary Diffuse GC. Although these are of interest in elucidating the molecular pathways of oncogenesis, discussion of these conditions is largely outside the scope of this review.

THE CORREA CASCADE

Chronic gastric inflammation

In 1975 Correa *et al*[18] described a stepwise progression of conditions within the stomach that were thought to result in GC. This was one of the first considerations of premalignant conditions in this disease and it was later found to be initiated by *Helicobacter pylori* (*H. pylori*). The initial step in the Correa cascade is the development of Chronic gastritis (ChG). *H. pylori* represents the archetypal cause of ChG, with infected patients in some studies having a greater than 10-fold higher chance of developing GC[19]. The effects of *H. pylori* on the gastric epithelium have been extensively studied, with one of the most important pathogenic factors being cytotoxin-associated gene A protein (CagA) positive strains. Virtually all of East Asian strains and 60% of Western strains of *H. pylori* strains are $cagA^+$, with infected patients developing more distinct inflammation, gastric ulceration and higher risk of GC[20-22]. Bacterial CagA protein interacts with a series of host epithelial proteins including ASPP2, RUNX3, PI3K, SHP2 and E-cadherin, resulting in the degradation and inactivation of p53 and RUNX3, deregulation of the PI3K-AKT, Ras-ERK and Wnt pathways and disruption of adherens junctions[23]. CagA has also been shown to alter DNA methylation patterns further deregulating normal epithelial gene expression patterns[24]. Intestinal metaplasia (IM) samples show higher levels of methylation than Atrophic gastritis (AG) samples, suggesting that DNA methylation pattern changes may play a vital role in the Correa model of IGC[24,25].

Autoimmune gastritis and atrophic gastritis

Autoimmune gastritis is a common aetiology of ChG, which results in activation of the adaptive immune system against parietal cells and intrinsic factor, leading to the destruction of the oxyntic gastric mucosa. As with other forms of chronic inflammation, autoimmune gastritis is a risk factor for GC through progression to intestinal metaplasia[26]. In a meta-analysis the overall relative risk of GC in patients with autoimmune gastritis was 6.8 (95%CI: 2.6–18.1)[27].

ChG leads to AG which refers to the atrophy and loss of gastric mucosal glands. Loss of specialised cells has significant implications on gastric function, with hypochlorhyria being one of the most recognised. In this state the loss of peptic acid production and raised gastric pH has implications on nutrient absorption (such as iron) and has significant implications on the gastric microbiome[28]. There has been considerable interest in the relationship between the gastric microbiome and GC, with a recent study uncovering dysbiosis of bacterial taxa along the Correa cascade[29]. At this stage it is uncertain if this dysbiosis represents a pre-malignant factor contributing to carcinogenesis in its own right, or simply a reflection of the change in the gastric microenvironment.

A key risk factor of chronic inflammation is the release of large amounts of reactive oxygen and nitrogen free species (ROS and NOS respectively), which are associated with DNA damage and increased mutation rates. Previous studies have shown that ROS and NOS released by inflammatory and epithelial cells can cause oxidative and nitrative DNA damage including the production of 8-oxo-7,8-dihydro-2′-deoxyguanosine (8-oxodG), a known mutagen and 8-nitroguanine[30,31]. The latter is formed by inducible nitric oxide synthase iNOS. Gene expression of iNOS is regulated by the NF-κB and STAT pathways among others[32]. These changes can result in DNA mutations thus promoting cellular changes and carcinogenesis.

GASTRIC STEM CELLS AND IM

In normal gastric epithelium stem cell populations give rise to nascent epithelial cells that mature and differentiate as they migrate to the apex of the gland[33]. Gastric and intestinal stem cells share an endodermal lineage, and through the process of chronic inflammation gastric stem cells may reprogram, producing metaplastic intestinal-type epithelium that replaces the normal gastric mucosa[34]. The continuing chronic inflammatory process results in further accumulation of genetic lesions in stem cells, ultimately resulting in dysplasia and cancer. As such IM can be thought of as a marker of stem cell stress and damage, with multiple inflammatory aetiologies converging to histologically identical metaplastic change. There have been multiple gastric stem cell populations characterised including $Lgr5^+$ stem cells in the adult antrum and the neonatal corpus and antrum, $Mist1^+$ stem cells found in the isthmus region of the corpus glands and $Troy^+$ stem cells that are thought to reside in the base of the corpus glands[33,35,36]. The role each of these plays in oncogenesis is an area of ongoing research. However, it is notable that different regions of the stomach have different stems cells and based on epidemiological evidence histological and molecular subgroups are found in different anatomic distributions suggesting a possible predetermined pathway for conversion to specific GC subgroups. For instance, TCGA found different anatomic distribution of molecular subgroups of GC with MSI being more likely to occur in the gastric corpus and antrum but rarely in the cardia[17].

IM

IM is usually found incidentally in patients undergoing upper endoscopy and is usually asymptomatic. While IM is defined by intestinal differentiation it is molecularly heterogeneous but can be histologically categorised as complete or incomplete subtypes (Figure 1). Complete IM (type I) resembles the small intestine epithelium with goblet cells, Paneth cells, eosinophilic enterocytes and a brush border[37]. It is associated with loss of markers of gastric mucin (MUC1, MUC5AC, MUC6) and expression of the intestinal sialic mucin, MUC2[38]. Incomplete IM more closely resembles the large intestine epithelium, lacking absorptive cells, but with columnar cells resembling gastric foveolar cells. It does not have a brush border and maintains expression of gastric mucin markers (MUC1, MUC5AC, MUC6) usually together with gain of MUC2[38]. Incomplete IM is further subdivided into Type II IM, with cells expressing a mixture of neutral mucins and intestinal sialomucins and Type III IM, with cells expressing sulfomucins[37]. In practice histopathological classification between complete and incomplete IM is often not mutually exclusive, with segments of tissue containing elements of both subtypes. The distinction between complete and

incomplete IM is clinically important as it appears incomplete harbours a higher risk of progression to cancer[39-42].

In the context of long-term *H. pylori* infection, IM possibly develops as an adaptive and protective lesion[43]. There has been extensive work into determining how *H. pylori* infection leads to IM with a number of genes implicated including SOX2 and CDX2. SOX2 is a transcription factor involved in gastric differentiation which negatively regulates intestinal differentiation, whereas CDX2 is a key intestinal transcription factor involved in establishing and maintaining IM[44]. SOX2 and CDX2 seem to be inversely regulated by *H. pylori*[45]. Complete IM has been shown to be predominantly SOX2 negative (93%) and incomplete IM mainly SOX2 positive (85%)[46]. Moreover CDX2 expression has been shown to be also induced in part through an NF-κB dependent mechanism following *H. pylori* infection[47].

Duodeno-gastric reflux is another proposed gastric insult contributing to ChG and IM formation, analogous to gastroesophageal acid reflux in Barrett's oesophagus[48]. There has been an association of increased incidence of IM after exposure to bile acids reported in a large-scale study involving a total of 2283 patients[49]. In this context the development of IM may represent a protective mechanism, with a metaplasia to an intestinal phenotype more capable of resisting the effects of bile than the normal gastric mucosa.

Risk factors in IM

H. pylori is a significant risk factor in the establishment of IM, however there are other clinical and environmental exposures that have been shown to be important risk factors for IM progression to GC. In a large-scale US study (n = 810821 patients) IM was more common in men, was more prevalent with increasing age and East Asian ancestry. This suggests IM may occur due to environmental exposures but in the context of hereditary risk[50]. Hereditary risk is relevant in GC even excluding major genetic syndromes with several studies showing intestinal-type GC is associated with a strong family history of GC[51-53]. With respect to premalignant lesions, it was shown that among siblings with a family history of any precancerous change there is an increase in risk of subsequent non-cardia GC with a hazard ratio of 2.5 compared with siblings of index persons with "normal or minor mucosal changes"[54]. The availability of siblings' precancerous data to the clinician could be useful in assessing a patient's risk of progressing to GC.

Once established, the degree of IM has been shown to be related to the risk of progression to cancer. Extensive IM with IM in the corpus, incomplete IM and IM located along the Maggenstrasse (along the lesser curve of the stomach) have been shown to increase the risk of progression towards cancer[40,42,55,56]. In one study of microsatellite instability (MSI), this molecular finding was enriched in GC and adjacent IM suggesting this may be an early event in MSI subtype GCs. It is notable that microsatellite unstable IM was of incomplete type in this study[57] which provides further evidence for the potential unique molecular pathways that begin in the premalignant context.

OLGA AND OLGIM

Both the Operative Link on Gastritis Assessment (OLGA) and on Gastric Intestinal Metaplasia (OLGIM) are based on histological assessment of random biopsies taken from designated areas of the stomach according to the Sydney protocol[58-60]. At least four sites are sampled from the stomach during upper gastroscopy (two antral and two corpus). Both OLGA and OLGIM are scoring standards used to grade and stage chronic gastric inflammation, gastric atrophy and intestinal metaplasia. They provide information with regards to topography and extent of atrophic gastritis and intestinal metaplasia, the latter being easier to assess and more consistent. Initially reported by Rugge *et al*[61,62] 2010 and 2011 for both OLGA and OLGIM and more recently by the meta-analysis carried out by Yue *et al*[63] 2018 OLGA and OLGIM stages Type III/IV are consistently associated with increased risk of progression to GC. These findings suggest that high risk patients with OLGA/OLGIM stages type III/IV would benefit from close and frequent monitoring to detect neoplastic lesions at the earliest possible stage.

POINT OF NO RETURN

The Correa cascade is often referred to as a linear progression, however in the majority of patients there may be little to no change along the Cascade cascade over

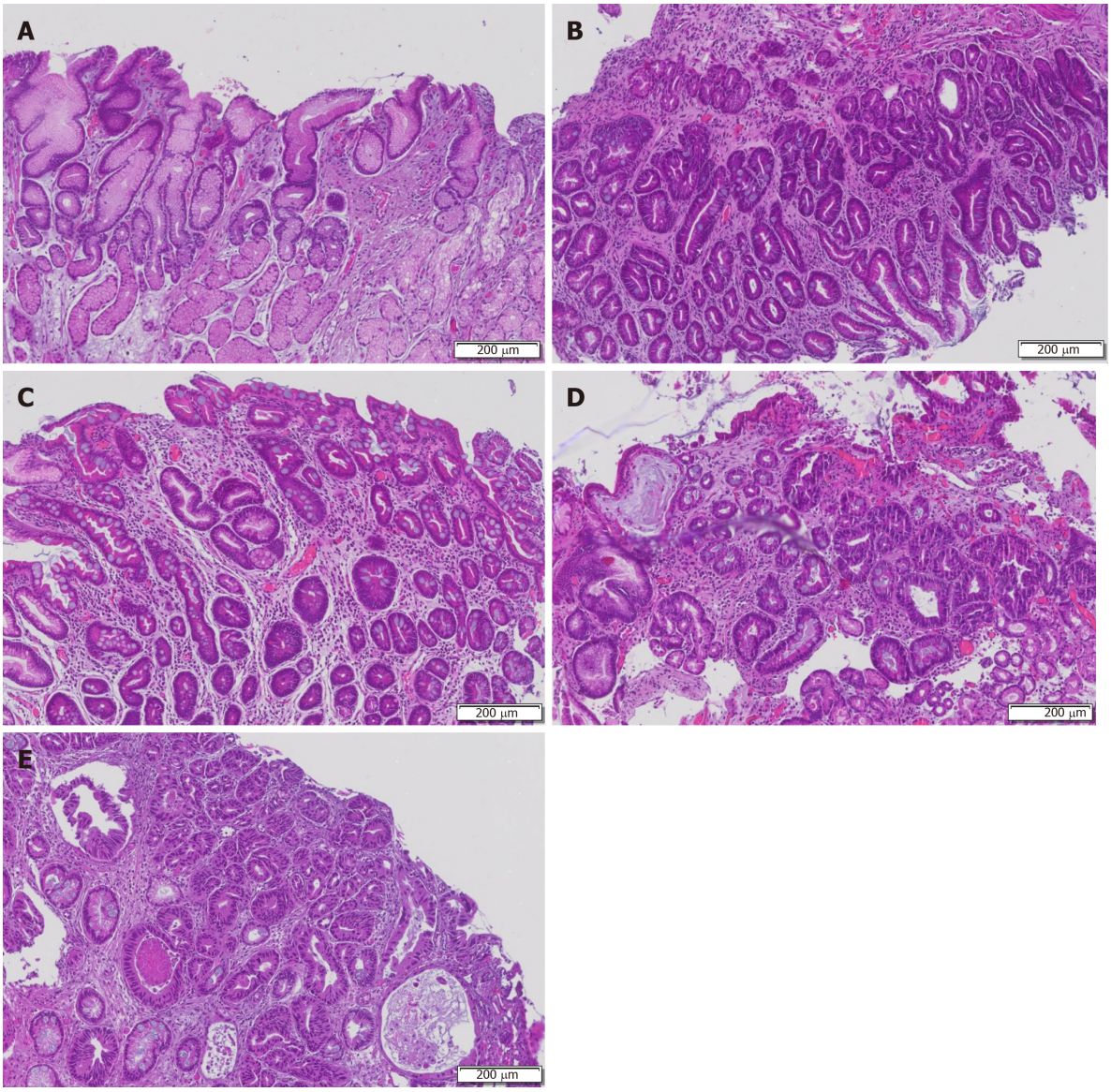

Figure 1 Complete or incomplete subtypes. A: Chronic gastritis with mucosal atrophy and lymphocytic infiltrate (asterix); B: Incomplete intestinal metaplasia resembling the colonic-type epithelium with irregular mucin droplets (arrowheads) and absence of a brush border; C: Complete intestinal metaplasia resembling the small intestinal epithelium with goblet cells alternating with eosinophilic enterocytes, brush border and Paneth cells; D: Low-grade dysplasia characterized by crowded glands with columnar cells and preserved polarity and pseudostratified nuclei; E: High-grade dysplasia with cuboidal cells, mitotic activity, prominent nucleoi, and high nuclear-cytoplasmia ratio.

many years. In other patients it can be a dynamic process with regression and/or progression of lesions, perhaps even rapid progression bypassing some of the putative stages. It is clearly evident that *H. pylori* infection and chronic inflammation in selected individuals causes progression of the cascade and it has been observed that successful eradication of *H. pylori* can lead to regression of histological features. There has been speculation that there is a point at which eradication is less effective at causing regression and indeed does not change the risk of progression in certain individuals. This has been referred as the "point of no return". *H. pylori* eradication results in complete resolution of histological inflammation and regression of atrophy in AG patients, with greater improvement seen in corpus AG compared to antral AG patients[64]. Unfortunately, the same effect is not seen in IM patients[64-67]. Once IM is established, eradication is only partially successful at reducing the risk of progression to GC. This suggests that IM may be the "point of no return" where genetic damage to gastric stem cells becomes irreversible. Although there is much evidence to support a point of no return, there has been evidence of regression from IM to AG or ChG in some cohorts[40,68]. A graphical summary of some of the larger IM progression studies is shown in Figure 2[40,68-73].

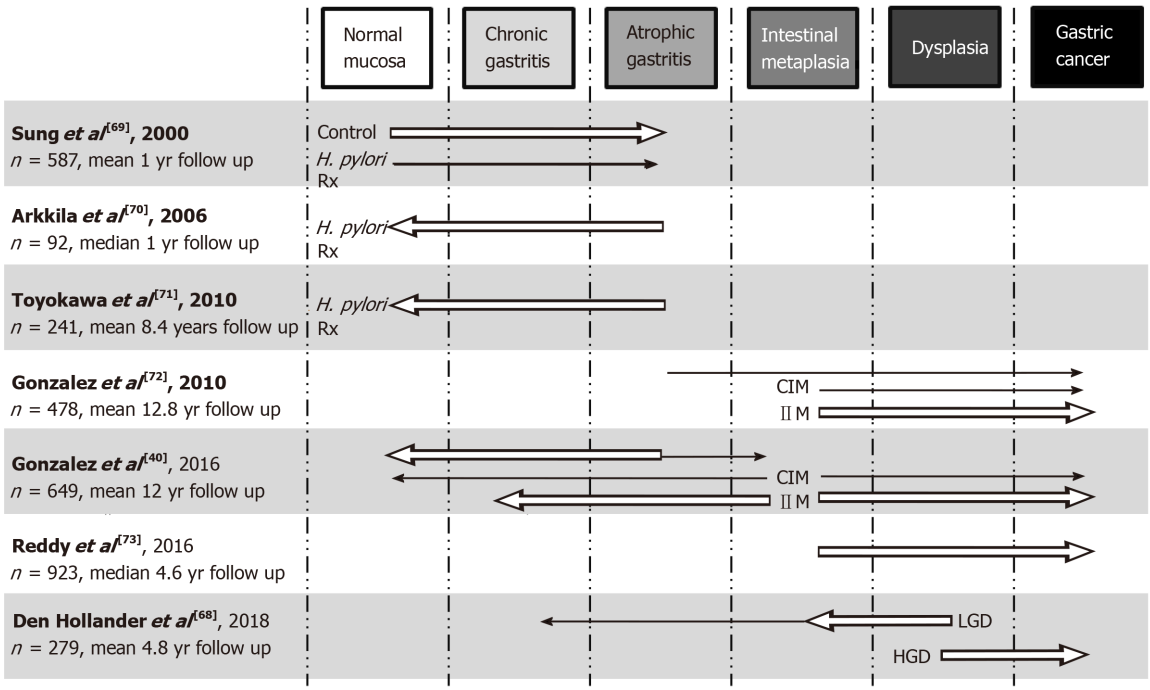

Figure 2 Graphical representation of selected large studies investigating progression/regression of premalignant gastric lesions across the stages of the Correa cascade. Arrows represent the direction of effect findings, with the size of the arrow the strength of effect (not to scale between cohorts and only major findings of trials represented). *H. pylori* Rx: *Helicobacter pylori* antibiotic therapy; CIM: Complete IM; IIM: Incomplete IM; LGD: Low-grade dysplasia; HGD: High-grade dysplasia; IM: Intestinal metaplasia.

SPASMOLYTIC PEPTIDE EXPRESSING METAPLASIA

Work from animal models of GC has introduced the concept of Spasmolytic peptide expressing metaplasia (SPEM). This is a cell lineage shown to be strongly associated with chronic gastritis in the fundus and gastric adenocarcinoma in animals[74]. It is often thought as an alternative metaplastic lineage to IM. SPEM is morphologically similar to Brunner's glands of the duodenum and expresses the trefoil spasmolytic polypeptide (SP or TFF2)[75]. It has been hypothesised that SPEM is an alternative precursor to GC and is associated with increased risk compared to IM[76]. Although SPEM is not a defined stage in the Correa cascade, it has been useful for studying the process of metaplasia formation in mice. To avoid confusion, SPEM is identified in the corpus and the fundus but not in the antrum as its characteristics are very similar to those of the deep antral and pyloric glands which also express TFF2. In mice infected with *Helicobacter felis*, SPEM develops after 6 to 12 mo of infection in the presence of active inflammation. First parietal cells are lost (oxyntic atrophy) and then the normal gastric lineages are replaced with metaplastic cells[77]. In two acute drug-induced SPEM models, with DMP-777 protonophore (abrogated inflammation) and L635 (prominent inflammation) as well as with *H. felis* infection in mice (chronic inflammation), it is suggested SPEM arises from the transdifferentiation of chief cells[77,78]. However more recently a study by Kinoshita *et al*[79] suggested that SPEM is the result of a regenerative process initiated by neck progenitor cells after chief cell loss. In another mouse model, SPEM in INS-GAS mice progressed to dysplasia after 1 year[80]. Following *H. pylori*-infection, Mongolian gerbils progressively develop ChG, followed by loss of parietal cells and metaplasia[81]. After 1 year of infection, SPEM is observed and mixed glands expressing both SPEM and IM are also seen. In humans there is growing evidence that suggests SPEM can either progress directly to dysplasia or become IM in the presence of continuous chronic inflammation[82]. These animal systems have been useful in studying the natural history of these lesions but it remains to be seen whether they are reliable models of the human condition.

THE ROLE OF THE IMMUNE MICROENVIRONMENT

We have continually reiterated the role of chronic inflammation in the development of

GC. In the context of a chronically inflamed microenvironment, there is some evidence that IM may arise due to the actions of specific immune cells. Using the murine model of L635-induced SPEM and following administration of clodronate, it was shown that macrophages are involved in the development of acute SPEM[83]. These macrophages were predominantly of the M2 subset (alternatively activated) and in the same study M2 macrophages were also shown to be increased in human SPEM and IM. Another prevalent immune cell in IM is neutrophils which were shown to be approximately 9-fold enriched compared to normal gastric tissue[84]. GC tissues were roughly 24 times enriched in neutrophils compared to normal gastric tissue. Thus macrophages and neutrophils may be vital immune cells required in the gastric microenvironment for SPEM and IM to develop and then to progress to GC. The role of the immune system in the process of gastric carcinogenesis has not yet been fully investigated.

CELLULAR AND MOLECULAR PATHWAYS OF PROGRESSION

IM progression to dysplasia and subsequently cancer occurs infrequently and the molecular mechanisms responsible for this progression are still not well understood. There are a number of challenges with studying this paradigm in view of the long duration over which these conditions progress, thus limiting prospective studies. This is compounded by the low rates of progression from each of the Correa stages and the potential confounder of tissue sampling when undertaking endoscopic follow up. Although the exact genomic or epigenomic pathways for IM progression to dysplasia are still being investigated, it is possible to postulate how certain events are necessary for progression by combining available data from a small number of key studies. It is known that: (1) IM is clonally derived from within the gastric mucosa[34,85]; (2) Gastric and IM glands divide by fission to form clonal patches[34,85,86]; (3) Over time, different gastric stem cells with accumulated genomic events (somatic mutations/chromosomal copy number gains and/or losses) can give rise to unique IM glands; (4) Further genomic changes may drive IM glands to proliferate or persist over a long period of time; (5) Dysplastic glands are formed that are genetically related to IM glands; entire dysplastic fields can share a foundation mutation[86] from which multiple subclones can result; and (6) This event can happen simultaneously in multiple regions of the stomach leading to an increased risk of GC developing across several locations and therefore providing a field cancerization effect.

To better understand how this process may unfold, studies on gastric adenoma (GA) and Barrett's oesophagus (BO) progression to GC and oesophageal adenocarcinoma (OAC), respectively, can be used as examples. In a recent study of gastric adenoma and paired GC from the same patients were used to determine clonal evolution. Clonal structure analyses showed that most GA/GC pairs exhibit parallel evolution with early divergence instead of a linear sequence of GA to GC progression[87]. Additionally, a small number of GC cases were clonally unrelated from paired GA suggesting the synchronous evolution of multiple clones that may progress to GC. BO is a premalignant intestinal metaplastic lesion that's often associated with gastro-oesophageal disease and predisposes patients to develop OAC[88]. Although exact values differ between studies, two population-based BO follow-up studies showed that the annual risk of progression of BO is 0.12%-0.14%[89,90]. BO has a higher mutation load (6.76 SNVs/Mb) than gastric IM but still lower than OAC (10.02 SNVs/Mb) and was shown to be polyclonal[91]. In one patient with BO, high grade dysplasia was shown to arise from multiple clones suggesting that the severity of intestinal metaplasia (a result of clonal expansion and cumulative molecular aberrations) may also play a key role in synchronous progression to GC[91].

Overall a holistic molecular approach is needed to elucidate the crucial events of how premalignant lesions actually cross the bridge to malignancy, a so-called "Pre-Cancer Genome Atlas"[92]. Using whole genome MBD-seq and RRBS analyses Kim *et al* showed that hypermethylation of gastrointestinal hormone receptors may play a key role in early gastric carcinogenesis[93]. Both gastrin and gastric acid secretion are thought to play important roles in cell differentiation and may play a part in creating a permissive environment or even directly involved in the process of carcinogenesis. A good summary review on the potential cellular and molecular pathways of gastric carcinogenesis was written by Rivas-Ortiz which added useful insight to this area[94]. It is very likely that GC is the result of multiple events co-occurring over time and space leading to various subtypes of GC (see TCGA molecular subtypes). If this is true, then it is also likely that differing sets of pre-cancerous events contribute to gastric carcinogenesis. Although some events may overlap across all GC subtypes *e.g.*,

chronic gastritis, others may be specific for a particular GC subtype *e.g.*, the breakdown of cellular mechanisms that keep diploidy intact leading to the chromosomal instability subtype.

HIGH RISK GENOMIC AND EPIGENOMIC ALTERATIONS IN IM

The median time for gastric intestinal metaplasia to progress to GC has been estimated to be 6.1 years, in contrast to low grade dysplasia which is only 2.6 years[95]. A recent study of genomic and epigenomic profiling of IM showed that IM has a low mutational burden compared to non-hypermutated GC (2.6 *vs* 6.9 mutations/Mb) and harbour recurrent mutations in certain tumour suppressor genes like FBXW7 (6/108 IM cases) but less in others, specifically TP53 and ARID1A (2/108 and 3/108 IM cases)[96]. However the presence of low frequency TP53 mutations in IM patients is in contrast to previous work within our group that showed an absence of TP53 mutations in IM samples paired with GC samples from the same patients[97]. A second finding of our study was that overexpression of p53 protein using immunohistochemistry has limited correlation to TP53 mutations. In the Huang *et al*[96] 2018 study, patients that progressed to dysplasia and GC had previously chromosome 8q amplifications and shortened telomeres. Interestingly, patients with IM that regressed had normal epigenomic patterns. DNA methylation profiling showed that the majority of IM patients in the high methylation group had relatively high mutational load, frequent chromosomal copy number variations and FBXW7 mutations and occurred mainly in the antrum.

LOW AND HIGH-GRADE DYSPLASIA DIFFER IN MUTATIONAL PATTERNS

The Padova classification was developed in 2000 to standardise histopathological reporting, which identifies five main categories for dysplastic lesions: (1) Negative for dysplasia; (2) Indefinite for dysplasia; (3) Non-invasive neoplasia; (4) Suspicious for invasive carcinoma; and (5) Invasive adenocarcinoma[98]. In practice, pathologists use categories 1 and 2 and subdivide category 3 as low (LGD) and high grade (HGD), the latter being associated with a higher risk of progression. A recent study using targeted deep DNA sequencing of 67 GC-related genes detected APC mutations in all LGD and also in some HGD cases[99]. However, APC and TP53 appeared to be mutually exclusive, the latter being present only in HGD and diminutive intramucosal GC (diameter < 10 mm). Analysis of tumor variant allele frequency suggested TP53 mutation is the initial event in TP53-mutated intramucosal GC. Importantly, this study suggested that linear evolution of LGD to HGD is rare and that early mutational events determine the evolution of dysplastic lesions. Early APC mutations lead to LGD whereas TP53 mutations lead to HGD which, following other genomic aberrations, subsequently evolve into early GC.

THE CORREA CASCADE AND DIFFUSE GC

Although there is considerable evidence of IM progressing to dysplasia and then to IGC, it is still debated whether any of the premalignant lesions that are part of the Correa cascade actually play a role in diffuse gastric carcinogenesis. In a prospective Japanese study, a proportion of patients that developed DGC had pangastritis (9/13), moderate to severe atrophy (9/13 and 1/13 respectively) and IM (8/13) at base line[100], suggesting a significant association between IM and DGC development[100]. A more recent study in South Korea showed that OLGA and OLGIM may have clinical utility in patients at risk of developing DGC[101]. Multivariate logistic regression analysis showed family history of GC, *H. pylori* infection, and OLGA/OLGIM stages III/IV were independent risk factors for both IGC and DGC[101]. Thus, atrophy and particularly IM likely play a dual role in gastric carcinogenesis dependent on context. If the right conditions are met, cellular and molecular changes within the stem cell compartment of these lesions lead to GC; if not, their presence creates a permissive environment for progression to GC to occur, possibly through hypochlorhydria and dysbiosis, and is also an indicator of increased patient progression risk. DGC may be the result of an "alternative" route to carcinogenesis, where a CDH1 mutation within the glandular stem cell compartment of a gastric gland or atrophic gland or even a

metaplastic gland/crypt produces a parent tumour cell.

Neuroendocrine cell dedifferentiation: An alternative route to GC?

An alternative paradigm to the stem cell theory as the tumour cell of origin has been gaining ground in recent years. In certain circumstances mature neuroendocrine cells may dedifferentiate, accumulate mutations and other genomic events and become tumour cells themselves[102]. Neuroendocrine tumours are the most likely results of such an event but also gastric adenocarcinomas. The enterochromafin like cell (ECL) is the main neuroendocrine cell in the oxyntic stomach, it produces and releases histamine and has gastrin receptors. Although the exact molecular and cellular mechanisms of this pathway to GC are not known, it is thought that loss of parietal cells and atrophy precedes cellular dedifferentiation of ECL cells. Thus, this pathway to GC may not fully follow the Correa cascade, going directly from an atrophic state to a hyperplastic, then dysplastic and then to either a neuroendocrine tumour or a gastric adenocarcinoma.

CONCLUSION

Our understanding of the molecular basis of GC and its premalignant lesions is accumulating rapidly, providing useful insights into the natural history of the disease (Figure 3). Knowledge remains lacking in many domains however, including the relationship between premalignant lesions and TCGA subtypes of GC. It could be the molecular changes characterising the TCGA GC subtypes represent disparate insults predisposing to initiation of the Correa cascade. Alternatively, the subtypes could represent accumulated "hits" following initiation with *H. pylori* infection. Insights into this pathway would stratify those at risk as well as inform prognosis and surveillance guidelines.

The observation that IM appears a relative point of no return along the Correa cascade, with only a small fraction of patients progressing to dysplasia and GC raises questions of the molecular determinants of progression. In the first study of its type, Huang *et al* laid the foundation for understanding this process, following an IM cohort for a minimum of 5 years and describing the molecular changes associated with progression in a large Chinese cohort. Validation of these findings in alternative cohorts is required for its use clinically.

In high-risk populations screening and surveillance has been successful in the early detection of GCs and improvements in 5-year survivals. However further work is required in low-risk populations to make strong evidence-based decisions regarding clinical screening or surveillance. In those known to have IM the risk factors discussed above should prompt the clinician to carefully consider surveillance including incomplete IM, dysplasia, extensive IM involving the corpus, male gender and those from high-risk ethnicities. The addition of molecular data such as TP53 mutation data, methylation patterns and chromosome 8 status would further improve risk-assessment algorithms, however larger population-based data is required for this to be accurate and practical.

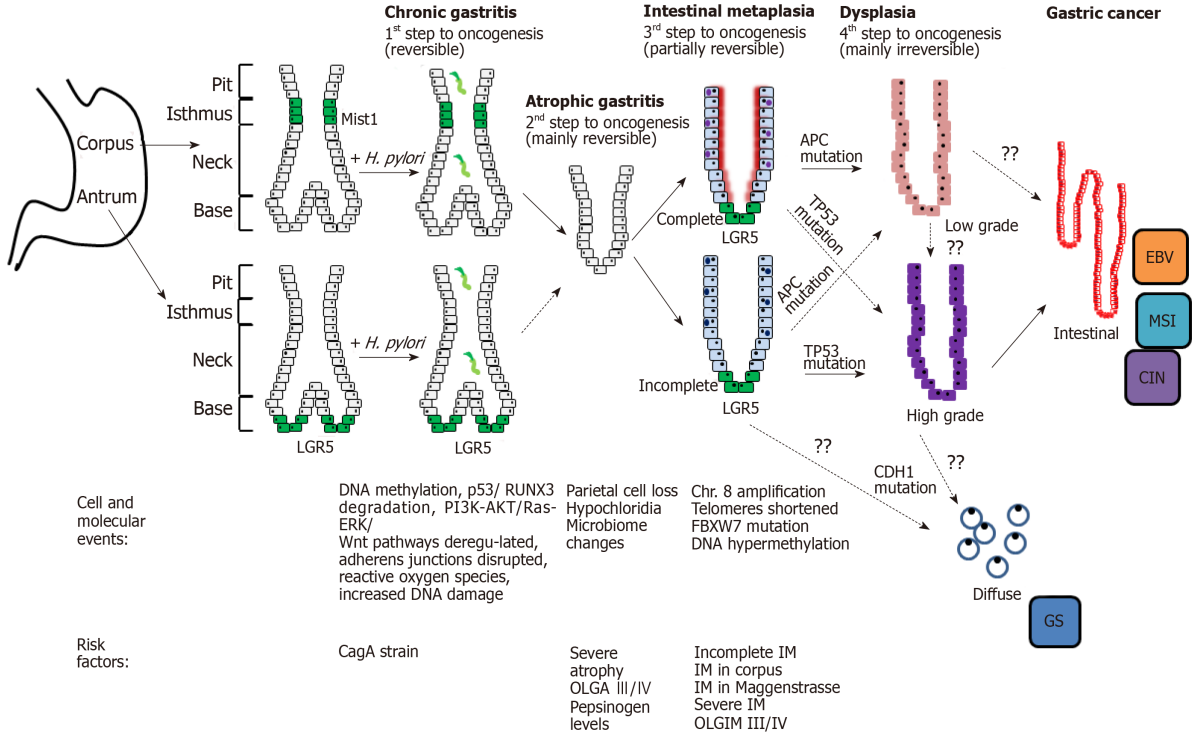

Figure 3 Summary of the cellular and molecular events associated with progression to cancer.*H. pylori*: *Helicobacter pylori*; EBV: Epstein Barr virus positive; MSI: Microsatellite unstable; CagA: Cytotoxin-associated gene A protein; IM: Intestinal metaplasia; GS: Genomically stable.

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REVIEW

Current status of adjuvant chemotherapy for gastric cancer

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Abstract

Although radical gastrectomy is a standard treatment for advanced gastric cancer, recurrence remains high. After several large-scale controlled studies have shown the beneficial effects of adjuvant chemotherapy, that treatment emerged as a standard option for advanced gastric cancer after gastrectomy. However, various guidelines from different countries have suggested different adjuvant chemotherapies. Understanding the differences between guidelines is very important for investigating further therapeutic strategies. Fortunately, because there are many ongoing studies about new regimens for adjuvant treatment, it is expected that patients with gastric cancer after surgery will have better outcome.

Key words: Gastric cancer; Adjuvant chemotherapy; Perioperative chemotherapy; Chemoradiotherapy; Guidelines

Core tip: There are differences in preferred adjuvant therapy for gastric cancer according to the guidelines from different countries. These include the National Comprehensive Cancer Network recommending chemoradiotherapy, the European Society for Medical Oncology recommending perioperative chemotherapy, and the Japanese recommending postoperative chemotherapy. Understanding the differences between guidelines can help in the future investigations of further regimens for adjuvant treatment of gastric cancer.

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INTRODUCTION

Although radical gastrectomy is a standard treatment for advanced gastric cancer, recurrence is a critical issue for long-term survival of patients. Gastric cancer is still one of the most common causes of death due to malignancy[1]. In the past, most researchers mentioned in their reviews that adjuvant treatment was not beneficial for improving survival after gastric cancer surgery. Some studies had found that adjuvant therapies did not improve the prognosis of patients with gastric cancer[2,3].

After the 2000s, several large-scale controlled studies showed beneficial effects of adjuvant chemotherapy on the survival of patients with gastric cancer[4,5]. These studies demonstrated better survival rates for patients who had received adjuvant therapies after gastrectomy for gastric cancer, as compared with patients who received surgery alone. Many subsequent studies also found the beneficial effects of adjuvant chemotherapy for patients with gastric cancer[6,7]. Therefore, adjuvant chemotherapy for gastric cancer has gained both research and clinical attention in the last 20 years, and it has become one of the standard treatment options for advanced gastric cancer after gastrectomy.

However, adjuvant therapies for gastric cancers are developed and studied separately among the various countries, without international guidelines. These various guidelines have suggested different adjuvant chemotherapies (Table 1). The differences among each might be the result of differences in race, epidemiology, etiologic factor, diagnostic tool, and clinical situation[8].

The purpose of this review is to summarize the previous studies about adjuvant chemotherapy for gastric cancer, describe the present treatment guidelines with regional differences, and discuss the ongoing studies and new regimens for adjuvant treatment of gastric cancer.

PREVIOUS STUDIES ON ADJUVENT CHEMOTHERAPY FOR GASTRIC CANCER

The SWOG INT-0116 trial by Macdonald *et al*[4] in 2001 was the first large-scale controlled study that provided positive results of adjuvant chemotherapy for gastric cancer. The investigators had made a comparison between surgery plus postoperative chemoradiotherapy and surgery alone for 556 patients with adenocarcinoma at the stomach or gastroesophageal junction. The results showed better overall and relapse-free survivals in the chemoradiotherapy group. However, that study had been criticized by many Asian researchers, in the aspect of low rates of D2 lymph node dissection. Most Asian surgeons believed that D2 lymph node dissection should be performed for locally advanced gastric cancer. Those authors have mentioned that results of the SWOG INT-0116 trial should be validated in patients who have undergone D2 lymph node dissection[9,10]. Despite its limitation, however, the SWOG INT-0116 trial has played a key role in furthering efforts to investigate postoperative therapy for gastric cancer. Its results are supported by several studies showing that postoperative chemotherapy plays a significant role in compensating undertreated surgery[11,12].

In 2006, the MAGIC trial showed that perioperative adjuvant chemotherapy using a triplet combination regimen produced a successful result in improving survival rates of patients with gastric cancer[5]. Ultimately, that trial supported a new concept in terms of using both preoperative and postoperative chemotherapy for gastric cancer. Neoadjuvant chemotherapy also has some advantages, such as relatively high dose with better compliance and down-staging before surgery[13,14]. After the MAGIC trial, perioperative chemotherapy became a standard treatment for locally advanced gastric cancer in the European society[15].

In Asian countries, radical gastrectomy with D2 lymph node dissection has been considered as a standard treatment for advanced gastric cancer applied earlier than that in Western countries. Therefore, many Asian studies have investigated postoperative adjuvant chemotherapy regimens after D2 lymph node dissection without radiotherapy or neoadjuvant therapy. The ACTS-GC trial has reported better survival rates with S-1 monotherapy in Japan[16,17]. The CLASSIC trial has shown beneficial effect of the capecitabine plus oxaliplatin chemotherapy regimen (XELOX) in Korea[18,19]. Both of these two regimens have been widely used in Asian countries for advanced gastric cancer after curative resection[20]. They have also been adopted in Western guidelines as a treatment option[15,21]. Kim *et al*[22] have reported that there are no differences in survival rates for patients with gastric cancer at earlier stages between these two chemotherapeutic regimens, although XELOX chemotherapy is more effective than S-1 for patients with higher stages of gastric cancer, including 3B

Table 1 Comparison of guidelines of adjuvant chemotherapy for gastric cancer

Guideline	Methods of adjuvant therapy	Regimen
NCCN	Postoperative chemoradiation (preferred)	5-FU plus irradiation
	Perioperative chemotherapy	ECF
		Modification of ECF
	Postoperative chemotherapy (only after D2 lymph node dissection)	XELOX
		Capecitabine plus cisplatin
ESMO	Perioperative chemotherapy (preferred)	ECF
		Modification of ECF
	Postoperative chemotherapy (patients without preoperative therapy)	S-1 monotherapy
		XELOX
	Postoperative chemoradiation (for undertreatment surgery)	5-FU plus irradiation
Japanese	Postoperative chemotherapy	S-1 monotherapy (preferred)
		S-1 plus oxaliplatin
		XELOX

5-FU: 5-Fluorouracil; ECF: Epirubicin, cisplatin, and 5-fluorouracil; ESMO: European Society for Medical Oncology; NCCN: National Comprehensive Cancer Network; XELOX: Capecitabine plus oxaliplatin.

and 3C.

GUIDELINES OF ADJUVENT TREATMENT FOR GASTRIC CANCER

There are several strategies for adjuvant treatments available, including chemoradiation, perioperative chemotherapy, and postoperative chemotherapy. Chemoradiation consists of intravenous 5-fluorouracil (5-FU) and irradiation being administered postoperatively. Oral administration of capecitabine is also accepted in combination with irradiation as an alternative to infused 5-FU[21]. Recommended regimens for perioperative chemotherapies are combination of epirubicin, cisplatin, and 5-FU (known as ECF) and its modification that includes the so-called ECX regimen of epirubicin, cisplatin, capecitabine, and EOX, which is epirubicin, oxaliplatin, capecitabine[15]. Postoperative adjuvant chemotherapies with S-1 monotherapy, or XELOX chemotherapy are accepted as treatment options for advanced gastric cancer after curative resection[20].

Because adjuvant treatments for advanced gastric cancer after gastrectomy were developed separately among countries, there are many differences in recommendations from the guidelines according to the various countries they were developed in.

National Comprehensive Cancer Network clinical practice guidelines

The National Comprehensive Cancer Network (NCCN) clinical practice guidelines provide standard treatment strategies for many cancers, including breast and colorectal cancers, for use not only in United States but also around the world[21]. Although the incidence of gastric cancer is low in the United States, its mortality is still high. Therefore, the NCCN guidelines for treatment of gastric cancer have some differences compared to others[23]. Although the NCCN guidelines agree that D2 lymph node dissection is associated with low mortality and reasonable survival benefit, they recommend gastrectomy with D1 or modified D2 lymph node dissection and emphasize that D2 lymph node dissection should be performed only by experienced surgeons because of its technical difficulty[24,25]. They recommend postoperative chemoradiation for cases that have not received preoperative chemotherapy, although the evidence of efficacy of using chemotherapy after R1 or R2 resection is unclear[26]. They also recommend perioperative chemotherapy, according to results from the MAGIC trial. For the postoperative chemotherapy, the NCCN guidelines state that it is difficult to apply results of the ACTS-GC and CLASSIC trials because D2 lymph node dissection is rarely performed in many of the United States cancer centers. They only include postoperative chemotherapy with XELOX as an option for cases that have undergone D2 or modified D2 dissection, with emphasis on chemoradiation[9]. S-1 is still regarded as an investigational agent in

north America.

European Society for Medical Oncology clinical practice guidelines

The European Society for Medical Oncology (ESMO) clinical practice guidelines suggest that D2 lymph node dissection should be performed for patients with stage IB-III gastric cancer. They recommend perioperative chemotherapy preferentially. Although fluoropyrimidine plus platinum-based doublet or triplet regimens are reasonable, combination of fluorouracil, epirubicin, and cisplatin is mostly recommended with strong evidence[27]. The ESMO guidelines recommend postoperative chemoradiotherapy or chemotherapy for patients who have undergone gastrectomy without preoperative therapy. Postoperative chemotherapy has been adopted according to results from the ACTS-GC and CLASSIC trials. Although postoperative chemoradiotherapy is a standard treatment in the United States, they state that this therapeutic option has not gained acceptance in Europe because of toxicity and difference in surgical quality. However, they recommend chemoradiotherapy in the case of suboptimal surgery with less lymphadenectomy or suspicious micrometastasis[26,28].

Japanese gastric cancer treatment guidelines

The Japanese gastric cancer treatment guidelines suggest that standard gastrectomy is curative gastric resection with D2 lymph node dissection for stage 1B or higher gastric cancer[20]. They recommend postoperative chemotherapy with S-1 monotherapy preferentially because the efficacy of S-1 has been proven in Japan. Although the CLASSIC trial showed good result from XELOX, oxaliplatin has not been approved for gastric cancer in Japan. After Japanese studies with oxaliplatin were published, combination therapy of capecitabine or S-1 plus oxaliplatin was adopted as an option for postoperative chemotherapy[29,30]. Because curative radical gastrectomy with D2 lymph node dissection has been the standard treatment in Asian countries for a long time, the Japanese guidelines did not mention radiotherapy as an adjuvant treatment at all, even for cases of noncurative resection.

ONGOING STUDIES AND NEW REGIMENS FOR ADJUVENT CHEMOTHERAPY

During the last 20 years, a variety of adjuvant therapies for gastric cancers have been investigated. Although the development of adjuvant treatment has been different among countries, there are several efforts to adopt different therapeutic strategies from the others. Most importantly, surgical treatment has become more standardized; for example, the European society has already accepted D2 lymph node dissection as a standard treatment. The NCCN clinical practice guidelines have also provided recommendation of D2 dissection in certain situations[15,20,21].

Today, several ongoing Asian studies, in Korea and China in particular, are investigating the efficacy of perioperative chemotherapy and/or postoperative chemoradiotherapy[31]. Studies investigating new regimens of combination with S-1 are ongoing. In Japan, the safety and feasibility of S-1 plus cisplatin as an adjuvant chemotherapy has been proven[32], and a phase II study of S-1 plus oxaliplatin as combination therapy has also been approved[30]. In China, a large-scale randomized controlled trial (referred to as the RESCUE-GC) is ongoing, to investigate the efficacy of S-1 plus oxaliplatin as adjuvant therapy for gastric cancer[33]. There is also a phase III study evaluating the significance of preoperative chemoradiation therapy for locally advanced gastric cancer. This study is designed to investigate addition of radiotherapy to the MAGIC trial[34].

After the ToGA trial found survival gain for advanced gastric cancer with trastuzumab combination[35], the significance of combination therapies with molecular target agents gained much interest in the field of adjuvant treatment. However, targeted agents have failed to show their efficacies as adjuvant treatment for gastric cancer. The ST03 trial showed no significant difference between the group who received chemotherapy alone and the group who received combination of chemotherapy plus bevacizumab but did find an increased rate of anastomotic leakage in the combination group[36]. To date, several studies are ongoing to investigate efficacies of target therapeutic agents as adjuvant treatment for gastric cancer[31].

Although adjuvant treatments have been developed and are widely used, recurrence and metastasis are still critical problems for survival of patients after gastrectomy. Drug resistance is one of the most important causes of therapeutic failure in gastric cancer patients. Although many researchers have studied the

mechanisms of drug resistance in gastric cancer, the regulation of these mechanisms has not been completely elucidated. Recently, several researchers have reported precise mechanisms of chemoresistance in gastric cancer and showed the possibility of advances in prediction of failure of chemotherapeutic agents[37,38]. These efforts can lead to the future development of individual therapeutic plans for patients with gastric cancer and novel strategies to overcome chemoresistance.

CONCLUSION

After the development of adjuvant chemotherapy for gastric cancer, there has been a years-long steady improvement in survival after gastric cancer surgery. In accordance with the many ongoing studies investigating new regimens as adjuvant therapy for gastric cancer, better prognosis of patients after surgery is expected in the future.

Because there are several differences in the various national and regional guidelines, it is very important to know and understand their differences and to make an effort to provide better treatment strategies by communication among each other. Unfortunately, this review cannot suggest the best strategy for the patients with gastric cancer; this is due to the results of studies between Western and Asian countries being difficult to compare directly in the present situation. For further advances and worldwide consensus in adjuvant treatment of gastric cancer, new studies are warranted, including studies about racial or genetic differences in patients with gastric cancer, worldwide studies to determine surgical and therapeutic standards, and studies to investigate the mechanisms of the oncology of gastric cancer.

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REVIEW

Tumor progression-dependent angiogenesis in gastric cancer and its potential application

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Abstract

Despite improvements in the early diagnosis, prognosis and therapeutic strategies for gastric cancer (GC), human GC remains one of the most frequently diagnosed malignant tumors in the world, and the survival rate of GC patients remains very poor. Thus, a suitable therapeutic strategy for GC is important for prolonging survival. Both tumor cells themselves and the tumor microenvironment play an important role in tumorigenesis, including angiogenesis, inflammation, immunosuppression and metastasis. Importantly, these cells contribute to gastric carcinogenesis by altering the angiogenic phenotype switch. The development, relapse and spreading of tumors depend on new vessels that provide the nutrition, growth factors and oxygen required for continuous tumor growth. Therefore, a state of tumor dormancy could be induced by blocking tumor-associated angiogenesis. Recently, several antiangiogenic agents have been identified, and their potential for the clinical management of GC has been tested. Here, we provide an up-to-date summary of angiogenesis and the angiogenic factors associated with tumor progression in GC. We also review antiangiogenic agents with a focus on the anti-vascular endothelial growth factor receptor (VEGFR)-mediated pathway for endothelial cell growth and their angiogenesis ability in GC. However, most antiangiogenic agents have reported no benefit to overall survival (OS) compared to chemotherapy alone in local or advanced GC. In phase III clinical trials, only ramucirumab (anti-VEGFR blocker) and apatinib (VEGFR-TKI blocker) have

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reported an improved median overall response rate and prolonged OS and progression-free survival outcomes as a 2nd-line agent combined with chemotherapy treatment in advanced GC. By providing insights into the molecular mechanisms of angiogenesis associated with tumor progression in GC, this review will hopefully aid the optimization of antiangiogenesis strategies for GC therapy in combination with chemotherapy and adjuvant treatment.

Key words: Gastric cancer; Angiogenesis; Vascular endothelial cell; Angiogenic phenotype switch; Anti-angiogenesis; Tumor dormancy

Core tip: Tumor angiogenesis in gastric cancer (GC) and antiangiogenic therapies for GC, including information from their preclinical and/or application to clinical trials, are discussed. The antiangiogenic strategies for advanced GC include decreasing the expression of proangiogenic ligands and their receptors, increasing the level of angiogenic inhibitors, and directly targeting the inner walls of endothelial cells. Here, the antiangiogenic strategies mainly focus on decreasing the expression of vascular endothelial growth factor-mediated pathway constituents for advanced GC in phase III clinical trials. Thus, this review provides a brief description of various tumor angiogenic factors for the purposes of diagnosis, prognosis and therapeutics and describes the antiangiogenic agents that are currently being investigated in preclinical and phase III clinical trials. Hopefully, according to the molecular mechanism of tumor angiogenesis, we highlight the accuracy of the diagnosis and prognosis and the selection of the most appropriate therapy for GC patients.

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INTRODUCTION

Gastric cancer (GC) has a high incidence throughout the world and a high mortality rate associated with malignant tumors[1-3]. GC might not cause any clinical symptoms at the early stage, resulting in the fact that GC is rarely detected at the early stage[2,3]. However, the five-year survival outcome for late-stage GC patients is only approximately 20%-30% after initial diagnosis[4], and gastrectomy is the major common treatment for GC. Thus, to improve the low survival outcome, it is necessary to develop novel therapeutic strategies for GC[5].

In recent decades, studies on the molecular mechanism of tumor development have focused on the genetic or epigenetic changes in tumor cells, such as the emergence of cancer stem cells (CSCs), epithelial-mesenchymal transition (EMT) and the expression of microRNAs (miRNAs)[6]. However, several studies conducted in recent years found that the tumor microenvironment (TME) strongly influences tumor growth and progression and revealed that the tumor-host interactions determine tumor progression[7,8]. The TME contains extracellular matrix and stromal cells, including ECs, tumor-associated fibroblasts and tumor-associated immune/inflammatory cells, which can regulate tumor progression through autocrine/paracrine cytokines or factors. Furthermore, cancer cells can support the angiogenesis of ECs, and ECs can also help cancer cell proliferation by releasing growth factors. Tumor-associated immune/inflammatory cells can control cancer cell proliferation and metastasis under different conditions, and cancer cells might induce immune cell dysfunction as well as proinflammatory cytokine release. Exosomal miRNAs can alter normal fibroblasts into TAFs for tumor survival, and TAFs can promote tumor proliferation and metastasis. Thus, the TME is also involved in multiple processes, including tumor angiogenesis, inflammation, immunosuppression and metastasis, as shown in Figure 1[9,10].

In 1971, Dr. Folkman and Klagsbrun[11] provided a novel theory stating that all phases of rapid tumor growth are dependent on tumor angiogenesis. At present, it is known that tumor angiogenesis plays a key role in tumor progression, and the

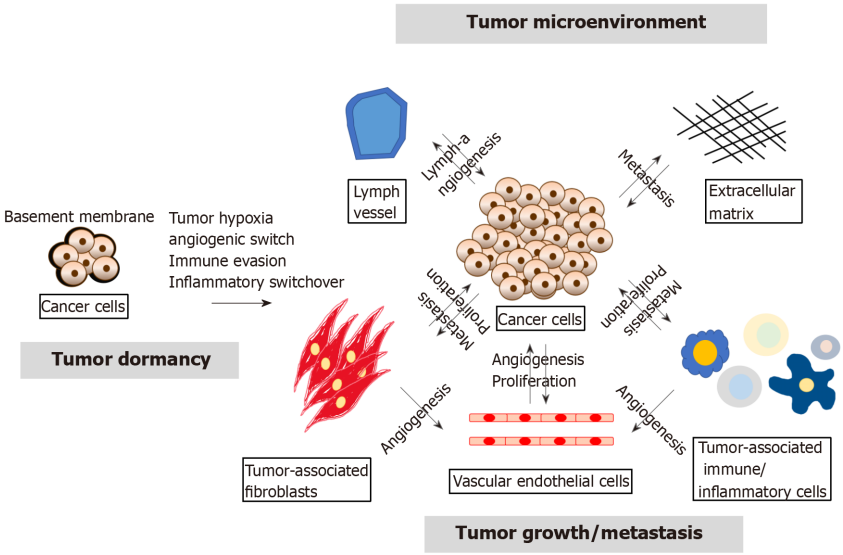

Figure 1 The tumor microenvironment regulates tumor growth, relapse and metastasis. Tumor dormancy can be induced in malignant cancer through several mechanisms, such as epigenetic or genetic changes (cancer stem cells, epithelial-mesenchymal transition, and miRNAs) in the tumor, tumor hypoxia, the angiogenic switch, immune evasion and inflammatory switchover. A change in the tumor microenvironment can facilitate tumor growth/relapse/metastasis and thereby permit the tumor to exit from dormancy through interaction with endothelial cells, tumor-associated fibroblasts, tumor-associated immune/inflammatory cells and the extracellular matrix.

angiogenic switch is necessary for tumor growth, relapse and metastasis. Herein, we provide a review of tumor-associated angiogenesis, explore the molecular regulation of angiogenesis, and discuss various antiangiogenic drugs and their potential applications based on preclinical and phase III clinical trials for GC.

MOLECULAR REGULATION OF TUMOR ANGIOGENESIS IN GC

An increasing number of studies has revealed that tumor growth is strongly associated with tumor angiogenesis[12]. Tumor growth, relapse and metastasis should turn on the "angiogenic switch" to induce tumor growth to a size greater than 1-2 mm. Numerous signals (*e.g.*, epigenetic changes, the TME, CSCs, EMT, and miRNAs) can disturb tumor dormancy, resulting in local tumor proliferation/recurrence or metastasis at a secondary site[13]. The "angiogenic switch" is regulated by angiogenic activators and inhibitors[14,15], and the timing of the "angiogenic switch" can occur before, during or after tumor progression. As will be discussed in the following sections (Table 1), recent studies have shown that the available knowledge on the induction and molecular regulation of tumor angiogenesis has grown rapidly, and several growth factors, growth factor receptors, cytokines and signaling pathways have been identified in GC.

TRANSCRIPTION FACTORS

Hypoxia and hypoxia-inducible factor

Preclinical trial: First, the basement membrane in growing tumor cells is injured locally, and tumor cells immediately experience destruction and hypoxia. Tumor hypoxia is a major force that triggers tumor angiogenesis and activates the expression of hypoxia-inducible factor-1 (HIF-1), which then induces the expression of various proangiogenic factors, including vascular endothelial growth factor (VEGF) and vascular endothelial growth factor receptor (VEGFR), in cancer cells[16-19]. Moreover, HIF-2 isoforms have similar functions as HIF-1, but HIF-2 mainly activates the expression of erythropoietin (EPO) in kidney and liver cells[20]. Overall, HIF-1 is known as a potential target of anticancer therapy in many cancers[21]. In addition, treatment with HIF-1-specific inhibitors has been studied in animal models, and it has been shown that this treatment results in slowed growth of tumors, decreased

Table 1 Regulators of tumor angiogenesis in gastric cancer and their use in antiangiogenic therapy

Biological category	Gene name	Regulator of pro-/anti angiogenic types	Antiangiogenic drug	Drug direct target	Preclinical trials; cell line (*in vitro*)/animal (*in vivo*)	Clinical application	
						Expression levels in GC patients	Prognostic factors (proangiogenic biomarker)
Transcription factor	Hypoxia HIF[16-19,22-25]	Activator	NSAID[82]	COX-1, COX-2 inhibitor	•	ND	ND
Growth factor	VEGF family[26-38]	Activator	Aflibercept[22]	Anti-VEGF-A	•	VEGF-A, C overexpression[21,41-47]	Lymph node metastasis (VEGF-A, C) Distant metastasis (VEGF-A) Poor survival (VEGF-A)
				Anti-PIGF			
			Bevacizumab[83-89]	Anti-VEGF-A	•	ND	ND
			IFN[90]	Anti-IFNR	•	ND	ND
			Rapamycin[91]	Anti-rapamycin kinase	•	ND	ND
			Neovastat[92]	Anti-VEGF	•	ND	ND
	PIGF[29,30,35,48]	Activator	Aflibercept[22]	Anti-VEGF-A	•	PIGF overexpression[49-51]	ND
				Anti-PIGF			
	FGF, EGF, HGF, IGF[31,52-55]	Activator	IFN[93]	Anti-IFNR	•	ND	ND
	PDGF[56,57]	Activator	SU6668	Multiple receptor TKI	•	ND	ND
			Orantinib[94]				
Growth factor receptor	VEGFR[32,33]	Activator	Ramucirumab[95-97]	Anti-VEGFR2	•	ND	ND
			Regorafenib[98,99]	VEGFR TKI	•	ND	ND
			Apatinib[98,99]	VEGFR TKI	•	ND	ND
			Foretinib[98,99]	VEGFR TKI	•	ND	ND
			SU5416 SU6668 Orantinib[94]	Multiple receptor (KDR/FGFR/PDGFR)	•	ND	ND
			Pazopanib[100]	Multiple receptor TKI	•	ND	ND
			Sorafenib (Nexavar)[101,102]	Multikinase inhibitor (the serine/threonine kinase Raf and receptor tyrosine kinases)	•	ND	ND
			Sunitinib (Sutent)[103,104]	Multitargeting TKI	•	ND	ND
			Telatinib Erbitux (Cetuximab)[105]	Multitargeting TKI	•	ND	ND
	GP130 IL-6R[58]	Activator	ND	ND	•	ND	ND
	Her2/Neu[59-62]	Activator	Trastuzumab[59,62]	Anti-Her2/Neu	•	ND	ND
Cytokine	Ang-1,3,4[63,64,66-73]	Activator	ND	ND	•	Ang-1,2 overexpression[74-77]	Lymph node metastasis Liver metastasis Poor survival
	Ang-2[65,66]	Activator Inhibitor					
	IL-6 [58]	Activator	ND	ND	•	ND	ND

	IL-8[37,106]	Activator	ND	ND	•	ND	ND
	IL-17[78]	Activator	ND	ND	•	ND	ND
	Tryptase[79,80]	Activator	ND	ND	•	Tryptase overexpression[81]	ND
ECM	MMP[92],[107,108]	Activator	Marimastat[107,108] Bay 12-9566 AG3340 Neovastat[92]	MMP inhibitor	•	ND	ND

GC: Gastric cancer; ND: Not determined; •: Determined; VEGF: Vascular endothelial growth factor; VEGFR: Vascular endothelial growth factor receptor; TKI: Tyrosine kinase inhibitor.

angiogenesis and minor vessel maturation[22]. Stoeltzing *et al*[23] obtained similar results using the dominant negative form of HIF-1 in GC. Chronic infection with *Helicobacter pylori* induces DNA damage by generating reactive oxygen species (ROS) in GC cells[24]. Overaccumulation of ROS might stimulate HIF-1 accumulation and aid tumor angiogenesis in GC[25].

PROANGIOGENIC LIGANDS AND RECEPTORS

VEGF family

Preclinical trial: Growing cancer cells encourage the growth of new blood vessels by secreting VEGF and VEGFR into the surrounding TME, and secreted VEGF binds to VEGFR on the outer surface of ECs. ECs are activated by the VEGF signaling pathway, and this activation induces the growth, survival, vascular permeability and migration of ECs to encourage tumor angiogenesis[26]. To date, various cytokines and a major proangiogenic factor of ECs have been found to be members of the VEGF-A family. The VEGF (homodimers) family of growth factors contains VEGF-A, B, C, D and E and placental growth factor (PlGF), and during angiogenesis[27,28], these growth factors bind to and activate the tyrosine kinase receptors (TKRs) VEGFR-1, VEGFR-2, and VEGFR-3, which are specifically expressed on the surface of ECs and have different affinities for the ligands. Consequently, the downstream TKR signaling proteins activate proliferation-mediating signaling pathways, such as the phosphatidylinositol 3 kinase (PI3K)/AKT, protein kinase C (PKC), and mitogen-activated protein kinase (MAPK; p38 and p42/44) pathways[29-31]. In general, VEGF-A binds to VEGFR-1 and VEGFR-2, PlGF and VEGF-B bind to VEGFR-1, and VEGF-C and VEGF-D bind to VEGFR-2 and VEGFR-3[32-34]. Carmeliet *et al*[35] reported that among the VEGFs, the *vegfa* gene can lead to embryonic lethality due to serious vascular defects after the loss of only a single allele in mice[34-36]. An *in vitro* tube formation assay using GC cells cocultured with human umbilical vein endothelial cells (HUVECs) demonstrated proangiogenesis function due to the upregulation of VEGF in GC cells[37]. In a rat model, the blockage of VEGF by a specific siRNA led to reduced proliferation and cell cycle arrest[38]. Moreover, the coreceptor of neuropilins in signaling pathways is activated by other growth factors or VEGFs, and neuropilins bind several growth factors and enhance their function; however, the molecular mechanisms affected by neuropilins remain unclear[39,40]. The above data indicate that GC cells possess proangiogenic abilities by secreting angiogenic cytokines to both stimulate ECs and to support their own growth in an autocrine manner. Furthermore, the growth and invasion of GC cells are mainly controlled by the VEGF-mediated pathway.

Clinical application: These discoveries from *in vitro* and animal models were confirmed in GC patients, and their diagnostic or prognostic abilities were tested in GC patients. Through ELISA, significantly higher preoperative plasma or serum VEGF levels were detected in GC patients compared with healthy control subjects. Importantly, a clinicopathological analysis revealed that higher VEGF expression in the plasma or serum of GC patients was significantly associated with advanced stage, distant metastasis and worse survival outcomes[21,41-47].

PlGF

Preclinical trial: PlGF is another member of the VEGF family and plays a proangiogenic role in the progression of some tumors[29,30,35,48]. Akrami *et al*[49,50] reported that the knockdown of PlGF in AGS and MKN-45 cells inhibited the

proliferation, self-renewal capacity, MMP activity, transcription activity and migration of these cells.

Clinical application: Higher PIGF and VEGF levels were detected by ELISA in GC tissues compared with paired noncancerous mucosa tissues. A clinicopathological analysis showed that higher expression of only PIGF in GC patients was significantly associated with tumor stage, distant metastasis and worse survival outcomes [51].

Fibroblast growth factors, epidermal growth factor, hepatocyte growth factor, and insulin-like growth factor

Preclinical trial: The fibroblast growth factor (FGF) family is a large cytokine family, and some of these cytokines, *e.g.*, FGF-1/-2, bind to different fibroblast growth factor receptors, *e.g.*, FGFR 1-4, to activate the PI3K/AKT/mTOR (mammalian target of rapamycin) pathway. Furthermore, these cytokines can regulate tumor angiogenesis, proliferation, migration and antiapoptosis/survival activities both *in vitro* and *in vivo*[31,52-54]. epidermal growth factor (EGF), hepatocyte growth factor (HGF) and insulin-like growth factor (IGF) reportedly stimulate proangiogenic, proliferation and survival activities similarly to those induced by VEGF[55].

Platelet-derived growth factor

Preclinical trial: Pericytes and smooth muscle cells secrete platelet-derived growth factor (PDGF)-BB, which then binds to PDGFR-β and thereby modulates tumor angiogenesis in ECs[56,57].

GP130, interleukin-6, and interleukin-6R

Preclinical trial: In a mouse model, the blockage of GP130 inhibits tumor development in the epithelium of the glandular stomach *via* the STAT 1/3-mediated angiogenesis pathway. These results suggest that the TME and cancer cells secrete interleukin-6 (IL-6) *via* autocrine or paracrine binding to GP130 or IL-6R[58].

Human epidermal growth factor receptor 2/Neu (HER-2/neu) and EGFR

Preclinical trial: In tumor cells, EGF binds to EGFR and HER-2/neu to activate the PI3K/AKT and RAS-MAPK-mediated pathways, which are involved in the overexpression of VEGF-A. The secretion of VEGF-A from cancer cells can be mediated through the activation of various signaling pathways. Furthermore, these factors act as central regulators of tumor growth and tumor angiogenesis in GC[59-62].

Angiopoietin-1, 2, 3, and 4 (Ang-1, -2, -3, and -4)

Preclinical trial: Ang-1, -2, -3, and -4 biologically serve as growth factors for ECs and can strongly regulate competitive interaction with TIE-2 (TKR), which is expressed on the surface of ECs[63,64]. The binding of Ang-1 to TIR-2 activates TIE-2 phosphorylation via the Ang-1/Tie2-cascade pathway and is involved in the proliferation, migration, inflammation and survival of ECs. Ang-2 is then released from activated ECs and serves as a significant antagonist[65,66]. Additionally, TIE-1 (an orphan receptor) can form a complex with TIE-2 to form heterodimers and compete with Ang-1/TIE-2 interactions and thereby promote inflammation in ECs[66-69]. Inhibition of Ang-1 or Ang-2 shows similar inhibition of cell proliferation in GC cell lines[70-73].

Clinical application: Blank *et al*[74] found that high expression levels of Ang in serum and tissue from GC patients are associated with poor survival. In addition, the Ang/VEGF ratio in GC and esophageal cancer patients serves as an independent proangiogenic biomarker for the clinical response to chemotherapy[75]. Another group of researchers found that Ang-2 can serve as an independent predictor of OS and liver metastasis in GC patients[76]. Moreover, Aktaş *et al*[77] found that VEGF, PIGF, and Ang-1 are strongly correlated with OS; thus, these angiogenesis prognostic indices (APIs) could predict survival outcomes in GC patients.

IL-8

Preclinical trial: Tumor-infiltrating macrophages secrete IL-8 and upregulate VEGF to activate EC angiogenesis in GC, as demonstrated in an *in vitro* assay[37].

IL-17

Preclinical trial: IL-17 stimulates the STAT3-mediated angiogenesis pathway to upregulate VEGF in GC[78].

Tryptase

Preclinical trial: Tumor-infiltrating mast cells (TIMs) secrete tryptase by binding to proteinase-activated receptor-2 (PAR-2) and then produce VEGF to stimulate tumor angiogenesis and EC proliferation, as demonstrated through *in vitro* and *in vivo*

assays[79,80].

Clinical application: TIMs can release tryptase *via* PAR-2 activation and are involved in tumor angiogenesis. Ammendola *et al*[81] suggested that an increased mast cell density positive for tryptase (MCDPT) and a higher general vascularized area are related to poor survival outcome and can thus serve as potential targets in both primary tumor and lymph node metastases in GC patients.

RESULTS FROM PRECLINICAL AND CLINICAL STUDIES OF ANTIANGIOGENIC THERAPIES FOR GC

According to the results of studies on the molecular mechanism of tumor angiogenesis, we can develop a novel antiangiogenic strategy that could reduce tumor angiogenesis and limit tumor growth instead of eradicate the tumors and thereby delay the progression of precancer/primary lesion to metastases/aggressive cancers. The purpose of antiangiogenesis therapy is not to directly target cytotoxic tumor cells but rather block the supply of oxygen, growth factors and nutrition from blood vessels[109]. Thus, this section will focus on several tumor angiogenic factors that could serve as potential targets for antiangiogenic drugs that are currently being investigated in preclinical (the section only highlights the most common antiangiogenic drugs; Table 1) and clinical studies on GC patients. Due to the metabolic changes and stemness of malignant cells lacking oxygen supply in various tumors, tumors appear to escape antiangiogenic therapy within a short time owing to the manipulation of alternative pathways[110], vasculogenic imitation[111] and recruitment of bone marrow-derived cells[112,113]. Various clinical trials have not shown a statistically significant extension of survival outcomes. Thus, most of the antiangiogenesis strategy can be ineffective. In phase III clinical trials, only ramucirumab (anti-VEGFR) and apatinib (VEGFR-TKI) have reported to improve ORR and prolong OS and PFS outcomes when used as a 2nd-line regimen combined with chemotherapy treatment in advanced GC (Table 2).

INHIBITORS OF PROANGIOGENIC LIGANDS AND RECEPTORS

Bevacizumab (avastin, genentech, rhumad)

Preclinical trial: As demonstrated in a preclinical model, this drug, which is a recombinant monoclonal antibody against VEGF-A, serves as a powerful and effective antiangiogenesis agent in several cancers[83-85]. An *in vitro* study revealed that treatment with bevacizumab reduced cell growth and pro-apoptosis in GC cell lines[86]. Yamashita-Kashima *et al*[87] performed an *in vivo* study and found that bevacizumab could be effective against GC and select biomarkers in the MKN-45 human gastric xenograft model. A study with mouse models revealed that treatment with bevacizumab significantly reduced the tumor size[88,89]. In the future, we will explore the effects of the antibody-mediated blockage of VEGF-mediated tumor angiogenesis in GC to obtain a more in-depth understanding.

Clinical trial: Ohtsu *et al*[114] explored the effect of bevacizumab, which is a VEGF blocker. The AVAGAST clinical trial indicated that the 1st line treatment of advanced GC patients (multiethnic population) with bevacizumab in combination with chemotherapy (Cisplatin; Cis/Capecitabine; Cap) resulted in significantly improved ORR (P = 0.0315) and extended PFS (P = 0.0037) outcomes compared with those achieved with chemotherapy alone (Table 2). However, the AVATAR clinical trial showed that the 1st line treatment of advanced GC patients (China) with bevacizumab in combination with chemotherapy (Cis/Cap) did not significantly prolong the survival outcomes compared with those achieved with chemotherapy alone[115]. In contrast, Ma *et al*[116] assessed the effects of bevacizumab in combination with chemotherapy (Docetaxel; Doc/Oxaliplatin; Oxa/5-FU) compared with those of the 1st line treatment of chemotherapy alone in advanced GC patients (China) and observed significantly improved ORR (P = 0.0436) and extended PFS (P = 0.013) outcomes compared with those achieved with chemotherapy alone. The other group, the ST03 clinical trial, showed that the perioperative treatment of advanced GC patients (United Kingdom) with bevacizumab in combination with chemotherapy (Cis/Cap/Epirubicin; Epi) had no positive results compared with those achieved with chemotherapy alone[117]. However, the differences in the outcomes achieved after bevacizumab treatment among the different populations remain unknown.

Table 2 Overview of phase-III clinical trials in gastric cancer including vascular endothelial growth factor, vascular endothelial growth factor receptor and vascular endothelial growth factor receptor tyrosine kinase inhibitor blockers

Tar-get C Cate-gory	Blockers	Country	Cancer type	Setting	Treat-ment	N	ORR (%)	DCR (%)	PFS (mo)	OS (mo)	Top 5 adverse events	Ref.
							HR (95% CI)*P* value	*P* value	HR (95% CI)*P* value	HR (95% CI)*P* value		
Anti-VEGF	Bevaci-zumab (Mono-clonal Ab)	Multieth-nic	•Metasta-tic GC	1st-line	Bevaci-zumab	387	46%	76.90%	6.7	12.1	Neutro-penia	AVA-GAST[114]
		Asia-Pacific	Unresec-table locally advanced GC		+Cis/Cap						Febrile neutrope-nia	
		Europe	Recurrent GC								Anemia	
		Pan-America	Gastro-esophagea l junction GC		Placebo	387	37.40%	67.70%	5.3	10.1	Decreased appetite	
					+Cis/Cap		*P* = 0.0315	ND	*P* = 0.0037	*P* = 0.1002	Diarrhea	
		China	Metastatic GC	1st-line	Bevacizu mab	100	40.70%	75.30%	6.3	10.5	Vomiting	AVA-TAR[115]
			Unresecta ble locally advanced GC		+Cis/Cap						Neutrope-nia	
			Recurrent GC								Nausea	
			Gastro-esophagea l junction GC								Anemia	
					Placebo	102	33.70%	72.10%	6	11.4	Intestinal obstruc-tion	
					+Cis/Cap		*P* = 0.348	ND	*P* = 0.4709	*P* = 0.5567		
		China	Unresecta ble locally advanced GC	1st-line	Bevacizu mab	40	65%	30%	15.2	17.6	Nausea	[116]
					+Doc/Ox a/5-FU						Vomiting	
											Sensory neuropa-thy	
					Placebo	40	42.50%	42.50%	12.3	16.4	Leukope-nia	
					+Doc/Ox a/5-FU		*P* = 0.0436	ND	*P* = 0.013	*P* = 0.776	Decreased hemoglo-bin	
		United Kingdom	Resectable GC	Peri-operative	Bevacizu mab	530	ND	ND	ND	48.10%	Lethargy	(United Kingdoms Medical Research Council ST03) [117]
			Esophago gastric junction GC		+Cis/Cap /Epi						Nausea	
			Lower esophagea l GC								Neutrope nia	
											Diarrhea	
					Placebo	533	ND	ND	ND	50.30%	Alopecia	
					+Cis/Cap /Epi		ND	ND	*P* = 0.56	*P* = 0.36		
Anti-VEGFR	Ramuciru mab	Multieth-nic	Advanced gastric GC	2nd-line	Ramuciru mab	238	3%	49%	2.1	5.2	Fatigue	REG-ARD[118]
	(Monoclo nal Ab)	North America, Europe, Australia,	Gastro-esophagea l junction GC		+ Pla/5-Fu						Abdomi-nal pain	
		New Zealand									Decreased appetite	
		Asia									Vomiting	

		South and Central America, India, South Africa, Middle East			Placebo	117	3%	23%	1.3	3.8	Constipation	
					+ Pla/5-Fu							
							ND	*P* = 0.76	*P* < 0.0001	*P* = 0.047		
		Multiethnic	Advanced gastric GC	2nd-line	Ramucirumab	330	28%	80%	4.4	9.63	Fatigue	RAINBOW[119]
		North and South America	Gastro-esophageal junction GC		+ Pac						Neuropathy	
		Europe									Decreased appetite	
		Australia,			Placebo	335	16%	64%	2.86	7.4	Abdominal pain	
		Asia			+ Pac						Nausea	
							P < 0.0001	*P* = 0.0001	*P* < 0.0001	*P* = 0.0169		
		Multiethnic	Metastatic GC	1st-line	Ramucirumab	326	41.10%	81.90%	10.2	11.2	Neutropenia	RAINFALL[120]
		North America	Gastro-esophageal junction GC		+ Cis/5-Fu						Anaemia	
		Europe									Hypertension	
		Japan			Placebo	319	36.40%	76.50%	9.2	10.7	Palmar-plantar erythrodysesthesia syndrome	
					+ Cis/5-Fu						Fatigue	
							P = 0.17	*P* = 0.095	*P* = 0.4	*P* = 0.68		
VEGF TKI	apatinib	China	Metastatic GC	3rd-line	Apatinib	176	2.84	42.05	2.6	6.5	Hand-foot syndrome	[121]
			Advanced GC								Proteinuria	
			Gastro-esophageal junction GC								Hypertension	
					Placebo	91	0	8.79	1.8	4.7	Myelosuppression	
							P < 0.001	*P* = 0.1695	*P* < 0.001	*P* = 0.0149	Nausea and vomiting	

A *P* value less than 0.05 indicates statistical significance according to the Mann-Whitney *U* test. VEGF: Vascular endothelial growth factor; VEGFR: Vascular endothelial growth factor receptor; TKI: Tyrosine kinase inhibitor; ORR: Median overall response rate; DCR: Median disease control rate; PFS: Median progression-free survival; OS: Median overall survival; Cis: Cisplatin; Cap: Capecitabine; Doc: Docetaxel; Oxa: Oxaliplatin; 5-FU: 5-Fluoropyrimidin; Epi: Epirubicine; Pla: Polylactic acid; Pac: Paclitaxel; HR: Hazard ratio; CI: Confidence interval.

Interferon, rapamycin, and neovastat

Preclinical trial: The interferon family contains multifunctional cytokines that exhibit antiviral and antitumor properties, induce regulatory cell apoptosis and immune responses and inhibit proangiogenic factors. Abdel-Rahman *et al*[90] evaluated bevacizumab in combination with other anticancer agents, such as mTOR inhibitors and interferon (IFN), as a more effective treatment for gastrointestinal tract and pancreatic tissues. Preclinical and clinical trials showed that other mTOR inhibitors, such as rapamycin, also display antiangiogenic activity in GC[91]. Moreover, Neovastat is a multifunctional drug that blocks VEGF, MMPs and proapoptotic activity in ECs. One MMP inhibitor (Marimastat) has been shown to induce positive outcomes in

phase III clinical trials with advanced GC patients. The other MMP inhibitors are continuing to be investigated in clinical trials[92].

Clinical trial: A clinical phase II trial showed that the treatment of advanced GC patients with interferon-alpha 2B (IFN) and folinic acid (FA) in combination with 5-fluorouracil (5-FU) chemotherapy also resulted in significantly prolonged PFS outcomes compared with those achieved with chemotherapy alone[122]. Al-Batran *et al*[123] demonstrated that mTOR-mediated inhibitors (*e.g.*, rapamycin) blocked the growth of GC cells and delayed tumor progression in cell lines and mouse models. Additionally, the mTOR inhibitor rapamycin has also yielded better survival outcomes in phase I/II studies of metastatic GC patients than do treatment without rapamycin.

Ramucirumab

Preclinical trial: Ramucirumab is a VEGFR-2-targeted monoclonal antibody that inhibit VEGFR-2 signaling. An *in vitro* study showed that treatment with ramucirumab also inhibited cell growth and promoted apoptosis in GC cell lines and animal models[95,96]. Thus, both bevacizumab and ramucirumab inhibit VEGF-mediated pathways in GC. Additionally, an *in vivo* study showed that the effects of combination therapy involving anti-VEGFR and anti-EGFR agents resulted in a significantly decreased tumor size in a GC mouse model[97].

Clinical trial: Fuchs *et al*[118] attempted to explore the effect of ramucirumab, which blocks VEGFR signaling. The REGARD clinical trial indicated that the treatment of advanced GC patients (multiethnic) with ramucirumab in combination with chemotherapy (Pla/5-Fu) resulted in significantly extended PFS ($P < 0.0001$) and OS ($P = 0.047$) outcomes compared with those achieved with placebo. Moreover, the RAINBOW clinical trial showed that the treatment of advanced GC patients (multiethnic) with ramucirumab in combination with chemotherapy (Paclitaxel; Pac) also resulted in significantly improved ORR ($P < 0.0001$) and DCR ($P < 0.0001$), extended PFS ($P < 0.0001$) and OS ($P = 0.0169$) outcomes compared with those achieved with chemotherapy alone[119]. In contrast, the RAUNFALL clinical trial showed that the treatment of advanced GC patients (multiethnic) with bevacizumab in combination with chemotherapy (Cis/5-Fu) had no positive results compared with those achieved with chemotherapy alone[120]. Ramucirumab was approved by the United States Food and Drug Administration (FDA) in 2014 as a 2nd-line treatment of advanced GC due to the REGARD and RAINBOW clinical trials and has beneficial effects on PFS and OS for advanced GC.

DIRECT ACTION ON ECs

Regorafenib, apatinib, and foretinib

Preclinical trial: Regorafenib, apatinib and foretinib belong to the family of multitargeting TKIs. Blockage of the effects of VEGF by silencing RNA in GC cell lines led to reduced tumor volume after implantation of these GC cells into nude mice[98]. The same effect was observed in mice treated with apatinib after tumor grafting[99].

Clinical trial: First, Li *et al*[121] explored the effect of apatinib, which VEGFR TKI blockade. A 116 clinical trial (3rd line) indicated that the treatment of advanced GC patients (China) with apatinib resulted in significantly improved ORR ($P < 0.001$), extended PFS ($P < 0.001$) and OS ($P = 0.0149$) outcomes compared with those achieved with placebo. In a phase II study, the tumor-angiogenesis inhibitor regorafenib, which targets VEGFR, TIE and multiple kinases, was evaluated in advanced GC patients, and the results showed that treatment with this inhibitor resulted in significantly prolonged PFS outcomes compared with those achieved with placebo[124]. Thus, regorafenib will be investigated in a phase III study. However, another antiangiogenic drug, foretinib, which inhibits VEGFR2 and TIE-2, did not yield any benefits in the survival outcomes of GC patients[125]. In addition, Shan *et al*[126] reviewed information from clinical trials evaluating antiangiogenic agents (with a focus on multitargeting TKIs) in advanced GC and found that only apatinib yielded a positive effect on PFS.

Orantinib (SU5416, SU6668), Pazopanib, Sorafenib (Nexavar), Sunitinib (Sutent), Telatinib (Erbitux, Cetuximab)

Preclinical trial: Orantinib (SU5416 SU6668)[94], pazopanib[100], sorafenib (Nexavar)[101,102], sunitinib (Sutent)[103,104] and telatinib (Erbitux, Cetuximab)[105] block tyrosine kinases and belong to the family of multitargeting TKIs. Suppressing the effects of VEGF by silencing RNA in GC cell lines led to decreased tumor

angiogenesis and growth after these cells were implanted into nude mice.

Clinical trial: Chen *et al*[71] summarized the results from clinical trial phase II studies of antiangiogenic drugs, including VEGF ligands, VEGFRs and multitarget TKIs, in advanced GC. The treatment of advanced GC patients with orantinib[127], pazopanib[128,129], sorafenib[130-133], sunitinib[134-136], telatinib[137-141] and vandetanib resulted in significantly extended OS and PSF.

Aflibercept

Preclinical trial: Aflibercept traps VEGF and PlGF *in vivo* and is currently being investigated in a clinical trial (NCT01747551) as a supplement to standard chemotherapy for GC patients[22]. In addition to VEGF-specific inhibition, the effect of HIF-1 blockage has been investigated in animal models in several studies. The treatment of subcutaneous xenografts with an inhibitory HIF-1 compound results in smaller and less vascularized tumors after implantation into nude mice.

Trastuzumab

Seidman *et al*[62] reported that the antibody trastuzumab blocks the Her2/neu receptor through the RAS-MAPK proliferation signaling pathway. A log-rank test showed improved survival outcomes in breast cancer patients. The comparison of two different Her2 and VEGF inhibitors revealed that the effect of tumor growth inhibition on Her2-overexpressing GC xenografts through the combination of Her2 and VEGF inhibitors was better than that achieved with either inhibitor alone[59].

Nonsteroid anti-inflammatory drugs

In an animal model, nonsteroid anti-inflammatory drug (NSAID)-mediated cyclooxygenase (COX) inhibition resulted in reduced tumor angiogenesis, and decreased HIF-1 expression was detected in GC cells after treatment with NSAIDs[25].

OTHER ASSOCIATED CHEMOTHERAPIES

In clinical phase trials, cancer patients are typically administered combination therapy consisting of antiangiogenic agents with chemotherapeutic agents. However, antiangiogenic therapy sometimes elicits several adverse effects, such as hypertension[142,143] or proteinuria[144], but the factors responsible for these adverse effects remain unknown. In general, the results from several studies on some antiangiogenic therapies, such as the inhibition of VEGF, Ang-1 and PlGF, indicate that antiangiogenic therapy not only inhibits EC migration and proliferation but also enhances chemotherapy ability. Hwang *et al*[145] indicated that the inhibition of VEGFR enhances paclitaxel sensitivity in GC cells. Another group of researchers showed that the upregulation of HIF-1 promotes chemotherapy and the antiapoptosis ability in GC cells by inducing miR-27a- or p53- and NF-kB-mediated pathways[146-148]. Additionally, compared with normal blood vessels, tumor vessels exhibit heterogeneity, versatility, high permeability and vascular properties that benefit chemotherapy[149]. Thus, antiangiogenic therapy could exert an adjuvant effect in chemotherapy.

CONCLUSION

Tumor angiogenesis involves a complex multistep process. In general, the available knowledge indicates that proangiogenic and pro-oncogenic (such as proliferation, anti-apoptosis, migration and invasion) pathways are linked to each other. Thus, tumor angiogenesis occurs at different stages of tumor progression, including tumor growth, metastasis and recurrence. This connection can be clearly observed by the administration of combination therapy against angiogenic and proliferative pathways, such as the VEGF-, EGFR- and STAT3-mediated pathways[16-19,31,52-54,58]. These transcription factors regulate cell growth, migration and angiogenesis in multiple ways.

First, we investigated the expression of angiogenic factors in GC through preclinical trials [cell line (*in vitro*)/animal model (*in vivo*)] and thus determined whether these factors could serve as predictive factors/biomarkers for proliferation, invasion or metastasis and/or have diagnostic or prognostic value[7,8]. An increasing number of studies has revealed that antiangiogenic agents attack tumor ECs as their target instead of tumor cells themselves, which is the final goal of tumor dormancy therapy. Moreover, the therapeutic target of antiangiogenic agents is tumor ECs, which are more genetically stable, show increased homogeneity and have a lower alteration level; antiangiogenic drugs can interact with ECs directly, resulting in

higher potency, decreased drug resistance and fewer side effects[150]. We explored the combination of antiangiogenic drugs and cytotoxic anticancer (chemical) drugs to develop a highly effective strategy for the management of advanced GC[13-15]. Thus, antiangiogenic drugs might be valuable for the long-term management of tumor dormancy because they do not induce the development of antiangiogenic drug resistance, and these drugs present fewer side effects. A few recent clinical trials have revealed that antiangiogenic therapy could potentially extend the survival outcomes of advanced GC patients[109].

DISCUSSION

In assessing the effectiveness of antiangiogenesis therapy, a clinical phase III trial showed that only ramucirumab (an anti-VEGFR antibody) and apatinib (VEGFR TKI blocker) achieved positive results (Table 2). Although both ramucirumab and bevacizumab are anti-VEGF drugs, bevacizumab (AVAGAST, AVATAR, ST03, Ma *et al*[116]) had no positive results on OS, while ramucirumab (REGARD, RAINBOW) was more effective targeted drug and exerted more positive results for OS in advanced GC. We suggested that this is because bevacizumab only binds to VEGF-A, whereas ramucirumab binds to VEGFR-2, which blocks more VEGFs. Therefore, ramucirumab could exert more effective antiangiogenic function due to the inhibition of more VEGF molecules. One possible reason is the differences in the targets of the antiangiogenic action. However, the differences in the ability of these two anti-VEGF drugs remain partially unknown. Furthermore, the different populations of GC patients might be another factor that affects the benefits of these drugs. In the AVAGAST and RAINBOW studies, the non-Asian subgroup (66.5%; RAINBOW) achieved a greater benefit in OS from antiangiogenic therapy than did the Asian subgroup (51%; AVAGAST). However, the effect of ramucirumab still lacks 1st-line chemotherapy evidence. The extent of the usefulness of ramucirumab still requires exploration in further trials in different ethnicities and upon delivery as a 1st-, 2nd- or 3th-line chemotherapy. Additionally, in evaluating the safety of antiangiogenic therapy, most adverse events related to antiangiogenesis are tolerable and controllable, including hypertension, neutropenia and wound healing (Table 2). Conversely, the Cougar-02 trial, a Doc+best supportive care (BSC) study, has a similar result for OS as the REGARD trial and was more cost effective[151]. Finally, of the VEGFR TKIs, only apatinib in the phase III clinical trial showed extended PFS and OS in advanced GC patients. We recommend that chemotherapy in combination with ramucirumab (anti-VEGFR) and apatinib (VEGFR TKI) significantly improves the outcome in ORR, expended PFS, and OS in the management of advanced GC.

Here, this review only included phase III clinical trials published in English. Previous studies have found that the combination of antiangiogenic agents with chemotherapy may be beneficial for advanced GC in OS, but potential publication bias should be considered when construing these results. To reduce possible publication bias, we tried to search in multiple databases. Nevertheless, some restrictions were present in this systemic review and statistical analysis (*e.g.*, meta-analysis)[152,153] such as the small size of included studies, multiple drugs implemented and the high heterogeneity between different studies. Therefore, a larger cohort size, more standardized research and high statistical quality should be implemented in future studies to identify patients who would most likely benefit from antiangiogenic treatment. Thus, this review will provide basic (tumor angiogenesis) and clinical (antiangiogenic drugs) research for the survey of the management of GC treatments.

FURTHER CHALLENGES OF ANTIANGIOGENIC THERAPY

Although several phase III clinical trials have reported positive results, new vessels in tumors have pleomorphic features, including heterogeneity, flexibility, penetrability, various vascular biomarkers, and turbulent blood flow with no lymphatic vessels, and these unusual features make the delivery of therapeutic drugs difficult. Hence, there remain several obstacles regarding the translation of antiangiogenic strategies from animal models to clinical trials[92,108,154].

The current problems regarding preclinical to clinical trials and the future directions for antiangiogenic therapy are discussed below.

In preclinical trials, we usually perform experiments in animals with xenografts of various tumor cells, but these models cannot represent spontaneous and orthotopic human cancers, particularly highly metastatic tumors[155]. Therefore, antiangiogenic drugs are not effective for every organ in the body. Antiangiogenic drugs often yield

different results or side effects in preclinical and clinical trials.

In advanced GC, the tumor develops several ways of escaping treatment and rapidly activating angiogenic pathways. Ebos *et al*[156] reported that enhanced metastasis was treated with sunitinib in a mouse model. Another group found a similar result[157]. This may partly fail to translate to a survival benefit of antiangiogenic drugs in localized or nonmetastatic GC. Therefore, it is crucial to develop novel biomarkers that are able to predict the prognosis of antiangiogenic treatments for advanced GC. In clinical trials, to assess antiangiogenic therapies, newer imaging systems and/or substitute biomarkers should be established for monitoring tumor vessel functions. Antiangiogenic drugs induce tumor dormancy, which is different from the results of chemotherapy[155].

The aims of managing GC are to reduce drug toxicity and adverse events and prolong survival. Therefore, the optimal biological dose and therapeutic schedule of antiangiogenic drugs should be established. Moreover, antiangiogenic drugs can be combined with chemotherapy and/or radiotherapy[149].

According to previous studies, the clinical effect is quite different in individuals due to heterogeneity of the tumor. It is unclear which patients benefit most from angiogenesis inhibitors. The race/ethnicity of patients seems to influence the efficacy of antiangiogenic treatments on OS. The patients should be selected, and angiogenic factors should be detected before the administration of antiangiogenic drugs. Individual angiogenic profiling according to an individual's genetic background remain a problem that need to be addressed.

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ORIGINAL ARTICLE

Basic Study

MicroRNA-331 inhibits development of gastric cancer through targeting musashi1

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Abstract

BACKGROUND

The molecular mechanisms involved in microRNAs (miRNAs) have been extensively investigated in gastric cancer (GC). However, how miR-331 regulates GC pathogenesis remains unknown.

AIM

To illuminate the effect of miR-331 on cell metastasis and tumor growth in GC.

METHODS

The qRT-PCR, CCK8, Transwell, cell adhesion, Western blot, luciferase reporter and xenograft tumor formation assays were applied to explore the regulatory mechanism of miR-331 in GC.

RESULTS

Downregulation of miR-331 associated with poor prognosis was detected in GC. Functionally, miR-331 suppressed cell proliferation, metastasis and tumor growth in GC. Further, miR-331 was verified to directly target musashi1 (MSI1). In addition, miR-331 inversely regulated MSI1 expression in GC tissues. Furthermore, upregulation of MSI1 weakened the inhibitory effect of miR-331 in GC.

CONCLUSION

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miR-331 inhibited development of GC through targeting MSI1, which may be used as an indicator for the prediction and prognosis of GC.

Key words: MicroRNA-331; Musashi1; Tumor growth; Metastasis; Gastric cancer

Core tip: Gastric cancer (GC) has become one of the main threats to human life and health. MicroRNAs (miRNAs) have been reported to act as promoters and inhibitors in GC. In this study, the effect of miR-331 on cell metastasis and tumor growth was illuminated in GC. The results showed that miR-331 was downregulated in GC, which predicted poor prognosis in GC patients. Moreover, overexpression of miR-331 inhibited cell metastasis and tumor growth in GC cells. Further, musashi1 (MSI1) is a direct target gene of miR-331. MiR-331 inhibited GC progression through the suppression of MSI1.

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INTRODUCTION

In recent years, gastric cancer (GC) has become one of the main threats to human life and health, of which more than 50% of cases occur in East Asia[1]. In China, the incidence rate of GC ranks first among all kinds of tumors. About 170000 people die of GC every year, almost one-fourth of the total number of malignant tumor deaths. Furthermore, more than 20000 new patients with GC are found every year[2]. The pathogenesis of GC is complex and still unclear, involving many etiological factors and genetic changes. Although great progress has been made in understanding the pathogenesis of malignant tumors, the treatment of GC still has limitations[3]. Moreover, low survival rate is found in GC patients, and cell metastasis is the main causes of poor prognosis in GC patients[4]. Despite the treatment strategy for GC has been improved, it is necessary to explore high-sensitivity and low-cost treatments.

It has been demonstrated that microRNAs (miRNAs) can act as promoters and inhibitors in various cancers including GC by inhibiting gene expression[5]. For example, miR-133a was downregulated in GC and inhibited tumor growth, migration, and EMT process by targeting PSEN1[6]. Conversely, upregulation of miR-592 was identified in GC and promoted cell proliferation, invasion and migration through the PI3K/AKT and MAPK/ERK signaling pathways[7]. Recently, the dysregulation of miR-331 in different cancers has aroused our concern. It has been demonstrated that miR-331 can function as a marker for diagnosis and prognosis of hepatocellular carcinoma patients[8]. It had been reported that miR-331 was overexpressed in malignant breast tumors[9]. Moreover, miR-331 promoted hepatocellular carcinoma cell metastasis and proliferation through regulating ING5[10]. In addition, miR-331 was found to enhance Epithelial-to-Mesenchymal Transition (EMT) in prostate cancer[11]. However, downregulation of miR-331 had been identified in esophageal adenocarcinoma and predicted tumor recurrence[12]. In addition, miR-331-3p regulated expression of NRP-2 to inhibit development and progression of glioblastoma[13]. In particularly, Guo *et al*[14] had shown that miR-331 directly targeted E2F1 and induced the inhibition of GC tumor growth. However, how miR-331 regulates GC cell viability and metastasis remains blurry and need to be investigated.

Musashi1 (MSI1) is a neural RNA-binding protein required for Drosophila adult external sensory organ development[15]. Moreover, the function of MSI1 has been identified in normal and cancer stem cells[16]. Recently, MSI1 has been found to regulate tumor growth and proliferation in several human cancers. MSI1 had been identified as carcinogenesis, progression, and poor prognosis related biomarker for gallbladder adenocarcinoma[17]. Furthermore, MSI1 can regulate breast cancer proliferation and is an indicator for poor survival[18]. More importantly, upregulation of MSI1 has been identified in human colorectal adenomas and GC[19,20]. Moreover, knockdown of MSI1 resulted in tumor regression in colon cancer[21]. However, the role

of MSI1 and its relationship with miR-331 have not been reported in previous studies.

Therefore, the regulatory mechanism of miR-331 with MSI1 was investigated in GC. We mainly focused on how miR-331 regulates GC tumor growth and metastasis. The findings will contribute to better understanding the pathogenesis of GC.

MATERIALS AND METHODS

GC clinical tissues and cell line

The GC tissues used in this experiment and their clinical and follow-up information were provided by Affiliated Hospital Taishan Medical University. All 78 patients involved in the experiment did not receive radiotherapy or chemotherapy prior to surgery. Participants provided written informed consent before designing the research, and Human Ethics Committee of Affiliated Hospital Taishan Medical University approved the experiment.

A normal gastric cell GES1 and SGC-7901, BGC-803, MKN-45 GC cell lines were purchased from Beijing Zhongke Quality Inspection Biotechnology Co., Ltd. (Beijing, China). The cells then were seeded in RPMI-1640 medium with 10% fetal bovine serum (FBS) and incubated in an atmosphere with 5% CO_2 at 37 °C.

Cell transfection

MiR-331 mimics, miR-331 inhibitors, and MSI1 plasmid (RiboBio Co, Ltd, Guangzhou, China) were transferred into MKN-45 cells respectively with Lipofectamine 2000 (Invitrogen, CA, United States) based on experimental needs. Untreated MKN-45 cells were set as the control. The nucleotide sequences were: miR-331 mimics, 5′-GCC CCU GGG CCU AUC CUA GAA-3′, antisense: 5′-CUA GGA UAG GCC CAG GGG CUU-3′; miR-331 inhibitor, 5′-UUC UAG GAU AGG CCC AGG GGC-3′.

Quantitative real-time polymerase chain reaction

We extracted total RNA from MKN-45 cell by using TRIZOL reagent (TaKaRa Bio, United States). The First-Strand cDNA Synthesis Kit (Promega, United States) was added to obtain cDNA. The mixture of the qRT-PCR standard reaction system was then added to SYBR Green PCR Master Mix (Applied Biosystems, CA, United States). The prepared PCR reaction solution was placed on ABI7300 real-time PCR machine (Applied Bio-systems) for PCR amplification reaction. U6 and GAPDH were respectively used as the controls for miR-331 and MSI1, which were quantified with the 2^{-ct} method. The forward and reverse primers of qRT-PCR are given in Table 1.

Cell viability assay

MKN-45 cells were pre-incubated in a 96-well plate for 24 h (at 37 °C, 5% CO_2). MKN-45 (2×10^4) cells were incubated for 24, 48, 72 and 96 h. 10 mL of CCK-8 (Dojindo, Kumamoto, Japan) solution was used to incubate the cells for 4 h. The absorbance at 450 nm was observed with a microplate reader (Molecular Devices).

Xenograft tumor formation assay

The xenograft study was approved by the Animal Care and Use Committee of Affiliated Hospital Taishan Medical University. We purchased nude mice (4 weeks old) from the Shanghai Lab Animal Research Center (Shanghai, China). Then 3×10^6 MKN-45 cells with stably overexpressing miR-331 or miR-NC were injected into mice subcutaneous. The tumor volume was observed every one week. After 4 wk, the mice were sacrificed and tumors were used for further study.

Ki67 immunohistochemistry

Ki67 Cell Proliferation Kit was purchased from Sangon Biotech Co., Ltd. (Shanghai, China). The experiment was performed based on their protocol. The section of gastric cancer tissues were dewaxed, hydrated, and washed twice with PBS for 5 min. After blocking with Blocking Buffer for 30-60 min at room temperature, Anti-ki67 Rabbit antibody (1:50) was added to incubate overnight at 4 °C in a humidified atmosphere. Then, secondary antibody HRP-conjugated Donkey anti-Rabbit IgG (1:500) was added and incubated for 60 min at room temperature. After washing 3 times with PBS, DAB mixture was added to each slide, and incubated for 5-10 min at room temperature protected from light. The section was washed, counterstained, dehydrated, transparentized and mounted. Images were captured using microscope.

Cell migration and invasion assay

The upper chamber surface of the bottom membrane of the Transwell chamber (8-μm pore size membranes) was coated with Matrigel (BD), and the Matrigel was polymerized into a gel at 37 °C for 30 min. Moreover, the transfected MKN-45 cells

Table 1 The forward and reverse primers of qRT-PCR

Gene	Primers sequence (5-3')
MiR-331	F: GCCCCTGGGCCTATCCTAGAA
	R: ACGCGTCGACTTTTAGGGCTAAGTTGCTTC
MSI1	F: CTCCAAAACAATTGACCCTA
	R: GCTCAAAATATTGCTTCACG
U6	F: AGAGCCTGTGGTGTCCG
	R: CATCTTCAAAGCACTTCCC
GAPDH	F: ATGGGGAAGGTGAAG GTCG
	R: GGGGTCATTGATGGCAACAAT A

were starved prior to invasion assay. 5 × 10^4 mL MKN-45 cell suspension (100 μL) was added to the Transwell chamber, and then a medium containing 20% FBS (600 μL) was added to the lower chamber with the 24-well plate. After routine incubation for 24 h, the Transwell chamber was fixed and stained. The number of invading cells observed under a microscope of 400 times. The Transwell cell migration assay performed without Matrigel, other process is basically same as the invasion assay.

The luciferase reporter assay

A pGL3 luciferase vector (Invitrogen) containing the wild or mutant type of 3'-UTR of MSI1 gene was prepared. The luciferase vector and miR-331 mimics were co-transfected into MKN-45 cells. After 48 h, Dual-Luciferase Reporter Assay System (Promega) was used to examine the luciferase activity. *MSI1* gene information was wild type (Ensembl number ENSG00000135097) and mutated sequence (5'-UGGCGAGGGCAGACCGGUCCCCA-3').

Western blot analysis

Protein samples were lysed using RIPA buffer (Beyotime, Shanghai, China). 10% concentrated SDS-PAGE protein loading buffer was added to the collected protein samples. After denaturing the protein, the protein sample was directly loaded into the SDS-PAGE gel and then transferred into PVDF membrane. PVDF membrane was incubated with the corresponding primary antibodies overnight at 4 °C, including rabbit polyclonal antibody to MSI1 (1:1000, ab21628, Abcam, Shanghai, China) and rabbit monoclonal antibody to GAPDH (1:1000, ab181602, Abcam, Shanghai, China). The washing solution was added for 5-10 min, and the diluted Goat anti-Rabbit. Goat anti-Rabbit IgG(H+L) HRP secondary antibody (1:500, ab205718, Abcam, Shanghai, China) was added and incubated at room temperature for 1 h. Finally, ECL reagent (Millipore, MA, United States) was used to detect proteins. In addition, E-cadherin, N-cadherin and Vimentin antibodies were all obtained from Abcam (1:1000, Shanghai, China).

Statistical analysis

Data were analyzed using SPSS 13.0 and Graphpad Prism 6. The difference between the groups was calculated through Chi-squared Test or Tukey's one-way ANOVA. The log-rank test Kaplan-Meier analysis was used to compare the survival differences. The data was shown as mean ± SD. When $P < 0.05$, the data is considered statistically significant.

RESULTS

Downregulation of miR-331 associated with prognosis was detected in GC

In GC tissues and cell lines, miR-331 expression was observed by qRT-PCR assay. First, low miR-331 expression was identified in GC tissues contrast to normal tissues (Figure 1A). Meanwhile, the reduction of miR-331 expression was found in SGC-7901, MGC-803, MKN-45 cells contrasted to GES1 cells (Figure 1B). MKN-45 cell line was selected for subsequent experiments because of the significant differences in expression of miR-331. Based on the expression of miR-331, these cases were divided into a high miR-331 expression group and a low expression group based on its median value in GC patients as a cutoff point (cutoff point = 0.75). Furthermore, abnormal miR-331 expression was correlated with lymph nodes metastasis and TNM stage in GC patients ($P < 0.05$, Table 2). In addition, shorter disease free survival (DFS)

and overall survival (OS) was correlated with low miR-331 expression in GC patients (Figure 1C and 1D). Therefore, miR-331 expression was reduced, which predicted poor prognosis of GC patients.

MiR-331 inhibited GC cell viability in vitro and vivo

MiR-331 mimic or inhibitor was transfected into MKN-45 cells to perform gain-loss experiment. MiR-331 expression was obviously increased by its mimics, but decreased by its inhibitor (Figure 2A). Next, we found that miR-331 mimics inhibited the proliferation of MKN-45 cells. And miR-331 inhibitor showed the opposite results (Figure 2B). Moreover, the effect of miR-331 on tumor growth was analyzed in GC. MiR-331 mimics was found to decline the tumor volume and suppressed tumor growth compared to control group (Figure 2C and 2D). Additionally, miR-331 overexpression led to a significant decrease in the number of hyperproliferative Ki-67$^+$ tumor cells (Figure 2E). Collectively, miR-331 inhibited cell proliferation and tumor growth in GC.

MiR-331 inhibited cell metastasis in GC

Then, how miR-331 regulates cell metastasis was investigated in MKN-45 cells. Transwell assay suggested that miR-331 overexpression suppressed cell migration, while miR-331 knockdown promoted MKN-45 cell migration (Figure 3A and 3C). For cell invasion in GC, the same effect of miR-331 overexpression and knockdown was also identified (Figure 3B and 3C). Next, the effect of miR-331 on EMT was explored in GC cells. Overexpression of miR-331 facilitated E-cadherin expression and hindered expressions of N-cadherin and Vimentin. In contrast, knockdown of miR-331 blocked E-cadherin expression and promoted expression levels of N-cadherin and Vimentin (Figure 3D), indicating that miR-331 blocked EMT in GC. Briefly, miR-331 inhibited cell metastasis in GC.

MSI1 is a direct target of miR-331

The target genes of miR-331 were searched in TargetScan databases to disclose how miR-331 suppresses GC progression. The binding sites between miR-331 and MSI1 were showed in Figure 4A. Next, luciferase reporter assay was designed to confirm that prediction. As predicted, miR-331 mimics inhibited the luciferase activity of Wt-MSI1, but had no effect on Mut-MSI1 luciferase activity (Figure 4B). Then, MSI1 expression regulated by miR-331 mimics or inhibitor was assessed in MKN-45 cells. The mRNA and protein MSI1 expression was inhibited by miR-331 mimics, but promoted by miR-331 inhibitor (Figure 4C and 4D). Thus, we believe that miR-331 directly targets MSI1 and regulates MSI1 expression.

Upregulation of MSI1 weakened the inhibitory effect of miR-331 in GC

In GC tissues, the abnormal MSI1 expression was then observed. MSI1 expression was dramatically increased in GC tissues in comparison with normal tissues (Figure 5A). Moreover, miR-331 expression was identified to negatively regulate MSI1 expression in GC tissues (Figure 5B). It indicates that the interaction between miR-331 and MSI1 may exist in GC. MiR-331 mimics and MSI1 vector were co-transfected into MKN-45 cells to verify the above conjecture. As we suspected, the reduction of MSI1 expression induced by miR-331 mimics was recovered by MSI1 vector in MKN-45 cells (Figure 5C and 5D). Functionally, MSI1 vector impaired the inhibitory effect of miR-331 on cell proliferation (Figure 5E). Upregulation of MSI1 also restored the inhibitory effect of miR-331 on EMT in MKN-45 cells (Figure 5F). For cell migration and invasion, the suppressive effect of miR-331 was impaired by upregulation of MSI1 (Figure 5G). Taken together, upregulation of MSI1 weakened the inhibitory effect of miR-331 on GC progression.

DISCUSSION

As a new research hotspot, the regulatory mechanism of miRNAs in GC has been widely reported, indicating the strong potential of miRNA in the future treatment of GC. In our research, miR-331 was downregulated in GC, which predicted poor prognosis of GC patients. Functionally, miR-331 acted as an inhibitor in GC through suppressing cell proliferation, metastasis and tumor growth. Further, miR-331 directly targets MSI1 and inversely regulates its expression. Furthermore, upregulation of MSI1 impaired the inhibitory effect of miR-331 in GC. Briefly, miR-331 inhibited GC progression through targeting MSI1.

In many human cancers, the deregulation of miR-331 is involved in their tumorigenesis. Decreased miR-331 expression has been identified in colorectal cancer, prostate cancer and glioblastoma[13,22,23]. The biological function of miR-331 was also

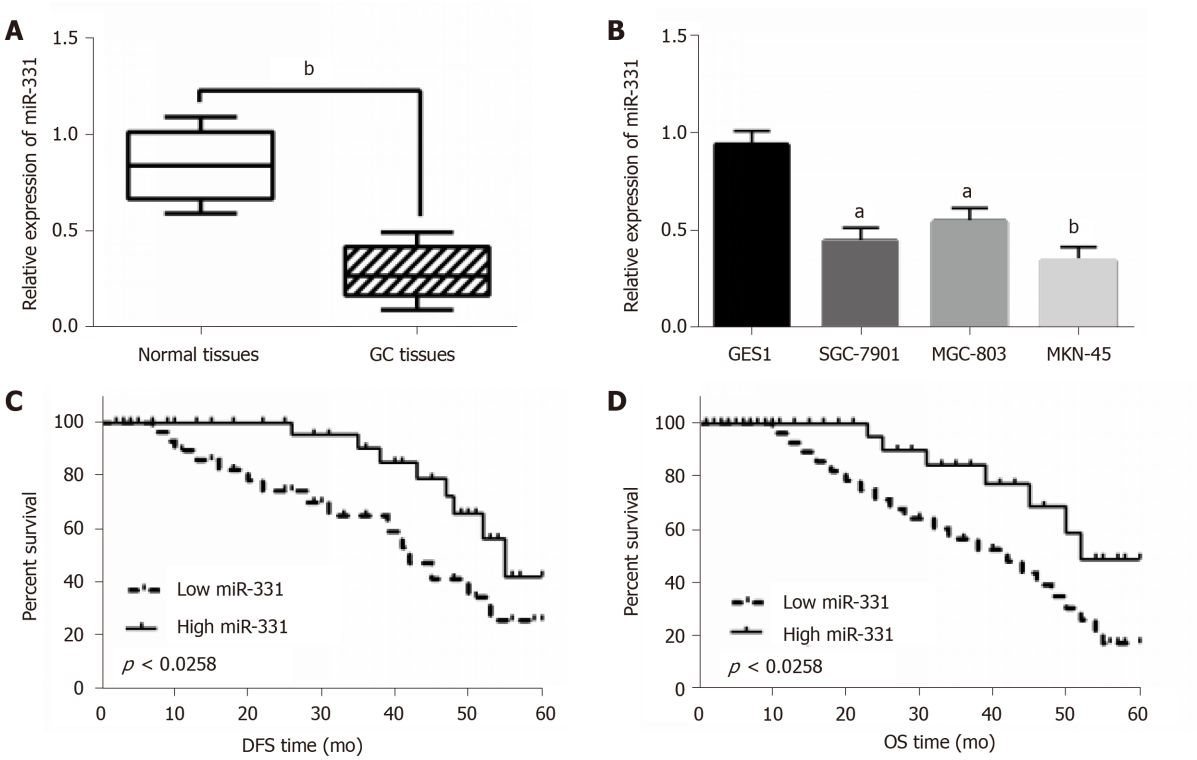

Figure 1 Downregulation of miR-331 associated with prognosis was detected in gastric cancer. A: The mRNA expression of miR-331 was examined in gafstric cancer (GC) tissues. B: MiR-331 expression was determined in MKN-45, MGC-803, SGC-7901, and GES1 cells. C and D: Low miR-331expression was correlated with shorter DFS and OS time in GC patients. [a]$P < 0.05$, [b]$P < 0.01$.

investigated in previous studies. For example, the inhibition of cell proliferation induced by miR-331 had been observed in urothelial carcinoma[24]. Chen *et al*[25] proposed that miR-331 inhibited melanoma cell proliferation and invasion through regulating AEG-1. Moreover, miR-331 overexpression reduced PCa cell migration and invasion, as well as xenograft tumor initiation[23]. Consistent with above previous studies, the inhibitory effect of miR-331 was also confirmed in GC. In addition, we also found that overexpression of miR-331 blocked EMT in GC cells, which has not been reported in other studies. These findings indicate that miR-331 can be used as a suppressor in GC progression.

Further, many target genes of miR-331 were confirmed in different cancers, such as ST7L and ERBB-2[26,27]. However, as far as we know, there is no research about the relationship between miR-331 and MSI1. Here, MSI1 was verified as a direct target of miR-331. And MSI1 expression was increased in GC tissues. The upregulation of MSI1 had also been found in GC tissues in previous report, which further confirm our results[28]. Moreover, overexpression of MSI1 was related to GC progression and poor prognosis in GC patients[29], indicating that MSI1 was involved in the development of GC. Additionally, upregulation of MSI1 has also been detected in cervical cancer, which promoted cell proliferation and tumor growth[30]. It is reasonable to know that miR-331 inhibits cell viability and metastasis via targeting MSI1 in GC. In line with previous studies, we testified above results in current research. Upregulation of MSI1 was found to weaken the inhibitory effect of miR-331 in GC.

In summary, it is firstly proposed the downregulation of miR-331 that associated with poor prognosis in GC patients in current research. Further, miR-331 directly targets MSI1 and inversely regulates its expression. Moreover, miR-331 inhibited cell viability and metastasis through targeting MSI1 in GC. Therefore, miR-331 may be a potential therapeutic target for GC.

Table 2 Relationship between miR-331 expression and clinic-pathological characteristics of gastric cancer patients

Characteristics	Cases	miR-331		*P* value
		High	Low	
Age (yr)				0.34
≥ 55	41	16	25	
< 55	37	8	29	
Gender				0.16
Male	43	13	30	
Female	35	11	24	
Tumor size (mm)				0.07
≤ 3	25	10	15	
> 3	53	14	39	
Lymph nodes metastasis				0.03[a]
Yes	21	8	13	
No	57	16	41	
TNM stage				0.02[a]
I-II	55	15	40	
III-IV	23	9	14	

[a]$P < 0.05$ was considered significant. Statistical analyses were performed by the χ^2 test.

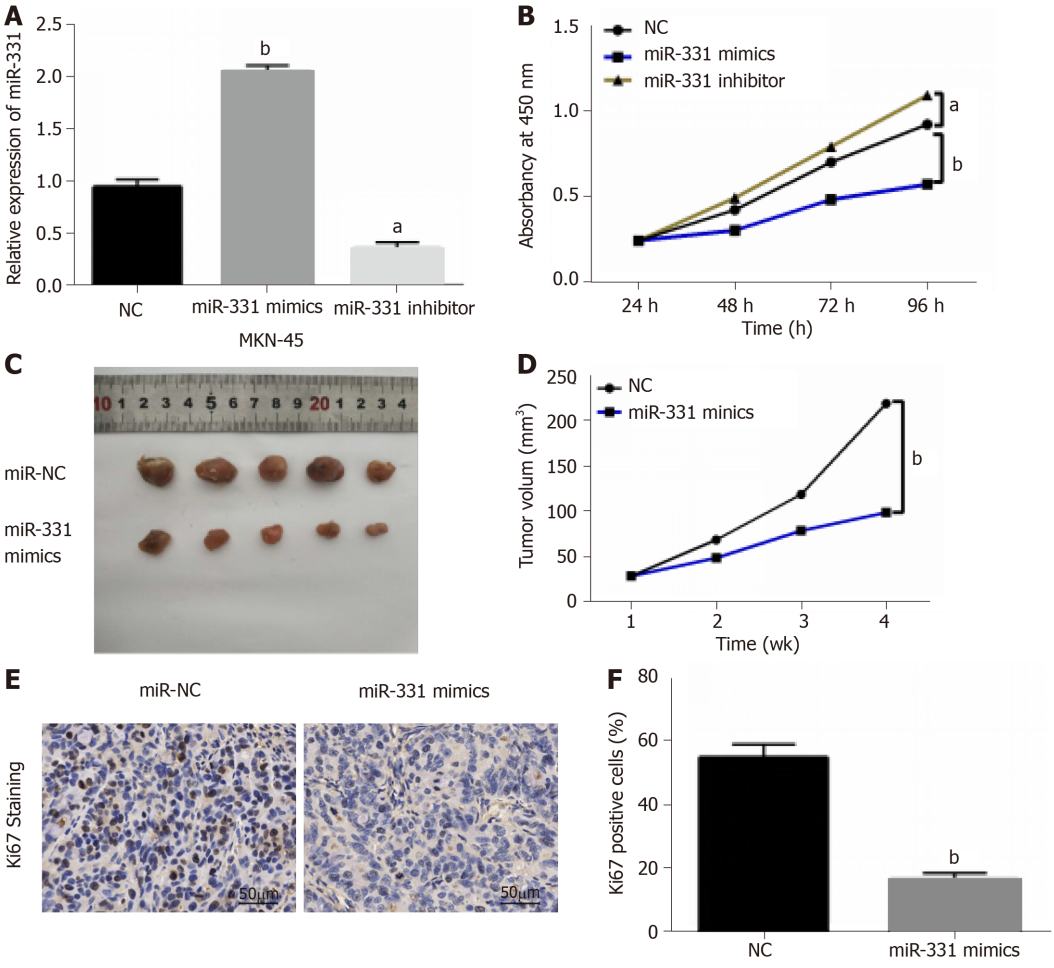

Figure 2 MiR-331 inhibited gastric cancer cell viability *in vitro* and *vivo*. A: MiR-331 mRNA expressions was determined in MKN-45 cells with miR-331 mimic or inhibitor. B: Cell proliferation was regulated by miR-331 mimic or inhibitor in MKN-45 cells. C: Photographs of gastric cancer (GC) tumors in miR-NC (n = 5) or miR-331 mimics (n = 5) group. Ten mice were used for xenograft tumor formation assay. Here are five of the results. D: In nude mice with miR-NC or miR-331 mimics, GC tumor volume was measured every week. E: Ki-67-stained sections of transplanted tumors in miR-NC or miR-331 mimics group. [a]$P < 0.05$, [b]$P < 0.01$.

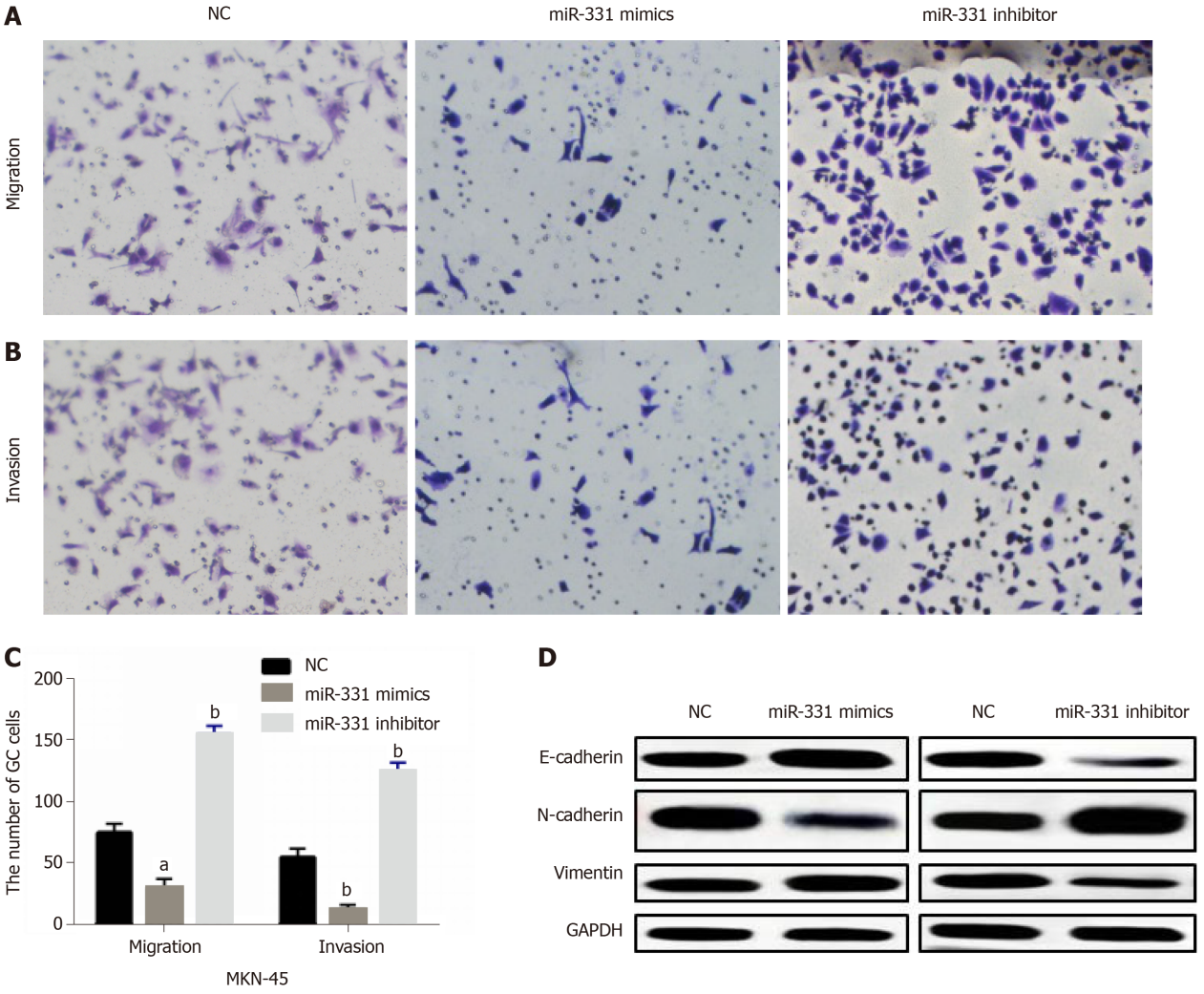

Figure 3 MiR-331 inhibited cell metastasis in gastric cancer. A-C: MKN-45 cell migration and invasion were regulated by miR-331 mimic or inhibitor. D: MiR-331 regulated expressions of E-cadherin, N-cadherin and Vimentin in MKN-45 cells. [a]*P* < 0.05, [b]*P* < 0.01.

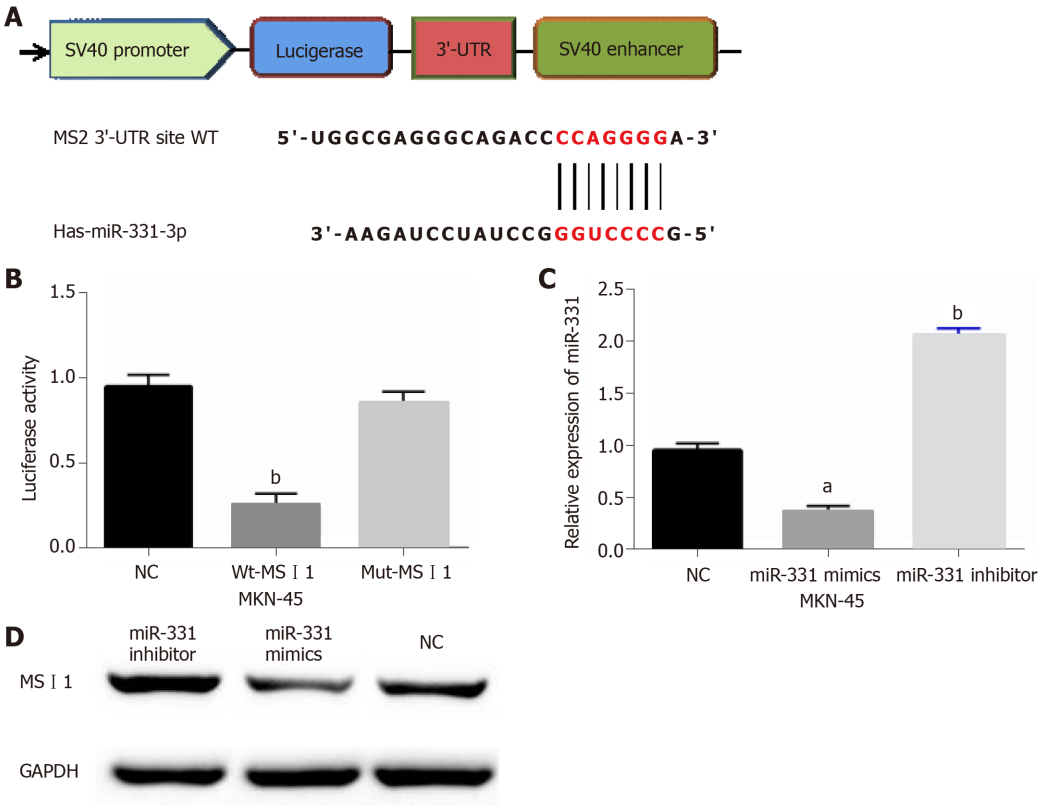

Figure 4 MiR-331 directly targets musashi1. A: There are binding sites between musashi1 (MSI1) with miR-331. B: Luciferase reporter assay (C, D) MSI1 expression regulated by miR-331 mimics or inhibitor was observed in MKN-45 cells. [a]*P* < 0.05, [b]*P* < 0.01.

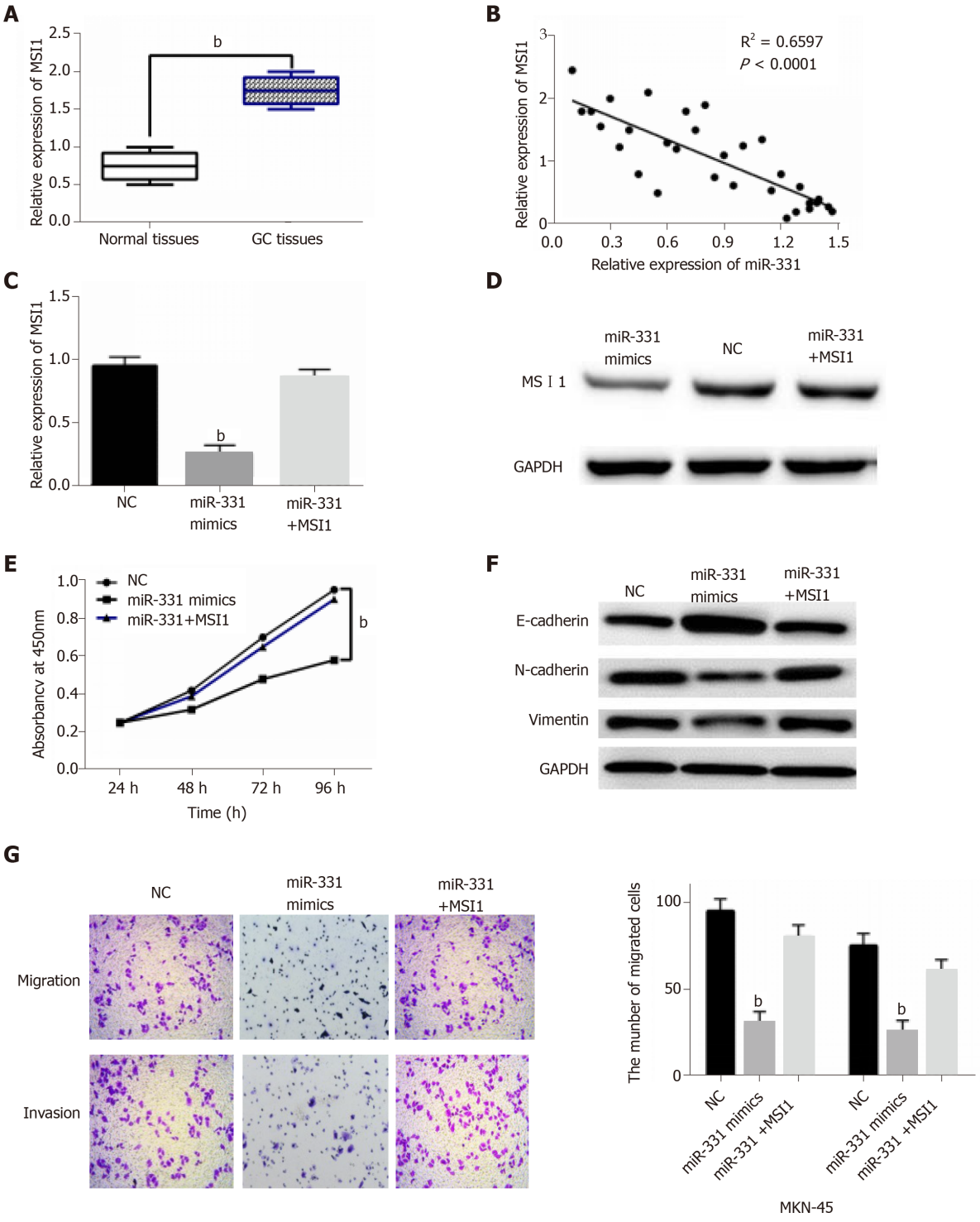

Figure 5 Musashi1 upregulation weakened miR-331 inhibitory effect in gastric cancer. A: Musashi1 (MSI1) expression was detected in gastric cancer (GC) tissues. B: MiR-331 negatively regulated MSI1 expression in GC tissues. C and D: The mRNA and protein MSI1 expressions were detected in MKN-45 cells containing MSI1 vector and miR-331 mimic. E: Cell proliferation was identified in MKN-45 cells containing MSI1 vector and miR-331 mimic. F: Protein expressions of E-cadherin, N-cadherin and Vimentin in MKN-45 cells with MSI1 vector and miR-331 mimic (G) Cell migration and invasion were identified in MKN-45 cells containing MSI1 vector and miR-331 mimic. $^{b}P < 0.01$.

ARTICLE HIGHLIGHTS

Research background

As one of the most frequent cancers, gastric cancer (GC) caused more than 700000 deaths in just 2012 worldwide. Although the molecular mechanisms involved in microRNAs (miRNAs) have been extensively investigated in GC, how miR-331 regulates GC pathogenesis remains unknown.

Research motivation
To find more molecular mechanism or biomarker for diagnosis and treatment of GC

Research objectives
This study aims to explore the anti-cancer effect of miR-331 in GC and investigate its molecular mechanism against GC cells.

Research methods
MiR-331 expression was observed by qRT-PCR assay in GC tissues and cell lines. MiR-331 mimic or inhibitor was transfected into MKN-45 cells to perform gain-loss experiment to observe effect of miR-331 on GC cell viability and migration. Bioinformatics analysis is used to predict the target gene of miR-331. The antagonistic effect between and MSI1 was confirmed by gain-loss experiment and detection of proliferation and migration. The expression of crucial proteins was measured by western blotting.

Research results
We found that downregulation of miR-331 was associated with poor prognosis in GC. In addition, miR-331 significantly inhibited GC cell growth, migration and invasion. Further, MSI1 was verified to directly target miR-331 and can effectively be regulated in GC tissues. Furthermore, upregulation of MSI1 weakened the inhibitory effect of miR-331 in GC. Western blotting analysis showed that E-cadherin, N-cadherin and Vimentin expression markedly affected by miR-331 and MSI1 in GC cell line, suggesting that EMT is a very direct regulated target of miR-331 and MSI1 in GC.

Research conclusions
Our study demonstrated that miR-331 can significantly inhibit GC cell growth, migration and invasion. Furthermore, it can work through MSI1. Therefore, our study provides some molecular mechanism and two new biomarkers for GC.

Research perspectives
In the future, research may reveal the important role of miR-331 that enhances the sensitivity of GC detection and further develop for its application in anti-cancer treatments. The identification of the miR-331/MSI1 molecular axis may further explain the underlying mechanism.

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ORIGINAL ARTICLE

Retrospective Study

Correlation between invasive microbiota in margin-surrounding mucosa and anastomotic healing in patients with colorectal cancer

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Abstract

BACKGROUND

Impaired anastomotic healing is one of the major complications resulting from radical resection in colorectal cancer (CRC). Accumulating evidence suggests that intestinal microbiota is correlated with anastomotic healing.

AIM

To explore the microbiota structural shift in margin-surrounding mucosa and evaluate the predictive ability of selected bacterial taxa for impaired anastomotic healing.

METHODS

Margin-surrounding mucosa samples derived from 37 patients were collected to characterize the microbial community structure by 16s rRNA gene sequencing. The patients were divided into two groups according to the healing status of anastomoses: well-healing group (n = 30) and impaired-healing group (n = 7). Statistic differences in bacteria taxa were compared by Wilcoxon test and chi-squared test. The predictive ability of the selected bacterial taxa for the healing status of anastomoses was evaluated by the area under the receiver operator characteristic curve.

RESULTS

Community structure shifts were observed in the impaired-healing group and

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well-healing group. Six bacterial species were found to be significantly correlated with anastomotic healing, and among these species, *Alistipes shahii*, *Dialister pneumosintes*, and *Corynebacterium suicordis* were considered as the predictive factors. Taking the known risk factor age into consideration, *Alistipes shahii*, *Dialister pneumosintes*, and *Corynebacterium suicordis* improved predictive ability for the healing status of anastomoses.

CONCLUSION
These data show that *Alistipes shahii*, *Dialister pneumosintes*, and *Corynebacterium suicordis* could be considered as supplementary factors in the prediction of anastomosis healing status in patients after CRC radical resection.

Key words: Intestinal microbiota; 16s rRNA gene sequencing; Anastomotic healing; Predictive ability; Colorectal cancer; Radical resection

Core tip: This study investigated the correlation between microbiota in mucosa tissues adjacent to surgical margin and anastomotic healing status. Bacterial community structure significantly varied in the impaired-healing group compared with the well-healing group. The current study was the first to demonstrate that six bacterial species were associated with the anastomotic healing in colorectal cancer (CRC) patients. Notably, *Alistipes shahii, Dialister pneumosintes*, and *Corynebacterium suicordis* in combination with age improved the accuracy for predicting the healing status of anastomoses. Thus, the three species could be used as the supplementary factors in predicting the healing status of anastomoses in CRC patients after radical resection of CRC.

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INTRODUCTION

Colorectal cancer (CRC) is one of the most frequently diagnosed cancers worldwide[1-3]. Impaired anastomotic healing is defined as a breach in a surgical join between two hollow viscera, with a leak of luminal contents that may emerge either through the wound or at the drain site, or near the anastomoses[4]. As one of the major complications after radical resection for CRC, impaired anastomotic healing may significantly increase hospital costs and prolong the length of hospital stay, and is also linked to cancer recurrence, metastasis, or even tumor-related death[5-11]. Clinical symptoms of impaired anastomotic healing often include abdominal pain/distension with fever, pus or fecal excretion, pelvic abscess, peritonitis, and even septic shock[12,13]. The International Study Group of Rectal Cancer[13] proposes the following system grading the severity of impaired anastomotic healing: A, no therapeutic intervention; B, active intervention is required but no re-laparotomy; and C, re-laparotomy is required. Impaired anastomotic healing rate has been found to vary from 1% to 19% depending on the anatomic location of the anastomosis[9,14-17]. Many risk factors have been identified to be associated with impaired anastomotic healing[10,18-20], for instance, a systematic review study[18] suggests that the main preoperative risk factors, which predict impaired anastomotic healing, are sex, age, tumor distal site, tumor size, advanced stage, renal disease, co-morbidity, and history of radiotherapy. Besides, blood loss/transfusion, duration of surgery, rectal contrast by computed tomography (CT), and C-reactive protein (CRP) level are considered as intraoperative risk factors or postoperative factors. The treatment strategies for impaired anastomotic healing after rectal cancer surgery can be divided into three stages according to the disease course as follows: Treatment strategies for the early stage (peritonitis stage, localized intra-abdominal abscess stage, and early stage fistula formation) mainly include identifying the necessity and opportunity of surgery,

establishing patency drainage, controlling infection, correcting electrolyte and acid-base imbalance, and providing nutritional support; in addition to the above measures, treatment strategies for the middle stage (fistula formation stage) include fistula management and closure treatment; in the later stage, deterministic remedial surgery is performed for unrecovered patients[13]. In terms of severity of specific symptoms, patients were treated either by systemic nutrition support, antibiotics, percutaneous drainage, and transanal revision or by re-laparotomy. Notably, antibiotic treatment should be implemented against Gram-negative bacteria and Gram-positive bacteria, and special attention is required for the anaerobic bacteria[21].

In most cases, the clinical symptoms of impaired anastomotic healing remain insidious, vague, and uncharacteristic, which are typically not recognized until postoperative days 5-8, sometimes even until postoperative day 12[15,22]. If not appropriately treated in time, impaired anastomotic healing in patients could easily evolve into severe postoperative complications and therefore affects morbidity, mortality, and functional and oncological outcomes[5-8]. Thus, early diagnosis and prediction of impaired anastomotic healing are of great significance. A pilot study suggests that combined changes of interleukin (IL)-4, IL-6, and IL-10 could accurately predict impaired healing of anastomoses[23]. Another study conducted on postoperative CRP in elective abdominal surgery shows that impaired anastomotic healing is unlikely to occur in patients with CRP < 135 mg/L on postoperative day 3[24]. Daams *et al*[25] carried out a study, in which peritoneal lactate concentration was continuously monitored by peritoneal microdialysis to characterize the ischemia and inflammation around the anastomosis, and they found a significant change of peritoneal lactate concentration in patients with impaired anastomotic healing. The results suggest that peritoneal microdialysis is predictive of impaired anastomotic healing after colorectal surgery[25-27]. However, these results need to be further confirmed by clinical trials. Intestinal microbes were first reported to be associated with impaired anastomotic healing over 60 years ago[28]. It is shown that directly using antibiotics on anastomotic tissues could promote the healing process and prevent leak in dogs undergoing colon resection and anastomosis. Recently, the mechanism has been confirmed to be linked to the direct effect of bacterial collagenases[12,29]. *Pseudomonas aeruginosa*, *Serratia marcescens*, and *Enterococcus faecalis*, which can express a collagenolytic phenotype, are reported to be associated with the impaired anastomotic healing[30-34]. Besides, Shogan *et al*[12] proved that *Enterococcus faecalis* is involved in the pathogenesis of impaired anastomotic healing by enhancing collagen-degrading activity and activating intestinal tissue matrix metalloproteinase 9 (MMP9). Preliminary evidence suggests that intestinal microbiota contributes to the occurrence and development of impaired anastomotic healing. Thus, it could be used as a potential predictor[12,20,29-34]. Recently, researchers reported the role of intestinal microbiota in the development of impaired anastomotic healing in the "donuts", in which a stapled colorectal anastomosis was made. Their results showed that a high abundance of *Lachnospiraceae* and *Bacteroidaceae* is strongly related to the impaired anastomotic healing, and the bacterial composition that consisted of 60% or more of these two families might be predictive of impaired anastomotic healing[35,36]. It can be seen that intestinal microbiota is emerging as a potential predictive factor for anastomotic healing. Nonetheless, it still lacks extensive clinical data and strong statistical evidence on the effect of intestinal microbiota on postoperative anastomotic healing. In this study, margin-surrounding mucosa samples derived from seven CRC patients with impaired anastomotic healing and 30 well-healed CRC patients were collected and the bacterial community was characterized by using 16s rRNA gene sequencing, with an aim to identify the specific bacteria related to the impaired anastomotic healing and to evaluate the ability of the selected taxa in predicting the healing status of anastomoses.

MATERIALS AND METHODS

Participant recruitment and sample collection

Thirty-seven patients with primary CRC who received surgical treatment from January 2017 to December 2018 at the First Affiliated Hospital, Zhejiang University School of Medicine were recruited to the study, and informed consent was obtained from all participants. The exclusion criteria for all participants were as follows: (1) Aged above 90 years old; (2) History of using antibiotics (excluding prophylactic antibiotic usage through intravenous infusion during the preoperative period) within two months; and (3) History of receiving chemotherapy or radiation treatments prior to the study, or personal history of chronic bowel disorders or metabolic diseases such as cirrhosis and diabetes. Specimens of mucosa tissues adjacent to surgical margin

derived from the participants were collected, immediately frozen in liquid nitrogen, and then stored at -80 °C for future DNA extraction and 16s rRNA gene sequencing. The study protocol was approved by the Medical Ethics Committee of the First Affiliated Hospital, Zhejiang University School of Medicine.

Anastomotic leak evaluation and confounders

Clinical manifestations of impaired anastomotic healing after rectal cancer surgery are diverse but could be roughly divided into two categories according to whether the clinical manifestations are typical[13,22]: (1) Typical manifestations: fever, which is defined as decline or rise of body temperature or persistent high fever during postoperative day 3-5; signs of rectal stimulation and acute diffuse peritonitis; increased pelvic drainage and changes in characteristics (*i.e.*, drainage of gas, mucilage, or feces); leak detected by digital rectal examination; severe paralytic intestinal obstruction and infectious shock; elevated levels of white blood cells, neutrophils, and CRP detected by laboratory examination; impaired anastomotic healing and surrounding fluid found by CT and magnetic resonance imaging examination through the anus or abdominal drainage tube angiography; and impaired anastomotic healing found on colonoscopy; and (2) Atypical manifestations: irregularly low or medium fever, frequent bowel movements, tenesmus, and gradually appearing local peritonitis and (or) paralytic intestinal obstruction in the hypogastria, showing flocculent matter by pelvic drainage. In this study, the impaired healing of anastomoses was defined according to the following clinical manifestations in patients who received radical resection for CRC: Abdominal or pelvic pain, fever (> 37.5 °C), discharge of feces, pus, or gas from pelvic drain, and discharge of pus from the rectum[13]. It should be noted that fever was not considered as impaired anastomotic healing in this study, as it is difficult to distinguish the clinical symptoms between postoperative infections from impaired anastomotic healing[15]. However, fever with abnormal drainage fluid (purulent, fecal, and long-term non-reduction) or with intestinal and abdominal abnormalities (such as abdominal pain without farting for a long time) was considered as impaired anastomotic healing.

According to the above indexes, the 37 patients were divided into two groups, namely, well-healing group (n = 30) and impaired-healing group (n = 7). The clinical characteristics (including age, sex, body mass index, tumor location, tumor size, TNM stage, tumor morphology, differentiation degree, intestinal obstruction, transfusion, blood loss, operation time, CRP, hemoglobin, and albumin) of the patients were recorded.

DNA extraction and characterization of bacterial community structure

Total DNA from the mucosa tissue samples was extracted using SDS/CTAB method. The purity and concentration of DNA were monitored on 1% agar gels (Thermo Fisher Scientific, Waltham, United States). The DNA sample was diluted into 1 ng/μL with sterile water and amplified using primers targeting the V4 region of the 16s rRNA gene (515F: 5′-GTGCCAGCMGCCGCGGTAA-3′ and 806R: 5′-GGACTACHVGGGTWTCTAAT-3′)[37]. Cycling conditions included preheating at 98 °C for 1 min, followed by 30 cycles of denaturation at 98 °C for 10 s, annealing at 50 °C for 30 s, elongation at 72 °C for 30 s, and final heating at 72 °C for 5 min.

PCR products were cleaned up using a GeneJETTM Gel Extraction Kit (Thermo Fisher Scientific, Waltham, United States). Sequencing libraries were constructed using an Ion Plus Fragment Library Kit (Thermo Fisher Scientific, Waltham, United States) following the manufacturer's instructions. The library quality was assessed on the Qubit@ 2.0 Fluorometer (Thermo Fisher Scientific, Waltham, United States). Finally, the library was sequenced on an Ion S5TM XL platform (Thermo Fisher Scientific, Waltham, United States).

Single-end reads were assigned to samples according to their unique barcodes and truncated by cutting off the barcodes from primer sequences. Qualities filtering on the raw reads were performed under specific filtering conditions to obtain the high-quality clean reads according to the Cut adapt quality controlled process (v1.9.1). The reads were compared with the Silva database (version 123) to detect the chimera sequences using UCHIME algorithm[38], and the chimera sequences were then removed to obtain the final clean reads[39].

Sequences analysis was performed with Uparse software (v7.0.1001). Sequences with a similarity ≥ 97% were assigned to the same OTUs. Representative sequence for each OTU was screened. The Silva database (version 123)[40] was used based on the MOTHUR algorithm to annotate taxonomic information. In order to study the differences of the dominant species in different samples (groups), multiple sequence alignment was conducted using the MUSCLE software (version 3.8.31). OTUs abundance information was normalized using a standard of sequence number corresponding to the sample with the least sequences. Subsequent analyses of alpha

diversity and beta diversity were all performed based on this output normalized sequencing data. Alpha and beta diversity analyses were calculated with QIIME software (version 1.7.0) and demonstrated with R software (version 2.15.3).

Statistical analysis

Statistical calculations were performed using SPSS (version 19.0). Wilcoxon test and chi-squared test were employed to analyze the correlation between the intestinal microbiota and anastomotic healing. The ability to discriminate impaired-healing and well healing was evaluated using the area under the receiver operator characteristic (ROC) curve (AUC). A P-value < 0.05 was considered statistically significant.

RESULTS

Characteristics of patients in well-healing and impaired-healing groups

A total of 37 CRC patients (age 67.97 ± 12.27 years old, 51.35% of males) were included in this study, and seven (18.9%) patients developed impaired anastomotic healing. Although it seemed to be a high rate of patients with impaired anastomotic healing, this is mainly because the method we adopted was more sensitive in order to ensure the minimum loss of impaired healing cases. The clinical characteristics of the patients are presented in Table 1. The patients were significantly older in the impaired-healing group (P < 0.05), and most of the tumors were found in the right colon (57.1%), while tumors located in the left colon and rectum were more commonly found in the well-healing group (36.7% and 46.7%, respectively). Patients in the impaired-healing group largely had stages I and III CRC (42.9% and 42.9%, respectively), while those in the well-healing group mainly had stage II CRC (50%). The differentiation degree concentrated in the moderate degree and mainly polyp adenocarcinoma and ulcerative adenocarcinoma were diagnosed in both groups. Intestinal obstruction occurred in two (28.6%) patients in the impaired-healing group and three (10%) patients in the well-healing group. Besides, no significant differences in operative time, blood loss, transfusion, CRP level, hemoglobin level, or albumin level were identified in the two groups.

Community structure shifts and correlation of intestinal microbiota with anastomotic healing

The structure shifts of microbiota in mucosa tissue were analyzed by 16s rRNA gene sequencing. A total of 4527 OTUs in all samples were identified, of which 1874 were common in the two groups. Besides, the results found 2293 unique OTUs in the well-healing group and 360 unique OTUs in the impaired healing group (Figure 1A). Rarefaction curve and species accumulation boxplot are shown in Figure 1B and C. The value of Good's coverage for each group was higher than 99.6%. Alpha diversity analysis was conducted to examine the estimators of community richness, diversity, and evenness by observed species index, Shannon index, Simpson index, Chao1 index, Goods coverage index, and PD whole tree index between the two groups (Figure 1D), however, no significant difference was detected. For beta diversity analysis, microbial community and composition were analyzed by using weighted UniFrac distance matrix for each group, and a significant difference was found between the two groups (Figure 1E, P < 0.01), suggesting that the community compositions of the two groups were different. LEfSe analysis showed that *Porphyromonas* genus and *Porphyromonadaceae* family were highly abundant in the impaired healing group (Figure 1E). Ten highest dominant phyla of the two groups are shown in Figure 1F. The dominant phyla of the impaired-healing group were *Proteobacteria* (33%), *Bacteroidetes* (25%), and *Firmicutes* (17%), which were same as those in the well-healing group (31%, 24%, and 21%, respectively). Figure 1G displays the ten highest dominant genera of the two groups. The dominant genera of the impaired-healing group were *Ignatzschineria* (11%), *Acinetobacter* (11%), and *Bacteroides* (11%), while *Bacteroides* (14%), *Stenotrophomonas* (9%), and *Ignatzschineria* (8%) were the dominant genera in the well-healing group.

To determine the association between the intestinal microbiota and anastomotic healing, Wilcoxon test was performed to analyze the abundance of bacteria species and genera in the two groups. Species and genera with significantly different abundances between the two groups are shown in Table S1 (P < 0.05). Thirty-three species and forty-five genera were found to be associated with the healing of anastomoses. Besides, six species (*Alistipes shahii*, *Dialister pneumosintes*, *Corynebacterium suicordis*, *Porphyromonas asaccharolytica*, *Vibrio diazotrophicus*, and *Clostridium leptum*) had a difference of carrier rate > 40%. As shown in Figure 2, *Alistipes shahii* and *Dialister pneumosintes* were significantly enriched in the well-

Table 1 Clinicopathological characteristics

Characteristic	Overall	Anastomotic healing		*P*-value[1]
	(*n* = 37)	Well (*n* = 30)	Impaired (*n* = 7)	
Age (mean ± SD), yr	67.97 ± 12.27	65.10 ± 11.36	80.29 ± 7.86	0.02
Body mass index (mean ± SD), kg/m^2	21.50 ± 2.73	21.70 ± 2.70	20.64 ± 2.71	N.S.
Sex: Male/female	19/18	16/14	3/4	N.S.
Tumor location: Right colon/left colon/rectum	9/12/16	5/11/14	4/1/2	N.S.
Tumor size (mean ± SD), mm	47.97 ± 22.87	47.17 ± 20.75	51.43 ± 30.08	N.S.
T stage: t1/t2/t3/t4	2/9/2/24	1/7/2/20	1/2/0/4	N.S.
N stage: n0/n1/n2	25/10/2	21/7/2	4/3/0	N.S.
M stage: m0/m1	37/0	30/0	7/0	N.S.
TNM stage: I/II/III	9/16/12	6/15/9	3/1/3	N.S.
Differentiation: Poor/moderate-poor/moderate /well	0/15/21/1	0/12/18/0	0/3/3/1	N.S.
Morphology: Elevated/ulcerative/invasive	17/19/1	14/15/1	3/4/0	N.S.
Intestinal obstruction: Present/absent	5/32	3/27	2/5	N.S.
Transfusion, *n*	1	0	1	N.S.
Blood loss (mean ± SD), mL	66.81 ± 31.60	67.76 ± 30.78	62.86 ± 34.52	N.S.
Operative time (mean ± SD), h	2.82 ± 0.68	2.71 ± 0.70	2.84 ± 0.67	N.S.
C-reactive protein (mean ± SD), mg/L	93.23 ± 48.75	101.88 ± 45.54	64.81 ± 48.18	N.S.
Hemoglobin (mean ± SD), g/L	105.36 ± 25.46	108.41 ± 25.05	92.29 ± 22.93	N.S.
Albumin (mean ± SD), g/L	34.30 ± 12.72	35.11 ± 13.88	30.80 ± 3.72	N.S.

[1]Wilcoxon test, chi-squared test, and paired *t*-test, as appropriate. N.S.: Not significant.

healing group ($P < 0.05$). However, the high abundance of *Corynebacterium suicordis*, *Porphyromonas asaccharolytica*, *Vibrio diazotrophicus*, and *Clostridium leptum* was significantly correlated with the impaired anastomotic healing ($P < 0.05$).

Predictive ability of selected bacterial taxa assessment for healing status of anastomoses

Univariate analysis and multivariate analysis showed that age was the only significant clinical variable for anastomotic healing (OR [odds ratio] = 1.223; 95% confidence interval [CI]: 1.032-1.449; $P = 0.020$), and age could be used as an independent factor predictive of the healing status of anastomoses (Figure 3, AUC = 0.838; 95%CI: 0.697-0.979; $P = 0.006$; the optimum cut-off value was 69.5). Six species (*Alistipes shahii*, *Dialister pneumosintes*, *Corynebacterium suicordis*, *Porphyromonas asaccharolytica*, *Vibrio diazotrophicus*, and *Clostridium leptum*) were selected as the potential predictive factors. Chi-squared analysis showed no correlation between these six bacteria and age. The 95%CIs of three bacteria (*Porphyromonas asaccharolytica*, *Vibrio diazotrophicus*, and *Clostridium leptum*) ranged from 0 to 1 in the ROC curve and were therefore excluded. In predicting the healing status of anastomoses in the two groups, we found that using *Alistipes shahii*, *Dialister pneumosintes*, and *Corynebacterium suicordis* were less useful than age (Figure 3, AUC = 0.824; 95%CI: 0.691-0.957; $P = 0.008$). However, the predictive ability was significantly improved if age was in combination with the three bacteria species, compared with the predictive model containing age only (age and *Alistipes shahii*, AUC = 0.886; 95%CI: 0.774-0.998; age and *Dialister pneumosintes*, AUC = 0.912; 95%CI: 0.807-1.000; age and *Corynebacterium suicordis*, AUC 0.874; 95%CI: 0.735-1.000) (Figure 3, $P < 0.01$). The predictive model combining age with *Dialister pneumosintes* proved to have the highest discriminatory ability (AUC = 0.912; 95%CI: 0.807-1.000; $P = 0.001$, the optimum cut-off value was 0.143), which was even higher than the model combining two bacterial species with age (age, *Alistipes shahii*, and *Corynebacterium suicordis*, AUC = 0.886; 95%CI: 0.774-0.998) (Figure 3, $P < 0.01$). Besides, age in combination with three bacterial species had a relatively equal predictive ability to the model in which age was combined with *Dialister pneumosintes* (Figure 3).

DISCUSSION

This study showed that the community structure was different between the impaired-

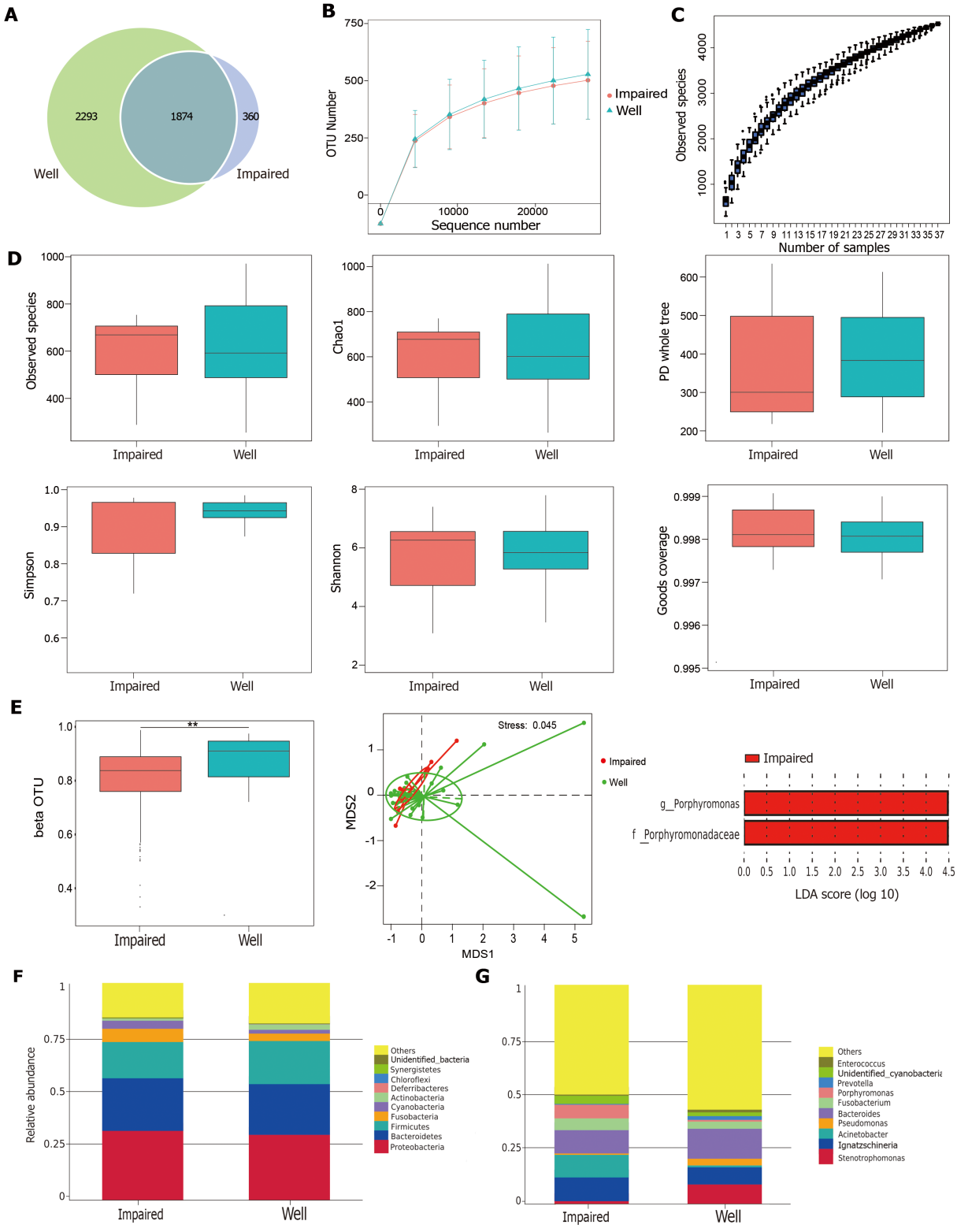

Figure 1 Structural change of the intestinal microbiota between impaired-healing group (*n* = 7) and well-healing group (*n* = 30). A: Venn diagram illustrating the total, unique, and shared numbers of OTUs predicted for impaired-healing group and well-healing group datasets; B: Rarefaction curve of OUT; C: Species accumulation boxplot; D: Alpha diversity analysis using observed species index, Shannon index, Simpson index, Chao1 index, Goods coverage index, and PD whole tree index; E: Beta diversity was significantly different between two groups by Wilcoxon test, $^{b}P < 0.01$; non-metric multi-dimensional scaling scores plot of weighted UniFrac distance matrix based on the relative abundance of OTU. Each symbol represents a sample. Colors represent different groups, stress = 0.045 < 0.2; LDA effect size (LEfSe) showed significant differences *in Porphyromonas* genus and *Porphyromonadaceae* family between two groups; F: Top-ten dominant phyla of two groups; G: Top-ten dominant genera of two groups.

healing group and the well-healing group. Six bacterial species were significantly correlated with anastomotic healing. *Alistipes shahii* and *Dialister pneumosintes* were significantly enriched in the well-healing group, which were not identified in the

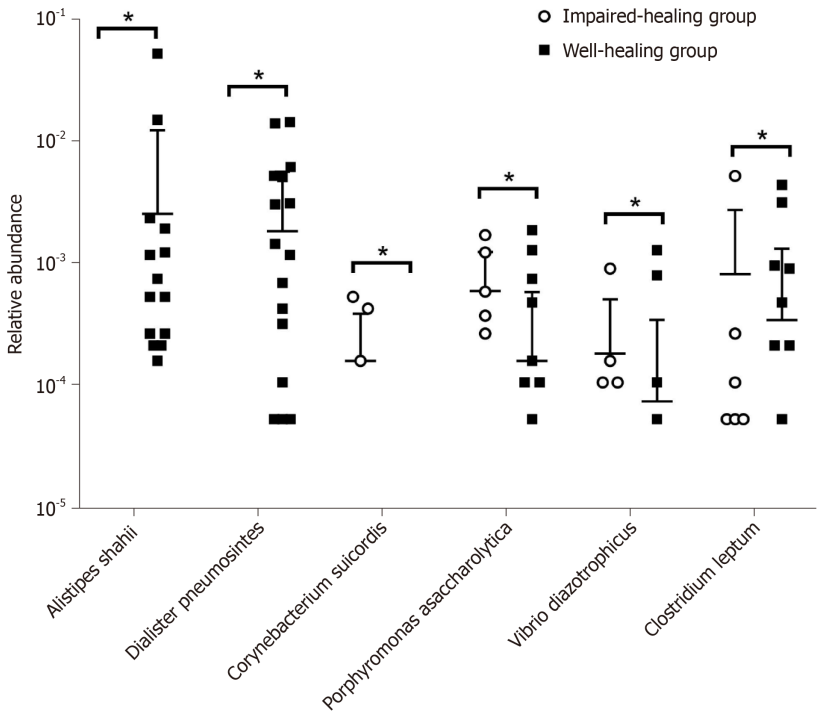

Figure 2 Correlation of intestinal microbiota with anastomotic healing. Relative abundance of six species (*Alistipes shahii*, *Dialister pneumosintes*, *Corynebacterium suicordis*, *Porphyromonas asaccharolytica*, *Vibrio diazotrophicus*, and *Clostridium leptum*) was significantly changed between the impaired-healing group and well-healing group. The significance was assessed by Wilcoxon test, $^{a}P < 0.05$.

impaired-healing group, indicating that *Alistipes shahii* and *Dialister pneumosintes* possibly contribute to anastomotic healing. However, the high abundances of *Corynebacterium suicordis*, *Porphyromonas asaccharolytica*, *Vibrio diazotrophicus*, and *Clostridium leptum* were strongly correlated with impaired anastomotic healing. *Corynebacterium suicordis* was only detected in the impaired-healing group, suggesting that it might be positively associated with impaired anastomotic healing. *Alistipes shahii* is a Gram-negative, strictly anaerobic, and rod-shaped bacterium[41]. *Dialister pneumosintes* is an obligate anaerobic Gram-negative rod associated with periodontal diseases and other oral infections[42]. Recently, it was reported that *Dialister pneumosintes* was related to hepatic abscess and bacteremia[42,43]. *Corynebacterium suicordis* is a Gram-positive, non-motile, non-spore-forming, catalase-positive, and rod-shaped bacterium[44]. *Porphyromonas asaccharolytica* has been previously reported to be associated with CRC and was correlated with lipopolysaccharide and energy biosynthetic pathways[45]. *Vibrio diazotrophicus* is a Gram-negative, facultatively anaerobic, halophilic, motile, and slightly curved rod-shaped bacterium[46]. *Clostridium leptum* is closely related to ulcerative colitis and significantly different in the ulcerated and the nonulcerated regions[47]. However, only a few studies investigated the role of these bacteria in anastomotic healing. To the best of our knowledge, this is the first report that these bacteria species were studied with the anastomotic healing in CRC patients. Based on our research and literature data, we speculated that these bacteria affect anastomotic healing through invasive ability and inflammatory mechanisms. It was revealed that *Enterococcus faecalis* contributed to the pathogenesis of impaired anastomotic healing by enhancing collagen-degrading activity and activating intestinal tissue MMP9[12,34]. *Pseudomonas aeruginosa*, *Serratia marcescens*, and *Enterococcus faecalis*, which express the collagenolytic phenotype, were reported to be associated with the impaired anastomotic healing[30-34]. Although there is a lack of direct evidence of the effects of intestinal microbes on anastomotic healing, several previous studies suggested that a correlation might exist between microbes and MMP activation. *Fusobacteria* (especially *Fusobacterium varium* and *Fusobacterium necrophorum*) were found to stimulate the secretions of MMP-9, MMP-13, and IL-8 from epithelial cells[48], while *Porphyromonas endodontalis* could produce lipopolysaccharides to induce the expression of MMP-9 through NF-κB signaling[49]. The mechanism by which these microbes affect anastomotic healing is worth further studying.

van Praagh *et al*[36] found that impaired anastomotic healing was linked to the

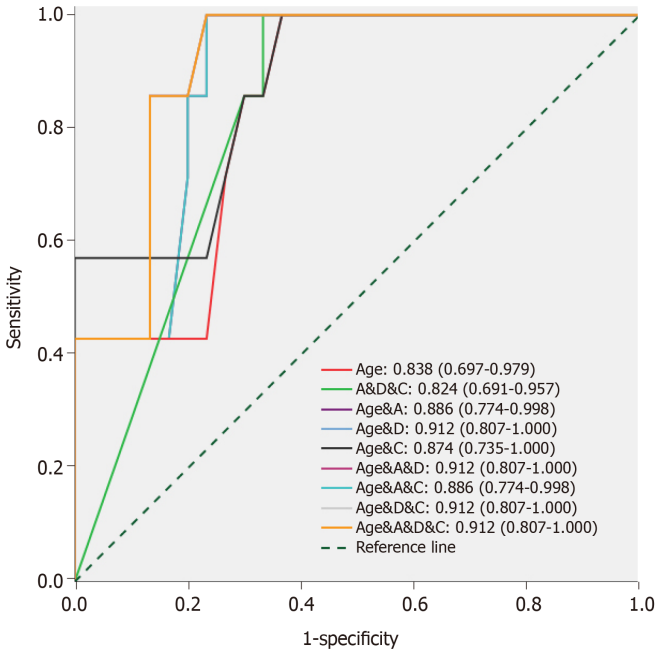

Figure 3 Microbial factors combined with clinical factor improve accuracy of predictive models for anastomotic healing. Receiver operator characteristic (ROC) curves for clinical factor (age) alone, microbial factors (*Alistipes shahii*, *Dialister pneumosintes*, and *Corynebacterium suicordis*) alone, and clinical factor with microbial factors. The value means the AUC (the area under the ROC curve) and 95%CI; A: *Alistipes shahii;* D: *Dialister pneumosintes*; C: *Corynebacterium suicordis*.

intestinal microbiota, particularly to a higher abundance of mucin-degrading members of the *Bacteroidaceae* and *Lachnospiraceae* families. Besides, a lower microbial diversity was found to be related to the impaired healing of anastomoses. Researchers speculated that a disturbed microbial composition could affect the metabolic balance and weaken colonization resistance to pathogenic bacteria involved in the occurrence and development of impaired anastomotic healing. The result was obtained from 123 "donuts" in which a stapled colorectal anastomosis was made. It was different from our sampling site (mucosa tissue adjacent to the surgical margin). Intestinal microbiota near the anastomotic site has been proved to interact with intestinal tissue and is likely to affect the healing[50]. Our study found that fewer OTUs were identified in the impaired-healing group, and the microbial diversity was lower, although the result was not statistically significant (which could be explained by the small sample size in this study). Patients might have a higher risk of developing impaired anastomotic healing when their microbial diversity was low[36], thus a full awareness of the role of intestinal microbiota in anastomotic healing is helpful in identifying high-risk patients and contributes to mitigating the potential severe clinical outcome caused by impaired anastomotic healing.

Sciuto *et al*[20] identified that older age (hazard ratio, 2.42), male sex (hazard ratio, 3.03), and lower anastomosis level (hazard ratio, 2.68) were the risk factors for impaired anastomotic healing. Consistent with the findings from previous reports, we also found that age (OR = 1.223) was significantly associated with the impaired healing of anastomoses and could be used as an independent risk factor to predict the healing status of anastomoses. We found that patients older than 69.5 years were more prone to develop impaired anastomotic healing. Notably, our results suggested that three associated bacteria species, especially *Dialister pneumosintes*, in combination with age significantly improved the predictive ability, compared with the model containing only age. van Praagh *et al*[36] suggested that samples were more likely to have impaired anastomotic healing if the total sum of *Lachnospiraceae* and *Bacteroidaceae* in them was higher than 60% and the Simpson diversity score was < 0.75. Thus, the effect of the intestinal microbiota on the predictive value of anastomotic healing is positively useful in clinical nursing and postoperative surveillance.

Technical factors such as the tension on the suture line, accurate suture placement, and blood supply were vitally important in ensuring the optimal healing of anastomoses[51]. Unfortunately, it was difficult to be controlled precisely, and this is true to the most experienced and technically proficient surgeons. Besides the preventive measures implemented during the bowel resection, the early detection and

diagnosis were equally important to prevent the patients from developing complications and severe clinical outcome caused by impaired healing of anastomoses. Our findings provided new clinical evidence for the theory that intestinal microbes are involved in the anastomotic healing and might contribute to the screening of the potential targets for the early diagnosis and treatment of impaired anastomotic healing.

In conclusion, the mucosa-invasive microbiota is associated with the impaired anastomotic healing in the patients enrolled in this study. *Alistipes shahii*, *Dialister pneumosintes*, and *Corynebacterium suicordis*, which are not related to age, could be used as the supplementary factors in the prediction of the healing status of anastomoses in CRC patients after radical resection of CRC.

ARTICLE HIGHLIGHTS

Research background

The clinical symptoms of impaired anastomotic healing are typically not recognized. However, if not appropriately treated in time, impaired healing could easily evolve into severe postoperative complications. Thus, early diagnosis and prediction of impaired anastomotic healing are highly necessary.

Research motivation

A large number of studies reported that intestinal microbiota contributes to the development of impaired anastomotic healing. A full understanding of the role of intestinal microbiota in anastomotic healing can help identify high-risk patients and alleviate the potentially serious clinical outcomes caused by impaired anastomotic healing.

Research objectives

To identify the specific bacteria related to impaired anastomotic healing and to evaluate the predictive ability of the microbiota taxa for the healing status of anastomoses.

Research methods

Margin-surrounding mucosa samples derived from seven colorectal cancer (CRC) patients with impaired anastomotic healing and thirty well-healed CRC patients were respectively collected and the bacterial community was characterized by 16s rRNA gene sequencing. Wilcoxon test and chi-squared test were performed to analyze the statistic differences of bacterial taxa in the two groups. The predictive ability of the bacterial taxa for the healing status of anastomoses was evaluated by the area under the receiver operator characteristic curve.

Research results

The community structure was different between the impaired-healing and the well-healing groups. Six bacteria species (*Alistipes shahii, Dialister pneumosintes, Corynebacterium suicordis, Porphyromonas asaccharolytica, Vibrio diazotrophicus*, and *Clostridium leptum*) were significantly correlated with anastomotic healing. Age was highly associated with the impaired healing of anastomoses. Three bacteria species (*Alistipes shahii, Dialister pneumosintes*, and *Corynebacterium suicordis*) in combination with age noticeably improved the accuracy for predicting the healing status of anastomoses.

Research conclusions

The mucosa-invasive microbiota was associated with the anastomotic healing in the research subjects. *Alistipes shahii, Dialister pneumosintes*, and *Corynebacterium suicordis* could be used as the supplementary factors in the prediction of the healing status of anastomoses in CRC patients after radical resection of CRC.

Research perspectives

Our findings provided new clinical evidence for the theory that intestinal microbiota is involved in the anastomotic healing, and it contributes to the screening of potential targets for the early diagnosis and treatment of impaired anastomotic healing.

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ORIGINAL ARTICLE

Observational Study

Colorectal cancer fecal screening test completion after age 74, sources and outcomes in French program

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Abstract

BACKGROUND

Elderly patients aged at least 75 years old (Elderly_75), represent 45% of colorectal cancer (CRC) incidence. As others, the French Colorectal Cancer Screening Program (CRCSP) does not include Elderly_75. To date, there is little evidence to justify stopping screening at 74 years of age.

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AIM
To describe CRC fecal screening test completion after age 74, source (CRCSP/Provider ordered) and outcomes of these tests.

METHODS
The study concerned 18704 Elderly_75 residing in eleven French districts (Ain, Doubs, Essonne, Haute-Saone, Hauts-de-Seine, Jura, Seine-Saint-Denis, Territoire-de-Belfort, Val-de-Marne, Val-d'Oise, Yonne), having performed a CRC screening test between January 2008 and December 2017. The tests performed in a circumstance of delayed response to a solicitation (DRS) from the local cancer screening managing center (Managing-Center) were distinguished from the tests non-solicited by the Managing-Center, performed after a recommendation by a General Practitioner (GP) or other provider ordered (RGP). DRS was any test realized by an Elderly_75 following an initial invitation from the Managing-Center with a maximum 24 mo after this invitation. Any Non-DRS test was considered RGP. The outcomes of these tests were described according to the circumstances of test completion.

RESULTS
Of 18995 screening-tests were performed at ages: 75 (83.5%), 76-80 (13.4%) and > 80 (3.1%) years old. Elderly_75 performed the screening test in a circumstance of DRS (71.9%) or RGP (28.1%). The proportion of the tests that could not be analyzed and not restarted was 13.2%. For these unanalyzed tests, the reason was age-related in 78.0% of cases, related to the laboratory's refusal to analyze the test of people aged ≥ 77. Reported colonoscopy completion rate was 81.3%. For those 575 people with reported colonoscopy, no complication was listed. 18.0% of the 366 Elderly_75 with lesions had no anteriority in the CRCSP. The neoplasia (124 Low-risk-polyps, 159 High-risk-polyps, 13 Unspecified-polyps and 70 CRCs) detection rate was 19.3/1000 Elderly_75 screened and the CRC detection rate was 3.7/1000 Elderly_75 screened.

CONCLUSION
The high rate of colonoscopy completion after a positive test and the high proportion of screened lesions observed suggest that the lengthening of the screening period could allow significant detection of CRC and polyps that occur in Elderly_75 excluded from CRCSP.

Key words: Colorectal cancer; Fecal screening test; Participation rate; Colonoscopy completion rate; Elderly

Core tip: Reported colonoscopy completion rate was 81.3% and the neoplasia detection rate was 19.3 per 1000 Elderly screened and the colorectal cancer detection rate was estimated at 3.7 per 1000 Elderly screened. These results remain significantly higher than that usually found in the French Colorectal Cancer Screening Program (CRCSP). The motivation to participate including for colonoscopy and the high proportion of screened lesions sufficiently demonstrate that the lengthening of the follow-up period in a screening program, up to about 80 years of age, could make it possible to detect many cases that occur after the exclusion of Elderly_75 from CRCSP.

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INTRODUCTION

In addition to its high incidence and high mortality (around 42000 new cases and 18000 deaths per year), colorectal cancer (CRC) meets all the conditions to be screened in France[1-3]. Screening every two years, allows to obtain a reduction in CRC mortality in tested population[2,3]. In Burgundy, this decrease in mortality was of the order of 16% after 11 years of follow-up of a cohort of 45000 individuals[2]. European Commission recommended the implementation of the Colorectal Cancer Screening Program (CRCSP) by the search for occult bleeding in stool to face the high morbidity and mortality associated with CRC[4]. Following the recommendations, the screening of the CRC started in Germany in 1977[5], was progressively implemented between 2000 and 2010 in other European countries, through CRC screening pilot programs, notably in England[6], in Italy[7] in Holland[8,9] and in France[10].

To date, most of the CRCSP policies target a medium risk population of CRC, defined on the age criterion with considerable variability from one program to another[11]. Since the effectiveness of screening depends on its application in an appropriate setting and at an appropriate frequency[12], most European programs, observe an interval of 2 years between two screenings campaign. The test was offered to people (all gender) aged between 40 and 60 in Bulgaria, between 50 and 74 in France, England and Belgium; between 50 and 70 in Italy, between 55 and 75 in Holland[13]. The justification of these age groups remains controversial in the current state of knowledge. Indeed, there is little objective data to justify discontinuation of screening at 75 years. Recent Modeling was proposed to estimate from what age it was desirable to no longer invite to do the screening. They estimate screening up to age 82 as relevant[14,15].

In France, as in other countries, the CRCSP does not include people aged ≥ 75 years, despite the high incidence of CRC after 74 years[16] and the constant increase in life expectancy since 2000[17]. It seems necessary to guide the debate on the problem of screening among the elderly. Indeed, in each French district, the sporadic participation of elderly patients aged at least 75 years old (Elderly_75), out of the national CRCSP's recommendation, is recorded every year. We describe here, these CRC fecal screening test completion after age 74, the source (screening program or provider ordered) and outcomes.

MATERIALS AND METHODS

Ethical considerations

Before analysis, all data were anonymized. The screening database had a favorable opinion from the institution that oversees the ethics of data collection ("Commission nationale de l'informatique et des libertés": CNIL). According to the current French legislation, a study that does not change the care of patients did not require the opinion of the Clinical Research Centers Ethics Committee. This article does not contain any studies with human participants performed by any of the authors. This study does not involve human participants and informed consent was therefore not required. This article does not contain any studies with animals performed by any of the authors.

Study design

A descriptive study of 18704 Elderly_75s, residing in eleven French districts (Ain, Doubs, Essonne, Haute-Saone, Hauts-de-Seine, Jura, Seine-Saint-Denis, Territoire-de-Belfort, Val-de-Marne, Val-d'Oise, Yonne), having performed a CRC screening test between January 1, 2008 and December 31, 2017. The circumstances of performing this test (source of the test, previous behavior in the CRCSP) and the outcome (test result, colonoscopy completion and detected lesions) were described.

Distribution of screening tests in the districts

On a voluntary basis, the eleven districts were chosen in three French regions (Bourgogne-Franche-Comté, Ile-de-France, Rhône-Alpes-Auvergne), among those using the same screening database management's materials. With a population (50-74 years) of 2355240 persons targeted by the 2015-2016 screening campaign, these eleven districts totaled 554294 participants in this campaign, with an average participation rate estimated at 23.5%[18].

In France, a nationwide population based CRCSP was rolled out from January 2008 to December 2009. The program was based on a biennial guaiac faecal occult blood test distributed to men and women aged 50-74 at average risk of developing CRC. If positive, the test was followed by a total colonoscopy[19,20]. CRCSP was organized at a district level by the local cancer screening managing center (Managing-Center), in accordance with CRCSP's national specifications[19,20]. Each year, people targeted by

the campaign receive a letter inviting them to take a free screening test from their General Practitioner (GP). The pattern of distribution of the screening test recommends that the GP gives the screening test to any person aged 50 to 74 years, provided with an invitation letter. In the absence of this letter, the GP may give the test after establishing the eligibility of the person, using a connection made available to doctors or by calling to the Managing-Center. Until July-November, 2014, people who did not tested after the first recall letter, received the test kit at home, six months after the initial solicitation. The only organizational change (related to the objective of the study) imposed by the introduction of a new screening test *i.e.*, OC-sensor© fecal immunochemical test (FIT) replacing guaiac test in May 2015, was the supplying mode of GPs in screening tests kit, the classic pattern of kit distribution remaining unchanged.

Operational definition of variables and descriptive analysis

For each Elderly_75 having performed a test out of recommendation, solicitation/participation data from previous campaigns were extracted from the Managing-Centers databases. These databases were regularly enriched by socio-demographic data (gender, age), colonoscopy, histopathology and follow-up data provided by partners (Health Insurance Plan, Medical Information Services, Gastroenterologists, Surgeons, GP, *etc.*). The tests performed in a circumstance of delayed response after a solicitation (DRS, initial or recall letter existing) from the Managing-Center, done before 75 years were distinguished from the tests non-solicited by the Managing-Center, performed after a recommendation by a GP or other provider ordered (RGP). DRS was any test realized by an Elderly_75 following an initial invitation from the Managing-Center with a maximum 24 months after this invitation. Any Non-DRS test was considered RGP.

Previous behavior in the CRCSP: Anteriority in the CRCSP was defined by the existence of at least one solicitation to participate in a CRCSP campaign between 50 and 74 years of age. The previous behavior in terms of the outcome of these earlier solicitations was described.

For each solicitation, the participation was defined as carrying out a fecal occult blood test according to the specifications of the French CRCSP[19,20]. For each person, the adherence to testing was evaluated as frequency of participation in previous campaigns. This frequency was expressed by the ratio between the observed number of participations in previous campaign and the expected number of participations in these previous campaigns. Considering the bi-annual organization of screening campaigns and a person age (X) at the date of first invitation in the district, the expected number of participation for a person aged Y year old, was (Y-X)/2. It was coded as a discrete variable: Without anteriority (The person was never solicited in the district program); Never participated (The person has not participated in any of the previous campaigns to which it was solicited); Participated with frequency < 30% of campaigns; frequency between 30 and 50%; frequency > 50% to 75% and frequency > 75%.

For Elderly_75 with a history of solicitation, the presence of a history of NLAI (Does Not Live at the Address Indicated) or refusal to participate in a campaign was collected and coded as a binary variable (No/Yes). Similarly, the presence of a history of colonoscopy, reported by a mailing response after solicitation, was described as a binary variable (No/Yes). If the person participated in at least one campaign, history of positive test and colonoscopy performed after this positive test was collected and coded as a discrete variable: No history of positive test, positive test without colonoscopy, positive test with normal colonoscopy, positive test with positive colonoscopy.

CRC fecal screening test completion after age 74: The outcome of screening test performed after 74 years was analyzed in terms of test quality (non-analyzable test proportion) and test result (Negative, Positive). In the event of a positive result, the diagnostic course has been analyzed in terms of frequency of colonoscopy completion and in terms of frequency and types of diagnosed lesions including: Low-risk-polyps, High-risk-polyps, Unspecified-polyp and CRC. High-risk-polyps included, Adenomas ≥ 10 mm (except hyperplastic polyps), Serrated adenomas, Adenomas with high grade dysplasia, Villous or Tubulo-villous adenomas. The diagnoses associated with CRC and polyps were those related to the C18-C20 and D12 codes of the 10th version of the WHO International Classification of Diseases[21]. The positive predictive value (PPV) of the test was estimated as the proportion of lesions among the Elderly_75 who completed a colonoscopy after a positive test.

Statistical analysis

Qualitative variables were described in frequency and quantitative variables were described in mean ± SD. Frequencies were compared between groups by a Pearson χ^2 test or Fisher's exact test. Means were compared between two groups by Student's *T* test. All the analyzes were carried out with version 13 of STATA software (College Station, Texas, United States) and $P < 0.05$ was considered for statistical significance.

RESULTS

Modality of participation

Of 18704 Elderly_75 (56.3% female), performed 18995 tests between January 1, 2008 and December 31, 2017. These 18995 screening-tests were performed at ages: 75 (83.5%), 76-80 (13.4%) and > 80 (3.1%) years old. Over this period, 250 Elderly_75 out of 18704 completed at least 2 tests after the age of 74. On average, over one year of campaign, 7 out of 1000 tests were realized by people aged ≥ 75 years. This proportion reached 15 per 1000 in 2015 (Table 1). 13442 people (71.9% of the sample), performed the first test after the age of 74, in a circumstance of DRS of the Managing-center. This response delayed by 7.8 ± 5.3 mo after the initial solicitation. For those who have performed a test under recommendation and have an anteriority in CRCSP, the delay between the last test in the CRCSP and this test was 37.6 ± 18.9 mo.

Mean age at the time of the test was 75.7 ± 1.4 years with extremes between 75 and 91 years (75.3 ± 0.3 years in the DRS group *vs* 76.9 ± 2.3 years in the RGP group, $P = 0.00001$). In the DRS group, 98.2% were 75 years old at the time of the test and 1.8% were 76-80 years. In the RGP group, 49.8% were 75 years old at the time of the test, 40.0% were 76-80 years and 10.2% were > 80 years ($P = 0.00001$). The proportion of females was not significantly different between DRS (56.5%) and RGP (56.0%, $P = 0.52$).

Previous behavior in the CRCSP

Seventy-nine point five percent of this population (76.9% of the 13442 DRSs *vs* 86.3% of the 5262 RGPs, $P = 0.60$) had an anteriority, i.e. at least one previous invitation in the CRCSP. Among these 14875 Elderly_75 with an anteriority (10336 in DRS and 4539 in RGP), 12678 had at least one previous screening test between 50 and 74 years old (9119 in DRS and 3559 in RGP) and they participated in 73.6% (77.2% in DRS *vs* 65.4% in RGP, $P = 0.00001$) of the campaigns to which they were solicited. Average number of this previous solicitations was 2.5 ± 1.3 (2.4 ± 1.2 in DRS *vs* 2.7 ± 1.3 in RGP, $P = 0.00001$). 11.8% of the Elderly_75 with an anteriority never participated in a previous CRCSP campaign (9.1% in DRS *vs* 18.6% in RGP, $P = 0.00001$) and 9.3% had a reported history of colonoscopy (8.0% DRS *vs* 12.1% RGP, $P < 0.001$). Only 1.9% (1.7% in DRS *vs* 2.1% in RGP, $P = 0.1$) had a reported history of NLAI or sent previously a form indicating their refusal to participate in the CRCSP. The frequency of participation in previous campaign and the results of previous tests by campaign year are summarized in Table 2. The proportion of Elderly_75 with a previous positive test in CRCSP among these with an anteriority was 1.1% (200 Elderly_75/18704). This proportion was higher in RGP (1.1% in DRS *vs* 2.8% in RGP, $P = 0.00001$). Whatever the source of the test and the year of screening, the frequency of participation in previous campaign was at least 60.0%. Peoples who performed the test under recommendation in 2012 were less regular in the CRCSP than those who performed the test the same year, in a context of DRS (Figure 1). Before 2010, the screening test was performed mostly by people without any anteriority (1339 out of 1721 Elderly_75 in 2009) in the CRCSP or having never participated in a campaign (157/1721 tests in 2009). From 2011 to 2017, the test was mostly performed by people who participated in at least 70% of campaigns.

CRC fecal screening test completion after age 74

Overall, 3.7% (3.7% in DRS *vs* 3.7% in RPD, $P = 0.79$) of the 18995 tests performed were positive (Table 3). The proportion of the tests that could not be analyzed and not restarted was 13.2%. For these unanalyzed tests, the reason was age-related in 78.0% of cases, related to the laboratory's refusal to analyze the test of people aged ≥ 77. 18.0% of the 366 Elderly_75 with lesions had no anteriority in the CRCSP and 10.4% had never participated. Reported colonoscopy completion rate was 81.3%. For those 575 people with reported colonoscopy, no complication was listed. 124 Low-risk-polyps, 159 High-risk-polyps, 13 Unspecified-polyps and 70 CRCs were detected, with an average neoplasia's PPV estimated at 0.64. This PPV varied from 0.46 (16 neoplasia out of 35 colonoscopy) in 2008 to 0.67 in 2017 (40 neoplasia out of 60 colonoscopy) (Table 4). The neoplasia detection rate was 19.3 per 1000 Elderly_75 screened and the CRC detection rate was estimated at 3.7 per 1000 Elderly_75

Table 1 Total tests performed in the ten districts and part of tests performed by elderly aged ≥ 75 years (elderly ≥ 75), by year of campaign

Campaign year	Total tests performed in the districts	Tests performed by elderly ≥ 75			
		75 yr	76-80 yr	> 80 yr	Total (Part in %)
2008	230467	1172	113	13	1298 (5.6)
2009	343468	1588	141	3	1732 (5.0)
2010	289120	1119	147	14	1280 (4.4)
2011	313982	1659	166	18	1843 (5.9)
2012	287818	1478	158	17	1653 (5.7)
2013	302392	1815	166	16	1997 (6.6)
2014	267342	1557	122	25	1704 (6.4)
2015	163976	2039	435	100	2574 (15.7)
2016	399039	1992	728	225	2945 (7.4)
2017	286039	1436	369	164	1969 (6.9)
Total	2883643	15855	2545	595	18995 (6.6)

screened. An increased detection rate (all neoplasia) was observed after the immunochemical test introduction in 2015 (Table 5). The mean age at neoplasia diagnosis was 75.7 ± 1.2 years (75.2 ± 0.2 years in DRS *vs* 77.1 ± 1.8 years in RGP, $P < 0.001$). This mean age was 75.4 ± 0.7 years when the lesion was a low risk polyp, 75.4 ± 0.5 years when it was a high-risk polyp and 77.0 ± 2.1 years when it was a CRC.

DISCUSSION

Few results of CRC fecal screening in elderly are available. This study present results of out of recommendation screening in 18704 elderly over 74 years of age. The results observed appear to be beyond expectations. Indeed, the proportion (3.7%) of positive tests remains significantly higher than that found (2.9) in these districts in the same period or that usually found (2.6%) in the CRCSP program[20]. In the national program, reported colonoscopy completion rate after a positive test was 76.9% with approximately major incidents, including perforation in 3 in 1000 colonoscopies[20]. In addition to a similar even higher rate of colonoscopy completion observed in this study (81.3%), no major incident was recorded in this elderly population. In France, the detection rate of CRC was estimated at 1.6/1000 in CRCSP[20], which is only half of the rate observed in this study. Certainly, the high detection rate observed could be explained, at least in part, by age. Indeed, the increase in the incidence of CRC with age has been well documented[1,22,23]. Between 40 and 80 years of age, the incidence doubles almost every ten years[24] and 45% of CRCs are diagnosed in people aged at least 75 years in Burgundy[16]. In this population, CRC were diagnosed on average age at 77 years. Considering these findings, it could be argued that extending the duration of CRCSP in healthy people up to 80 years, could allow the screening of the many cases that occur after the exclusion of the Elderly_75 from CRCSP.

The sample described in this analysis consisted mainly of people regularly invited by the local cancer screening managing center with delayed answer. Although the age group concerned by the CRCSP is 50 to 74 years old, the logistic approach implemented for the last invitation challenges this approach. Indeed, people receive their last invitation until the eve of the 75th birthday. This timing inevitably has the consequence of carrying out a screening test beyond the extreme ages recommended in CRCSP.

The motivation of people previously devoted in the CRCSP, to continue the screening after 74 years has been previously discussed and ending screening at 75 years is a real ethical issue[25]. Previous modeling studies have recommended screening up to 82 years, however, heterogeneity of the Elderly_75 population limit this implementation[14,15]. Indeed, in addition to the fact that the gain in years of life remains very modest, heterogeneity of the population with both healthy subjects and subjects in precariousness or with comorbidities makes it difficult to choose the type of appropriate screening[25]. The United States Preventive Services Task Force advocated screening for CRC in healthy people up to age 84. Beyond this age, it suggested that the disadvantages of screening outweigh profits[22,26].

In order to sustain the CRCSP extension after 74 years, the availability of the

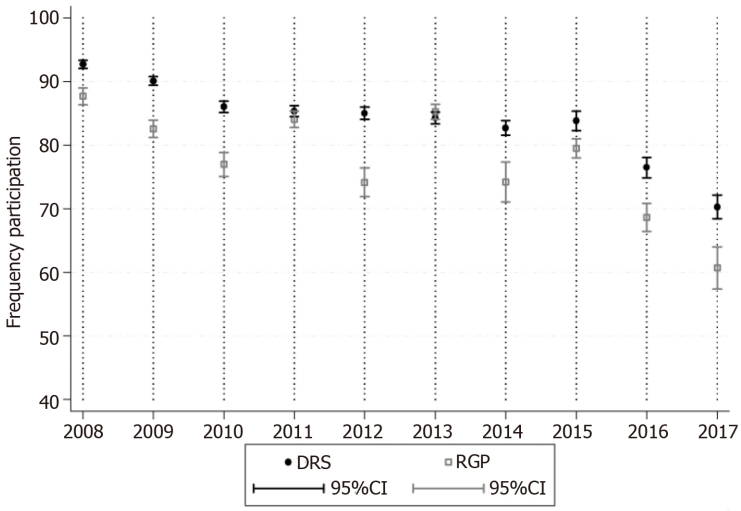

Figure 1 Frequency of participation in previous campaigns, by circumstances and by screening year. DRS: Delayed response after solicitation; RGP: Recommendation of a General Practitioner/Provider ordered; CI: Confidence interval.

medical profession must be acquired for medical monitoring and a continuous evaluation of the disadvantages and benefits of screening. It has been documented that the adherence of the GP is a determining factor in the acceptability and effectiveness of a screening campaign[27]. Although the inequalities in access to cancer care are still visible in France[28,29], the results of this study show the possible availability of GPs to do a shared decision making process with patients regarding the pros and cons, and individualized risk assessments. Indeed, more than a quarter of the test were performed under medical recommendation which would testify not only an adherence of GPs but especially their concern for the follow-up of patient excluded from CRCSP. However, two major hypotheses could explain the test delivery to the Elderly_75 by the GPs. The hypothesis of a catch-up for the less regular persons in the CRCSP between 50 to 74 years, is concordant with the low frequency of participation in previous campaign of the people having performed a test after provider ordered recommendation (compared to the DRS). It is not excluded that GPs also use the screening test as an additional means of diagnosis. The bond in Elderly_75 participation after FIT introduction (in 2015), which is more sensitive and more user-friendly (only one sample) is also an essential point to be mentioned.

The proportion of non-analyzable tests remains abnormally high, especially among those who have taken the test under provider recommendation and after the introduction of FIT. Indeed, the proportion of non-analyzable tests was 1.6% in the CRCSP[19,20]. When we subtract these numerous cases related to the refusal of the laboratory, the elderly could have a rate of non-analyzable tests lower than that observed in the CRCSP. The fundamental question with this main reason could be: "How many cancers have not been detected because of this age-based refusal of the person who made the tests"? The answer to this question is predicted by the high incidence of CRC (0.6/1000 Person-years) among people performing their last screening tests (between 70 and 74 years) in the CRCSP[23].

The study sample consisted mainly of 83.4% of tests performed at the age of 75, which could lend interpretation almost exclusively to this age. In addition, outside of the hypotheses evoked, the study could not bring more precise details on the real circumstances of realization of the test after 74 years. Indeed, a major concern regarding the RGP group is the possible self-selection bias: The considered population could have been motivated to participate in the screening by high risk factors or symptoms. In this group, screening test could have been used as a diagnostic tool. However, performing a colonoscopy after a positive screening test is a classic pattern in the French organized screening program. Certainly, the obtained results do not provide enough evidence on the benefits of screening in this age group, i.e. decrease in mortality from CRC if screening would be recommended above the currently accepted age limit. The results could, however, constitute a starting point (or even a reference) for a possible experimentation of the screening program for CRC in the elderly, in France.

In conclusion, despite a regulatory framework that is not favorable to their participation in CRCSP campaigns, a motivation to continue CRCSP after 74 years of

Table 2 Distribution of 18704 elderly aged ≥ 75 years, according to the test performing contexts and history in Colorectal Cancer Screening Program, by year of campaign

Test achieving contexts	Campaign year										
	Total	2008	2009	2010	2011	2012	2013	2014	2015	2016	2017
Total	18704	1298	1721	1264	1815	1627	1966	1687	2525	2882	1919
Circumstances											
Delayed response to solicitation	13442	1108	1509	982	1548	1363	1689	1496	814	1645	1288
Recommendation of a GP	5262	190	212	282	267	264	277	191	1711	1237	631
Average number of previous solicitation[1]	2.5 ± 1.3	1.2 ± 0.4	1.2 ± 0.5	1.3 ± 0.6	1.4 ± 0.8	1.8 ± 0.9	2.3 ± 0.9	2.5 ± 1.0	3.1 ± 1.1	3.2 ± 1.2	3.5 ± 1.3
Fidelity in CRCSP											
Without anteriority	3829	1174	1339	417	164	114	80	76	99	212	154
Never participated	2197	57	157	278	384	212	200	143	183	330	253
< 30%	248	0	0	1	3	5	17	24	34	74	90
30%-50%	1824	4	28	50	130	205	312	268	236	324	267
50%-75%	1573	0	1	27	53	64	145	180	357	431	315
75%-100%	9033	63	196	491	1081	1027	1212	996	1616	1511	840
NLAI/refusal history[1]	277	3	5	8	25	25	28	32	40	59	52
Colonoscopy history in mailing response[2]	1378	10	22	44	106	97	138	155	271	300	235
History of positive test [T(+)][2]											
T(+) without colonoscopy	22	1	0	0	0	2	4	0	8	5	2
T(+) with normal colonoscopy	3	0	0	0	0	1	0	2	0	0	0
T(+) with colonoscopy (+)	175	0	0	0	4	3	16	19	39	51	43

This was about the first test for people who performed at least 2 tests after 74 years.
[1]Persons without anteriority are excluded;
[2]Persons without anteriority or who have never participated in the previous Colorectal Cancer Screening Program are excluded. NLAI: Does Not Live at the Address Indicated; CRCSP: Colorectal Cancer Screening Program; GP: General Practitioner.

age was observed. To optimize CRC fecal screening for these Elderly_75, in addition to existing communication tools, the implementation of other means to better select patients and minimize the proportion of non-analyzable tests could be beneficial. The motivation to participate including the high colonoscopy participation rates and the high proportion of screened lesions demonstrate eloquently that the lengthening of the follow-up period in a screening program, up to about 80 years of age, could make it possible to detect many cases that occur after the exclusion of Elderly_75 from CRCSP. In a period of implementation of proposals for improving national program of CRCSP, it seems necessary to feed the debate on screening of the elderly to alert decision-making bodies. More in-depth studies on the feasibility of organized screening in Elderly_75 seem necessary to confirm or not, the current screening age limit.

Table 3 Distribution of the 18995 tests performed by elderly aged ≥ 75 years, according to the circumstances of realization (It was about the circumstances of the first test for people who performed at least 2 tests after 74 years) and the results of the test

Results	Circumstances of realization		
	Delayed response after solicitation	Recommendation of a prescriber doctor	Total
Total tests performed after 74 yr	13596	5399	18995
Negative test	12529	3262	15791
Non-analyzable and non-restated test	559	1938	2497
Positive without colonoscopy	107	25	132
Positive with normal colonoscopy	147	62	209
Positive with colonoscopy (+)	254	112	366
Total lesions screened	254	112	366
Unspecified polyps	8	5	13
Low risk polyps	94	30	124
High risk polyps	109	50	159
Colorectal cancer	43	27	70

Table 4 Distribution of 18995 tests performed by elderly aged ≥ 75 years (elderly ≥ 75), according to the results of the test and year of screening

Results	Screening year										
	Total	2008	2009	2010	2011	2012	2013	2014	2015	2016	2017
Total test performed by elderly ≥ 75	18995	1298	1732	1280	1843	1653	1997	1704	2574	2945	1969
Test results											
Negative test	15791	1227	1600	1204	1720	1549	1855	1590	1878	1837	1331
Non-analyzable and non-restated	2497	23	70	23	69	70	89	82	544	972	555
Positive without colonoscopy	132	13	13	11	5	7	10	9	16	25	23
Positive with normal colonoscopy	209	19	20	15	15	7	22	5	49	37	20
Positive with colonoscopy (+)	366	16	29	27	34	20	21	18	87	74	40
Type of lesions screened											
Unspecified polyps	13	0	2	0	1	0	0	0	6	1	3
Low risk polyps	124	6	7	11	12	7	10	6	24	29	12
High risk polyps	159	5	13	10	16	7	5	7	44	33	19
Colorectal cancer	70	5	7	6	5	6	6	5	13	11	6

Table 5 Colorectal cancer detection rate in the 11 districts and part of tests performed by elderly aged ≥ 75 years (elderly ≥ 75), by year of campaign

Campaign year	Tests performed in the 11 districts				Tests performed by elderly ≥ 75 in the 11 districts			
	No. of tests (% of T+)	No. of colonoscopy (Completion rate in %)	No. of neoplasia (Detection rate in %)	No. of CRC (Detection rate in %)	No. of tests (% of T+)	No. of colonoscopy (Completion rate in %)	No. of neoplasia (Detection rate in %)	No. of CRC (Detection rate in %)
2008	230467 (2.7)	5392 (85.0)	3020 (13.1)	420 (1.8)	1298 (3.7)	35 (72.9)	16 (12.3)	5 (3.9)
2009	343468 (2.5)	7324 (83.7)	4025 (11.7)	609 (1.8)	1732 (3.6)	49 (79.0)	29 (16.7)	7 (4.0)
2010	289120 (2.5)	6117 (85.4)	3250 (11.2)	473 (1.6)	1280 (4.1)	42 (79.3)	27 (21.1)	6 (4.7)
2011	313982 (2.3)	6283 (86.9)	3327 (10.6)	458 (1.5)	1843 (2.9)	49 (90.7)	34 (18.4)	5 (2.7)
2012	287818 (2.1)	5010 (84.5)	2618 (9.1)	355 (1.2)	1653 (2.1)	27 (79.4)	20 (12.1)	6 (3.6)
2013	302392 (1.8)	4646 (84.7)	2420 (8.0)	325 (1.1)	1997 (2.7)	43 (81.1)	21 (10.5)	6 (3.0)
2014	267342 (1.9)	4146 (81.0)	2153 (8.1)	238 (0.9)	1704 (1.9)	23 (71.9)	18 (10.6)	5 (2.9)
2015	163976 (4.4)	6222 (85.8)	4107 (25.0)	474 (2.9)	2574 (5.9)	136 (89.5)	87 (33.8)	13 (5.1)
2016	399039 (4.7)	155109 (80.9)	9993 (25.0)	1126 (2.8)	2945 (4.6)	111 (81.6)	74 (25.1)	11 (3.7)
2017	286039 (4.4)	8928 (71.5)	5764 (20.2)	632 (2.2)	1969 (4.2)	60 (72.3)	40 (20.3)	6 (3.0)
Total	2883643 (2.9)	69177 (81.9)	40677 (14.1)	5110 (1.8)	18995 (3.7)	575 (81.3)	366 (19.3)	70 (3.7)

T+: Positive test; CRC: Colorectal cancer.

ARTICLE HIGHLIGHTS

Research background

In France, as in other countries, the Colorectal Cancer (CRC) Screening Program (CRCSP) does not include people aged ≥ 75 years, despite the high incidence of CRC after 74 years and the constant increase in life expectancy since 2000. Indeed, elderly patients aged at least 75 years old (Elderly_75), represent 45% of CRC incidence. To date, there is little evidence to justify stopping CRC screening at 74 years of age.

Research motivation

In this period of implementation of the proposals for reform of the National CRCSP, our motivation was to feed the debate on the problem of screening in the elderly, in order to alert the decision-making bodies. By noting that in each French district, the sporadic participation of Elderly_75, out of the national CRCSP's recommendation, is recorded every year.

Research objectives

Our objective was to describe the sources and outcomes of these screening test performed out of recommendation. Indeed, in the absence of a CRC screening program involving this age group, the elderly and/or their attending physicians decide sporadic participation in the campaigns organized by the local cancer screening managing center. The analysis of these sources and outcomes could argue the need for the implementation of an experimental program on the benefits and risk of CRC screening after the age of 75 years.

Research methods

The study concerned 18704 Elderly_75 residing in eleven French districts (Ain, Doubs, Essonne, Haute-Saone, Hauts-de-Seine, Jura, Seine-Saint-Denis, Territoire-de-Belfort, Val-de-Marne, Val-d'Oise, Yonne), having performed a CRC screening test between January 2008 and December 2017.

Research results

Of 18995 screening-tests were performed by these 18704 Elderly_75 at ages: 75 (83.5%), 76-80 (13.4%) and > 80 (3.1%) years old. Elderly_75 performed the screening test in a circumstance of delayed response to a solicitation from the local cancer screening managing center (71.9%) or following a recommendation by a General Practitioner or other provider ordered (28.1%). The proportion (3.7%) of positive tests remains significantly higher than that found (2.9) in these districts in the same period or that usually found (2.6%) in the CRCSP program. It's obvious that the high risk of colonoscopy complication after 75 years is a barrier to screening CRC in the elderly. With a reported colonoscopy completion rate estimated at 81.3%, this study recorded no complications. On reminder, in the national program, reported colonoscopy completion rate after a positive test was 76.9% with approximately major incidents, including perforation in 3 in 1000 colonoscopies. The neoplasia (124 Low-risk-polyps, 159 High-risk-polyps, 13 Unspecified-polyps and 70 CRCs) detection rate was 19.3/1000 Elderly_75 screened and the CRC detection rate was 3.6/1000 Elderly_75 screened. In this population, CRC were diagnosed on average age

at 77 years.

Research conclusions

Despite a regulatory framework that is not favorable to their participation in CRCSP campaigns, a motivation to continue CRCSP after 74 years of age was observed. Considering these findings, it could be argued that extending the duration of CRCSP in healthy people up to 80 years, could allow the screening of the many cases that occur after the exclusion of the Elderly_75 from CRCSP.

Research perspectives

We are confident that more in-depth studies on the feasibility of CRC screening in Elderly are needed to confirm or not, the current age limit of screening at 74 years. Our perspectives converge in this direction because a pilot project for the implementation of CRC-screening after the age of 75, is currently in the process of identifying strategic and financial partners. One of the main objectives of this study will be to assess the risk associated with performing colonoscopy. Awaiting the implementation of this project, we intend to open the debate in France with the results of this study.

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SYSTEMATIC REVIEWS

Detection and management of oligometastatic disease in oesophageal cancer and identification of prognostic factors: A systematic review

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Abstract

BACKGROUND

Oesophageal cancer is the eighth most common cancer worldwide. The prognosis of oesophageal cancer patients still remains poor. The 5-year survival rate rarely exceeds 5% in case of metastatic disease. Some patients may however present with oligometastasis which can be treated with loco-regional therapy.

AIM

To assess the current practice regarding the management of patients with oligometastatic oesophageal cancer and identify prognostic factors affecting survival following treatment for oligometastasis.

METHODS

A systematic search of the literature was performed in Cochrance Library, MEDLINE and EMBASE databases from September 1950 to January 2019. Relevant electronic databases were searched for studies assessing the clinical outcome of oligometastasis.

RESULTS

A total of 14 publications were included, of which 12 studies assessing metachronous oligometastasis and 2 on synchronous oligometastasis. All included articles evaluated the specific outcomes of metastasis, management modality and survival outcomes. The majority of the patients presented with oesophageal squamous cell carcinoma. The median disease free interval (time to recurrence) in patients was 19.6 mo and the overall survival reached 30.8 months. Unfavourable prognostic factors were assessed in eight studies and included time to recurrence < 12 mo, large diameter pulmonary lesions (> 20 mm), disease free interval (DFI) < 12 mo, extra-pulmonary metastasis, primary tumour pathological

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stage III/IV.

CONCLUSION
Oligometastatic oesophageal cancer in selected patients is amenable to loco-regional treatment, and the overall survival of this patient cohort may be improved with patient and tumour-specific treatments.

Key words: Oligometastasis; Oesophageal cancer; Neoplasm; Liver; Pulmonary

Core tip: Oesophageal cancer often presents with early metastatic spread, which carries a poor prognosis. Some patients may have limited metastatic disease that can be treated with loco-regional therapy. The guidelines for the management of oligometastasis in oesophageal cancer are however not clearly established and survival outcomes remain unclear. The aims of this review were to assess the current practice for the treatment of oligometastatic oesophageal cancer and factors affecting survival following treatment of oligometastasis. A total of 14 publications were included assessing the management and survival outcomes and the majority of these studies opted for aggressive treatment in appropriate patient selection.

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INTRODUCTION

Oesophageal cancer is the eight most common cancer and the sixth leading cause of death from cancer with 400200 deaths[1-3]. Metastatic spread can occur early, and symptoms often only become apparent in the later stage of the disease. Hence, the prognosis of oesophageal cancer still remains poor with an overall 5-year survival rate of 18%[4]. Surgical resection is the standard treatment for patients presenting with early stage cancer. However, the survival is still low due to high incidence of either loco-regional or distant recurrence, ranging from 29% to 59%[5-11]. Moreover, approximately half of the patients present primarily with distant metastasis at the time of diagnosis with a 5-year survival of less than 5%[6]. Combined treatment modalities are used for the management of locally advanced disease, consisting of neoadjuvant chemotherapy with or without radiotherapy followed by surgery[12]. Meanwhile, patients with recurrent or metastatic disease most commonly undergo systemic palliative therapy[13-15].

Several factors are believed to influence the long-term survival in patients undergoing curative treatment. One study found that presence of regional lymph node metastasis and chemoradiation, compared to surgery, were associated with poor 5-year survival, whilst female gender and patients receiving neoadjuvant therapy had better outcomes[16]. Nevertheless, the specific prognostic factors in patients treated loco-regionally for oligometastatic disease in oesophageal cancer remain unclear. Type and extent of recurrence may also affect survival as distant recurrence and more than three recurrent locations were associated with worse post-recurrence survival compared to loco-regional and solitary recurrence respectively[17]. Most recurrences occur in the first postoperative year, and approximately 90% develop by the end of the third year[9]. Metastatic oesophageal cancer has been regarded as end-stage disease with the most commonly affected sites for metastasis being the distant lymph nodes, liver, brain, lung and bone[18]. However, some of these patients may present with oligometastatic cancer. Oligometastasis is defined as a state of limited metastatic disease characterised by fewer than 5 metastasis[19]. Oligometastasis can be synchronous oligometastasis, which are detected at the time of primary cancer diagnosis or metachronous, which occur following treated primary cancer site [20]. The clinical implication of oligometastasis lies largely in the possible improvement in disease control and survival when patients with oligometastatic disease are treated

with definitive loco-regional therapy. Early detection of oligometastasis enables early intervention and may thus potentially improve survival. Careful surveillance is therefore one of the key components in the management of oesophageal cancer. However, to date there is a lack in specific guidelines regarding optimal management of patients presenting with oligometastatic oesophageal cancer.

For the purposes of our study, we defined oligometastasis as a single solid organ recurrence. This systematic review focuses on the current practice regarding treatment of oligometastatic oesophageal cancer and factors affecting survival following treatment of oligometastasis.

MATERIALS AND METHODS

Search strategy and study selection

A systematic literature search of MEDLINE (January 1950 to September 2018), EMBASE (January 1974 to September 2018), Web of Science (January 1990 to September 2018), and the Cochrane Library databases was performed. The following search terms were used "(o)esophageal cancer", "oligometastasis" and "oligo-recurrence" and the Medical Subject Headings (MeSH) term "esophageal neoplasms". The search was expanded by identifying synonyms or closely related terms and a manual search of the references of included studies was performed to identify any missing articles. The full search strategy is shown in Supplementary Table 1. Two reviewers (SJ and KT) independently assessed titles and abstracts for inclusion of relevant references, followed by screening of the full text. Articles were included if the following elements were evaluated: (1) Assessment of survival outcomes and/or prognostic factors in patients presenting with solid organ metastasis following treatment for oesophageal cancer; and (2) Synchronous or metachronous oligometastasis. Only articles published in English were included. Review articles were excluded. Articles focusing on solely lymph node recurrence without solid organ metastasis were excluded. The following data was extracted: study design, sample size, mean age, diagnostic tool, type of treatment modality, histological subtype, site of metastatic lesion, disease free survival and overall survival for synchronous oligometastasis and metachronous oligometastasis.

Quality assessment

The methodological quality of included studies was assessed by means of the Newcastle-Ottawa scales for cohort and case-control studies. The quality is rated by awarding stars in each domain with three domains in total (selection, comparability and exposure)[21]. Articles are graded as "good quality" if 3 or 4 stars in the selection domain and 1 or 2 stars in the comparability domain and 2 or 3 stars in the outcome/exposure domain, "fair quality" if 2 stars in the selection domain and 1 or 2 stars in the comparability domain and 2 or 3 stars in the outcome/exposure domain or "poor quality" if 0 or 1 star in the selection domain OR 0 stars in comparability domain OR 0 or 1 stars in the outcome/exposure domain. The methodological quality assessment of the case series was reported using a novel tool based on modifications of the Newcastle-Ottawa, Pierson and Bradford Hills scales[22]. Eight items are categorised into four domains (selection, ascertainment, causality and reporting). A total score on these 8 items can be created by adding up the binary response to a sum score.

RESULTS

Literature search

The systematic search yielded 399 initial results. After removal of duplicates, 235 references were screened on title and abstract. Subsequent assessment of full text resulted in inclusion of 14 articles. A graphical representation of the review process is demonstrated in a PRISMA flow chart (Figure 1)[23].

Methodological quality assessment

The results of the quality assessment of the studies included are summarised in the Supplementary Tables 1, 2 and 3 respectively. The majority of included studies were cohort studies. For all studies, both the case and control population consisted of patients treated for oesophageal cancer and data was retrospectively obtained from existing hospital databases. Furthermore, patients in the control group were not obtained as a random sample in the population and were selected from the same source as the cases. However, no information was given in the methodology of the

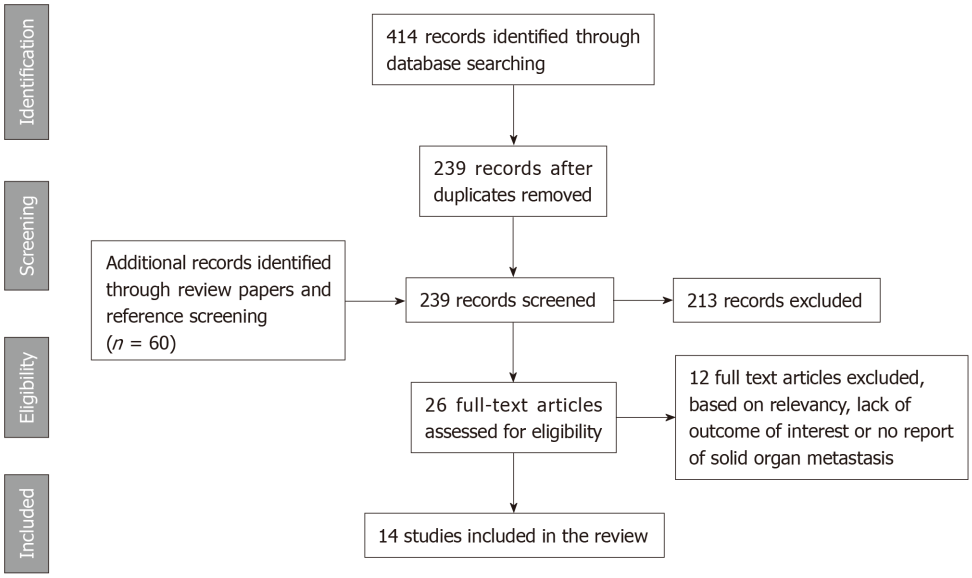

Figure 1 PRISMA flow chart of the selection process.

studies regarding matching of both groups. The remaining articles had a retrospective cohort design or were case series. Again, the study population consisted of a selected group of patients. The comparability of the cohorts was well established, but the outcome of interest was already present at the start across all studies due to retrospective design.

Management of metachronous oligometastasis

Twelve studies included[24-35], eight of those assessed the survival outcomes of patients treated with resection of pulmonary metastases, three included multiple oligometastasis sites and one study only on liver oligometastasis (Supplementary Tables 4 and 5). The histological subtype of primary oesophageal cancer was squamous cell carcinoma (75.2%), adenocarcinoma (23.0%) and sarcoma/basaloid tumour (1.8%). The mean age was 63.3 years across all studies. The majority of patients has undergone resection for the primary oesophageal cancer with either chemotherapy or radiotherapy or a combination. In only one study patients were managed with definitive chemotherapy[27]. The Disease-Free Interval (DFI) was reported in 8 of the studies and the mean DFI was 19.6 months. The DFI duration was established as a prognostic factor is four studies. A disease-free survival (DFI) of less than 12 mo resulted in a 5-year survival rate of 15.7%, which was much lower than for patients with a DFI exceeding 12 months (39.2%, $P = 0.048$)[25].

Patients were investigated with various imaging modalities including Chest X-ray (CXR), computed tomography (CT) and positron emission tomography (PET) scan. However, the follow-up regimes were not specified in the studies included to assess variation in clinical practice and impact on detection. The management of oligometastasis involved surgery and mostly either chemotherapy or chemo-radiotherapy. The main reported outcomes were overall survival (OS) and the mean was 30.8 mo across all studies. Oligometastasis recurrence occurred in 32.1% of cases. Time to recurrence was identified as a predictor of survival and patients presenting with recurrence within 12 mo of definitive therapy for the primary tumour, had worse survival ($P = 0.034$)[35].

Chen *et al*[24] showed that patients with multiple pulmonary metastasis developed recurrence in comparison to those with solitary lesions, however, this was a small study of 5 patients. Regarding pulmonary metastasis, a larger diameter of the lesion (> 20 mm) was marginally associated with worse outcomes ($P = 0.087$)[27]. Presence of extra pulmonary metastases was established as unfavourable prognostic factor in five of the studies and Ichikawa *et al*[28] showed that none of the patients with extrapulmonary metastasis survived beyond 3 years, compared to 54.7% for patients presenting with a solitary pulmonary lesion ($P = 0.0411$).

A 2013 retrospective cohort study assessed survival following resection of liver and lung metastases. Patients with pulmonary recurrences had better outcomes (median survival of 13 mo) than metastases in the liver (medial survival of 5 mo) or other sites (median survival of 3 mo) and a surgical approach of these pulmonary lesions also seemed to beneficial with a median survival of 48 mo compared to 10 mo if not

treated with resection (P = 0.009). Hepatic metastasectomy failed to establish a significant survival benefit (P = 0.06) [32]. The latter results were similar to the findings of Huddy *et al*[33] who assessed the outcomes of 4 patients treated with liver resection and to Hiyoshi *et al*[34]. In addition, the latter author could not demonstrate an improvement in survival following resection of lesions in the brain and bone. However, patients treated with pulmonary metastasectomy (solitary, bilateral or multiple lesions) showed a trend towards better outcomes. A recent study conducted by Ghaly *et al*[35] evaluated prognostic factors for survival of 56 patients following multimodal therapy of oligometastasis in the liver, bone, brain or adrenal glands. The median survival was not significantly different between both groups (P = 0.661). Time to recurrence was identified as a predictor of survival and patients presenting with recurrence within 12 mo of definitive therapy for the primary tumour, had worse survival (P = 0.034)[35].

Management of synchronous oligometastasis

Two studies assessed survival in oesophageal cancer patients presenting with synchronous oligometastasis[36,37] (Supplementary Tables 4 and 5). Onal *et al*[36] assessed the impact of an aggressive treatment approach of both primary tumour and solitary brain metastasis. Patients underwent definitive CRT of the primary tumour locally ablative treatment of the brain metastasis, consisting of radiotherapy, surgery or radiosurgery. The median time to progression was 8 mo and median survival was 18.9 mo, suggesting that this approach might improve survival in selected patients. A more recent study investigated the impact of suspicious lesions on pre-treatment imaging on the survival of patients undergoing oesophagectomy. The presence of suspected liver metastases had a 5-year survival rate of only 9.9% compared to 26.1% in patients with suspicious lesions at other sites or with no evidence of metastases on pre-treatment imaging (P = 0.014)[37].

DISCUSSION

Despite advances in diagnostic tools and treatment modalities, loco-regional and distant recurrences still occur frequently in oesophageal cancer. Survival rate is worse in the presence of haematogenous metastases (16 mo) compared to loco-regional recurrence (25.5 mo)[38]. Standard treatment modality for recurrences in oesophageal cancer often consists of systemic therapy. Patients presenting with oligometastatic disease may however benefit from aggressive local therapy with improvement in survival rates. To date, there is no guideline on the management of distant oligometastasis in oesophageal cancer and currently resection of distant metastases is mostly a personalised treatment.

The lungs are amongst the most common affected sites for metastasis in oesophageal cancer. Patients often present with multiple lesions and might have metastases at other sites as well. Furthermore, primary lung tumours commonly coexist with oesophageal cancer as smoking is a known risk factor in both malignancies[39,40]. Distinguishing metastases from second primary lung cancer requires genetic analysis, which was not performed in the included studies. Kanamori *et al*[31] excluded lesions suggestive of second primary lung cancer based on histological findings and Kozu *et al*[27] applied several clinical criteria. The latter author found poor long-term survival in these patients, confirming the aggressive nature of metastatic disease in oesophageal cancer. Consequently, survival rates could be affected in the other included studies as the number of patients with pulmonary metastases might be fewer than reported[41]. Pulmonary metastasectomy has proven its efficacy in other types of cancer, including colorectal, renal and head and neck malignancies. Both initial as repeated resections were encouraged in colorectal cancer as it could significantly improve survival rates [42]. This is consistent with the findings in the studies included in our review as the vast majority of the papers believed that pulmonary metastasectomy was a promising treatment option for improvement in survival following resection of a solitary pulmonary lesion. Ichikawa *et al*[28] has shown that it was a safe and feasible approach as the incidence of pulmonary complications remained low and no in-hospital mortality occurred. Hiyoshi *et al*[34] suggested that not only patients with a solitary lesion are good candidates for metastasectomy, but resection of bilateral and metachronous pulmonary multiple lesions might improve the prognosis as well. However, the study population only consisted of 9 patients with a solid organ metastasis. Furthermore, resection of pulmonary metastasis may have an improved prognostic value in metastatic gastric cancer[43].

Resection of liver metastases is common practice in colorectal cancer with a 5-year

survival rates of more than 50% compared to patients receiving palliative treatment[44,45]. None of our included studies demonstrate a survival benefit of resection of liver metastasis, however, it is important to note that patient numbers were small. In addition, there is contrary evidence to survival benefit in patients undergoing resection for brain metastasis. Hiyoshi *et al*[34] did not show any benefit in the resection of lesions in the brain or bone. In contrast with this, Onal *et al*[36] suggested an improvement in survival outcome in patients with oligometastatic brain metastasis when treated with an aggressive approach of both the primary tumour and the metastatic lesion.

Recently, Kanamori *et al*[31] showed that the risk of re-recurrence of metastasis was 70% in those undergoing pulmonary metastectomy. However, smaller studies had lower recurrence rates of 22% Kobayashi *et al*[30], 50% Huddy *et al*[33] and 60% in Chen *et al*[24]. The rate of recurrence following treatment of oligometastasis was not reported in other studies. In all the included reports patients had surgical resection of their oligometastasis and either chemotherapy or chemoradiotherapy. It is thus unclear whether these patients significantly benefit from resection of metastases.

Hsu *et al*[9] showed that patients with more risk factors such as liver recurrence, early recurrence, and no treatment for recurrence would suffer from poorer post-recurrence survival. Therefore, patients with isolated, oligometastasis of EC after multimodality therapy may represent a subset of patients who will benefit from aggressive treatment of their metastatic disease and survival might be extended in this patient population. The majority of patients included had oesophageal squamous cell carcinoma (OSCC). The pattern of metastasis is different as OSCC has a higher incidence rate of lung metastasis in comparison to oesophageal adenocarcinoma which had a higher incidence rate of liver metastasis. The median disease free interval in patients was 18.6 months. The overall survival was 31.1 months. Furthermore, to our knowledge, there are currently no commonly accepted prognostic factors of metastatic oesophageal cancer indicating an improved prognosis. Several reports have reported favourable prognostic factors depending on their patient cohort, this included solitary metastasis, absence of extrapulmonary metastases and lack of nodal involvement and greater disease free interval > 12 months. In addition, the concept of the "test of time" is a convincing indicator of a more favourable biological cancer behaviour. However, there is an increasing shift toward individualized, multidisciplinary management of oligometastasis because it is difficult to conduct randomized controlled trials due to the variety of presentations[7]. The multicenter FLOT3 study with metastatic tumours of the oesophagogastric junction and gastric cancer suggests that well-selected patients may benefit from surgery following chemotherapy at the stage of limited metastases[46]. The results of the FLOT5 study is still awaiting to be published, assessing the effect of chemotherapy alone versus chemotherapy followed by surgical resection on survival and quality of life in patients with limited metastatic adenocarcinoma of the stomach or oesophagogastric junction. Only two papers assessed the presence of synchronous oligometastasis in patients with oesophageal cancer[36,37]. Hence, no comparison in the difference of between the presence of synchronous or metachronous oligometastatic disease could be made regarding survival outcomes.

The main limitation to this review is that all the studies included were retrospective observational studies. The majority of patients were OSCC and therefore not representing the other major oesophageal cancer subtype of adenocarcinoma. Therefore, there was clinical heterogeneity was present due to majority of patients being of Asian-predominant studies. Most of the studies included the proportion of patients receiving metastasectomy in their assessment. Hence, the sample size in the majority of the studies was small, which may introduce selection bias. Another limitation is that only papers published in English were included.

Aggressive treatment of oligometastatic disease in oesophageal cancer is performed on an individual basis. The lung and liver are amongst the most common sites of metastasis in oesophageal cancer. Several factors have been identified which might influence survival and should be taken into consideration in the management of oligometastasis. Most studies advocate a personalised approach to patient management until there are more studies to guide future decision making.

ARTICLE HIGHLIGHTS

Research background

Oesophageal cancer is the eighth most common cancer worldwide with an associated poor prognosis. The 5-year survival rate rarely exceeds 5% in case of metastatic disease. Combined treatment modalities are used for the management of locally advanced disease, consisting of neoadjuvant chemotherapy with or without radiotherapy followed by surgery. Meanwhile,

patients with recurrent or metastatic disease most commonly undergo systemic palliative therapy. However, to date there is a lack in specific guidelines regarding optimal management of patients presenting with oligometastatic oesophageal cancer. The European Society for Medical Oncology, suggests that patients with metastasis can be considered for different options of treatment depending on the clinical case. It is unclear for current studies whether resection improves the overall survival and what is the optimal management.

Research motivation
This systematic review focuses on the current practice regarding treatment of oligometastatic oesophageal cancer and factors affecting survival following treatment of oligometastasis.

Research objectives
This review aims to assess the current practice regarding the management of patients with oligometastatic oesophageal cancer and identify prognostic factors affecting survival following treatment for oligometastasis.

Research methods
An extensive systematic search of the literature was performed in Cochrance Library, MEDLINE and EMBASE databases on January 4th, 2019. Relevant electronic databases were searched for studies assessing the clinical outcome of oligometastasis.

Research results
The main finding of this systematic review is that Oligometastatic oesophageal cancer in selected patients is amenable to loco-regional treatment, and the overall survival of this patient cohort may be improved with patient and tumour-specific treatments. However, there is an increasing shift toward individualized, multidisciplinary management of oligometastasis because it is difficult to conduct randomized controlled trials due to the variety of presentations.

Research conclusions
The lung and liver are amongst the most common sites of metastasis in oesophageal cancer. Most studies advocate a personalised approach to patient management until there are more studies to guide future decision making. Aggressive treatment of oligometastatic disease in oesophageal cancer is performed on an individual basis. Several factors have been identified which might influence survival and should be taken into consideration in the management of oligometastasis. Most studies advocate a personalised approach in the management of oligometastatic oesophageal cancer.

Research perspectives
The current management advocated by most studies is based on a personalised approach to patient management until there are more studies to guide future decision making. Larger scale future studies or randomised controlled trials to assess optimal management plan for oligometastatic disease is required to guide management of this patient cohort.

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SYSTEMATIC REVIEWS

Clinical characteristics and surgical treatment of schwannomas of the esophagus and stomach: A case series and systematic review

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Abstract

BACKGROUND

Gastrointestinal schwannomas are slow-growing benign mesenchymal neoplasms that originate from Schwann cells of the nerve sheath of Auerbach´s plexus or less frequently from Meissner´s plexus. The main differential diagnosis of gastric schwannomas are the gastrointestinal stromal tumors (GISTs), which are classified by their immunohistochemistry. The treatment of choice for gastric schwannomas is surgery where laparoscopy plays an important role. Wedge resection, subtotal or total gastrectomy can be done. In its counterpart, esophageal schwannomas are benign tumors of the esophagus that are very uncommon since they comprise less than 2% of all esophageal tumors. The main differential diagnosis is the leiomyoma which corresponds to the most common benign esophageal tumor, followed by GIST. The treatment consists on tumoral enucleation or esophagectomy.

AIM

To review the available literature about gastrointestinal schwannomas; especially lesions from de stomach and esophagus, including diagnosis, treatment, and follow up, as well as, reporting our institutional experience.

METHODS

A systematic review was conducted following the Preferred Reporting Items for Systematic Reviews and Meta-Analyzes guidelines. The following databases were used for reviewing process: PubMed, Ovid, MEDLINE, and Scopus. Only English language manuscripts were included. All gastrointestinal schwannomas specifically located in the esophagus and stomach were included. Cases that did

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not report long-term follow-up were excluded.

RESULTS
Gastric localization showed a higher prevalence in both, the literature review and our institution: 94.95% (n = 317) and 83% (n = 5) respectively. With a follow-up with disease-free survival greater than 36 mo in most cases: 62.01% (n = 80) *vs* 66.66% (n = 4). In both groups, the median size was > 4.1 cm. Surgical treatment is curative in most cases

CONCLUSION
Schwannoma must be taken into account in the differential diagnosis of gastrointestinal mesenchymal tumors. It has a good prognosis, and most are benign. A disease-free survival of more than 36 mo can be achieved by surgery.

Key words: Schwannoma; Esophagus; Stomach; Surgery; Systematic review

Core tips: We performed a systematic review of the literature searching two types of rare tumors; esophageal and gastric schwannomas. We review its form of presentation, diagnosis, differential diagnosis and treatment. We also performed a systematic review trying to gather all case reports and case series in a single paper. We have found 16 cases of esophageal schwannomas and 301 cases of gastric schwannomas in all literature. We also reviewed our institutional experience with the report of 6 cases of gastrointestinal schwannomas, 1 is esophageal and 5 are gastric.

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INTRODUCTION

Gastrointestinal schwannomas are slow-growing benign mesenchymal neoplasms that originate from Schwann cells. The most frequent locations are the stomach and colon, with the esophagus being the least frequent site.

Despite being tumors widely known by clinicians, gastrointestinal schwannomas are rare. It is accepted that the treatment of choice is laparoscopic or open resection, where in wedge, subtotal or total gastrectomy can be performed.

We present an institutional case series, and a systematic review of esophagic and gastric schwannomas. The aim of this study is to review the available literature about gastrointestinal schwannomas; especially lesions from de stomach and esophagus, including diagnosis, treatment, and follow up, as well as, reporting our institutional experience.

Literature review

Gastric schwannomas: Gastrointestinal schwannomas are slow-growing benign mesenchymal neoplasms that originate from Schwann cells of the nerve sheath of Auerbach´s plexus or less frequently from Meissner´s plexus[1]. They were first described by Daimaru *et al*[2] since 1988 to date, only 300 cases approximately have been reported in the literature[3]. It has been suggested that gastric schwannomas play a different genetic mechanism compared to soft tissue schwannomas by not expressing monosomy on chromosome 22 and very rarely mutations in NF2[4]. Mesenchymal tumors include leiomyomas, gastrointestinal stromal tumors (GIST), and schwannomas, with the latter being very rare since it represents 0.2% of all gastric tumors, 6.3% of gastric mesenchymal tumors, and 4% of all benign gastric tumors[5-7]. Gastric schwannomas tend to appear as single lesions in the stomach with the following subdivisions: the body (59.3%), antrum (26.7%) fundus (12%) and cardia (2%), followed by colon as submucosal tumors[1,3]. They have an average reported diameter of 4.69 cm ranging from 0.8 to 15.5 cm. Female predominance has been

observed with a male to female ratio of 1: 2.64 and can occur at any age, although predominantly between the fifth and eighth decades of life, with 86.43% diagnosed in people over 40 years of age, with an average age of 56.82 years[1-4,6].

The main differential diagnosis of gastric schwannomas are GISTs which careful differentiation of these two entities should be done, since clinical, histological and demographic presentation are very similar, but the treatment and prognosis of each one is very different[1,4]. It is estimated that for every 45 cases of gastric GISTs there is gastric schwannoma[4]. Gastric schwannomas have an excellent prognosis after surgical resection, while GIST can recur, have malignant potential ranging from 10% to 30% and in certain cases can be treated with imatinib achieving great response[1].

Gastric schwannomas can rarely progress into malignant tumors of the peripheral nerve sheath (MTPNS), known as malignant schwannomas, mainly when they have a mitotic index greater than 10/50 HPF[1,4]. Only 10 cases (4.5%) of all gastric schwannomas reported in the literature have been described as MTPNS wich are characterized by a greater number of mitotic index, presence of necrosis and nuclear atypia[1,3]. However, this idea of malignant transformation has been questioned in recent research, since most of the malignant transformation were reported prior to modern immunohistochemistry, which they probably corresponded to GISTs instead of schwannomas[4].

Patients with gastric schwannomas are usually asymptomatic with incidental findings in 43.3% during endoscopies for unrelated conditions[1,3,6]. Symptomatic patients typically present with abdominal pain or discomfort followed by upper gastrointestinal bleeding and less frequently with a palpable abdominal mass (3.05%), poor appetite (3.05%), dyspepsia (1.82%), weight loss (1.21%), nausea or vomiting (0.6%)[1,3,6]. There's only one case of gastroduodenal intussusception due to gastric schwannoma reported in the literature[8].

Preoperative diagnosis is a challenge for the surgeon because of the difficulty to differentiate gastric schwannomas from GISTs. In upper gastrointestinal endoscopy, gastric schwannomas are frequently seen as firm, protruding submucosal masses and in patients with active bleeding the ulcerated mucosa is observed[1,6]. Endoscopic biopsies have limited usefulness since false negatives can occur when the mucosa of the lesion is intact[1]. Fine needle aspiration biopsy (FNA) guided by endoscopic ultrasound (EUS) is a reliable diagnostic method although with a reported diagnostic accuracy of 50 to 85.2% for mesenchymal gastric tumors, which have a hypoechoic appearance in the EUS[6,9].

In computed tomography (CT), gastric schwannomas are distinguished by having homogeneous density, leiomyomas often show calcifications and leiomyosarcomas are usually more heterogeneous[10]. However, the radiological findings in both CT and magnetic resonance imaging (MRI) of gastric schwannomas are not specific and can be confused with GISTs even with positron emission tomography with 18-fluorodeoxyglucose (PET-FDG), since they present as hypermetabolic[6,11]. The diagnosis is based on histology and confirmed with the immunohistochemical report which is the gold standard. Histopathology of these tumors shows a fascicular arrangement with spindle-shaped nuclei. Immunohistochemistry is positive for S-100 protein(Figure 1), vimentin and glial fibrillary acidic protein (Figure 2), CD34 positive or negative, and negative for smooth muscle actin, desmin, DOG-1 and c-Kit (CD117); the latter positive in GIST[12,13]. It is important to distinguish gastric schwannomas from malignant tumors with positivity for S-100 such as clear cell gastrointestinal sarcoma and metastatic melanoma[4].

The treatment of choice for gastric schwannomas is surgery where laparoscopy plays an important role. Wedge resection, subtotal or total gastrectomy can be done since they have low malignant potential[1,3,4]. Lymphadenectomy is not usually performed unless enlarged lymph nodes are seen, since gastric schwannoma rarely metastasizes to lymph nodes. Only minimally invasive endoscopic approaches should be performed with or without laparoscopic approach, when the diagnosis of gastric schwannoma is definitively confirmed[3]. Given its benignity, the recurrence of gastric schwannoma is only associated with positive surgical margins. Frequent CT follow-up is not recommended[1,14].

Schwannomas of the esophagus: These benign esophageal tumors are very rare since they comprise less than 2% of all esophageal tumors[15-17]. The most common submucosal esophageal tumor is leiomyoma, diagnosed in 50% of every benign case, followed by GISTs, which have malignant potential[18-20]. Other less frequent benign submucosal esophageal tumors are lipomas, granular cell tumors and schwannomas[21]. Gastrointestinal schwannomas comprise between 0.4 and 1% of all submucosal tumors of the gastrointestinal tract, and most of them are found in the stomach, making them extremely rare in the esophagus[15,21,22].

Schwannomas are the most common neurogenic tumor, which are derived from

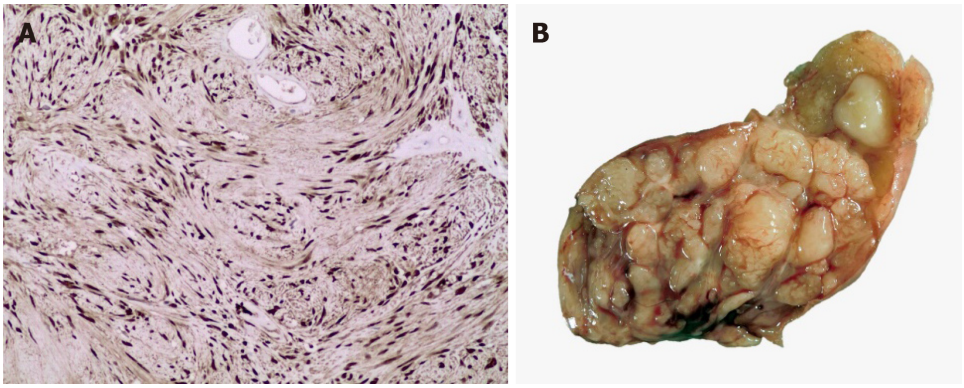

Figure 1 Immunohistochemistry and macroscopic images. A: The immunohistochemistry was diffusely positive for S100 (in the nucleus and cytoplasm) in the proliferative cells; which confirms its histomorphogenesis of the cells of schwann. This image corresponds to the esophagic schwannoma resected at our institute; B: Macroscopic image of the esophageal schwannoma.

Schwann cells hence its name[15]. Schwannomas typically originate in the posterior mediastinum, followed by the chest wall and lung parenchyma, and extremely rare from the esophagus[15,20]. Esophageal schwannomas are usually found in the upper thoracic segment[17,23]. An average size of 5.6cm has been reported, with a range of 0.5 to 10 cm[24]. They are predominant in the female gender with a female to male ratio of 19: 8 and a mean age at diagnosis of 50 years[25,26].

Most patients are asymptomatic and are diagnosed incidentally[17]. The most common symptom is moderate to severe dysphagia followed by dyspnea[15,17,20]. Symptoms usually correlate with tumor size due to the effect of mass on neighboring structures which can also result in chest pain, pneumonia or hemoptysis[17]. There are two reported cases of benign schwannoma in the upper esophagus with compression to the trachea[23,25].

The preoperative diagnosis of esophageal schwannoma is a challenge for the surgeon since there are no special characteristics on imaging studies and even endoscopic biopsies can be useless because schwannomas are found in the submucosal tissue[15,21]. A successful preoperative diagnosis can lead to a less invasive surgical treatment[26]. CT and MRI have limited diagnostic utility; however, its use in conjunction with PET-FDG has been reported to be useful in the diagnosis of esophageal tumors [27].

On CT, esophageal schwannoma tends to show a homogeneous density while leiomyomas often show calcifications; on the other hand, leiomyosarcomas tend to be more heterogeneous[10]. Currently, fine FNA biopsy guided by EUS is used to diagnose submucosal tumors because it has a diagnostic accuracy of 85.2%, allowing more adequate samples to be obtained than upper gastrointestinal endoscopy biopsy[10]. Some unlikely risks of EUS-guided FNAB are bleeding, infection, trachea perforation; however, it is considered a safe, reliable and accurate method[9,17,21]. Through PET, schwannomas prove to be hypermetabolic; however, this has no clinical correlation with malignancy since these tumors are derived from nerve cells that express the type 3 glucose transporter which increases FDG uptake[28]. When the preoperative diagnosis is not reliable, a transoperative pathological diagnosis may be useful[17]. Schwannomas are usually benign and can show one or two histological patterns: Antoni A and B. The Antoni A pattern shows compact areas with palisaded spindle cells, while the Antoni B pattern consists of poorly organized tissue with variable cystic changes and hemorrhage. MTPNS are the counterpart of benign schwannomas. These tumors are very rare and their histopathological report is characterized by greater mitotic cells, necrosis and atypia[15]. Positive immunohistochemistry for S-100 protein and negative for smooth muscle markers such as actin and desmin support the diagnosis of MTPNS[11]. Immunohistochemistry is essential to differentiate a GIST from a schwannoma[20,25].

The treatment of esophageal schwannoma is tumoral enucleation or esophagectomy[17,25]. Local resection versus esophagectomy is preferred since the former has lower morbidity and is curative for benign schwannomas while the latter has a higher rate of postoperative complications such as recurrent laryngeal nerve palsy, pulmonary involvement or chylothorax[29-31]. Tumor enucleation is usually viable because esophageal schwannoma is usually limited to the submucosa; however, this technique is not recommended for very large tumors due to an increased risk of esophageal stenosis[31,32].

Surgery is indicated for symptomatic lesions, lesions with a diameter greater than 4

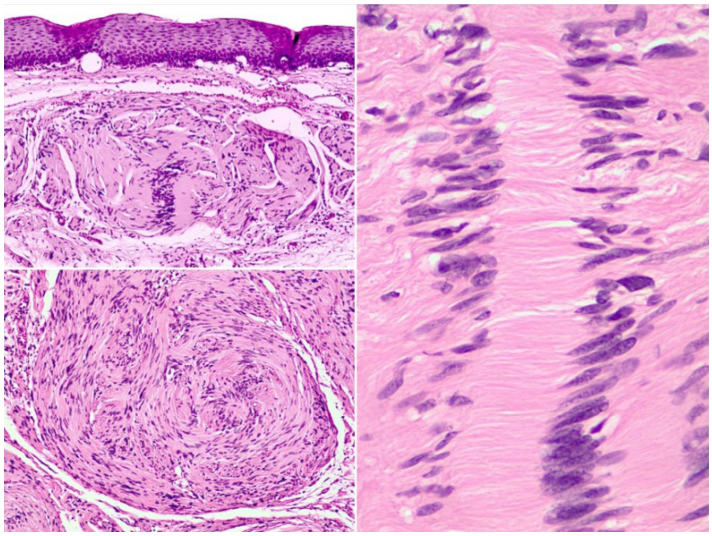

Figure 2 Nulcear palisading around fibrillary process (verocay bodies) is often seen in cellular areas. This image corresponds to the esophagic schwannoma resected at our institute.

to 5 cm, suspicion of malignancy and lesions that have increased in size during follow up[15]. The surgical approach should be considered based on the tumor size, the location of the lesion and the patient's condition[17].

When there is suspicion of malignancy, such as tumor larger than 10 cm, biopsy with mitotic figures, esophageal muscle invasion or cellular atypia; the indication is esophagectomy with negative margins and lymph node dissection to prevent recurrences or lymph node metastases[33,34]. For sporadic tumors in the upper thoracic segment, a right thoracotomy is preferred, and less often a cervical approach[15,23,33]. There are case reports of successful resections of schwannomas by minimally invasive thoracic surgery (VATS) with less pain, fewer postoperative complications such as pneumonia and shorter recovery time compared to standard thoracotomy[15,18,35]. However, VATS is not recommended for large submucosal tumors due to an increased risk of mucosal damage when performing extensive dissections[36].

In general, esophageal schwannomas have a good prognosis after surgical resection and a long term with CT and esophagoscopy is recommended[35].

MATERIALS AND METHODS

A comprehensive literature search was conducted using controlled vocabulary and key words in the following databases: PubMed, MEDLINE, Cochrane and Ovid. The following MeSH terms were used "schwannoma" and "gastrointestinal schwannoma". The detailed search strategy is shown in the study flow diagram (Figure 3). The references of identified studies were also searched to identify additional studies for inclusion. Only English language manuscripts were included. All Gastrointestinal schwannomas specifically located in the esophagus and stomach were included. Cases that did not report long-term follow-up were excluded. Only one researcher carried out the review of the cases reported in the literature. The following variables were extracted for each case report: age, sex, race of the patient, tumor site, tumor size, surgical procedure, long-term follow-up, and disease-free time.

RESULTS

Initially, 90 case reports and series of cases were found in the literature, of which 39 publications were excluded due to lack of complete data according to the methodology previously described, and finally 51 articles were included. We extracted a total of 317 cases of esophageal and gastric schwannomas, with a total of 16 and 301 cases respectively[2-4,8,12,34,37-75]. The clinical characteristics of these 317 cases [including the documented cases of our center (INCMNSZ)] are summarized in Table 1.

Institutional experience

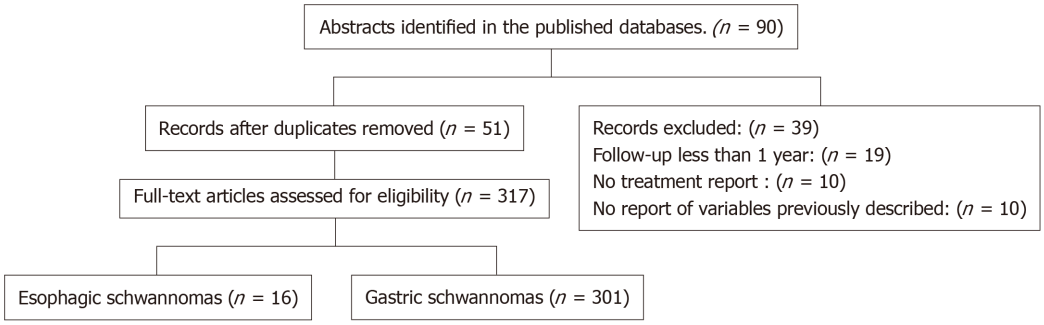

Figure 3 Flowchart.

We included a total of 6 upper gastrointestinal schwannomas (esophagus and stomach) (Figure 4). Only one case was at the esophageal level and the remaining 5 at gastric level. The clinical and pathological characteristics of these patients are shown in Table 2. In all 6 cases, complete surgical resection was attempted, with only one case reporting positive surgical margins in the histopathologic report.

DISCUSSION

Schwannomas or neurinomas are benign mesenchymal neoplasms that originate from Schwann cells located at the sheath nerve of the Auerbach's plexus or less frequently at the Meissner plexus. Its presentation as gastrointestinal tumors are uncommon; being the most frequent location at the level of the stomach, representing 4% of benign tumors at this level, and 2% of all esophageal tumors; a female predominance has been reported, and tend to appear in the 6th decade of life. Most of them are asymptomatic[3,6].

Among the most important differential diagnoses to be ruled out is GIST, which often has a malignant behavior (10%-30%) with different prognosis and treatment. The definitive diagnosis is made through immunohistochemistry which plays a fundamental role, since it allows distinguishing them from other tumors such as GIST and leiomyoma[8,9,10].

Our research tend to review the existing literature in a systematic way and report the clinical experience of INCMNSZ. A total of 317 cases were reported in the main databases while we present a total of 6 cases. Gastric localization showed a higher prevalence in both, the literature review and our institution: 94.95% (n = 317) and 83% (n = 5) respectively. With a follow-up with disease-free survival greater than 36 months in most cases: 62.01% (n = 80) *vs* 66.66% (n = 4). In both groups, the median size was > 4.1 cm. However, there was a slight discrepancy regarding the age of presentation, which was manifested earlier in the patients of our center with a predominance of 51-60 years of age.

Surgical treatment is curative in most cases and usually a wedge resection, subtotal or total gastrectomy without lymphadenectomy is enough to achieve negative margins with a low recurrence rate.

In conclusion, schwannoma is a clinical entity that must be taken into account in the differential diagnosis of gastrointestinal mesenchymal tumors. It has a good prognosis, and most are benign.

The clinical characteristics reported in the literature are very similar to our series of cases, gastric localization is more prevalent than esophageal location. A disease-free survival of more than 36 mo can be achieved by surgery.

Table 1 Clinical characteristics of esophageal and gastric schwannomas, *n* (%)

Characteristics	Institutional experience (*n* = 6)	Literature review[1] (*n* = 317)
Location		
Stomach	5 (83.3)	301 (94.9)
Esophagus	1 (16.6)	16 (5)
Size		
1-2 cm	Stomach: 2 (33)	Stomach: 20
2.1 – 4 cm	Stomach: 1 (16.6)	Stomach: 66; Esophagus: 1
> 4.1 cm	Stomach: 2 (33); Esophagus: 1 (16.6)	Stomach: 88; Esophagus: 3
Follow-up with disease- free survival in months		
< 12	Stomach: 1 (16.6); Esophagus: 1 (16.6)	Stomach: 18
43823	0	Stomach: 21; Esophagus: 2
25 – 36	0	Stomach: 8
>36	Stomach: 4 (66.6)	Stomach: 78; Esophagus: 2
Age		
< 30 yr	Stomach: 1 (16.6)	Stomach: 6 (2.47); Esophagus: 3 (18.75)
30-40 yr	Stomach: 1 (16.6)	Stomach: 18 (7.43); Esophagus: 1 (6.25)
41-50 yr	Esophagus: 1 (16.6)	Stomach: 49 (20.24); Esophagus: 3 (18.75)
51-60 yr	Stomach: 2 (33.3)	Stomach: 72 (29.75); Esophagus: 4 (25)
>60 yr	Stomach: 1 (16.6)	Stomach: 97 (40.08); Esophagus 5 (31.25)

[1]Only the cases in which the data is reported explicitly are shown.

Table 2 Clinical and pathological characteristics

Case	Sex	Age, yr	Tumor	Size	Location	Margins	IHQ
1	F	41	Schwannoma	7.5 cm x4.5 cm x1.9 cm	Esophagus	Positive	S100+, GFAP-, CD117-, COD1-, KI67 <1%
2	F	37	Schwannoma	4.2 cm x3.1 cm	Stomach	Negative	S100+, CD117-, DOG1-, CD34-, Actina-, Desmina-, CD56-.
3	F	29	Schwannoma	2.3 cm x2.3 cm	Stomach	Negative	S100+, CD56+, DOG1-, CD117 ,CD34-, Desmina-, Actina-.
4	F	67	Schwannoma	1.9 cm x1.5 cm	Stomach	Negative	DOG1-, CD117-, CD34-, Actina-, Calponina-, S-100+, CD56-.
5	F	54	Schwannoma	4.5 cm x4.3 cm x4 cm	Stomach	Negative	S100+, CD34+, CD117-, Actina-.
6	F	55	Schwannoma	8 cm x5 cm	Stomach	Negative	DOG1-, CD117-, S100+, KI671%.

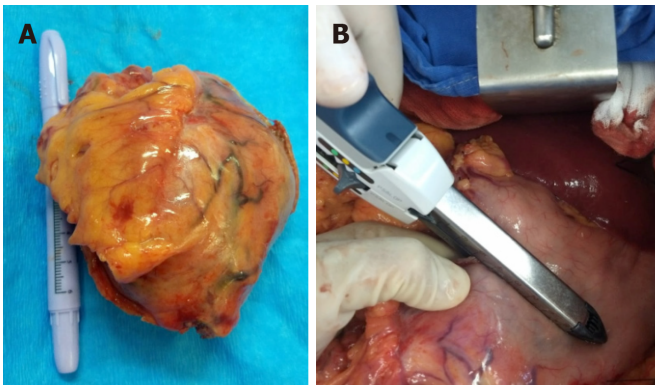

Figure 4 Resection of a gastric schwannoma. A: Tumor of approximately 10 cm; B: Wedge resection by linear cutting stapler of the greater gastric curvature.

ARTICLE HIGHLIGHTS

Research background

Schwannomas are benign neoplasms originated from Schwann cells. Schwannomas are more commonly locate in the stomach. Schwannomas are usually asymptomatic lesions.

Research objectives
We have reviewed the available literature about gastrointestinal schwannomas, especially lesions from de stomach and esophagus (diagnosis, treatment, and follow up).

Research methods
Following the Preferred Reporting Items for Systematic Reviews and Meta-Analyzes guidelines, a systematic review was conducted. The PubMed, Ovid, MEDLINE, and Scopus databases were used for reviewing process. Only English language manuscripts were included. All gastrointestinal schwannomas specifically located in the esophagus and stomach were included, and cases that did not report long-term follow-up were excluded.

Research results
Gastric localization showed a higher prevalence in both, the literature review and our institution. With a follow-up with disease-free survival greater than 36 mo in most cases. In both groups, the median size was > 4.1 cm. Surgical treatment is curative in most cases.

Research conclusions
Schwannoma must be taken into account in the differential diagnosis of gastrointestinal mesenchymal tumors. Schwannoma has a good prognosis, and most are benign.A disease-free survival of more than 36 mo can be achieved by surgery.

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CASE REPORT

Gallbladder cancer harboring *ERBB2* mutation on the primary and metastatic site: A case report

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Abstract

BACKGROUND

Bile duct cancer constitutes gallbladder cancer (GBC), intrahepatic cholangiocarcinoma (ICA), and extrahepatic cholangiocarcinoma (ECA). These three entities show morphological and immunohistochemical resemblance so that it is difficult to differentiate between primary ICA and liver metastasis of GBC, which sometimes becomes a point of discussion in clinical practice. Although these cancers demonstrate significant differences in their mutational landscape, several reports demonstrated shared genomic alteration in paired primary and metastatic site aids in distinguishing metastatic recurrence from second primary cancers.

CASE SUMMARY

We present a 73-year-old female patient who underwent curative resection for GBC harboring epidermal growth factor receptor 2 (ERBB2) activating mutation on next-generation sequencing (NGS)-based genomic testing. One year later, a

Satoh reports other from Merck Serono Co., Ltd, other from Takeda Pharmaceutical Company, other from Eli Lilly and Company, other from Bristol Myers Squib, other from Yakult Honsha, other from Ono Pharmaceutical Co., Ltd, other from Chugai Pharmaceutical Co., Ltd, from null, other from Takara Bio INC, outside the submitted work.

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hepatic lesion was observed on follow-up imaging and she underwent surgical resection for a pathological diagnosis. The histological findings of the hepatic lesion were similar to those of the primary lesion. Additionally, using NGS panel testing, the hepatic lesion was found to have *ERBB2* activating mutation, which is the identical mutation detected in the sequencing result of the primary site. ERBB2 activating mutation occurs more frequently in GBC than ICA and ECA. Therefore, in the present case, we think this molecular finding potentiated the diagnosis of the liver mass toward a metastatic recurrence. Additionally, this patient underwent *HER2*-targeted treatment with lapatinib in combination with capecitabin and obtained clinical benefit.

CONCLUSION
This case illustrated NGS panel usefulness in distinguishing GBC recurrence from second primary cancer and *HER2*-targeted agent efficacy on *ERBB2* mutated GBC.

Key words: Gall bladder cancer; Bile duct cancer; *ERBB2* mutation; Precision medicine; Mutation-driven targeted treatment; Case report

Core tip: We present a case report of a patient with gallbladder cancer (GBC) harboring epidermal growth factor receptor 2 (*ERBB2)* hotspot extracellular domain mutation (Ser310Phe) on both the primary site and metachronous liver metastasis. Given that pathological differentiation between hepatic metastasis and primary cancer of the liver is often difficult, next-generation sequencing panel could be a novel option for patients who need to distinguish a metastatic lesion from a second malignancy, which would affect staging and the treatment strategy. This case also illustrated the benefit of the *HER2*-targeted agent in the treatment of GBC harboring *ERBB2* activating mutation.

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INTRODUCTION

Gallbladder cancer (GBC) is an uncommon malignancy with an aggressive clinical course. Its prevalence varies among geographical areas and is higher in Asia and the Andes region[1]. GBC constitutes bile duct cancer along with intrahepatic cholangiocarcinoma (ICA) and extrahepatic cholangiocarcinoma (ECA). However, these are distinct entities with significant differences in their mutational landscape[2]. For example, isocitrate dehydrogenase 1 or 2 mutations, breast cancer *1* associated protein-1 mutations, and fibroblast growth factor receptors fusions are frequently seen in ICA, whereas Kirsten rat sarcoma viral oncogene homolog and, mothers against decapentaplegic homolog 4 mutations are more likely to occur in ECA. However, GBC has a high frequency of avian erythroblastosis oncogene B2 (ERBB2), transformation-related protein 53 (TP53), and cyclin-dependent kinase inhibitor 2A mutations.

Generally, surgical resection is the only curative option for localized GBC, and chemotherapy is the primary treatment for unresectable or recurrent disease. Despite recent advances in the treatments, more than half of the patients have experienced a recurrence after radical resection and prognosis of metastatic disease is very poor with 5-year survival around 5%[3,4]. The liver is the most common site of recurrence in GBC[4]. The histological differentiation between primary ICA and liver metastasis of GBC is often difficult owing to morphological and immunohistochemical resemblance, whereas the distinction between the metastasis of the primary malignancy and newly developed second primary malignancy is clinically important for accurate staging and tailoring treatment strategies[5]. Reflecting recent technical

advances in high-throughput next-generation sequencing, there are several reports describing the utility of genomic profiling in the differential diagnosis of a metastatic recurrence and distinguishing it from second primary malignancy[6].

Here, we present a case report of a patient with GBC harboring *ERBB2* activating mutation on both the primary site and metachronous liver metastasis, which aids in the differentiation from secondary malignancy. Additionally, this patient was treated with human epidermal growth factor receptor-2 (*HER2*)-targeted agent, lapatinib, and achieved clinical benefits.

CASE PRESENTATION

Chief complaints

The patient was a previously healthy 73-year-old female who underwent curative resection for GBC (pT2N0M0 according to the eighth International union against cancer TNM classification). We performed next-generation sequencing (NGS)-based genomic profiling of the resected specimen using the NGS gene panel, Oncomine® Comprehensive Assay version 3 (OCA v.3, Thermo Fisher Scientific), which revealed *ERBB2* Ser310Phe (c.929C>T; VAF, 18%) and *TP53* Ser241Tyr (c.722C>A; VAF, 19%) mutations. One year later, a hepatic lesion was observed on follow-up imaging and she underwent surgical total biopsy for a pathological diagnosis.

History of present illness

A patient had no symptoms and was in good health at the time of total biopsy.

History of past illness

The patient had no previous medical history.

Physical examination and laboratory testing

The patient's physical examination was not remarkable and laboratory testing was within normal limits, including tumor markers, such as *CA19-9* and *CEA*.

Imaging Examination

Contrasted computed tomography (CT) showed an ill-defined low attenuation lesion in the posterior lobe of the liver (Figure 1).

Further diagnostic work-up

The hepatic lesion was histologically diagnosed as well-differentiated adenocarcinoma and the histological findings of the hepatic lesion were similar to those of GBC (Figure 2). Therefore, the lesion was considered a metastasis. Moreover, we performed genomic profiling from the liver tumor using the NGS panel, Oncomine® Target Test system (OTT, Thermo Fisher Scientific). This revealed *ERBB2* Ser310Phe (c.929C>T; VAF, 26%), which was identical to the mutation detected in the sequencing result of the primary site; thus, the liver tumor was the most consistent with a metastasis of GBC rather than localized ICC. To evaluate *HER2* overexpression in tumor cells, we performed immunohistochemistry of *HER2*, which was negative (*HER2* score 0). Since *TP53* was not included in the gene list of OTTs, *TP53* mutation status at the metastatic site was not assessed.

FINAL DIAGNOSIS

The final diagnosis of the presented case is hepatic recurrence of GBC.

TREATMENT

After the total biopsy of liver metastasis, she was treated with two standard chemotherapy regimens, namely gemcitabine and cisplatin, and TS-1; however, her disease did not obtain clinical benefit from these treatments. After six months from hepatic resection, she was confirmed to have a progressive disease during second-line chemotherapy. At that time, she had liver and pulmonary recurrence, as well as pulmonary and inferior vena cava tumor embolism, which caused tachycardia and peripheral edema.

Considering no standard treatment beyond second-line for GBC, we treated the patient with lapatinib with a combination of capecitabine (lapatinib at a dose of 1250 mg per day continuously plus capecitabine at a dose of 2000 mg per square meter of

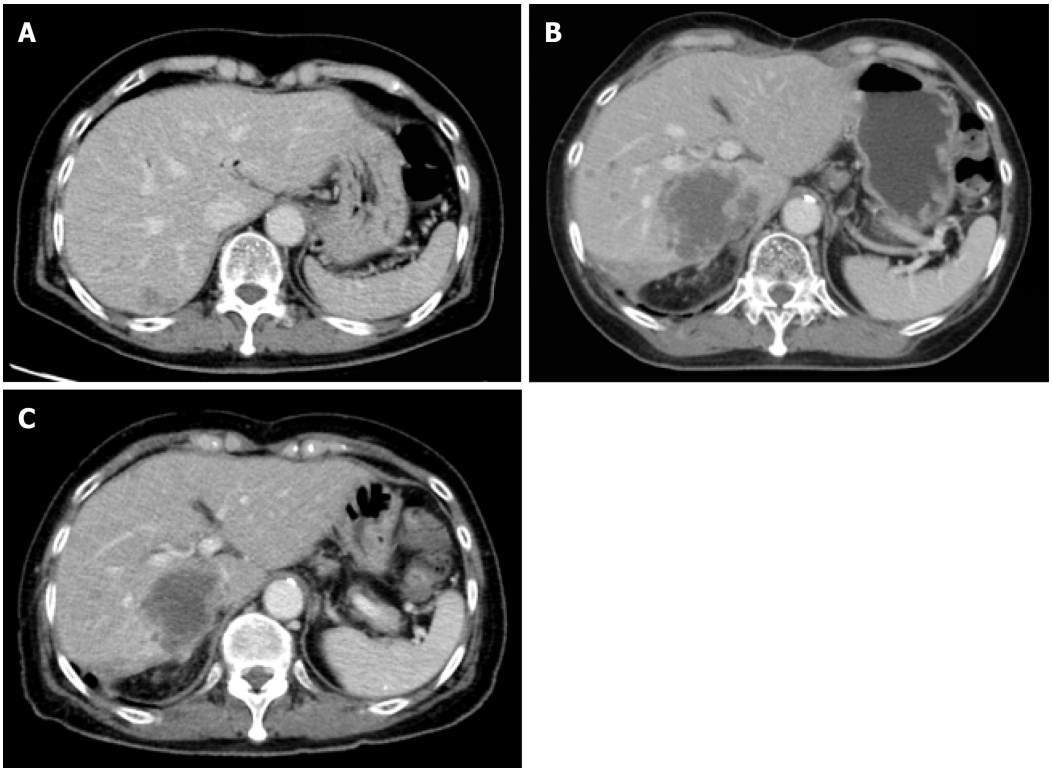

Figure 1 Contrasted computed tomography images. A: Contrasted computed tomography scan before surgical biopsy. A hypoattenuating lesion with ill-defined margins was observed in the posterior lobe of the liver; B and C: Comparative contrasted computed tomography scan before (B), and after (C) 2 cycles of lapatinib and capecitabine treatment. Hepatic metastases were reduced in size and tumor emboli in inferior vena cava (arrow heads) was mostly disappeared with the therapy.

body-surface area on days 1 through 14 of a 21 d cycle) based on the accumulating preclinical and clinical evidence that tumors with *ERBB2* mutation benefit from *HER2*-targeted treatment.

OUTCOME AND FOLLOW-UP

Within a week of treatment, she experienced major subjective clinical improvement, which included resolution of peripheral edema. After 2 cycles of treatment, contrasted CT imaging showed a decrease in the size of tumor emboli and hepatic lesions (Figure 1). However, after 4 cycles of treatment, the patient discontinued treatment due to grade 3 mucositis. Mucositis was gradually subsided over two weeks after discontinuation of the treatment. One month after discontinuation, her disease progressed, and she chose best supportive care.

DISCUSSION

We observed the same *ERBB2* Ser310Phe mutation in the primary tumors, as well as the metachronous hepatic lesion of this patient using NGS panels. We believe this molecular finding potentiated the diagnosis of the liver mass toward a metastatic recurrence. In addition, this patient exhibited a favorable effect of the *HER2*-targeted agent on GBC with *ERBB2* activating mutation.

Histologically, metastatic adenocarcinoma of the biliary tract cannot be distinguished from ICA or pancreatic origin owing to similarities in appearance and immunohistochemical staining patterns[5]. In addition, there is no particular method established to assess genetic relationships and clonality in primary and metastatic sites in malignancy. However, limited studies have shown the potential of genetic profiling to distinguish between a metastatic recurrence of the primary cancer and newly developed second primary cancer in several malignancies[6-8]. Previous reports demonstrated shared genomic alteration in paired primary and metastatic sites was useful in differentiating multifocal non-small cell lung cancer from intrapulmonary metastasis[7,8]. Moreover, Vignot *et al*[9] showed that genomic profiles of the first metastatic recurrent sites are highly concordant to the primary site in colorectal

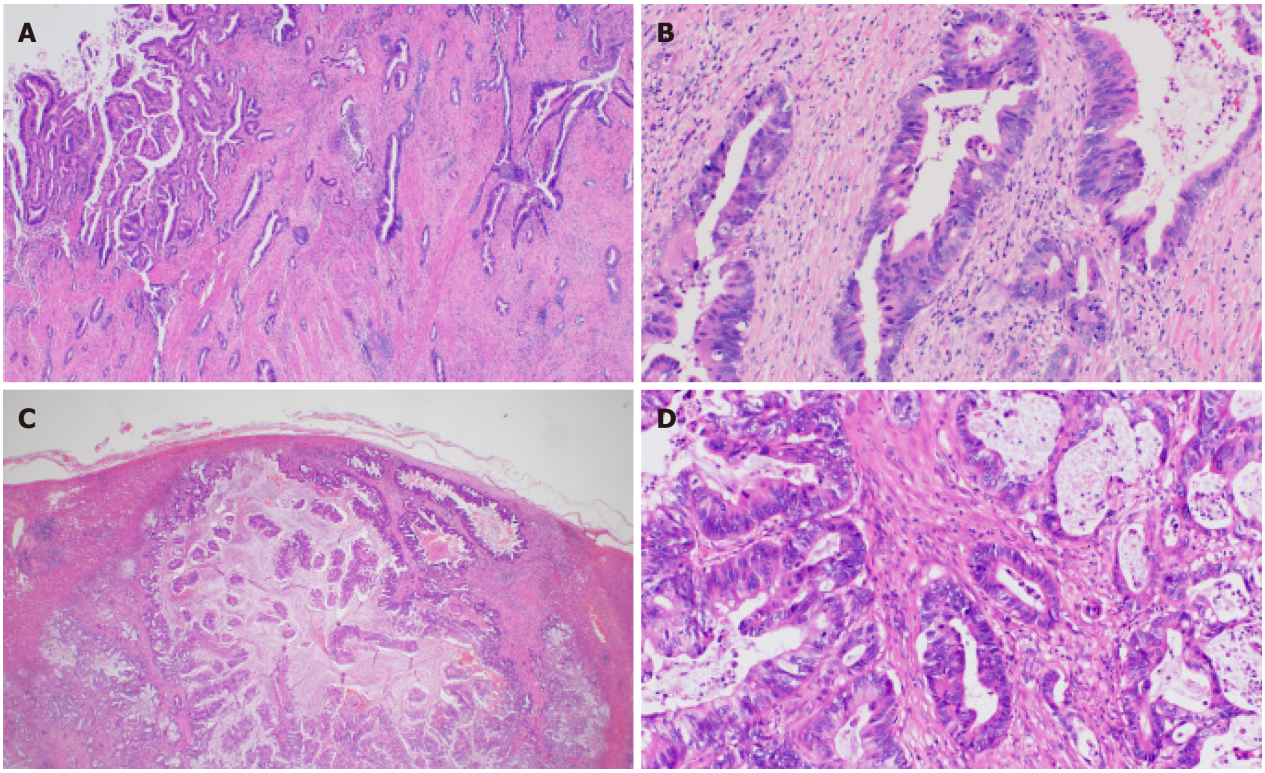

Figure 2 Tumor histology. A: Low-power microscopic view of the gallbladder cancer. The tumor cells form tubules of variable sizes. The tumor infiltrates deeply into the gallbladder wall; B: High-power microscopic view of the gallbladder cancer. Atypical columnar cells with enlarged nuclei grow in tubular structures. Stromal fibrosis and inflammation are also observed; C: Low-power microscopic view of the hepatic lesion. Adenocarcinoma with vaguely nodular contour involves the liver parenchyma; D: High-power microscopic view of the hepatic lesion. Glandular structure is predominant. The tumor cells have enlarged nuclei with coarse chromatin. Multiple mitoses are observed.

cancer. In the present case, the primary site and hepatic lesion shared the identical *ERBB2* mutation. *ERBB2* mutations are relatively frequent (9%-10%) in GBC, in contrast to ICA, as shown in previous studies[10-12]. Currently, there are no reports evaluating the concordance between primary and metastatic sites in CA; however, we considered this molecular finding supported the diagnosis of metastatic recurrence rather than the primary carcinoma of liver origin.

Additionally, *ERBB2* Ser310Phe is a known activating hotspot mutation in the extracellular domain[13]. The growing body of preclinical evidence and early phase trials supports *HER2*-targeted therapy for cancers harboring *ERBB2* activating mutations, whereas standardized molecular treatment has not been determined for this population[14-16]. Furthermore, the efficacy of *HER2*-targeted treatment on *ERBB2*-mutated tumors seems to vary between tumor types and mutation loci. Neratinib is an irreversible pan-HER tyrosine kinase inhibitor and clinical efficacy of neratinib for various *ERBB2*-mutated cancers was evaluated in the basket trial[17]. Neratinib exhibited the greatest activity in patients with breast cancer [Overall response rate; ORR 32% (8/25)], followed by biliary tract cancer [ORR 22.2% (2/9)). When stratified by a mutant allele, response was greatest in patients with kinase domain hotspot mutation [ORR 21.4% (9/42)], followed by Ser310 mutation [ORR 10% (3/30)] and exon 20 insertion mutation [ORR 7.1% (2/28)]. Among two biliary tract cancer patients with *ERBB2* Ser310 mutation included in this trial, one patient responded to neratinib. Ado-trastuzumab emtansine, a *HER2*-targeted antibody-drug conjugate linking trastuzumab with emtansine, demonstrated ORR of 44% (8/18) for patients with lung cancer harboring *ERBB2* mutation, including Ser310, in phase II basket trial; however, to the best of our knowledge, there are no reports evaluating its benefit for GBC with *ERBB2* mutation[18]. Javle *et al*[19] reported a case series of biliary tract cancer harboring *ERBB2* mutations. In this report, one cholangial cancer patient with *ERBB2* Ser310 mutation treated with trastuzumab, a humanized monoclonal antibody directed to *HER2*, in combination with FOLFOX, was not effective. Our patient obtained clinical benefit from lapatinib and capecitabin combination treatment. Lapatinib is a dual tyrosine kinase inhibitor that targets epidermal growth factor receptor and *HER2* and the combination treatment of lapatinib and capecitabin was evaluated initially in *HER2* positive breast cancer patients and showed prolonged survival with tolerable toxicity[20]. A previous case report indicated substantial efficacy

of this combination treatment in a patient with metastatic extramammary Paget's disease harboring *ERBB2* Ser310 mutation[21]. Given that TS-1 monotherapy, which is oral fluoropyrimidine as with capecitabin, was prescribed as second line treatment and was not effective to this patient, modest benefit from this combination treatment would be attributed to lapatinib. As both lapatinib and capecitabin are off-label use in Japan for patients with cholangiocarcinoma, we prescribed these agents following patients' written consent.

CONCLUSION

This case highlighted the usefulness of NGS panels in distinguishing hepatic metastasis from primary cancer of the liver, which sometimes becomes a point of discussion in daily practice. Although we need a large cohort for verification, NGS panel may be a novel option for patients who need to distinguish a metastatic lesion from a second malignancy, which would affect staging and treatment strategies.

This case also illustrated the value of lapatinib in combination with capecitabine in the treatment of GBC harboring *ERBB2* activating mutation. We recognized *HER2*-targeted agent as a potential treatment for *ERBB2* mutated tumors. Further investigation of *HER2*-targeted agent in this population is warranted.

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