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ABOUT COVER

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AIMS AND SCOPE

The primary aim of *World Journal of Gastrointestinal Oncology* (*WJGO*, *World J Gastrointest Oncol*) is to provide scholars and readers from various fields of gastrointestinal oncology with a platform to publish high-quality basic and clinical research articles and communicate their research findings online.

WJGO mainly publishes articles reporting research results and findings obtained in the field of gastrointestinal oncology and covering a wide range of topics including liver cell adenoma, gastric neoplasms, appendiceal neoplasms, biliary tract neoplasms, hepatocellular carcinoma, pancreatic carcinoma, cecal neoplasms, colonic neoplasms, colorectal neoplasms, duodenal neoplasms, esophageal neoplasms, gallbladder neoplasms, *etc*.

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REVIEW

Gastrointestinal neuroendocrine tumors in 2020

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Abstract

Gastrointestinal neuroendocrine tumors are rare slow-growing tumors with distinct histological, biological, and clinical characteristics that have increased in incidence and prevalence within the last few decades. They contain chromogranin A, synaptophysin and neuron-specific enolase which are necessary for making a diagnosis of neuroendocrine tumor. Ki-67 index and mitotic index correlate with cellular proliferation. Serum chromogranin A is the most commonly used biomarker to assess the bulk of disease and monitor treatment and is raised in both functioning and non-functioning neuroendocrine tumors. Most of the gastrointestinal neuroendocrine tumors are non-functional. World Health Organization updated the classification of neuroendocrine tumors in 2017 and renamed mixed adenoneuroendocrine carcinoma into mixed neuroendocrine neoplasm. Gastric neuroendocrine tumors arise from enterochromaffin like cells. They are classified into 4 types. Only type I and type II are gastrin dependent. Small intestinal neuroendocrine tumor is the most common small bowel malignancy. More than two-third of them occur in the terminal ileum within 60 cm of ileocecal valve. Patients with small intestinal neuroendrocrine tumors frequently show clinical symptoms and develop distant metastases more often than those with neuroendocrine tumors of other organs. Duodenal and jejuno-ileal neuroendocrine tumors are distinct biologically and clinically. Carcinoid syndrome generally occurs when jejuno-ileal neuroendocrine tumors metastasize to the liver. Appendiceal neuroendocrine tumors are generally detected after appendectomy. Colonic neuroendocrine tumors generally present as a large tumor with local or distant metastasis at the time of diagnosis. Rectal neuroendocrine tumors are increasingly being diagnosed since the implementation of screening colonoscopy in 2000. Gastrointestinal neuroendocrine tumors are diagnosed and staged by endoscopy with biopsy, endoscopic ultrasound, serology of biomarkers, imaging studies and functional somatostatin scans. Various treatment options are available for curative and palliative treatment of gastrointestinal neuroendocrine tumors.

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Key words: Gastrointestinal neuroendocrine tumors; Gastric neuroendocrine tumors; Small intestinal neuroendocrine tumors; Colonic neuroendocrine tumors; Rectal neuroendocrine tumors; Carcinoid syndrome

Core tip: Neuroendocrine tumors are increasingly being seen in our clinical practice. There has been excellent progress in the understanding of tumor biology. Currently, we have various ways of diagnosing and treating neuroendocrine tumors. This article will discuss the epidemiology, pathogenesis and clinical aspects as well as the current treatment protocol and follow up recommendations in patients with neuroendocrine tumors.

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INTRODUCTION

Neuroendocrine tumors (NETs) arise from the diffuse system of neuroendocrine cells *i.e.* cells with features of both nerve cells (which can receive message from the nervous system) and endocrine cells (which have the ability to synthesize and secrete monoamines, peptides and hormones)[1]. Neuroendocrine cells do not have any axons or nerve terminals. The electrical signals from the nervous system can be converted into hormonal signals with production of hormones, peptides and amines. As neuroendocrine cells are ubiquitous in our body, NETs can form in different organs including the gastrointestinal tract (GI), pancreas, lungs, gallbladder, thymus, thyroid gland, testes, ovaries and skin. Most of the NETs are in the GI (55%) or in the bronchopulmonary system (25%). NETs can develop throughout the GI (GI-NETs) in the following areas: The small intestine (45%), rectum (20%), appendix (16%), colon (11%), and stomach (7%)[2]. The diagnosis of rectal NETs has surpassed the diagnosis of small intestinal NETs (SI-NETs) since the year 2000 (except year 2001) when screening colonoscopy was implemented[3]. NETs are a heterogenous group of benign or malignant tumors with various morphologies and functions. The incidence and prevalence of NETs have been increasing over the last few decades[4]. About 40% NETs are hormone secreting[5]. Most NETs are slow growing with a small percentage of NETs being rapidly growing[6]. About 20% of NETs are associated with hereditary genetic syndromes like multiple endocrine neoplasia type 1 (MEN1) and neurofibromatosis type 1 (NF-1)[7]. We will review the epidemiology, classification, biology, clinical aspects, and management of GI-NETs in this article.

EPIDEMIOLOGY

NETs constitute only 0.5% of all malignant conditions and 2% of all malignant tumors of the GI[8]. In the United States, the incidence and prevalence of NETs have been increasing over the last few decades possibly due to early-stage detection, increased awareness, and widespread use of endoscopy and imaging studies for various gastrointestinal diseases. There was a 6.4-fold increase in annual age-adjusted incidence of NETs from 1973 (1.09 per 100000 persons) to 2012 (6.98 per 100000 persons). This increased incidence was found in all organs. 2000-2012 Surveillance, Epidemiology, and End Results (SEER) 18 registry showed the highest incidence of GI-NET to be 3.56 per 100000 population[9]. The prevalence also increased from 0.006% in 1993 to 0.048% in 2012. NETs are more prevalent in females than in males with a ratio of 2.5:1[10]. Bronchopulmonary NETs are more common in Caucasians[11] whereas GI-NETs are more common in African Americans[12].

CLASSIFICATION OF GASTROINTESTINAL NET

In 2017, the World Health Organization (WHO) updated the classification of NET. The histologic grading is based on mitotic index and Ki-67 index which are recorded in hot spots of the tumor. During cell division, Ki-67 protein is found in the cell nucleus. The proportion of Ki-67 – positive tumor cells (Ki-67 index) correlates with cellular proliferation, clinical course and its prognosis. Higher grade is considered if there is any discrepancy between mitotic index and Ki-67 index. The mixed adenoneuroendocrine carcinoma was renamed as MiNEN (mixed neuroendocrine neoplasm) considering that the mixed neoplasms may contain non-endocrine component other than adenocarcinoma, for example acinar cell carcinoma or squamous cell carcinoma. Each component must be at least 30% to fall into the category of MiNEN[13]. The 2017 WHO Classification of GI-NETs is outlined in the Table 1[14].

BIOLOGY OF NET

NETs are slowly growing tumors. As mentioned before, neuroendocrine cells have both neural and endocrine characteristics. They have cytoplasmic dense core granules which contain chromogranin A (CgA), synaptophysin and Neuron-specific enolase (NSE) and can synthesize and secrete various physiologically active monoamines, peptides and hormones. CgA and synaptophysin are necessary for diagnostic confirmation but proliferative index of Ki-67 and mitotic index are necessary for prognostic information. CgA is released from the cytoplasmic chromaffin granules into the blood, and as a result, serum CgA is raised in both non-functioning and functioning NETs. Serum CgA is the most commonly used biomarker to assess the disease burden and monitor treatment response[15]. The type of hormone secreted by functioning NETs varies with different organs. While GI-NETs synthesize and secrete serotonin and other vasoactive amines, Pancreatic-NETs (P-NETs) produce and secrete gastrin, insulin, glucagon and somatostatin[16]. Recently, there has been tremendous progress in the understanding of tumor microenvironment (TME) of NETs. TME consists of stromal cells, extracellular matrix (ECM), endothelial cells and inflammatory cells. NET cells activate and proliferate stromal cells *i.e.*, fibroblasts by secreting various soluble factors which include serotonin, fibroblast growth factor (FGF), platelet-derived growth factor (PDGF) and transforming growth factor β (TGF-β). Fibroblast activation leads to local and distant fibrosis. Some peculiar changes are seen in the ECM of NETs. In SI-NET, focal desmoplasia is common[17] and this is due to the presence of plenty of myofibroblasts/stellate cells producing collagen III fibers, desmin and vimentin[18]. Somatostatin receptors and their downstream pathways have been found to be primary regulators of neuroendocrine cell proliferation, protein synthesis and hormone secretion[19]. Various proangiogenic factors are secreted by NET cells. These include vascular endothelial growth factor, FGF, PDGF, semaphorins and angiopoietins. These lead to endothelial cell recruitment, proliferation, and neovascularization making the tumor highly vascular (density of microvessels becomes 10 times higher than that in epithelial tumors)[20]. Different immune cells (T cell, B cells, macrophages, dendritic cells, NK cells and mast cells) infiltrate the NETs making the TME immunosuppressed; this is more pronounced in P-NETs than SI-NETs probably due to a higher mutation rate in P-NETs[21]. CD+FoxP3+ T regulatory (Treg) and tumor-associated macrophage infiltration have been associated with high-grade NET[22,23] and poor prognosis. Soluble inhibitory factors secreted by NETs impair the maturation and function of dendritic cells, and as a result, antigen presentation to dendritic cells becomes impaired. NK cells also show impaired cytolytic activity in patients with GI-NETs. Tumor-infiltrating neutrophils, mast cells and macrophages can cause complex inflammatory and angiogenic responses. It is not known whether tumor-infiltrating lymphocytes have anti-tumor activity in the TME that can lead to an indolent course of NETs. Checkpoint proteins are heterogeneously expressed in G1/G2 NETs. NEC and 3% of P-NETs express enough checkpoint proteins to become appropriate candidates for immunotherapy[16]. Thus, TME not only controls the behavior, growth, invasive and metastatic capabilities, and local and systemic immune suppressive effects of NETs but also response to treatment.

Table 1 World Health Organization classification of gastrointestinal neuroendocrine tumors

Well-differentiated neuroendocrine neoplasms (NENs)		
	Ki-67 index (%)	**Mitotic index/10 HPF**
NET grade 1 (G1)	< 3	< 2
NET grade 2 (G2)	3-20	2-20
NET grade 3 (G3)	> 20	> 20
Poorly differentiated neuroendocrine neoplasms (NENs)		
	Ki-67 index (%)	**Mitotic index/10 HPF**
NEC grade 3	>20	>20
-Small cell type		
-Large cell type		
Mixed neuroendocrine neoplasms (MiNEN)		
Source: Adapted from WHO Classification of Tumors of Endocrine Organs, Fourth edition (2017)[14]		

HPF: High-power field; NET: Neuroendocrine tumor; NEC: Neuroendocrine carcinoma.

CLINICAL ASPECTS OF GI-NETS

Gastric NETs

Most of them develop from enterochromaffin-like cells (ECL cells) while a small proportion develop from non-ECL cells of gastric mucosa. G-NETs constitute 7% to 8% of all NETs. The incidence of gastric NETs (G-NETs) has been increasing (more than 10-fold over the last 30 years)[24]. As per the SEER 9 registry, the incidence of G-NETs increased from 0.31 per 1000000 persons in 1975 to 4.85 per 1000000 persons in 2014[25]. The increased incidence is probably due to multiple factors including the extensive use of upper endoscopies, evaluation of subepithelial lesions by endoscopic ultrasonographies (EUS), improved immunohistochemical staining, imaging modalities, tumor biomarkers, molecular markers and increased awareness of the diagnosis. There are rare reported cases of well-differentiated NENs (gastric carcinoids) developing after long-term use of proton pump inhibitors[26,27]. G-NETs are classically categorized into 4 types[28] as described below and summarized in Table 2.

Type I: It is the most common type of G-NETs accounting for 70%-80% of all G-NETs. It occurs in response to hypergastrinemia in the setting of achlorhydria (gastric pH > 7) typically seen in autoimmune chronic atrophic gastritis (CAG) where gastric parietal cells in the gastric body and fundus are destroyed by an autoimmune process. About 5% of autoimmune CAG may develop type I G-NET. It can also occur in *Helicobacter pylori*-induced CAG with hypergastrinemia[29]. Hypergastrinemia leads to ECL cells hyperplasia and promotes the formation of G-NETs in patients with CAG[30]. Most of the time, G-NETs are diagnosed incidentally in the investigation of patients with anemia or dyspepsia or other gastrointestinal symptoms. Endoscopically, they generally appear as smooth, rounded, subcentimeter, subepithelial multiple polypoid lesions with or without central depression in the gastric fundus or gastric body[31]. EUS may show a hypoechoic or isoechoic lesion with regular margins in the lamina propria (2nd echo layer) or submucosa (3rd echo layer)[32]. EUS is also helpful in finding out local metastasis to lymph nodes which generally occurs in 5% of cases. Computerized tomography (CT) or magnetic resonance imaging (MRI) should be done to rule out any distant metastasis which can happen in 2% of cases. Histologically, most G-NETs are positive for CgA, NSE, and vesicular monoamine transporter 2 (characteristic of histamine producing cells). A multi-disciplinary team should be involved to individualize treatment. Endoscopic resection either by polypectomy, endoscopic mucosal resection (EMR) or endoscopic submucosal dissection (ESD) is the treatment of choice if the lesions are not extensive[33]. But if the lesions are large (> 1 cm), extensive (involving the muscularis propria on EUS), multifocal (> 5) and recurrent on a previous endoscopic resection site, wedge resection of the stomach or even gastric antrectomy should be considered to eliminate the source of gastrin[34]. But all NEC should be treated by radical gastrectomy[35]. Patients should have surveillance endoscopy every 6 mo following endoscopic resection or surgery. The prognosis of

Table 2 Summary of different types of gastric neuroendocrine tumors

	Type I	Type II	Type III	Type IV
Distribution	70% to 80% of all GNETs	5% to 6% of all GNETs	15% to 20% of all GNETs	Most rare
Cell of origin; And location	ECL; Gastric body and fundus	ECL; Gastric body and fundus	ECL in most cases; Anywhere in stomach	Non-ECL; Anywhere in stomach
Gastrin status	Hypergastrinemia	Hypergastrinemia	Normogastrinemia	Hypergastrinemia -1/3rd of cases
Gastric mucosa	Atrophic	Hypertrophic	Normal	Atrophic most of the time but can be hypertrophic
Endoscopically	Multiple subcentimeter polypoid lesions	Multiple small (1 to 2 cm) polypoid lesions	Large (> 2 cm), solitary polypoid lesion	Large (> 4 cm) polypoid lesion
Treatment	Polypectomy, EMR, ESD, wedge resection of stomach, gastric antrectomy	Surgical resection of gastrinoma and aggressive gastrectomy	Partial or total gastrectomy and regional lymphadenectomy, chemotherapy	Partial or total gastrectomy with regional lymphadenectomy followed by adjuvant chemotherapy

ECL: Enterochromaffin-like cells; EMR: Endoscopic mucosal resection; ESD: Endoscopic submucosal dissection; GNETs: Gastric neuroendocrine tumors.

type I G-NETs is excellent with a 5-year survival of almost 100% (90%-95%)[36]. One study suggested that Netazepide (a gastrin/cholecystokinin 2 receptor antagonist) could be a potential medical treatment of type I G-NETs as it decreased the number and size of type I G-NETs as well as serum CgA[37].

Type II: It is the least common type of G-NETs accounting for 5%-6% of all G-NETs. It occurs in response to hypergastrinemia in the setting of hyperchlorhydria (gastric pH ≤ 2) typically associated with MEN1-Zollinger-Ellison syndrome (ZES) and rarely sporadic ZES[38]. In patients with normal gastric mucosa, hypergastrinemia causes gastric mucosal hypertrophy, ECL hyperplasia and dysplasia. However, a defect in the suppressor protein menin due to mutation of MEN1 gene located on chromosome 11q13 leads to transformation of G-NET[39]. As a result, G-NETs occur in < 1% of sporadic ZES and 13%-43% of MEN1-ZES[40]. Endoscopically, they appear as multiple, small (1-2 cm) polypoid lesions in the stomach. Histologically, they are generally well differentiated NENs limited to muocsa and/or submucosa. Metastasis can occur in 10% to 13% of cases. Treatment includes surgical resection of gastrinoma and aggressive gastrectomy. There are some case series showing somatostatin analogue octreotide could regress the type II G-NETs and serum gastrin levels[41]. The prognosis of type II G-NETs is good with a 5-year survival of 70%-90%.

Type III: These are sporadic G-NETs accounting for 15%-20% of all G-NETs. They occur most commonly in men over the age 50 years in the presence of normogastrinemia and normal gastric mucosa. They develop from ECL cells in most cases in the absence of ECL hyperplasia and are not dependent on gastrin. Patients are often asymptomatic or may present with abdominal pain, weight loss and iron deficiency anemia (IDA)[42]. Hepatic metastasis can be the initial presentation. Endoscopically, they appear as a large (> 2 cm), solitary, polypoid tumor arising from the gastric body, fundus or gastric antrum. Histologically, they are aggressive grade 3 NECs with high potential for local and distant metastasis (> 50%) regardless of their size. Treatment of non-metastatic type III G-NET is surgical resection (partial or total gastrectomy) and regional lymphadenectomy[43]. Treatment options for metastatic lesions include octreotide (for carcinoid syndrome) systemic chemotherapy (streptozocin, 5-fluorouracil with leucovorin, cyclophosphamide, doxorubicin, oxaplatin, dacarbazine), molecular targeted agents (bevacizumab, sunitinib, sorafenib, everolimus), targeted radionucleotide therapies (indium-DTPA-octreotide, Lutetium-DOTA-Tyr3-octreotide, Yttrium-DOTA-Tyr3-octreotide), transarterial chemoembolization (TACE) and radiofrequency ablation (for symptomatic hepatic metastasis)[44]. Type III G-NET carries a worse prognosis with a 5-year survival rate of less than 35%.

Type IV: They are the most rare of all G-NETs. They occur more commonly in males above the age of 60 (mean age 63-70 years). They are of non-ECL cell origin and gastrin-independent. Hypergastrinemia is seen in one third of cases and CAG is frequently (82% of cases) present[10]. Patients may present with dyspepsia, gastrointestinal bleed, IDA and weight loss. Endoscopically, the tumor appears as a

large (usually > 4 cm) polypoid tumor anywhere in the stomach. At the time of diagnosis, type IV G-NETs may have already metastasized to the lymph nodes and liver. Histologically, they are aggressive NECs grade 3 almost identical to gastric adenocarcinoma except for the presence of endocrine cells in the tumor matrix. Angioinvasion, lymphoinvasion and deep wall invasion are also present. Immunohistochemically, CgA may be absent but NSE and synaptophysin are strongly expressed[45]. Treatment of localized type IV G-NET includes partial or total gastrectomy with regional lymphadenectomy followed by adjuvant chemotherapy. Cisplatin-based chemotherapy (etoposide plus platinol) is offered as the first line treatment for metastatic type IV G-NET. FOLFOX (folinic acid, fluorouracil and oxaliplatin) and FOLFIRI (folinic acid, fluorouracil and irinotecan) are considered as second-line treatment options when Cisplatin-based therapy fails[46,47]. The prognosis is extremely poor with a mean survival of 6.5-14.9 mo[48].

SI-NETs

Their incidence has surpassed that of small bowel adenocarcinomas. Currently the most common primary small bowel malignancy accounting for 25% of all GI-NETs[1], SI-NETs arise from eneterochromaffin cells located at the base of the intestinal crypts in the submucosa. The incidence of SI-NET has increased probably due to increased diagnostic modalities. As per SEER registry, the age-adjusted annual incidence of jejunal and ileal NETs is 0.67 per 100000 population in the United States[49]. SI-NETs are indolent, often multifocal and have a distal predilection. More than two thirds of SI-NETs are in the terminal ileum within 60 cm of ileocecal valve. The approximate distribution of SI-NET is duodenum–2%, jejunum-7% and ileum-89%[50]. Patients with SI-NETs frequently experience clinical symptoms. SI-NETs metastasize to distant locations more often than other types of NETs[51]. Duodenal and jejuno-ileal NETs are biologically and clinically distinct[52].

Duodenal NETs

They are becoming more prevalent. They represent 2% to 3% of all GI-NETs. More commonly seen in males, the mean age of diagnosis is 6th decade of life. Most of the duodenal NETs (d-NETs) are solitary, small lesions limited to the duodenal mucosa and submucosa. The majority remain silent and are diagnosed incidentally during routine investigations. At the time of diagnosis, 40% to 60% of d-NETs are already metastatic to regional lymph nodes and 10% to the liver. Tissue diagnosis is generally done by endoscopic biopsy or EUS with fine needle aspiration (FNA). All patients with d-NETs should be checked for fasting serum gastrin, serum CgA and screen for MEN1 syndrome. 5-types of d-NETs are found. These are described as follows and summarized in Table 3[53].

Gastrinomas: They are subcentimeter multiple tumors originating from G-cells in the submucosal layer of proximal duodenum (D1-57%, D2-31%, D3-6% and D4-3%)[54] and secrete excessive gastrin. They account for about 10% of all d-NETs. They are the most common functional d-NETs followed by somatostatinoma > 80% of gastrinomas arise in the gastrinoma triangle (arbitrarily defined - superiorly confluence of cystic duct and common bile duct, inferiorly 2nd and 3rd portion of duodenum, and medially body and neck of pancreas). Duodenal wall gastrinoma is seen in 40%-50% of all gastrinoma. They are the most common cause of ZES. They could be sporadic (75%) or part of MEN1-ZES. Clinically they present with chronic, recurrent and refractory peptic ulcer disease (PUD), chronic diarrhea, and gastroesophageal reflux disease (GERD)[55]. 54% of duodenal gastrinomas can be malignant[56]. Gastrinomas are generally diagnosed biochemically by the presence of high fasting serum gastrin level, basal acid output (BAO)/maximal acid output (MAO) > 0.6 and positive Secretin suppression test. Duodenal gastrinomas can be localized by various investigations which include EUS, somatostatin receptor scintigraphy (SRS), CT, MRI, selective angiography, Indium 111-labeled diethylenetriamine penta-acetic acid (DTPA) octreotide and (68)Ga-DOTATE PET/CT scan[57]. Recent studies suggest that (68)Ga-DOTATE PET/CT scan is more sensitive and specific than ^{111}In-DTPA-Octreotide scan in detecting primary and metastatic NETs[58]. Intraoperative endoscopic transillumination of duodenal wall (transillumination from the serosal side by the surgeon while examining the mucosal side by the endoscopist) is also very helpful in detecting duodenal wall gastrinomas[59]. The treatment of nonmetastatic duodenal gastrinoma is surgical resection or enucleation of the tumor without pancreaticoduodenectomy. In patients with duodenal gastrinoma with hepatic metastasis, treatment options include hormonal therapy with octreotide,

Table 3 Summary of different types of duodenal neuroendocrine tumors

	Gastrinomas	Somatostatinoma	Gangliocytic paraganglioma	Non-functioning d-NETs	Duodenal NECs
Location	Proximal duodenum. > 80% gastrinoma triangle	Ampullary or peri-ampullary region	Peri-ampullary region	Proximal duodenum	Peri-ampullary region
Presenting symptoms	Chronic diarrhea, recurrent and refractory peptic ulcer disease, gastroesophageal reflux disease	Nausea, abdominal pain, weight loss, obstructive jaundice or very rarely somatostatinoma syndrome	Asymptomatic, gastrointestinal bleeding, anemia, abdominal pain	Asymptomatic or nausea, vomiting	Asymptomatic, nausea, vomiting, gastrointestinal bleeding
Diagnosis	BAO/MAO > 0.6, positive Secretin suppression test, EUS, somatostatin receptor scintigraphy (SRS), CT, MRI, selective angiography, Indium 111-labeled diethylenetriamine penta-acetic acid (DTPA) octreotide and (68)Ga-DOTATE PET/CT scan	CT, MRI, endoscopy, EUS-FNA	Endoscopy, EUS-FNA, CT	Endoscopy, EUS-FNA	Endoscopy, EUS-FNA
Treatment	Surgical resection or enucleation of the tumor without pancreaticoduodenectomy for nonmetastatic duodenal gastrinoma. In patients with duodenal gastrinoma with hepatic metastasis treatment options include hormonal therapy with octreotide, chemotherapy (streptozocin, doxorubicin, 5- fluorouracil), radiotherapy with yttrium 90-DOTA-lanreotide, hepatic embolization alone or with chemoembolization, cytoreductive surgery and liver transplantation	Endoscopic resection should be adequate if the NET is less than 1 cm. Transduodenal excision should be done for 1-2 cm tumor. But Whipple's surgery with local lymph node resection should be considered for more than 2 cm tumor	Endoscopic resection or radical excision including pancreaticoduodenectomy depending on the size, depth of invasion and lymph node metastasis	Transduodenal resection is indicated for d-NETs invading the muscularis propria. Radial surgery is advocated for d-NETs > 2 cm in diameter, d-NETs with lymph nodes involvement and all peri-ampullary d-NETs	radical surgery or chemotherapy

BAO: Basal acid output; MAO: Maximal acid output; (68)Ga-DOTATE PET/CT scan: Gallium -68 DOTATE positron emission tomography/computerized tomography scan; d-NETs: Duodenal neuroendocrine tumors; CT: Computerized tomography; MRI: Magnetic resonance imaging; EUS: Endoscopic ultrasonography; FNA: Fine needle aspiration.

chemotherapy (streptozocin, doxorubicin, 5-fluorouracil), radiotherapy with yttrium 90-DOTA-lanreotide, hepatic embolization alone or with chemoembolization, cytoreductive surgery and liver transplantation[60-63].

Somatostatinoma: They originate from D-cells in the ampullary or periampullary region of the duodenum and secrete excessive amount of somatostatin. They can be sporadic or part of MEN1 syndrome or associated with NF-1. They occur in up to 10% of patients with NF-1. Somatostatinomas are generally solitary, large, malignant tumors and have metastasized to lymph nodes or the liver at the time of diagnosis. Clinically, duodenal somatostatinomas may present with non-specific or mechanical symptoms like nausea, abdominal pain, weight loss, obstructive jaundice or very rarely somatostatinoma syndrome which consists of the triad of diabetes mellitus, cholelithiasis and steatorrhea[64]. Most of the time, duodenal somatostatinomas are detected incidentally during imaging studies like CT or MRI or endoscopy. They can be further evaluated by EUS with FNA or FNA biopsy (FNAB). Histologically, psammoma bodies are present inside the tumor cells in 68% cases of duodenal somatostatinoma[65]. Endoscopic resection should be adequate if the NET is less than 1 cm. Transduodenal excision should be done for 1-2 cm tumor. But Whipple's surgery with local lymph node resection should be considered for tumors that exceed 2 cm[66].

Gangliocytic paraganglioma: They are rare duodenal NETs with a predilection for the second part of duodenum near the ampulla. The tumor mostly exhibits a benign nature except regional lymph node metastasis in 5% to 7% of cases. The tumor size varies from 0.5 cm to 10 cm (average 2.5 cm). They can remain asymptomatic or present with gastrointestinal bleeding and anemia due to mucosal ulceration or abdominal pain due to mass effect[67]. They are generally detected during imaging studies done for other indications. Endoscopically, they look like subepithelial tumors, deeming mucosal biopsy nondiagnostic. The tumors are isoechoic on EUS. CT can identify them as soft tissue masses. Histologically, they consist of spindle, epitheloid and ganglion cells and the diagnosis is confirmed by immunohistochemical staining[53]. Treatment includes endoscopic resection or radical excision including pancreaticoduodenectomy depending on the size, depth of invasion and lymph node metastasis[68,69].

Non-functioning d-NETs: The majority (90%) of d-NETs are non-functional and are detected during routine endoscopy done for other reasons. Patients may remain asymptomatic or present with obstructive symptoms like nausea, vomiting or jaundice. EMR should be considered for d-NETs < 2 cm confined to submucosa. Transduodenal resection is indicated for d-NETs invading the muscularis propria. Radial surgery is advocated for d-NETs > 2 cm in diameter, d-NETs with lymph nodes involvement and all peri-ampullary d-NETs[70,71].

Duodenal NECs: They are extremely rare aggressive tumors proximal to the ampullary region. Patients may present with abdominal pain, nausea, vomiting and gastrointestinal bleeding. Upper endoscopy may show a polypoid mass near the ampulla[72] and this is further evaluated by EUS-FNA/FNAB. Histologically solid "sheetlike" proliferation of tumor cells with high mitotic index is found[73]. In comparison to well-differentiated NENs, duodenal NECs are more invasive in terms of lymphovascular invasion, duodenal wall invasion beyond submucosa, local lymph node metastasis and distant metastasis[74]. Its course of deterioration is rapidly progressive despite radical surgery or chemotherapy[75].

Jejuno-Ileal NETs

They account for 23% to 28% of all GI-NETs[76]. Most of the Jejuno-Ileal NETs (JI-NETs) are nonfunctioning. The mean age of diagnosis is 6th or 7th decade of life with no sex predilection[77]. The JI-NETs are generally > 2 cm in size, and consist of multiple tumors in up to 40% of cases[78]. At the time of diagnosis, 70% of them have invaded the muscularis propria with metastasis to the regional lymph nodes, and 50% of patients may have hepatic metastasis regardless of tumor size[79]. The hallmark of JI-NETs is desmoplastic reaction leading to mesenteric fibrosis which may manifest in about 50% of cases[80]. Fibrosis around the metastatic lymph nodes causes mesenteric contraction which can kink the small bowel resulting in intestinal obstruction. Mesenteric fibrosis can also impinge on the mesenteric blood vessels giving rise to mesenteric ischemia in about 10% of affected patients[81]. Desmoplastic reaction can also involve the retroperitoneum leading to retroperitoneal fibrosis, obstructive uropathy and hydronephrosis. Clinically, patients may be completely asymptomatic or may present with abdominal pain, intestinal obstruction, gastrointestinal bleeding and decreased urination. Radiologically, mesenteric fibrosis appears as a mesenteric mass with linear soft tissue opacities and possible calcification radiating outwards in a "wheel spoke" pattern. Mesenteric fibrosis does not depend on the NET size or Ki-67 proliferative index. It is associated with not only various comorbidities but also distant metastasis and poor prognosis[82]. Diagnostic modalities include: (1) Biomarkers: Serum CgA, serum NSE and urinary 5-hydroxy indole acetic acid (as a marker of carcinoid syndrome); (2) Diagnostic endoscopy: Capsule endoscopy and balloon-assisted or spiral endoscopy; and (3) Diagnostic imaging: SRS (Octreoscan), (68)Ga-DOTATE PET/CT or ^{111}In-DTPA-Octreotide scan.

Treatment of JI-NET includes surgical resection of primary NET with regional lymphadenectomy even in the presence of hepatic metastasis. There is no role of chemotherapy in well- differentiated JI-NEN. Combination chemotherapy - capecitabine and temozolomide for metastatic poorly differentiated JI-NEN[83], and combination of cisplatin or carboplatin and etoposide for JI-NEC[84] have been found to be helpful. Hepatic metastasis can be treated by octreotide therapy, transarterial embolization with microparticles (bland embolization), TACE, radiotherapy (peptide receptor radionucleotide therapy) with yttrium 90-DOTA-lanreotide or 177-lutetium-DOTA-lanreotide, and radiofrequency ablation. The 5-year survival rate of JI-NET is 60% in non-metastatic disease but becomes 18% when metastatic to the liver.

Carcinoid syndrome

It is the combination of symptoms which occur in about 20%-30% of cases of JI-NETs when they metastatize to the liver. The syndrome occurs when bioactive amines and peptides (about 40 different types) produced by the NETs enter the systemic circulation. 90% of carcinoid syndrome have metastatic NETs to the liver except bronchopulmonary NETs, ovarian NETs and GI-NETs with extensive retroperitoneal lymph node metastasis as they can release their bioactive amines directly into the systemic circulation and do not need to be metastatic to the liver to produce carcinoid syndrome. Clinically, the syndrome is characterized by chronic flushing (occurring in 94% of patients), and/or diarrhea (occurring in 80% of patients). Other manifestations include wheezing (occurring in 10%-20% of patients) due to bronchospasm, pellagra due to niacin deficiency and carcinoid heart disease (occurring in 40%-50% of patients). Flushing is due to excessive release of tachykinins (substance P, neurokinin A, neuropeptide K) and histamine. Diarrhea is mainly due to excessive secretion of serotonin which increases gastrointestinal motility and secretion[85]. Bronchospasm is histamine-induced but carcinoid wheezing should not be confused with bronchial asthma as administration of beta-2 agonist may cause severe and prolonged vasodilation[86]. As most of the dietary tryptophan (70% instead of only 1% normally) is converted to serotonin by the NETs leading to deficiency of tryptophan necessary for niacin synthesis, niacin deficiency occurs. Carcinoid heart disease is due to histamine-induced plaque-like deposit of fibrous tissue on the endocardium and valves of right heart leading to restrictive cardiomyopathy, and tricuspid and pulmonary regurgitation with or without coexistent stenosis and ultimately right heart failure[87]. Diagnosis of carcinoid syndrome is supported by elevated 24 h urinary 5 hydroxylindoleacetic acid (5-HIAA) which has a sensitivity and specificity of > 90%[88] and elevated serum CgA which is released from well-differentiated NETs. The level of 5-HIAA reflects tumor burden and decreases with treatment response. There are various food and medications that can affect 5-HIAA level. Tryptophan rich food (like banana, plum, pineapple, kiwi, eggplant, avocado, peanut, walnut, pecan, oats, beans, lentils, seeds, tofu, cheese, eggs, fish, chicken, turkey and red meat) can yield a false positive result. Acetaminophen, nicotine, caffeine, guaifenesin, phenobarbital and methamphetamine can increase 5-HIAA levels. Alcohol, aspirin, imipramine, methyldopa, levodopa, monoamine oxidase inhibitors, corticotropin and INH can decrease 5-HIAA level. Patients should be advised to stop taking these medications 24 h before and during urine collection.

Treatment options for carcinoid syndrome: (1) Long-acting somatostatin analog: Octreotide LAR 20 mg to 30 mg[89] or lanreotide 60 mg to 120 mg intramuscularly every 4 wk[90]. Flushing and diarrhea are improved in 80% of patients by this therapy[91]. If the symptoms are not adequately controlled, Octreotide LAR or lanreotide can be given every 3 wk instead of every 4 wk; (2) Hepatic resection: considered in neuroendocrine liver metastasis when 90% or more of the disease bulk can be resected keeping adequate functional hepatic reserve[92]. Prophylactic octreotide therapy should be given preoperatively and intra-operatively to prevent carcinoid crisis; and (3) Hepatic artery bland embolization or chemoembolization can reduce flushing and diarrhea in carcinoid syndrome[93]. Prophylactic octreotide therapy should be given pre and post-embolization to prevent carcinoid crisis.

In refractory symptomatic cases, other treatment options include: (1) Telotristat ethyl (tryptophan hydroxylase inhibitor) 250 mg by mouth 3 times day in combination with somatostatin analog therapy can control diarrhea in patients with carcinoid syndrome not responding to somatostatin analog therapy[94]; (2) Interferon-alpha: 3 to 5 millions up to 3 to 5 times per week can improve the symptoms of carcinoid syndrome (flushing, diarrhea) in 40% to 50% of cases refractory to somatostatin analog therapy[95,96]. Interferon has multiple antitumor effects as it can stimulate T cells, induce cell cycle arrest and inhibit angiogenesis. But Interferon is rarely used because of its tremendous side effects; (3) Everolimus - a mammalian target of rapamycin inhibitor in combination with octreotide can improve flushing and diarrhea in patients with carcinoid syndrome refractory to octreotide therapy[97]; (4) 177-Lutetium dotatate (peptide receptor radioligand therapy): Can improve diarrhea in patients with carcinoid syndrome refractory to octreotide[98]; and (5) Anti-diarrheal agents – lomotil, loperamide and cholestyramine are good adjunctive therapies to control diarrhea.

Carcinoid crisis

Carcinoid crisis is a critical complication of carcinoid syndrome characterized by wide fluctuation of blood pressure (hemodynamic instability) with a predominance of hypotension, severe flushing, dyspnea and confusion due to release of huge amount of

bioactive amines from the NET into the systemic circulation[99]. The crisis is triggered by either exposure to anesthetic agents or manipulation of the tumor during biopsy or surgery or embolization. Treatment is administration of mega dose of octreotide (500 μg to 1000 μg intravenous bolus followed by infusion of octreotide 50 μg to 200 μg per hour[100]. Administration of intravenous fluid alone may not be effective. Calcium and adrenergic agents should be avoided to improve blood pressure as paradoxical effect can occur in these patients as they can increase release of bioamines from the NETs. Prophylactically, octreotide 300 μg to 400 μg is given intravenously or subcutaneously prior to biopsy, surgery and embolization of NETs to reduce the occurrence of carcinoid crisis[101].

Carcinoid heart disease

Patients with carcinoid syndrome generally present with symptoms and signs of right heart failure with systolic murmur along the left sternal edge. Diagnosis is established by doing 24 h urinary 5-HIAA and transthoracic echocardiography[102]. N-terminal pro-brain natriuretic peptide (NT-proBNP) > 260 ng/mL is also useful as a biomarker of the presence of carcinoid heart disease[103]. Management includes administration of somatostatin analogs and other measures to control carcinoid syndrome as well as treatment of right heart failure with salt and water restriction, loop diuretics and digoxin. Tricuspid and pulmonary valve should be replaced in case of symptomatic valve disease and progressive ventricular dysfunction[104]. Annual clinical evaluation with serum NT-proBNP should be done for early detection of carcinoid heart disease. Carcinoid heart disease should be managed by a multidisciplinary team which includes gastroenterologists, oncologists, NET experts, endocrinologists, cardiologists and cardiothoracic surgeons.

Appendiceal NET

Appendiceal NET represents the 3rd most common NET in the GI. Most of the patients are asymptomatic and diagnosed incidentally with 0.3% to 0.9% cases undergoing appendectomy. The average age of diagnosis is 42 years and it occurs more commonly in females than in males[105]. They are generally submucosal and have a predilection to be located at the tip of the appendix[106] but in about 10% of cases, they can develop at the base of the appendix leading to obstruction and appendicitis[107]. Histologically, appendiceal NETs are EC-cell (serotonin-producing) NETSs, L-cell-type NETs and MiNENs (goblet cell cancer and adenocarcinoid). The local and distant metastatic potential depends on the size and histology of the NET. NET size > 2 cm, NEC and MiNEN have higher incidence of metastasis[108,109]. Consensus guideline (Table 4) suggests that simple appendectomy should be enough if the NET size is < 1 cm. If the NET size is 1 cm to 2 cm, appendectomy and periodic post-operative follow up is recommended for 5 years. Right hemicolectomy should be considered in this category if any of the following criteria is present: involvement of the base of the appendix, cecal infiltration, invasion into the mesoappendix or serosa, involvement of tumor margin, positive lymph nodes, lymphovascular invasion, presence of goblet cells or poorly differentiated cells, Ki67 index > 2%, MiNEN)[110]. If the NET is > 2 cm, treatment is right hemicolectomy within 3 mo from the time of appendectomy but staging work up is required. The National Comprehensive Cancer Network recommends multiphasic CT or MRI of abdomen and pelvis. SRS-based scan (Octreoscan) or (68)Ga-DOTATE PET/CT, serum CgA, 24 h 5-HIAA should also be considered[111]. Colonoscopy is also indicated to evaluate for synchronous colorectal cancer[112].

Colonic NETs

The second most prevalent advanced gastrointestinal cancer after colorectal cancer. As per SEER registry, the incidence of colonic NETs increased from 0.02 per 100000 in 1973 to 0.2 per 100000 in 2004[113]. The mean age of presentation of colonic NETs is 7th decade of life and female to male ratio is about 2:1[114]. Colonic NETs arises from Kulchitsky cells or enterochromaffin cells located within the crypts of Lieburkuhn of colon. Nearly 70% of colonic NETs are in the right colon, particularly in the cecum[115]. The patients remain asymptomatic until the NET size becomes large because of increased diameter of right colon than left colon. At the time of diagnosis, the average size of the NETs is about 5 cm and most have local or distant metastasis. Patients generally present with abdominal pain due to mass effect or tumor-induced desmoplastic reaction, gastrointestinal bleeding and weight loss. Sometimes, colonic NETs are detected as a mass lesion during screening colonoscopy. Treatment is segmental colectomy with wide regional lymphadenectomy. The overall 5-year

Table 4 Appendiceal neuroendocrine tumor: Size and surgery

Appendiceal NET size	Surgery
< 1 cm	Simple appendectomy
1 cm to 2 cm	Appendectomy and periodic post-operative follow up is recommended for 5 yr. Right hemicolectomy should be considered in the presence of involvement of base of the appendix, cecal infiltration, invasion into the mesoappendix or serosa, involvement of tumor margin, positive lymph nodes, lymphovascular invasion, presence of goblet cells or poorly differentiated cells, Ki67 index > 2% or MiNEN
> 2 cm	Right hemicolectomy within 3 mo from the time of appendectomy but staging work up is required. This includes multiphasic computerized tomography or magnetic resonance imaging of abdomen and pelvis. SRS-based scan (Octreoscan) or (68)Ga-DOTATE PET/CT, serum CgA, 24 h 5-HIAA and colonoscopy to evaluate for synchronous colorectal cancer

NET: Neuroendocrine tumors; (68)Ga-DOTATE PET/CT scan: Gallium -68 DOTATE positron emission tomography/computerized tomography scan; CgA: Chromogranin A; 5-HIAA: 5 hydroxylindoleacetic acid; MiNEN: Mixed neuroendocrine neoplasm.

survival is 33% to 42%. Imaging studies should be done to stage colonic NETs.

Rectal NETs

There is 10-fold increased incidence of rectal NETs over the last 30 years. The incidence of rectal NETs is approximately 1 per 100000 populations per year[116]. The mean age of diagnosis of rectal NETs is about 56 years and they are slightly more common in males than in females[117]. They also have higher incidence and prevalence in both Asian Americans and African Americans as compared to Caucasians. Most of the rectal NETs remain asymptomatic and are diagnosed incidentally during screening colonoscopy or when lower endoscopy is done for another reason[118]. Symptomatic patients may present with rectal bleeding, rectal discomfort, pruritis ani and change in bowel habit. Endoscopically, rectal NETs appear as smooth, round, sessile, polypoid lesions with overlying normal appearing or yellow- discolored mucosa, usually located within 5 to 10 cm of the anal verge. But as the diameter of the NET exceeds 5 mm, atypical endoscopic findings are noted and these include semipedunculated appearance, hyperemia, central depression, erosion and ulceration[119]. Most of the rectal NETs (80% to 90%) are < 1 cm in size, confined to the submucosa and well-differentiated NENs at the time of diagnosis. EUS and MRI of the pelvis play an important role in the evaluation of depth of rectal NETs and regional lymph node involvement. MRI is more sensitive in detecting nodal disease, and EUS in differentiating submucosal from muscularis propria involvement. Conventional polypectomy is ineffective as most of the rectal NETs are submucosal. In one study, complete resection rate by conventional polpectomy was 30.9%[120]. Piecemeal biopsy removal of rectal NETs should be discouraged as histological assessment of lateral and deep margins cannot be done. Traditional EMR (submucosal injection to lift the lesion followed by snare polypectomy) is effective for lesions < 0.5 cm. Curative resection of rectal NETs ≤ 1 cm in size can be done by device-assisted EMR (cap-assisted EMR or ligation-assisted EMR) or ESD as long as EUS examination does not show muscularis propria invasion and pararectal lymph node metastasis[121,122]. If the rectal NET is 1 cm to 2 cm in size and there is no muscularis propria invasion and pararectal lymph node metastasis, ESD or wide surgical excision is recommended. As the metastatic potential is high with rectal NET > 2 cm in size, low anterior resection or abdominoperineal resection is advocated in those cases. SRS-based scan (Octreoscan) or (68)Ga-DOTATE PET/CT should be done to detect any distant metastasis. Treatment options for metastatic rectal NETs include systemic therapies, liver directed therapies and palliative surgery. As per European Neuroendocrine Tumor Society, patients should have surveillance following complete resection of rectal NETs as follows: (1) Rectal NET < 1 cm (grade 1 or 2): No surveillance needed; (2) Rectal NET < 1 cm (grade 3): Annual colonoscopy for 5 years; (3) Rectal NET 1 cm to 2 cm (irrespective of grade): Colonoscopy, EUS and MRI at 12 mo, then colonoscopy every 5 years; (4) Rectal NET > 2 cm (grade 1 or 2): Annual colonoscopy, EUS and MRI for 5 years; and (5) Rectal NET > 2 cm (grade 3): Colonoscopy, EUS and MRI every 4 mo to 6 mo during the first year, then annually for 5 years.

Serum CgA can give additional information during surveillance if elevated at time diagnosis and normalized after resection of the NET so that increase in CgA level may indicate recurrence of the NET. Rectal NETs have the best prognosis among all the GI-NETs with 5-year survival rate of 74% to 88% as per SEER database and Norwegian

Register of Cancer for the period 1993-2004[123].

CONCLUSION

The GI-NETs are rare but their incidence and prevalence have been increasing. They have characteristic biology, histopathology and clinical behavior. Most of the time, they are slow growing tumors but can be rapidly growing at times depending on the site, size and grade of the tumor. Majority of the GI-NETs are non-functioning except a few which can secrete bioactive amines and hormones and produce hormonal syndrome. Patients tend to be asymptomatic but can sometimes present with symptoms from mechanical causes as the tumor enlarges or causes fibrosis along with GI bleeding. GI-NETs are generally diagnosed and staged by endoscopy with biopsy, serology of biomarkers, EUS, imaging studies and functional somatostatin scans. Histologically, diagnosis is confirmed by positive immunohistochemical staining of CgA and synaptophysin. Treatment and prognosis depend on the grade and stage of the tumor. Current treatment modalities include endoscopic resection, surgery, somatostatin analog therapy, Peptide receptor radioligand therapy, chemotherapy, liver targeted therapy (radiofrequency ablation, bland embolization and chemoembolization) and symptomatic treatment. Immunotherapy will serve as a future treatment modality. Patients should be kept under surveillance program following treatment of GI-NETs.

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REVIEW

Early stage colon cancer: Current treatment standards, evolving paradigms, and future directions

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Abstract

Colon cancer continues to be one of the leading causes of mortality and morbidity throughout the world despite the availability of reliable screening tools and effective therapies. The majority of patients with colon cancer are diagnosed at an early stage (stages I to III), which provides an opportunity for cure. The current treatment paradigm of early stage colon cancer consists of surgery followed by adjuvant chemotherapy in a select group of patients, which is directed at the eradication of minimal residual disease to achieve a cure. Surgery alone is curative for the vast majority of colon cancer patients. Currently, surgery and adjuvant chemotherapy can achieve long term survival in about two-thirds of colon cancer patients with nodal involvement. Adjuvant chemotherapy is recommended for all patients with stage III colon cancer, while the benefit in stage II patients is not unequivocally established despite several large clinical trials. Contemporary research in early stage colon cancer is focused on minimally invasive surgical techniques, strategies to limit treatment-related toxicities, precise patient selection for adjuvant therapy, utilization of molecular and clinicopathologic information to personalize therapy and exploration of new therapies exploiting the evolving knowledge of tumor biology. In this review, we will discuss the current standard treatment, evolving treatment paradigms, and the emerging biomarkers, that will likely help improve patient selection and personalization of therapy leading to superior outcomes.

Key words: Adjuvant; Circulating tumor DNA; Immunoscore; Minimally invasive; Neoadjuvant; FOxTROT; Minimal residual disease; International duration evaluation of adjuvant chemotherapy

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Core tip: Although the majority of patients with colon cancer are diagnosed in an early stage, cancer recurrence after initial curative therapy is frequent, underscoring the need for novel approaches. The challenges in the current treatment paradigm include the lack of precise patient selection tools for adjuvant therapy, disabling toxicities, and modest efficacy of the adjuvant therapies. Herein we provide a contemporaneous appraisal of the early stage colon cancer treatment and discuss how evolving technologies, including circulating tumor DNA, can potentially transform the standard of care.

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INTRODUCTION

Colorectal cancer (CRC) continues to be a major global health problem, with approximately 1.09 million new cases diagnosed and 551000 deaths from it each year[1]. Globally the burden of CRC is expected to increase by 60% resulting in more than 2.2 million new cases and 1.1 million deaths annually by the year 2030[2]. Recent data from the western countries suggest that the incidence of CRC is increasing in population under age 50[3]. Approximately 75% of newly diagnosed CRC patients present with non-metastatic early stage disease[4], which presents an opportunity of curative-intent treatment. Despite surgery and adjuvant therapy, 5% to 30% patients with colon cancer (CC) endure recurrence[5,6].

Colorectal carcinogenesis is a protracted multistage process which evolves over several decades. Most CRCs arise from adenomatous polyps that gradually progress to dysplasia and eventually to carcinoma over a period of 5-15 years[7], which opens up an opportunity for early detection and cure. Screening can identify early stage CRC that is easier to treat and has a lower mortality rate than advanced CRC. In addition, screening can prevent CRC by detecting and removing premalignant polyps before they progress to carcinoma. CRC incidence and mortality rates have been declining in the United States, likely due to widespread adoption of screening[8]. However, conventional colonoscopy has about 25% of false-negative results due to flat or depressed precancerous lesions[9]. A systematic review of colonoscopy studies reported a pooled miss rate of 22% for all polyps and 26% for polyps smaller than 5 mm in size[10].

While current treatment modalities with proven efficacy save thousands of lives, short- and long-term toxicities of the treatment often significantly compromise the quality of life. To improve efficacy and reduce toxicity, contemporary research is focusing on the following areas: (1) Minimizing the invasiveness of surgical resection and improving surgical recovery; (2) Refining patient selection for adjuvant therapy based on novel biomarkers; (3) Precise risk stratification to calibrate treatment type, intensity and duration; and (4) Exploration of new systemic therapies incorporating targeted agents. In this article, we present a review of the current standard treatment strategies and evolving treatment paradigms that might improve outcomes in the near future.

EARLY STAGE COLON CANCER: OVERVIEW OF TREATMENT STRATEGIES

Current standard treatment of early stage CC consists of upfront resection of the primary tumor along with regional lymph nodes and, in selected patients, administration of adjuvant chemotherapy (AC) 4 to 8 wk after the surgery[11-13]. For stage I CC, the current standard of care is surgery alone, which results in a 5-year disease-free survival (DFS) rate of 95%[6], and AC is not recommended. In stage II CC, the reported 5-year DFS rate with surgery alone ranges from 82% to 88%[6,14], and the

benefit of AC remains controversial. Current major guidelines recommend AC guided by risk stratification based on clinicopathologic features for patients with stage II CC[11-13]. AC, preferably with a combination of fluoropyrimidine and oxaliplatin, is recommended for all resected stage III patients[11]. Of note, surgery alone can achieve a 5-year DFS rate of 45%-50% in stage III patients[14,15], and the administration of oxaliplatin-based AC after surgery results in a 5-year DFS rate of 67%-70%[5,16,17]. These data highlight that only 17%-20% of stage III patients survive long term because of AC. The gain in survival with oxaliplatin-based AC needs to be considered in the light of treatment-related toxicities, especially 12.5% incidence of grade 3 neuropathy after 6 months of treatment[5]. Table 1 summarizes the role of surgery and chemotherapy in early stage CC.

SURGERY FOR EARLY STAGE COLON CANCER

The techniques for surgical resection have changed dramatically in the last three decades with the invention of minimally invasive techniques such as laparoscopy and robotic surgery. Endoscopic techniques that can be employed for select stage I tumors are currently an active area of research and offer the potential to significantly reduce the risk of complications, which averages about 20% in patients undergoing traditional surgical resection[18]. The expansion of laparoscopy for colectomy, along with the rapid growth of robotics has allowed surgeons to perform colectomy with significant reductions in complications and faster recovery for patients. In general, the goal of surgical resection is three-fold: To resect visible malignant disease, to remove the tumor in the wall of the colon and to remove the lymph nodes in the drainage basin for the tumor. By accomplishing this, accurate pathologic staging can be determined, and patients can be stratified into risk categories based on histologic and pathologic features. Such risk stratification is nearly impossible to perform without detailed histopathologic information.

Endoscopic resection

In select cases of large non-invasive premalignant polyps or early invasive tumors with favorable features, endoscopic resection can be employed. Clearly, for advanced adenomas such as tubulovillous adenomas or intramucosal adenocarcinoma, lymph node resection is not indicated, and only complete removal of the mucosal based dysplastic tissue is needed. Additionally, certain malignant polyps with favorable features, such as well or moderate differentiation, pedunculated morphology, at least 2 mm from the cauterized edge, without lymphovascular invasion and no evidence of distant or nodal metastases, are amenable to endoscopic resection with very low risk of lymph node metastasis and excellent long-term overall survival[19]. In a study of patients with malignant polyps who were lacking only one listed favorable feature, the risk of lymph node metastasis was 8% and residual carcinoma was 3% following surgery; with the risk of surgical complications at 13%, the balance remained even suggesting that only patients with multiple poor prognostic features would benefit from surgery (Table 2)[20,21]. Furthermore, some features are high-risk enough on their own to warrant resection even if others are lacking, specifically poorly differentiated tumors or mucinous or signet ring histology or those with deeper submucosal invasion, as these morphologies are associated with rates of lymph node metastases as high as 17%-46%[21,22]. Thus, for malignant polyps and very small stage I disease, the recommendation for full segmental colonic resection should be an individualized decision based upon the patients' tumor risk factors and surgical risk factors. In some guidelines, specific recommendations are laid out for when such treatments should be employed to help guide surgeons on risk management in such complicated settings[23].

There are three general advanced endoscopic techniques: Endoscopic mucosal resection (EMR), endoscopic submucosal dissection (ESD), and combined endoscopic-laparoscopic surgery (CELS). EMR, which involves the injection of fluid to "lift the polyp" from the submucosa followed by polypectomy using snare technique, differs from ESD, where endoscopic knives are used to create an incision in the bowel wall after fluid injection, and the lesion is removed circumferentially[24]. Both EMR and ESD allow higher rates of *en bloc* resection of the colon lesion and minimize piecemeal resection, which can make margin identification difficult and can lead to higher polyp recurrence rates[25]. EMR is technically less challenging, has lower complication risks, and can be repeated multiple times if necessary in the case of recurrent non-invasive dysplasia[25]. ESD is employed with larger lesions and for those where invasion into the submucosa is suspected as this technique allows resection into the deeper

Table 1 Role of surgery and adjuvant chemotherapy in early stage colon cancer (American Joint Committee on cancer stages I to III)

	Stage I	Stage II	Stage III
Definition	The tumor has grown through the colonic mucosa and has invaded the muscular layer of the colon	The tumor has grown through the wall of the colon or invaded adjacent organ, but has not involved the regional lymph nodes	The tumor has spread to the regional lymph nodes, but not to the distant organs
Contribution of surgery	5-yr DFS rate of 95% with surgery alone[6]	5-yr DFS rate of 82% to 88% with surgery alone[6,14]	5-yr DFS rate of 45%-50% with surgery alone[14,15]
Contribution of adjuvant chemotherapy	Adjuvant chemotherapy not recommended	Only offered to "high-risk" group-magnitude of benefit is uncertain	Recommended for all patients. Absolute improvement of 5-yr DFS rate is about 20% because of adjuvant chemotherapy[5,16,17]

DFS: Disease free survival.

Table 2 Prognostic features of malignant polyps

Features consistent with low risk of lymph node metastases (Low risk/favorable features)[26]	Features consistent with high risk of lymph node metastases (Poor prognostic features)[26]
Margins with no dysplasia or malignancy	Poorly differentiated
Well or moderately differentiated	Mucin/mucinous
No angiolymphatic invasion	Signet ring or cribriform histology
Superficial invasion into submucosa (≤ 2 mm)	Tumor budding
	Lymphovascular invasion
	Deeper invasion into submucosa (> 2 mm)

(submucosal) layers of the bowel wall[25]. If deeper invasion is a concern, endoscopic ultrasound or chromoendoscopy during the procedure can be performed with good accuracy in predicting the depth of submucosal invasion, and this can help guide the choice of endoscopic resection[22]. Once the lesions are removed, if poor prognostic features are present (as defined in Table 2) then surgery is recommended due to elevated risk of nodal metastases[26]. Overall, the results of ESD have been very good, with local recurrence rates of 2% in one single-institution high volume center, all of which were high-grade dysplasia without invasion and piecemeal resection was shown to be the significant predictor of recurrence[27].

In cases where endoscopic resection is difficult, or the risk of complications is high, a combined surgical and endoscopic approach, known as CELS, can be utilized with great effect. Because the occult rate of invasive cancer for patients with benign appearing endoscopically unresectable polyps is low, 8.4% in one study, surgical resection may be avoided in select patients[28]. In CELS, the surgeon laparoscopically mobilizes the colon, offers assistance with positioning the colon in redundant patients, and repairs any perforation or controls bleeding when needed while the endoscopist performs the mucosal resection to remove the lesion. This technique has been shown to have acceptable risks with complication rates of 11% and failure rates of 6% in one study[29]. Furthermore, the cost of such procedures are lower than formal colectomy, most due to reductions in hospital length of stay for the CELS procedure[30].

Principles of surgical resection

The goal of surgical resection is three-fold: To resect visible malignant disease, to remove the affected segment of intestine, and to remove the correlating draining lymph nodes with vascular ligation and mesocolon integrity[31]. In the absence of synchronous lesions, the surgeon should inspect the abdominal cavity for evidence of other disease, and plan operative resection based upon the location of the tumor in the colon and its lymphovascular drainage such that a margin of colon 5-7 cm proximal and distal to the tumor is removed *en bloc* with the associated mesentery extending to the origin of the named primary blood vessel feeding the segment of bowel[26]. A minimum of 12 lymph nodes should be resected to allow accurate pathologic staging and improved survival[32]. When feasible, anastomosis of the proximal and distal resection margins should be considered to allow bowel continuity.

In the last few years, the idea of a complete mesocolic excision (CME) has gained popularity. This idea is similar to the complete mesorectal excision for rectal cancer–a sharp dissection along anatomic embryologic planes to dissect the colon mesentery from the retroperitoneum and isolate the angiolymphatic drainage to its most central location[33,34]. Studies indicate that the rate of central nodal metastasis is approximately 2%-3%, even when other nodes closer to the tumor location do not harbor metastases (*i.e.* skip metastases), thus if surgical resection reduced even just this recurrence, CME would be as effective as AC for low-risk stage II patients, in part by identifying micro-metastatic disease and optimizing lymph node harvest[35]. This dissection is not without cost, as overly aggressive clearance of lymphatic tissues around origin vessels on the aorta can not only damage the vessels, but also the nerve plexus resulting in diarrhea, delayed gastric emptying, as well as urologic and sexual dysfunction[35,36]. While several retrospective cohort studies have shown favorable oncologic outcomes, there remains no randomized controlled study to support the benefit of CME at this time, and a recent meta-analysis did not find any significant difference in complications or oncologic outcomes[37-39]. A corollary to CME, sentinel lymph node biopsy (removing the first draining node for a given tumor to determine if additional nodal resection is needed) has been commonly used in many other malignancies, including breast cancer and melanoma; however, in CC *in vivo* sentinel node biopsy has not been routinely utilized due to technical considerations with the procedure. Sentinel lymph node mapping (identifying the location of the first draining node within the resected lymphatic tissue) may have more utility. While it is not routinely recommended as part of the pathologic assessment, there is the potential to identify nodal micro-metastases and thereby more accurately stage patients, yet even in doing so long-term outcomes may not be appreciably affected[40,41]. Future studies will need to be performed to understand the full benefits of costs of CME and nodal mapping techniques.

Minimally invasive surgical resection

Laparoscopic surgical techniques were first described in the late 1980s and has spread widely throughout the surgical community with its principles impacting every facet of surgical care; CRC treatments are no exception. In the early 2000s, several randomized controlled trials validated the safety and oncologic utility of laparoscopic surgery for CC[42-44]. Laparoscopic resections have been shown to have less operative blood loss, faster return of bowel function, fewer complications, shorter hospital stays, with no differences in oncologic outcomes such as positive margins, lymph node harvest, or survival[45-47]. This interest in reducing the impact of surgery with ever smaller incisions and ever less invasive approaches has led to a number of novel surgical techniques including hand-assisted laparoscopy (using a smaller approximately 4 cm port to allow a single hand into the abdominal cavity), single-incision surgery (all ports through one incision about 2-3 cm long), and robotic surgery (using a "robotic" platform with fine and flexible instruments). These various techniques, which use the same oncologic principles discussed previously, are appropriate options with comparable oncologic outcomes, and the choice of technique ultimately lies with the surgeon[26]. Despite enthusiasm and recommendations from multiple societies, the rates of minimally invasive surgery utilization in many countries only reaches 50% with considerable geographic variability; it is not entirely clear why this is the case, but the long training needed for mastery of complex laparoscopic procedures and higher equipment costs are certainly contributory[26,31].

ADJUVANT CHEMOTHERAPY: GOAL, ENDPOINTS AND TIMING

The primary goal of adjuvant therapy is eradication of micro-metastatic residual disease after surgical removal of the primary tumor to achieve a cure. Since micro-metastatic disease cannot be reliably identified or monitored, historically improvement in 5-year overall survival (OS) had been the gold standard to confirm the benefit of AC. Overall, the 5-year OS correlates well with the long term disease control, as demonstrated in 2 large retrospective analyses[48,49] including an ACCENT (Adjuvant Colon Cancer End Points) database analysis of 20898 patients enrolled in 18 randomized trials. The ACCENT database analysis reported recurrence rates of less than 1.5% per year after 5 years and less than 0.5% per year after 8 years from the study enrollment[49]. These data support the view that 5-year OS is a reliable surrogate marker of long-term survival and provides the "evidence for cure". However, a long follow up period is needed to demonstrate an improvement in 5-year OS with the

newer therapies in clinical trials, which underscored a need for an alternative strategy. A separate ACCENT database analysis of patients treated with 5-FU-based AC suggested that the 3-year DFS rate is an excellent predictor of 5-year OS, especially for stage III patients[50], and the 3-year DFS rate could be a surrogate endpoint for adjuvant CC trials. Subsequent retrospective analyses, which included patients receiving oxaliplatin-based AC, supported this view[51,52] although extended survival after recurrence as a result of improved therapy of metastatic disease weakened the strength of association between 3-year DFS and 5-year OS. Overall, 3-year DFS rate is considered a reliable endpoint to assess the efficacy of adjuvant therapy. The Drugs Advisory Committee of the United States Food and Drug Administration (FDA) has accepted a 3-year DFS rate as a regulatory endpoint for adjuvant therapy trials in CC. The adjuvant therapy with FOLFOX (5-FU, LV and oxaliplatin) was approved in the United States for stage III CC based on the improvement of the 3-year DFS rate reported in the MOSAIC trial[53]. The IDEA (International Duration Evaluation of Adjuvant Chemotherapy) pooled analysis[54], which evaluated the non-inferiority of AC administered for 3 mo *vs* 6 mo in stage III CC patients, also chose 3-year DFS rate as the primary endpoint.

The ideal time interval between surgery and initiation of AC is unknown, and a randomized clinical trial has not been conducted to date to address this question. Although the major guidelines do not specifically recommend a time window after the surgery, initiation of AC within 6 to 8 wk of surgery is required in most adjuvant clinical trials and has been accepted as a preferred practice. However, AC often does not begin within 8 wk of surgery in routine clinical practice due to a variety of reasons, including delay in recovery from the surgery. In this regard, laparoscopic surgery has an advantage over open resection, as recovery from the surgery is faster[55]. The impact of delaying initiation of AC on survival has been investigated in several retrospective studies and meta-analyses, which reached conflicting conclusions. A recent SEER-Medicare database analysis of 18491 patients reported significantly worse OS with initiation of AC after 8 wk of surgery, although benefit still persisted with a delay of up to 5 mo[56]. Two meta-analyses of fluoropyrimidine-based AC trials reported a higher risk of mortality with delayed initiation of AC beyond 8 wk[57] and 12 wk[58]. Conversely, a population-based analysis by the British Columbia Cancer Agency reported no adverse impact on outcome with a delay beyond 8 wk in patients with stage III CC who received oxaliplatin-based AC, implying that analyses based on fluoropyrimidine-based AC may not apply to patients who receive oxaliplatin-based AC[59]. The results of retrospective studies should be viewed in the light of possible biases, most important of which is the possibility that adverse tumor biology may have been responsible for both delays in initiation of AC as well as adverse survival outcome. For example, surgery of T4 CC is associated with higher post-operative morbidity[60] which can potentially delay the initiation of AC, and at the same time, the T4 disease is an independent predictor of poor survival[61]. In absence of conclusive data, we recommend initiation of AC within 8 wk of surgery. However, it is important to recognize that delayed initiation of AC, even up to 24 wk from the surgery, is associated with some degree of benefit[62].

DURATION OF ADJUVANT CHEMOTHERAPY

Duration of AC has evolved over the last 3 decades through a series of clinical trials[63-66] from 18 mo in the 1980s to 3 mo currently for a select group of patients. The MOSAIC trial, which established FOLFOX as the preferred adjuvant therapy for stage III CC, used chemotherapy for 6 mo[53]. However, oxaliplatin-based regimens for 6 mo are associated with several disabling toxicities, especially the oxaliplatin-induced peripheral sensory neuropathy. Some degree of neuropathy occurs in nearly all patients[53], and approximately two-thirds will have symptoms one-year post-treatment or beyond[67,68]. Moreover, the neuropathy often peaks several months after the last dose of oxaliplatin, which makes the preemptive dose adjustment to prevent neuropathy difficult[69]. In consideration of the potential curability and long survival of patients undergoing AC, the efficacy of a shorter duration of adjuvant therapy was explored in a pooled analysis of six large randomized trials with stage III CC patients (IDEA study) which evaluated the primary hypothesis that 3 mo of adjuvant oxaliplatin-based therapy would be non-inferior to standard 6 mo with a primary endpoint of 3-year DFS rate[54]. This pooled analysis had a non-inferiority design in which non-inferiority of 3 mo *vs* 6 mo would be established if the upper limit of the two-sided 95% confidence interval (CI) of the hazard ratio (HR) did not exceed 1.12.

The rationale behind choosing this non-inferiority margin was it corresponded to a worsening of the 3-year DFS rate by 2.7% compared to the standard therapy (from 72% to 69.3%), an outcome that was considered acceptable. Overall, about 40% of the patients received CAPOX (capecitabine and oxaliplatin), and 60% received FOLFOX. After a median follow-up of 41.8 months, although there was only 0.9 % difference in the 3-year DFS rate (74.6% *vs* 75.5%), the non-inferiority of 3 mo *vs* 6 mo was not confirmed in the overall study population (HR 1.07; 95%CI: 1.00-1.15). In a preplanned subgroup analysis by chemotherapy regimen, the non-inferiority of 3 mo was observed for CAPOX but not for FOLFOX. Of the patients who received CAPOX, 3 mo was found to be non-inferior to 6 mo (DFS rates of 75.9% *vs* 74.8%, respectively; HR 0.95; 95%CI: 0.85-1.06). Conversely, for patients receiving FOLFOX, 6 mo was found to be superior to 3 mo (DFS rate of 73.6% for 3 mo *vs* 76% for 6 mo; HR 1.16; 95%CI: 1.06-1.26; P = 0.001). Furthermore, an exploratory analysis revealed that in the 'low risk ' patient group (T1–3 and N1; 58.7% of patients), 3 mo of therapy was non-inferior to 6 mo for both CAPOX and FOLFOX regimens, with the 3-year DFS rates of 83.1% and 83.3%, respectively (HR 1.12; 95%CI: 0.90-1.12). Conversely, in patients with "high risk" tumors (T4/N1-2 or any T/N2 ; 41.3% of patients), the 6-month therapy was superior to the 3-month (3-year DFS rate of 64.4% *vs* 62.7% for the treatments combined; HR 1.12; 95%CI: 1.03-1.23; P = 0.01 for superiority). As expected, there was a substantial reduction in neurotoxicity with the 3-mo treatment. The incidence of neurotoxicity of grade 2 or higher with the 3-month regimens was 16.6% with FOLFOX and 14.2% with CAPOX compared to the 6-mo regimens, 47.7% with FOLFOX and 44.9% with CAPOX. Thus, the IDEA analysis provided a basis for treating low-risk stage III CC patients with 3 mo of therapy, especially if CAPOX is utilized. Based on this data, the most recent National Comprehensive Cancer Network (NCCN) guidelines recommend CAPOX for 3 mo as the preferred regimen for patients with low-risk stage III CC. For patients with high-risk stage III CC, CAPOX for 3 to 6 mo (with category 1 evidence for 6 mo) or FOLFOX for 6 mo (category 1) are recommended. Although CAPOX appears to have superior efficacy than FOLFOX in IDEA analysis, the evidence is not conclusive. The choice of using CAPOX vs. FOLFOX was not randomized, which increased the potential for selection bias. This is an important consideration in view of the fact that capecitabine is often poorly tolerated in the US population[70].

Four trials in IDEA collaboration (SCOT, TOSCA, ACHIEVE-2, and HORG) enrolled patients with high-risk stage II CC, with a total of 3273 patients randomly assigned to 3 mo *vs* 6 mo of adjuvant therapy, of whom 2019 received CAPOX and 1254 received FOLFOX[71]. The overall analysis failed to establish the non-inferiority of 3 mo *vs* 6 mo of treatment in terms of efficacy. In the entire population, five-year DFS rate was 80.7% *vs* 84% for 3 mo *vs* 6 mo of therapy, respectively (HR 1.18; 95%CI: 1.05-1.31; absolute difference of 3.3%). A subset analysis by regimen showed that 3 mo of CAPOX was non-inferior, with a 5-year DFS rate of 81.7% for 3 mo *vs* 82.0% for 6 mo (HR 1.02; 95%CI: 0.88-1.17). By contrast, the 5-year DFS rate for FOLFOX was 79.2% for 3 mo of treatment *vs* 86.5% for 6 mo, an absolute 7.3% difference in favor of longer treatment duration (HR 1.42; 95%CI: 1.19-1.70). It was concluded that 3 mo of CAPOX is a reasonable choice for high-risk stage II CC patients.

ADJUVANT CHEMOTHERAPY: CURRENT STANDARD

AC following the surgery is routinely recommended for all patients of resected stage III CC based on the unequivocal survival benefit demonstrated in numerous clinical trials, both with the 5-FU monotherapy[49] and oxaliplatin-based regimens[72]. The benefit with AC for the stage II group as a whole is debatable. Table 3 summarizes landmark adjuvant chemotherapy trials conducted in stage II and III CC patients.

Stage II colon cancer

Despite several randomized trials and meta-analyses, an unequivocal robust survival benefit from AC has not been demonstrated in stage II CC patients. The challenges to show a clear benefit with AC in stage II patients include marked prognostic heterogeneity within this patient group (5-year survival rate of 66.5% in T3N0 tumors *vs* 37.3% in T4bN0 tumors[73]), stage migration as a result of improved lymph node sampling over the years[74], excellent prognosis with the surgery alone[6] and a smaller number of stage II patients enrolled in randomized studies. The important studies which evaluated AC in stage II patients include QUASAR[75], MOSAIC[76], NSABP C-07[17], IMPACT B2 analysis[77] and the Cancer Care Ontario group analysis[78]. The

Table 3 Landmark adjuvant trials in early stage colon cancer

Study (Reference)	Study population	Patients (*n*)	Experimental arm	Control arm	Study result/Conclusion
Intergroup (INT) 0035[64]	Stage II and III	1296	5-FU bolus + Levamisole for 1 yr.	Observation.	Stage III: 5-FU/Levamisole reduced recurrence rate by 41% ($P < 0.0001$) and the death rate by 33% ($P = 0.006$). Stage II- No survival benefit with 5-FU/Levamisole. One year of 5-FU based adjuvant chemotherapy became the standard for stage III patients.
NSABP C-03[66]	Duke stage B and C	1081	Bolus 5-FU plus LV for 1 yr.	MOF for 1 year.	5-yr DFS rates- 54% *vs* 66% in favor of 5-FU/LV, $P = 0.0004$. 5-yr OS rates - 66% *vs* 76% in favor of 5-FU/LV, $P = 0.003$.
IMPACT B2[77]	Stage II	1016	Bolus 5-FU/LV for 6 mo.	Observation.	Pooled analysis of B2 CC in 5 randomized trials. No significant improvement in survival with the adjuvant chemotherapy. The 5-yr EFS: 73% for controls and 76% for 5-FU + LV (HR, 0.83; 90%CI: 0.72-1.07). The 5-yr OS: 80% for controls and 82% for 5-FU + LV (HR, 0.86; 90%CI: 0.68-1.07).
Intergroup (INT) 0089[63]	High-risk stage II and stage III	3794	(1) Low-dose LV plus 5-FU (Mayo Clinic regimen); (2) High-dose LV plus 5-FU (Roswell Park regimen); and (3) Low-dose LV plus Levamisole plus 5-FU. Each for 30-32 wk.	Bolus 5-FU plus levamisole for 1 year.	None among the 4 arms was statistically superior in terms of DFS or OS. Roswell park regimen was better tolerated than Mayo Clinic regimen in terms of diarrhea. 6 mo of 5-FU/LV replaced 12 mo of 5-FU/Levamisole as standard of care.
GERCOR C96.1[85,86]	Stage II and stage III	905	Semimonthly infusional 5-FU/LV (de Gramont regimen). Duration- 24 *vs* 36 wk.	Monthly bolus 5-FU /LV (Mayo Clinic regimen). Duration- 24 *vs* 36 wk.	DFS and OS were not statistically different between treatment groups and treatment durations. Semimonthly infusional 5-FU/LV regimen had better toxicity profile and was adopted as the standard arm for the MOSAIC trial.
QUASAR[75]	Stage I-III	3239 (Colon stage II = 2291)	5-FU/LV monthly bolus (Mayo clinic regimen) for 6 mo.	Observation.	3.6% (95%CI: 1.0–6.0) absolute improvement in 5-year OS with adjuvant chemotherapy in stage II CC patients.
X-ACT trial[92]	Stage III	1987	Capecitabine- 6 mo.	5-FU/LV (Mayo Clinic regimen)- 6 mo.	5-yr OS rates 71.4% with capecitabine *vs* 68.4% with 5-FU/LV (P = 0.06). Capecitabine was at least equivalent to 5-FU/LV in terms of OS and DFS.
MOSAIC[53,76]	High-risk Stage II and stage III	2246	FOLFOX4 for 6 mo.	de Gramont regimen (infusional 5-FU/LV) for 6 mo.	10-year OS rates for stage III - 67.1% *vs* 59.0 % (HR, 0.80; $P = 0.016$) in favor of FOLFOX. 10-year OS rates for stage II - 78.4% *vs* 79.5% (HR, 1.00; $P = 0.980$). FOLFOX replaced 5-FU/LV as the standard adjuvant therapy in resected stage III CC.
NSABP C-07[17,90]	Stage II and stage III	2407	FLOX for 6 mo.	Bolus 5-FU/LV (Roswell Park) for 6 mo.	5-yr DFS 69.4 *vs* 64.2% favoring FLOX (HR, 0.82; 95%CI, 0.72–0.93; $P = 0.002$) corresponding to an 18% relative reduction in the risk of a DFS event. 5-yr OS was similar between treatment groups.
NO169968/ XELOXA[16]	Stage III	1886	CAPOX- 6 mo.	bolus 5-FU/LV (Mayo Clinic or Roswell Park regimen) for 6 mo.	7-yr DFS rates 63% versus 56% in favor of CAPOX (HR, 0.80; 95%CI, 0.69–0.93; $P = 0.004$). 7-year OS rates 73% *vs* 67% in favor of CAPOX (HR, 0.83; 95%CI, 0.70–0.99; $P = 0.04$).
IDEA meta-analysis[54]	Stage III	12834	FOLFOX or CAPOX for 3 mo.	FOLFOX or CAPOX for 6 mo.	Noninferiority of 3 mo versus 6 mo treatment was not confirmed in the overall study population. Among the patients with low-risk tumors (T1-T3, N1), 3 mo of therapy with CAPOX was noninferior to 6 mo, with a 3-year rate of disease-free survival of 85.0% versus 83.1% (hazard ratio, 0.85; 95%CI, 0.71-1.01).

CC: Colon cancer; 5-FU: 5 Fluorouracil; LV: Leucovorin; MOF: Lomustine + vincristine + 5-FU; NS: Not significant; DFS: Disease free survival; EFS: Event free survival; OS: Overall survival; HR: Hazard ratio; CAPOX: Capecitabine and oxaliplatin.

QUASAR trial randomized 3239 patients with CRC (1073 patients of stage II CC in each arm) to observation *vs* monthly bolus 5-FU/LV for 6 mo. Among the patients with stage II CC, there was only a trend towards better OS in favor of the group who received AC with a five-year OS of 83.9% *vs* 81.5 % (HR 0.86; 95%CI: 0.54-1.19). The major criticism of the QUASAR trial was the small number of lymph nodes harvested (median number 6). The IMPACT B2 and the Cancer Care Ontario group analysis, both designed to evaluate the benefit of 5-FU-based AC, also failed to show a clear survival benefit. Conversely, in an ACCENT database analysis of nearly 6900 patients, 5-FU-based AC was associated with a 5% absolute improvement in survival at eight years (72% *vs* 66.8%, $P = 0.026$)[49]. A National Cancer Database (NCDB) analysis, which included 153110 patients of stage II CC diagnosed between 1998 and 2011, also showed a benefit with AC[79]. The 5- and 10-year OS rates were 73% and 51% with chemotherapy, as opposed to 62 % and 35% without chemotherapy.

The impact of adding oxaliplatin to 5-FU/LV backbone in stage II patients was explored in two prospective randomized trials, the MOSAIC[76] and the NSABP- C07[17] trials. The final report of the MOSAIC trial[76] reported identical 10-year OS rates with 5-FU/LV *vs* FOLFOX4: 79.5% *vs* 78.4% (HR 1.00; $P = 0.98$), respectively. NSABP-C07 trial also did not show any benefit of oxaliplatin containing regimen FLOX over 5FU/LV (5-year DFS rate 82.1% *vs* 80.1%, respectively; $P = 0.67$). Of note, no prospective randomized trial has been conducted to date comparing oxaliplatin-based AC with observation alone in resected stage II CC patients. In summary, evidences are lacking to support the routine use of AC in stage II CC patients.

Several studies have suggested that certain clinicopathologic high-risk features might be predictive of benefit from AC in stage II CC patients[80,81]. The current NCCN guideline recommends consideration of AC in stage II CC patients with following high-risk features[11]- T4 primary tumor, poorly differentiated histology (exclusive of tumors with deficient mismatch repair), lymphovascular invasion (LVI), perineural invasion (PNI), bowel obstruction, localized perforation, inadequately sampled lymph nodes (< 12 nodes) and close, indeterminate, or positive margin. The MOSAIC[5] trial included 569 patients with high-risk stage II CC- 282 patients randomized to the FOLFOX4 arm and 287 patients to the 5-FU/LV arm. The 5-year DFS rate was numerically higher with FOLFOX4, 82.3% (95%CI: 77.2%-86.28%) *vs* 74.6% (95%CI: 69.1%-79.34%), a difference that was not statistically significant. The NCDB analysis[79] mentioned above demonstrated a benefit with AC with a 5-year OS improvement from 57% to 76% ($P < 0.001$) in the high-risk group.

An important limitation of the studies described above is that these studies analyzed the high-risk stage II patients collectively as a group, despite the possibility that biologic heterogeneity among the various high-risk features may exist. A retrospective study, which analyzed the patients based on a single predominant high-risk feature[82], showed that AC was associated with improved OS only among the patients with T4 tumor as the single high-risk feature (HR 0.51; 95%CI: 0.34–0.78) or combinations involving T4 tumors as T4/< 12 sampled lymph nodes (HR 0.31; 95%CI: 0.11–0.90), T4/high grade histology (HR 0.26; 95%CI: 0.11-0.61), and T4/LVI (HR 0.16; 95%CI: 0.04–0.61). A prospective randomized trial to evaluate the benefit of AC exclusively in the high-risk stage II CC patients has not been conducted to date.

Stage III colon cancer

Once the NSABP C-01 trial[65] demonstrated a survival benefit with 5-FU-based AC in patients with resected Duke B and C colon cancer and the enhancement of the antitumor activity of 5-FU by leucovorin (LV) was reported[83], clinical trials over the next decades were conducted with three major schedules of 5-FU and LV combinations: (1) Monthly bolus 5-FU and LV (Mayo clinic regimen); (2) Weekly bolus FU and LV, 6 wk out of 8 wk (Roswell Park Memorial Institute regimen, RPMI); and (3) Semimonthly infusional 5-FU/LV regimen (de Gramont schedule)[63,66,84-87]. These clinical trials led to two important conclusions : (1) Stage III CC patients derived unequivocal survival benefit from the AC whereas stage II patients did not; and (2) All three 5-FU/LV schedules had comparable efficacy, but the semimonthly regimen had better tolerability[85,86,88,89]. These trials established 5-FU/LV based regimens as the standard adjuvant therapy for stage III CC in the pre-oxaliplatin era. The GERCOR C96.1 trial[85,86] compared semimonthly regimen with monthly bolus 5-FU/LV in stage II and III patients, each given over 24 wk and 36 wk. There were no significant differences in DFS with either treatment arm (HR, 1.04) or between 24 wk or 36 wk of therapy (HR, 0.94) at a median follow up of 6-year. The semimonthly regimen was less toxic, particularly with regards to hematologic and gastrointestinal adverse events ($P <$ 0.001). As a result, the semimonthly regimen was adopted as the standard arm in the subsequent MOSAIC trial[53].

In the next phase, several randomized adjuvant trials were conducted in which oxaliplatin was added to the 5-FU/LV backbone[16,17,76]. The MOSAIC trial, which randomized resected stage II and III patients to semimonthly 5-FU/LV *vs* oxaliplatin-based FOLFOX4 for 6 mo, demonstrated a superior 3-year DFS in stage III patients treated with FOLFOX4[53] and the benefit sustained long term. Most recent publication of MOSAIC data, after a median follow up of 9.5 years, reported a 10-year OS of 67.1% with FOLFOX4 *vs* 59% with 5-FU/LV (HR 0.80; 95%CI: 0.66-0.96; P = 0.016)[76]. In the XELOXA trial[16], resected stage III CC patients were assigned to CAPOX *vs* bolus 5-FU/LV (as Mayo Clinic regimen or RPMI) for 6 mo. After a median follow up of about 7 years, the 7-year DFS rates (the primary endpoint of the study) were 63% and 56% with CAPOX and 5-FU/LV, respectively (HR 0.80; 95%CI: 0.69-0.93; P = 0.004). In the NSABP C-07 trial[17,90], oxaliplatin was added to the weekly bolus 5-FU/LV (FLOX) and was compared to the RPMI regimen for 6 mo in stage II and III patients. This trial reported outcome after 8 years of median follow up which showed a favorable 5-year DFS with FLOX in the combined stage II and III population- 69% *vs* 64% (HR 0.82; 95%CI: 0.72-0.93; P = 0.002), but no OS benefit (5- year OS of 80% *vs* 78 % with an HR of 0.88; 95%CI: 0.75-1.02; P = 0.08). Based on these trial results, FOLFOX and CAPOX emerged as the preferred adjuvant regimens for resected stage III CC. FLOX regimen is rarely used in the current clinical practice because of toxicities, particularly diarrhea and neutropenia. However, the FLOX regimen could be a logical alternative for patients who experience chest pain with capecitabine or infusional 5-FU[91].

Capecitabine as adjuvant therapy was evaluated in stage III CC patients in the Xeloda in Adjuvant Colon Cancer Therapy (X-ACT) study[92] which randomly assigned 1987 patients to six months of capecitabine alone (1250 mg/m^2 twice daily for 14 of every 21 d) or monthly bolus 5-FU/LV . With a median follow-up of 6.9 years, capecitabine was at least equivalent to 5-FU/LV in terms of DFS (HR 0.88; 95%CI: 0.77-1.01) and OS (HR 0.86; 95%CI: 0.74-1.01). This pattern was maintained in all subgroups, including patients aged 70 years or older.

AC in the elderly population (aged ≥ 70 years) poses a number of unique challenges, which include limited bone marrow reserve, impaired functional capacity, co-morbidities, and increased risk of toxicities from chemotherapy. Analysis of pooled clinical trial data[93] as well as population-based studies[94-96] have provided evidence that 5-FU/LV based AC confers as much OS benefit in elderly population as in younger population and the rate of toxicities are not higher in the older population. However, the benefit from the addition of oxaliplatin to 5-FU/LV in the elderly population is controversial. Post-hoc analyses of MOSAIC[97] and NSABP C-07[17] trials, as well as an ACCENT database analysis[98] have failed to demonstrate a significant survival benefit with oxaliplatin-based regimens in patients aged ≥ 70 years. On the other hand, a benefit was suggested in a pooled analysis of four randomized trials comparing an oxaliplatin-containing *vs* a non-oxaliplatin containing regimen[99]. In this analysis, OS was significantly improved in all age groups, although the benefits of oxaliplatin were attenuated in those aged ≥ 70 years (HR 0.78; 95%CI: 0.61-0.99, *vs* HR 0.62; 95%CI: 0.54-0.72). Furthermore, patients aged ≥ 70 years are more likely to discontinue oxaliplatin earlier than younger patients[17]. As a result, oxaliplatin-based regimens are not routinely recommended for patients aged ≥ 70 years, although not contraindicated for those in good general health. For elderly patients considered to have low-risk disease and/or considered unsuitable for oxaliplatin-based chemotherapy, capecitabine or 5-FU based regimens are reasonable alternatives. A subgroup analysis of the X-ACT trial confirmed the efficacy of capecitabine in stage III patients aged ≥ 70 years[92]. If tolerance to capecitabine is poor, which is prevalent in the United States[70], intravenous 5-FU/LV based regimens are reasonable alternatives, especially the semimonthly regimen, because of its favorable toxicity profile[86].

Oxaliplatin based AC is the current standard of care for stage III patients with dMMR/MSI-H tumors, which is supported by a retrospective study[100]. 5-FU monotherapy is contraindicated in this group, as discussed in the following section. The role of immunotherapy in this setting is currently being investigated in clinical trials[101].

Several drugs active in metastatic setting have failed to show any benefit in the adjuvant setting, including the addition of irinotecan to 5-FU/LV[102-104], the addition of bevacizumab to oxaliplatin-based regimens[105,106], the addition of bevacizumab to capecitabine[107] and finally the addition of cetuximab to FOLFOX in the N0147[108] and PETACC8[109] trials. Figure 1 illustrates 5-year DFS rates with standard adjuvant regimens in stage III CC.

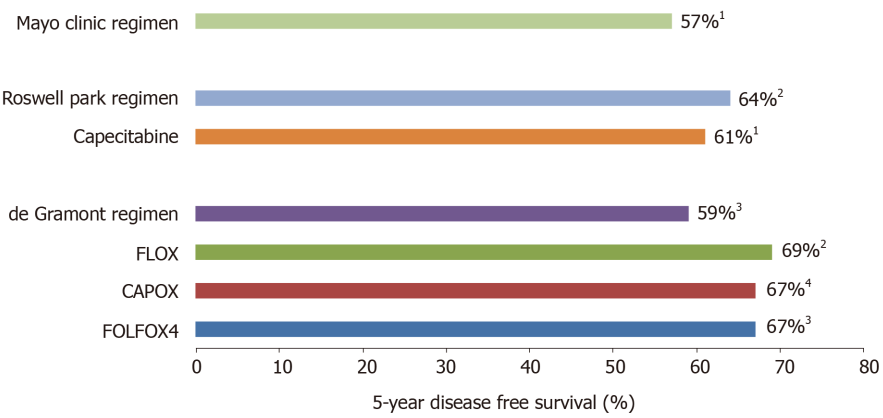

Figure 1 5-year disease free survival rate in stage III colon cancer patients treated with standard adjuvant chemotherapy regimens. [1]X-ACT trial[92]; [2]NSABP-C07[17,90]; [3]MOSAIC trial[53,76]; [4]NO16968/XELOXA[16].

REFINING PATIENT SELECTION AND PERSONALIZATION OF ADJUVANT THERAPY

The most important challenge in the current treatment paradigm of early stage CC is the inability to detect micro-metastatic residual disease after the surgery. Clinicopathologic characteristics currently utilized to optimize adjuvant therapy imperfectly prognosticate the risk of cancer recurrence. As a result, AC is recommended in all resected stage III CC patients, although only about 20% of these patients are the actual beneficiary of the adjuvant therapy, as discussed earlier. Conversely, AC is withheld in all average risk stage II patients, and 12%-18% of these patients endure cancer recurrence[6,14]. Recent research has unveiled a variety of promising tools and biomarkers which might enable precise patient selection and therapy personalization. These biomarkers/tools broadly belong to the following categories: (1) Circulating tumor DNA (ctDNA) based assays; (2) Tools based on immune contexture of the primary tumor ("immunoscore"); and (3) Molecular markers and genomic profiling. Table 4 summarizes the leading prognostic and/or predictive biomarkers.

Circulating tumor DNA

The ctDNA is the fraction of cell-free DNA in the circulation that originates from the apoptotic or necrotic tumor cells and carries tumor-specific genetic or epigenetic alterations. A rapidly increasing body of research indicates that the presence of tumor-specific ctDNA in the bloodstream after completion of the curative surgery can identify patients with residual, radiographically occult cancer who are at a substantially higher risk of cancer recurrence[110-115]. Two recently reported cohort studies, designed to determine the prognostic value of ctDNA in newly diagnosed resected stage II and III CC patients who had at least one tumor-specific DNA mutation commonly found in CC, are of particular importance[113,114]. The first study analyzed 230 patients with stage II CC using a next-generation sequencing panel on blood collected 4-10 wk after surgical resection[114]. The study showed that, among the patients who did not receive AC, 79% (11 out of 14) with detectable ctDNA post-surgery had a cancer relapse at a median follow-up of 27 mo. On the other hand, recurrence occurred in only 16 (9.8 %) of 164 patients with negative postoperative ctDNA (HR 18; 95%CI: 7.9-40; $P < 0.001$). Kaplan-Meier estimates of relapse-free survival at 3 years were 0% for the ctDNA-positive and 90% for the ctDNA-negative groups. Detectable ctDNA following resection had a positive predictive value of 100% and a negative predictive value of 92%. Among the stage II patients who received AC, the presence of ctDNA after completion of chemotherapy was also associated with an inferior recurrence-free survival (HR 11; 95%CI: 1.8-68; $P = 0.001$). On multivariate analysis, the detection of ctDNA was associated with the highest risk for recurrence (HR 28; $P < 0.001$), and the other well-known high-risk clinicopathologic features (*i.e.*, < 12 lymph nodes examined, presence of lymphovascular invasion, microsatellite status) did not meet statistical significance. In the other study with stage III patients[113], 47% of patients with detectable ctDNA post-surgery were disease-free at 3 years compared with 76% of those with undetectable ctDNA (HR 3.8; 95%CI: 2.4-21.0;

Table 4 Evolving tools and biomarkers which may help precise patient selection for adjuvant therapy and therapy personalization in early stage colon cancer

Biomarker/tool	Clinical significance	Potential use and relevance	Ref.
ctDNA	Prognostic	ctDNA detection in the bloodstream after surgical resection and adjuvant chemotherapy provides direct evidence of residual micro-metastatic disease and correlates with a very high risk of cancer recurrence in resected stage II and III patients. Sensitivity, specificity, positive and negative predictive values are 48%, 100%, 100% and 91%, respectively. Reported studies suggest that ctDNA can potentially serve as a real time marker of adjuvant therapy efficacy in stage II and III patients.	[110-115]
Immunoscore	Prognostic	High immunoscore is associated with favorable prognosis in both stage II and III patients independent of patient T stage, N stage and microsatellite instability. High-risk stage II patients with high Immunoscore had similar time to recurrence compared with average risk stage II patients in a recent report.	[118-122]
dMMR	Prognostic and predictive	Associated with favorable prognosis in stage II and possibly low-risk (IDEA defined) stage III patients. Predicts lack of benefit and possibly harm with 5-FU based adjuvant chemotherapy in both stage II and III patients.	[124-137]
KRAS and $BRAF^{V600E}$ mutation	Prognostic	KRAS and $BRAF^{V600E}$ mutations have been reported to be associated with a worse prognosis in several large retrospective studies, in both stage II and III patients. dMMR status attenuates adverse prognostic impact of $BRAF^{V600E}$ mutation, possibly except in IDEA defined high-risk stage III CC.	[133,137-141]
Genomic profiling (Oncotype Dx Colon Cancer®)	Prognostic	Prognostic discrimination capacity is insufficient to guide therapy in routine clinical practice.	[142-147]
PIK3CA mutations	Predictive	Retrospective analysis suggests an association between the use of aspirin and improved survival among the patients with mutated-PIK3CA colorectal cancer including stage I-III patients.	[152]
CDX2 expression	Prognostic and predictive	Retrospective analysis suggested lack of CDX2 expression was associated with worse outcome in stage II and III CC. Lack of CDX2 expression appears to be predictive of benefit from adjuvant chemotherapy in stage II patients.	[153]
CMS	Prognostic	CMS1 tumors have a good prognosis, the CMS4 tumors have a poor prognosis, and the CMS2 and CMS3 types have an intermediate prognosis. Not validated to guide therapy in routine clinical practice.	[148-151]

ctDNA: Circulating tumor DNA; dMMR: Deficient mismatch repair status; CC: Colon cancer; CMS: Consensus molecular subtypes.

$P < 0.001$). On multivariate analysis, ctDNA status after surgery had the strongest independent association with cancer recurrence among the clinicopathological variables studied, including T and N stage. Disease recurrence at 3 years was also higher in the patients with detectable ctDNA after AC than in those without ctDNA after AC (77% *vs* 30%; HR 6.8; 95%CI: 11.0-157.0; $P < 0.001$). Furthermore, conversion from positive to negative ctDNA status after AC resulted in a lower recurrence rate compared to the patients with persistent ctDNA (HR 3.7; $P = 0.04$). In both studies, the risk of cancer recurrence was substantially higher in those who had detectable ctDNA post-surgery, which did not turn undetectable after standard AC, suggesting the possibility that ctDNA can potentially serve as a real-time marker of efficacy of the adjuvant therapy. A recently reported analysis of the IDEA-France data (presented at the ESMO 2019 Congress) also confirmed that the presence of ctDNA post-operatively is an independent adverse prognostic marker (adjusted HR 1.85; $P < 0.001$) in stage III patients[116]. These data, taken together, suggest that ctDNA can serve as a tool to detect minimal residual disease following resection and AC in early stage CC patients,

independent of known clinicopathologic risk factors.

The ctDNA, although looks promising, has several important limitations, which include modest sensitivity in the adjuvant setting (50%-60%)[117], a lack of standardization across the platforms, and a lack of validation cohorts in the reported studies. Moreover, among the stage III patients[113] who completed at least 12 wk of prescribed adjuvant therapy (78 out of 96), 9 patients had detectable ctDNA post-surgery which turned undetectable after AC, and 3 of these 9 patients had disease recurrence with a time to recurrence between 15.7 to 20 mo. This observation highlights a potential drawback of ctDNA as a marker of efficacy of adjuvant therapy.

Immunoscore

Immunoscore, derived from the density of CD3+ and CD8+ T-cells within the tumor and its invasive margin, is an emerging tool that may play an important role in the near future to risk-stratify early stage CC patients into distinct prognostic groups with significant therapeutic implications[118-121]. Immunoscore has recently been validated prospectively in a large trial population of stage I-III CC patients and has been demonstrated to have a stronger association with survival characteristics than a variety of other risk parameters, including the AJCC/UICC TNM classification system[120]. A separate study reported that high-risk stage II patients with high Immunoscore had a time to recurrence similar to the low-risk stage II patients implying that Immunoscore can potentially risk-stratify high-risk stage II CC patients and help precise patient selection for adjuvant therapy[119]. A meta-analysis to evaluate the prognostic value of immunoscore in CC, which included 8 studies, confirmed that low immunoscore was significantly correlated with poor OS (HR 1.74; 95%CI: 1.43-2.13) and DFS (HR 1.82; 95%CI: 1.64-2.03)[122]. Clinical trials are needed to assess the value of Immunoscore in guiding therapeutic decision making. Immunoscore, once prospectively validated, has the potential to help select patients for observation who would otherwise be candidates for AC based on current guidelines. Perhaps of even greater importance is the potential for the immunoscore to be used to identify the subset of patients who might be responsive to immunotherapy-based adjuvant therapy.

Molecular markers and genomic profiling

A variety of molecular markers are reported to have prognostic and predictive value with important therapeutic implications in early stage CC. Microsatellite instability (MSI), a characteristic genetic signature of deficient mismatch repair mechanism (dMMR), is an important prognostic and predictive biomarker which currently influences treatment decision. High levels of MSI (MSI-H), defined as instability in ≥ 30% of microsatellite loci, occurs approximately in 15% to 20% of early stage CRC patients[123] with higher prevalence in stage II as compared with stage III CC (21 *vs* 14% in one study)[124]. Patients with dMMR stage II CC have an excellent prognosis with surgery alone, and AC does not improve survival[125-128]. Current NCCN guideline does not recommend AC in MSI-H/dMMR stage II patients, even in patients with high-risk features such as T4 tumors[11].

For the patient group with stage III disease, the MSI-H/dMMR status has also been shown to be associated with favorable prognosis in some[129,130] but not in all studies[131]. Furthermore, it has been suggested in a retrospective analysis that the favorable effect of dMMR status is limited to patients with right-sided stage III tumors treated with FOLFOX -based AC[132]. A recently reported pooled analysis of stage III CC patients (n = 5337) enrolled in 2 adjuvant trials with FOLFOX ± cetuximab [N0147 (Alliance) and PETACC-8] reported that the prognostic advantage of MSI-H status is limited to IDEA study defined low-risk stage III patients[133].

A number of retrospective analyses support the view that MSI-H phenotype predicts the lack of efficacy or even potential harm with 5-FU based AC[126,127,134,135]. Furthermore, in vitro studies suggest that dMMR CC cell lines are less susceptible to 5-FU induced cytotoxicity[136]. Based on these data, AC with 5-FU/LV alone is not recommended for stage II or III CC patients. Conversely, both DNA mismatch repair-proficient and –deficient CC cell lines are sensitive to oxaliplatin[137], and AC with oxaliplatin-based regimens retains its efficacy in MSI-H stage III CC patients[100].

Poor survival associated with the presence of KRAS[138-140] and $BRAF^{V600E}$ mutations[140,141] in early stage CC patients have been reported in several large retrospective studies. In a recently reported retrospective analysis, KRAS mutation was found to be a strong predictor of shorter time to relapse in both IDEA analysis defined low- and high-risk stage III patients who received FOLFOX-based AC for 6 mo[133]. However, sufficient data do not exist at this time to use KRAS or BRAF mutation status to guide adjuvant therapy.

Several multigene assays have been explored as prognostic and predictive tools to identify higher-risk patients in a given TNM stage group. Oncotype dx colon is the most extensively studied gene panel[142-146]. The validation studies with stage II and III patients in QUASAR and NSABP C-07 trials showed that the Oncotype dx recurrence scores are prognostic for recurrence, DFS, and OS but not predictive of benefit from AC[142,146]. ColoPrint, a gene expression classifier similar to Oncotype dx, has been shown to significantly improve prognostic accuracy in stage II patients independent of other clinical factors[147]. However, sufficient data do not exist to recommend these tools for routine clinical use at this time.

Consensus Molecular Subtypes (CMS), proposed by the CRC Subtyping Consortium based on unsupervised gene expression profile to refine the classification of CRC and facilitate prognostication and development of expression signature-based targeted therapies[148], is another area of development. Among the four subtypes, CMS4 or the mesenchymal subtype has the worst survival rate. Although CMS system has been demonstrated to have prognostic significance[149-151], this system has not been extensively validated for clinical use at this time.

A few other molecular markers deserve a mention, which include PIK3CA mutation and CDX2 expression. A retrospective analysis of 964 rectal or CC patients in Nurses' Health Study and the Health Professionals Follow-up Study revealed that PIK3CA mutation status could predict a survival advantage from adjuvant therapy with aspirin[152]. The loss of CDX2 expression was identified as a negative prognostic marker in a retrospective cohort of patients with stage II and stage III CC[153]. Furthermore, a lack of CDX2 expression identified a subgroup of patients with stage II CC who appeared to benefit from AC. However, these hypothesis-generating results need prospective validation before being deployed into routine clinical practice.

PERIOPERATIVE CHEMOTHERAPY

Accumulating preclinical and clinical data suggest that the surgical trauma can influence several pathophysiological processes potentially leading to tumor metastasis and recurrence[154], which provides a biologic basis for exploration of an alternative strategy in which a part of the systemic chemotherapy is delivered for "chemical debulking" prior to the surgery. The rest is delivered after the surgery, referred as "perioperative" chemotherapy. Potential benefits of administration of chemotherapy before surgery are several, which include earlier treatment of occult micro-metastatic disease, improved tolerability, and dose intensity, opportunity to assess response to preoperative chemotherapy to inform adjuvant therapy, reduction of tumor cell shedding during surgery and improved R0 resection rates. A retrospective NCDB analysis reported a 23% lower risk of death at 3 years in T4b non-metastatic CC patients treated with preoperative chemotherapy followed by surgery compared to patients who had upfront resection followed by AC (HR 0.77; 95%CI: 0.60-0.98; P = 0.04)[155]. Several single-arm studies, including the pilot phase of the randomized FOxTROT trial, have explored the feasibility of perioperative chemotherapy in operable, locally advanced CC and reported significant tumor downstaging with acceptable toxicity[156,157]. Recently two studies that explored the efficacy of perioperative chemotherapy have reported their results- the phase II PRODIGE 22 trial[158] and the phase III FOxTROT trial[159].

The preliminary result of the ongoing FOxTROT trial (NCT00647530) has been presented at the American Society of Clinical Oncology annual meeting (2019). In this trial, 1052 patients (median age of 65 years) with operable, non-obstructed early stage CC (T3 to T4, N0 to N2 and M0 based on CT scan) who were fit for modified FOLFOX (mFOLFOX) and surgery, were randomized in a 2: 1 ratio to the novel neoadjuvant treatment arm consisting of 6 wk of mFOLFOX followed by surgery and 18 wk of mFOLFOX post-operatively (n = 698) or control arm (n = 354). Patients in the control arm underwent upfront surgery, followed by 24 wk of adjuvant mFOLFOX. The trial allowed physicians to replace mFOLFOX with CAPOX as the chemotherapy backbone and to shorten the duration of chemotherapy from 24 wk to 12 wk in older, low-risk patients. Attempted curative surgery was successful in 98% of patients in both treatment groups. In this trial, the perioperative therapy arm was associated with a trend towards an improved 2-year rate of failure, the primary endpoint of the study, defined as relapse or persistent disease at 2 years (13.6% in the perioperative arm *vs* 17.2% in the control arm). This difference, however, did not reach the target statistical significance (HR 0.75; 95%CI: 0.55–1.04; P = 0.08). The absence of statistically significant benefit in this trial was attributed to the lower than expected failure rate in

control arm-18% *vs* expected 25% to 32% used for the power calculation. The perioperative treatment protocol was well-tolerated and safe, with no increase in perioperative morbidity, and a trend toward fewer serious postoperative complications (4.7% *vs* 7.4% rate of anastomotic leak or intra-abdominal abscess, 4.3% *vs* 7.1% rate of complications requiring further surgery, and 12% *vs* 14% rate of complications prolonging postoperative stay). Furthermore, perioperative arm had marked reduction in the rate of incomplete resections, 5% *vs* 11% (P = 0.001).

An exploratory subgroup analysis of the FOxTROT trial provided important information regarding the patients with dMMR tumors. In this analysis, exclusion of the patients with dMMR tumors (n = 173) resulted in a drop of the HR for a 2-year treatment failure rate, suggesting that the neoadjuvant therapy was less effective in patients with dMMR tumors. On pathological examination of the resected tumors from patients who received pre-operative chemotherapy, tumor regression induced by chemotherapy was absent in nearly 74% of the dMMR tumors as compared to 26.6% in the pMMR (proficient mismatch repair) tumors. This result suggests that upfront surgery probably would be the preferred option for the early stage CC patients with dMMR tumors. The role of pre-operative immunotherapy is unknown at this time for this patient group.

The phase II randomized study, PRODIGE 22[158], had the similar design in which the patients with resectable localized stage III or high-risk stage II CC determined by CT scans were randomized to receive either 6 months of adjuvant FOLFOX after colectomy (control) or 4 cycles of FOLFOX before surgery and 8 cycles after surgery (perioperative arm). The primary endpoint of the study was the histological tumor regression grade (TRG). In this trial, TRG was not significantly improved in the perioperative arm, but overall mortality and morbidity rates were similar in both arms. It is important to note that the CT scan criteria were associated with a 33% rate of over staging in the control group. Based on these results, it can be inferred that perioperative chemotherapy should not be adopted as a standard treatment option at this time. However, these trial results provide a rationale for using perioperative chemotherapy in selected patient groups, such as T4b patients who are at risk of incomplete resection.

CONCLUSION

Although the treatment of early stage CC has evolved at a slower pace in last decades, research involving novel and biomarker-guided therapies is likely to advance this field in the near future. The likely areas of focus are: (1) Personalization of therapy, based on clinicopathologic and molecular characteristics, in terms of type, duration, and intensity; and (2) Discovery of novel treatment with improved efficacy.

Although oxaliplatin-based chemotherapy is the current standard adjuvant therapy for resected stage III dMMR patients, the efficacy of chemotherapy in this tumor type is limited[160,161]. Based on the data confirming the efficacy of checkpoint inhibitors in patients with metastatic dMMR CC[162], investigators have moved on to evaluating immunotherapy agents in the adjuvant setting. The ATOMIC trial (NCT02912559) is currently ongoing whichcompares mFOLFOX for 6 mo plus 12 mo of atezolizumab *vs* 6 mo of mFOLFOX in patients with resected stage III dMMR CC. In the POLEM trial (NCT03827044), patients who have undergone surgical resection for stage III dMMR or POLE exonuclease domain–mutant CC will be assigned to chemotherapy (CAPOX for 12 wk or capecitabine for 24 wk) or chemotherapy followed by avelumab for 24 wk.

The PIK3CA mutated CC patients are another molecular subgroup of patients currently under study. A retrospective study, which primarily included stage I-III CRC patients, reported a potential benefit of aspirin on CC specific mortality in PIK3CA mutated patients. Several trials are underway to assess the impact of aspirin as an adjuvant treatment in stage III or high-risk stage II patients with PIK3CA mutation [PRODIGE 50-ASPIK trial (NCT02467582), Add-Aspirin (ISRCTN74358648)].

As discussed above, it is uncertain if the addition of oxaliplatin to 5-FU benefits elderly patients with stage III CC. The PRODIGE 34-ADAGE trial (NCT02355379) is currently underway to assess the benefit of adjuvant chemotherapy with or without oxaliplatin in patients over 70 years who have stage III CC.

Recently published encouraging data with HER2[163,164] and BRAF[165] directed therapy in metastatic CC may translate into new trials in the adjuvant setting. A trial is currently assessing dual HER2 inhibition (with pertuzumab plus trastuzumab) in unresectable CC, including non-metastatic locally advanced patients (NCT03365882). A recently reported pooled analysis of stage III CC patients treated with FOLFOX-

based AC showed that the IDEA meta-analysis defined high-risk stage III patients with $BRAF^{V600E}$ mutant tumors had a much worse prognosis compared to the rest of stage III patients[133]. This patient group should possibly be the target of adjuvant trial with $BRAF^{V600E}$ directed therapy.

The high risk stage III patients have a 3-year DFS rate of around 65%, even with 6 months of adjuvant oxaliplatin-based chemotherapy[54]. This group represents a population in need of more effective treatments. A trial is exploring the intensification of adjuvant treatment for this group (IROCAS, NCT02967289) with the addition of irinotecan to the FOLFOX backbone. The evolving perioperative chemotherapy approach utilized in the FOxTROT trial described above, which led to an increase in R0 resection rate with no increase in postsurgical complications, may also potentially improve the outcome of high-risk stage III patients.

The ability of traditional clinicopathologic characteristics to define the risk of cancer recurrence and optimize the adjuvant therapy for patients with resected early stage CC is limited. In this regard, ctDNA is a promising tool that has shown a very high prognostic value in both stage II and III CC patients. One of the major obstacles to utilizing this platform is the need to have a marker mutation unique for a given patient in order to determine that ctDNA is actually pathologic. Each patient will need to have unique mutation profile, limiting the applicability of this tool. Furthermore, mutations may not be present in all clones of a malignancy. Thus, each marker must be patient-specific and highly conserved across all clones of a patient's tumor. At this time, the use of ctDNA technology is limited by the absence of prospective data confirming its value as a predictive biomarker for adjuvant therapy. Nonetheless, the early results are promising, and several randomized clinical trials are underway to further evaluate the prognostic value of ctDNA (NCT02842203, NCT03312374, NCT03637686), and to explore the value of ctDNA-directed adjuvant therapy in resected stage II and III CC [DYNAMIC-II (ACTRN12615000381583), DYNAMIC-III (ACTRN12617001566325), NRG-GI005 (COBRA) for stage IIA CC, CIRCULATE-IDEA].

In conclusion, despite a lack of newer agents with improved efficacy, a number of advances have altered the treatment landscape of early stage CC. Existing treatment regimens have been modified and refined to decrease the impact on patients, improve tolerability and optimize patient outcomes. As we move to an era dominated by the utilization of advanced surgical technologies, targeted therapies, and immunotherapy, it is likely that outcome will continue to improve with a reduction in treatment-related complications. The use of biomarkers and genomic signatures to risk stratify individual patients presents an enormous opportunity to personalize treatment. We anticipate that the use of ctDNA-based tools will improve patient selection for adjuvant therapy and help the detection of early, curable recurrences.

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REVIEW

One size does not fit all for pancreatic cancers: A review on rare histologies and therapeutic approaches

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Abstract

Exocrine pancreatic neoplasms represent up to 95% of pancreatic cancers (PCs) and are widely recognized among the most lethal solid cancers, with a very poor 5-year survival rate of 5%-10%. The remaining < 5% of PCs are neuroendocrine tumors that are usually characterized by a better prognosis, with a median overall survival of 3.6 years. The most common type of PC is pancreatic ductal adenocarcinoma (PDAC), which accounts for roughly 85% of all exocrine PCs. However up to 10% of exocrine PCs have rare histotypes, which are still poorly understood. These subtypes can be distinguished from PDAC in terms of pathology, imaging, clinical presentation and prognosis. Additionally, due to their rarity, any knowledge regarding these specific histotypes is mostly based on case reports and a small series of retrospective analyses. Therefore, treatment strategies are generally deduced from those used for PDAC, even if these patients are often excluded or not clearly represented in clinical trials for PDAC. For these reasons, it is essential to collect as much information as possible on the management of PC, as assimilating it with PDAC may lead to the potential mistreatment of these patients. Here, we report the most significant literature

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regarding the epidemiology, typical presentation, possible treatment strategies, and prognosis of the most relevant histotypes among rare PCs.

Key words: Rare pancreatic cancers; Pancreatic acinar cell cancer; Pancreatic adenosquamous cancer; Undifferentiated pancreatic cancer; Pancreatoblastoma; Pseudopapillary pancreatic cancer

Core tip: Due to their rarity and lack of consistent literature, rare subtypes of exocrine pancreatic cancer are often assimilated with the more frequent pancreatic ductal adenocarcinoma, even if they have peculiarities in their presentation and treatment strategy. The aim of this review is to summarize the most relevant literature regarding these rare subtypes of pancreatic cancers.

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INTRODUCTION

Traditionally, when speaking about pancreatic cancer (PC), we refer to exocrine pancreatic neoplasms, which represent up to 95% of all PCs and are widely recognized among the most lethal solid cancers, with a very poor 5-year survival rate of 10%[1]. Exocrine PCs account for 7.8 new cases every 100000 people and is the 11th most common cancer worldwide[2]. The remaining approximately < 5% of PCs are neuroendocrine tumors, which are characterized by a better overall survival (OS), with a median OS (mOS) of 3.6 years (ranging from 15 mo for grade 3 disease to 140 mo for grade 1 disease)[3].

Since roughly 85% of exocrine PCs are pancreatic ductal adenocarcinomas (PDACs), the vast majority of literature and data from clinical trials and basic research are focused on this particular histotype. However, up to 10% of PCs have a rare histotype such as adenosquamous carcinoma, undifferentiated carcinoma (UC), acinar cell carcinoma (ACC), cystic tumors, papillary adenocarcinoma, and other exocrine variants[4]. As reported in Table 1, the World Health Organization (WHO) has identified more than 10 subtypes of PCs according to their anatomopathological characteristics[4] (Figure 1). Due to their very low incidence, the biological and clinical features of these rare types of PC are still poorly understood. Furthermore, since patients with rare histotypes of PC are often excluded or not well represented in clinical trials for PDAC, there are limited data regarding the best treatment strategy and most information is from case reports or small case series. The aim of this review was to discuss the most relevant rare subtypes and their peculiarities in terms of epidemiology, diagnosis, prognosis, and treatment.

ACC

Epidemiology and prognosis

ACC of the pancreas represents < 2% of all PCs. As for most rare cancers, there are no prospective data and nearly all of the information is from small single-center series and the United States National Registry of PC[5-8]. Compared to patients with PDAC, those with ACC appear to be younger, with a median age of 60-67 years, and more frequently male. Additionally, patients with ACC tend to have larger tumors, with a median diameter ranging from 8 to 10 cm. Despite previous evidence stating that these tumors are more frequently located in the tail of the pancreas, the most recent WHO classification states that this specific histotype is more often located in the head of the

Table 1 World Health Organization classification of exocrine malignant epithelial tumors 5th edition

Histotype	Subtype	Frequency, %
Ductal adenocarcinoma	NOS	85%
	Carcinoma undifferentiated	1%-7%
	Adenosquamous carcinoma	1%-4%
	Undifferentiated carcinoma with osteoclast-like giant cell	< 1%
	Colloid carcinoma	1%-3%
	Poorly cohesive carcinoma	Extremely rare
	Signet-ring cell carcinoma	Extremely rare
	Medullary carcinoma NOS	Extremely rare
	Hepatoid carcinoma	Extremely rare
	Large cell with rhabdoid phenotype	Extremely rare
Acinar cell carcinoma	NOS	< 2%
	Acinar cell cystadenocarcinoma	Extremely rare
	Mixed acinar-neuroendocrine carcinoma	Extremely rare
	Mixed acinar-endocrine-ductal carcinoma	Extremely rare
	Mixed acinar -ductal carcinoma	Extremely rare
Pancreatoblastoma		Extremely rare
Solid pseudopapillary neoplasm	NOS	3%
	With high-grade carcinoma	Extremely rare

NOS: Not otherwise specified.

pancreas. However, these cancers do not usually cause jaundice, and patients typically have several non-specific symptoms, such as weight loss and abdominal pain. Despite this, compared to PDAC, ACC is less likely to have distant disease at diagnosis and is more frequently diagnosed at an earlier stage, allowing for surgical resection in about 38% of patients[7,8].

These tumors seem to be less aggressive than the most common PDAC, even if their prognosis is still poor. In particular, in previously reported series, the mOS of ACC patients ranged from 17 to 19 mo, reaching 47 mo in those who underwent surgical resection[5-9]. Based on a more recent analysis of 57804 PC patients who underwent surgical resection, ACC achieved an mOS of 67.5 mo (51% 5-year OS)[10]. In a few case reports, OS reached up to 123 mo. To date, there are no variables that can help select these patients with long OS. These data might encourage a more aggressive approach for evaluating and treating these tumors[11,12].

Pathology and molecular biology

Macroscopically, ACCs are large tumors, which are frequently well circumscribed and partially encapsulated. The cut surface is usually homogeneous, and pink to tan with a fleshy or friable consistency. Necrosis, cystic evolution, and hemorrhage are sometimes observed. Upon histological examination, the most frequent feature is an acinar pattern, with neoplastic cells arranged in small glandular units, followed by a trabecular, glandular, and solid architecture. Nonetheless, some case series have also shown that ACC can rarely show pleomorphic or spindle cells[4,13,14]. Furthermore, neoplastic cells have a moderate amphophilic to eosinophilic granular cytoplasm rich in zymogen granules, which are usually positive with periodic-acid Schiff and are resistant to diastase. When present, this feature is highly supportive of ACC diagnosis. However, the milestone for diagnosis of ACC is the immunohistochemical identification of pancreatic enzyme. Upon analysis with a specific antibody, trypsin and chymotrypsin can be useful for diagnosis, but B-cell lymphoma/leukemia 10 (BCL-10) shows high specificity[4]. Cytokeratin (CK) 19 and CK 7 can also be expressed, as in PDAC.

Recent studies with whole-exome sequencing, even with the limits of small

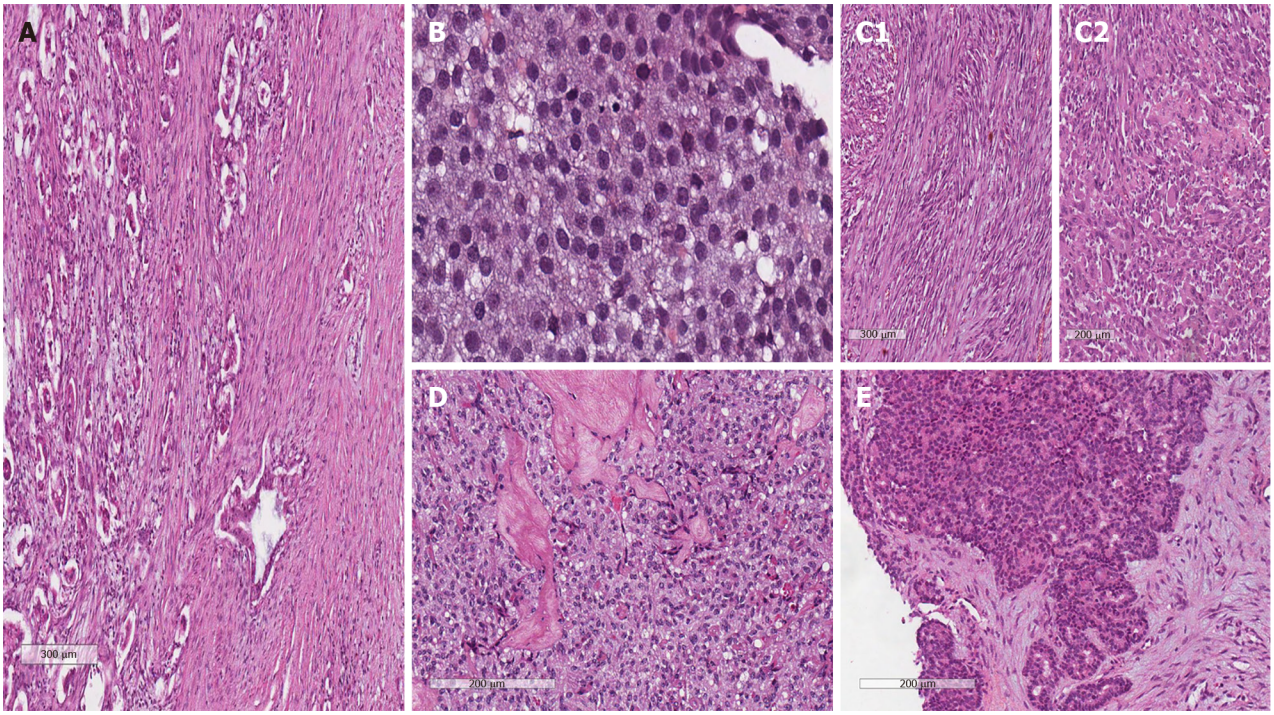

Figure 1 Microscopic anatomopathological characteristics (hematoxylin and eosin stain). A: Ductal adenocarcinoma; B: Acinar cell carcinoma; C1: Undifferentiated carcinoma with spindle cell features; C2: Undifferentiated carcinoma with rhabdoid cells; D: Pseudopapillary; E: Pancreatoblastoma.

numbers, have revealed specific molecular patterns of ACC that apparently differ from those known for PDAC[15-17]. First of all, ACC seems to be characterized by a higher mutational frequency than PDAC, comparable to those of other digestive tract cancer such as colorectal cancer. Additionally, typical PDAC mutations such as *KRAS* are not as frequent in these small series of ACC. In particular, Jiao *et al*[15] analyzed 17 ACCs, identifying somatic mutations in *SMAD4* (23%), *JAK1*, *RB1* or *TP53* (17%) *APC*, *ARID1A*, *GNAS*, *MLL3*, *PTEN*, *FAT4*, and *CTNNB1* (11%) as the most frequent alterations[15]. In addition, Furukawa *et al*[16] described somatic or germline mutations of *BRCA2* (3 of 7 cases) and *FAT* genes (4 of 7 cases)[16]. Finally, Jäkel *et al*[17] showed that ACC is also characterized by chromosomal alterations with a numerous copy number variations, an aberrantly DNA methylation, and downregulation of the tumor suppressor genes *ID3*, *ARIDI1A*, *APC*, and *CDKN2A*, which may be related to the loss of function of DNA repair systems[17]. Chromosomal alterations were well explored in several other studies, which demonstrated *BRAF* (about 20%)[18,19] and *RET* (7.5%) rearrangements[20]. Although these alterations were found in very small series, some of them may be susceptible to targeted therapy, opening the door to possible new treatment developments in ACC.

Imaging

On computed tomography (CT), ACC lesions tend to be large, partially, or completely exophytic and mostly hypodense due to relative hypovascularity in comparison to the surrounding pancreas, on both arterial and venous phase images. A sizeable proportion has an enhancing capsule, cystic, hemorrhage or necrotic component, which may be related to the digestive effect of the pancreatic enzymes released by neoplastic cells[21]. Moreover, lesions located in the pancreas head or uncinate process unfrequently cause severe pancreatic or biliary ductal dilatation[21-24].

On magnetic resonance imaging (MRI), ACC predominantly presents as an oval, large, well-marginated exophytic mass with moderate and heterogeneous enhancement after intravenous administration of contrast. It is frequently characterized by cystic and necrotic areas, and hemorrhage can be observed. Interestingly, ACC is clearly visible in diffuse-weighted MRI, which may be a promising useful tool for ACC diagnosis[23,24].

MRI yields excellent soft tissue contrast and appears to be superior to CT in showing the tumor margin, cystic and necrotic areas, peripancreatic extension, and vascular involvement even without the administration of contrast. Imaging of ACC is so characteristic that it may have a key role in suggesting the diagnosis of this rare

histotype. However, some exceptions have been reported[23].

Treatment

Overall, surgical resection for localized disease remains the only curative treatment. To date, the contribution of adjuvant chemotherapy and radiotherapy has not been well investigated. However, considering the available data, resection and adjuvant chemotherapy are associated with the longest rate of mOS, whereas in some cases, chemoradiation has shown activity in both the neoadjuvant and locally advanced settings[5,8,9,25-28].

In metastatic patients, various first-line treatments have been reported, even if only in small series and case reports. Most of the time, these patients are treated with regimens commonly used for PDAC, such as capecitabine or gemcitabine monotherapy or Gemcitabine- (gemcitabine/nab-paclitaxel, GEMOX) or fluoropyrimidine-based (FOLFOX, FOLFIRI, FOLFIRINOX) regimens. There are very few data available for the second-line setting. In 2002, Holen *et al*[5] published a retrospective monocentric series of 18 patients treated with various regimens: Only 2 patients had a partial response (PR) obtained respectively with FOLFIRI and the combination of cytarabine plus cisplatin and caffeine[5]. In 2011, Lowery *et al*[29] published a new retrospective study, from the same institution, describing the results obtained in 25 patients treated with various regimens in first- and/or second-line. The mOS of patients with metastatic disease was 19.6 mo, with survival up to 57 mo in the 11 patients who had PR or confirmed prolonged stable disease (SD). These patients were treated with GEMOX, gemcitabine-docetaxel-capecitabine, cisplatin plus gemcitabine, gemcitabine plus erlotinib and cisplatin plus irinotecan[29]. The potential activity of regimens containing fluoropyrimidine and/or platin-based compounds was confirmed by Kruger *et al*[30], who reported PR to first- and second-line treatments. In first-line setting, the authors reported PR with FOLFIRINOX in two patients (maintained for up to 14 mo) and with GEMOX and capecitabine in two other patients (maintained for up to about 6 and 13 mo, respectively). In the second-line setting, the objective response was reported in three patients treated with FOLFOX and FOLFIRINOX, with progression-free survival ranging from 9 to 12 mo[30]. This work is not the only report of FOLFIRINOX activity in this setting, as there are at least three case reports in which prolonged SD (time to progression [TTP] of 9 mo) and two PRs were observed[31-33]. Similarly, Yoo *et al*[28] observed a PR in three of four patients treated with FOLFOX in second-line, with 6.5 mo (95%CI, 2.8 to 10.2 mo) of progression-free survival[28]. Finally, in a recent multicenter Italian retrospective study including patients with rare pancreatic histotype tumors, Brunetti *et al*[26] showed that PR was achieved in first-line in 2 of 23 patients treated with GEMOX and gemcitabine-fluorouracil, and in second-line in 1 patient treated with FOLFIRINOX[26].

Regarding the use of single-agent gemcitabine, three small series reported poor activity[28,30,34]. Finally, there have been reports of the potential activity of S-1 in first and second-line settings[34-36].

In conclusion, the published literature shows promising activity of combination treatments, particularly regimens based on fluoropyrimidine and oxaliplatin. In addition, studies have also shown signs of activity for gemcitabine-based regimens, whereas no data support the efficacy of its use as a single agent in the first-line treatment of patients with ACC. As more data regarding molecular classification and alterations involving DNA repair genes arise such as *BRCA2* mutations[16], there is interest in the development of novel therapeutic strategies that can exploit these characteristics.

PSEUDOPAPILLARY

Epidemiology and prognosis

Pseudopapillary tumors (PTs) account for about 3% of all exocrine PCs, with increasing prevalence due to improvements in imaging devices. PTs are more frequent in adolescent girls and young women, with a median age of 28 years at diagnosis, whereas less than 10% of PTs are diagnosed in slightly older men (median age 35). There is no known association with ethnic origin or clinical or genetic syndromes, although very rare cases have been reported in the setting of familial adenomatous polyposis[37]. At diagnosis, PT is characterized by a large round solid or mixed solid cystic lesion frequently located in the pancreatic tail, which may be the reason that patients infrequently experience jaundice. Additionally, there are no specific symptoms of PT and it often presents as a palpable abdominal mass, abdominal

discomfort and pain, nausea, vomiting, asthenia, weight loss, back pain, or pancreatitis.

Despite being counted among malignant pancreatic neoplasms, PTs are considered to be a low-grade, indolent disease. Indeed, only 10% to 15% of cases recur or metastasize and the overall 5-year survival is about 97% even in the presence of metastasis. These survival rates are definitely better and more encouraging than those of PDACs or other aggressive histotypes. Furthermore, the more aggressive cases are often tumors that harbor an undifferentiated component or have peculiar pathological features usually associated with an aggressive behavior such as diffuse growth pattern, high mitotic activity, nuclear atypia, and tumor necrosis[4,38,39].

Pathology and molecular biology

Grossly, PTs appear as large lesions with solid and cystic components, they are usually very soft, but may be firm and sclerotic. They are well-demarcated with a rim of fibrous capsule and adjacent organs invasion is rare[4,40,41]. Cut sections of PT show alternate solid and yellow areas with cystic, necrotic, and hemorrhagic zones which sometimes may be as large as a pseudocyst. Calcifications are also frequently observed[39].

Histologically, the solid component of PT consists of poorly cohesive epithelioid cells with oval nuclei, finely dispersed chromatin low nucleus and either eosinophilic or clear vacuolated cytoplasm and perivascular pseudo papillae. Some of the neoplastic cells contain eosinophilic, diastase-resistant PAS-positive globules of varying size, which may also occur extracellularly, sometimes in large amounts. Glycogen is not prominent and mucin is absent. Mitotic figures might be present, although they are usually rare[4,39,42].

Immunohistochemistry analysis has shown that PT has a low Mib1/Ki67 rate and is positive for beta-catenin (nuclear and cytoplasmic), vimentin, synaptophysin, progesterone receptor (nuclear), CD56, neuron-specific enolase, CD10, and nuclear E-cadherin. Extracellular expression of e-cadherin is lost due to mechanisms that are not clear yet[4,42,43], E-cadherin is a transmembrane protein that has a pivotal role in cell adhesion through interactions with catenins, and its extracellular loss may explain the loss of cell cohesiveness of pseudopapillary pattern[44], making it useful in distinguishing PT from other histotypes[45].

Immunohistochemical beta-catenin overexpression is strongly correlated with mutations of its gene which frequently occur in PT, mostly on exon-3. This mutation leads to hyperactivation of the beta-catenin/Wnt pathway and subsequent activation of the transcription of several oncogenic genes, such as cyclin D1. The consequent deregulation of cell cycle plays an important role in PT development[46-48]. However, several studies have shown that PTs are also characterized by the overexpression of cyclin-dependent kinase inhibitors p21 and p27, which have inhibitory effects on cyclin D1 and its cyclin-dependent kinases complex. Although the mechanism of p21 and p27 upregulation is unknown, it may explain the low growth rate of this rare histotype of PC[48]. By contrast, molecular changes often detected in PDAC, such as alterations of p53 and K-RAS, have not been detected in PT[46].

Imaging

CT features of PT include both solid and cystic lesions without any internal septation, secondary to hemorrhagic degeneration, which are well demarcated by a surrounding capsule. At the margin of the mass, calcification and solid areas can be identified[49,50]. During the CT pancreatic phase, there is weak enhancement compared to the surrounding pancreatic parenchyma, which gradually increases in the hepatic venous phase[51]. Atypical PTs on CT have no surrounding capsule, solid or cystic component, with hyperattenuation during the pancreatic phase and dense internal calcification with no defined margin[51].

Otherwise, on MRI, PT is defined as an encapsulated lesion with both a solid and cystic component as well as hemorrhage without internal septation[52]. Interestingly, Yu *et al*[52] proposed an MRI classification in which PT lesions were separated in three main classes by specific MRI features on T1- and T2-weighted images related to the predominance of solid or hemorrhagic areas. According to their study, MRI may be considered superior to CT in terms of correlation of clinicopathological and radiological findings of PT[52].

Treatment

For localized disease, surgery is the treatment of choice and more than 95% of patients can be cured after radical resection. Due to the favorable behavior of this disease,

surgery with organ preservation is indicated when feasible[53]. Moreover, since evidence of lymph node metastasis is extremely rare[42,54] a formal lymphadenectomy should not be routinely performed[42,53]. Considering the excellent long-term prognosis even for metastatic disease, which is mostly confined to the liver, mesentery, and peritoneum, surgical approaches[53,55] or locoregional treatments[56] are reported to be potentially effective even in this setting, with a high 5-year survival rate with en bloc resection of locally progressed PTs or with synchronous or metachronous resection of distant metastases[57-60]. Furthermore, in highly selected cases, even orthotopic liver transplantation (OLT) may be taken into account for patients with unresectable liver metastases. To date, four cases of patients with liver metastatic PT who underwent OLT have been published. In the first two reports, young 14- and 21-year-old patients with PTs and multiple unresectable hepatic metastases were successfully treated with partial liver transplantation from a living donor without any sign of disease recurrence after 2 years of follow up[61,62]. Longer recurrence-free survival was later reported by Łągiewska *et al*[63], whose patient was free of disease after 5 years from cadaveric OLT. However, in two other patients, disease recurred within 1 and 4 years[64,65].

There are only anecdotal data on the role of systemic treatment in neoadjuvant, adjuvant and metastatic settings. Tumor response to preoperative chemotherapy using cisplatin in combination with 5-fluorouracil (5-FU) or gemcitabine were reported[66-68]. Similar results were observed in a patient treated with a combination of etoposide, cisplatin, cyclophosphamide, doxorubicin, and vincristine[54] and combination of cisplatin, ifosfamide, etoposide, and vincristine followed by intraoperative radiofrequency ablation[69]. By contrast, no response to multiple agents was seen in another case report[58]. Radiotherapy showed activity in a single case of a locally advanced unresectable disease[70].

Regarding the metastatic setting, in a small series, Brunetti *et al*[26] treated two patients with GEMOX, achieving 1 SD and 1 progressive disease (PD) with a TTP of 5 and 2 mo, respectively: One patient received first-line treatment with gemcitabine plus 5-FU with SD, and the last one interestingly achieved a 22-mo SD with single agent gemcitabine[26]. In the same study, those patients who progressed to GEMOX received second line with gemcitabine plus 5-FU with SD reaching a TTP of 9 and 8 mo, respectively. Similar results were also observed with capecitabine used as second line after progression to gemcitabine plus 5-FU with SD and a TTP of 6 mo[26]. Moreover, Morikawa *et al*[71] described a case of a patient treated with paclitaxel as second line after S1 and gemcitabine, who was alive and without progression after 20 mo of follow-up[71]. The low malignant potential of PT was also confirmed by an interesting case of a patient with long survival after several lines of treatment: gemcitabine alone (6 cycles), gemcitabine plus irinotecan (3 cycles), oxaliplatin plus irinotecan and capecitabine (8 cycles), gemcitabine plus capecitabine (6 cycles), weekly 5-FU and lastly, until publication of the data, capecitabine alone[72].

UC

Epidemiology and prognosis

UC is a histological variant of PDAC that accounts for 1% to 7% of all exocrine PCs. Anaplastic, sarcomatoid, and carcinosarcomas are recognized morphological variants of UC composed of pleomorphic mononuclear cells admixed with bizarre giant cells or spindle and rhabdoid cells. UC of the pancreas has been defined as an epithelial neoplasm without a definitive direction of differentiation with a diffuse sheet-like growth pattern without overt glandular differentiation. UC with osteoclast like giant cells (OCGC) is another histologic subtype of PDAC, with different morphologic features and clinical behavior. UC is usually diagnosed at a median age of 61 years and tends to be more frequent in males and Caucasians. At diagnosis, it is observed as a bulky tumor, usually involving the pancreatic head. Its clinical presentation is similar to that of PDAC and is characterized by abdominal pain, jaundice, weight loss, and fatigue[73-76]. In addition, anemia and elevated leukocyte count have been reported, possibly related to hemorrhage and necrosis that follow the rapid and uncontrolled tumor growth[75]. Some authors also reported increased secretion of granulocyte colony-stimulating factor, which may contribute to leukocytosis and is correlated with a worse prognosis[75,77,78]. However, even with some discrepancies in the literature, cancer antigen 19-9 (CA 19-9) and carcinoembryonic antigen (CEA) levels are reportedly lower than those found in PDAC[74].

Considering the rarity and aggressive behavior of this histotype, survival data about UC are mostly represented by single case reports or small case series. To date, there

are no large prospective clinical studies. The overall prognosis of patients affected by UC appears to be worse than those affected by PDAC, with a median OS of about 5.5 mo. There are reports of advanced patients dying within weeks from the onset of symptoms. However, patients who undergo surgical resection have a mOS similar to that of PDAC[74,75,79]. Several reports have shown that among UC patients, those with OCGC tend to have a longer survival. This may be related to the more indolent biological behavior of this specific subtype, which allows a higher rate of curative resections or slower disease progression in the metastatic setting[73,75,79,80].

Pathology and molecular biology

Since the first description in 1954, UC has been described using various terms including anaplastic, spindle cell, giant cell, pleomorphic giant cell, and round cell. In 2019, the WHO classified this heterogeneous neoplasm as a subtype of PDAC under the name of UC[4]. As mentioned above, however, OCGC is described as a separate variant of PDAC due to its different biological and clinical behaviors.

Macroscopic examination of UC has shown that it can be a large solid or cystic lesion, or a mixture of both. Cystic evolution is consequent to the rapid uncontrolled growth of neoplastic cells that are highly prone to degeneration, hemorrhage, and necrosis. This feature may help to distinguish UC from PDAC, in which cystic components are infrequent (< 1%). The histological examination of UC is remarkably heterogeneous and characterized by the presence of pleomorphic and/or spindle cells, whose predominance differentiates the two main subtypes (anaplastic UC/sarcomatoid UC), which grow in poorly cohesive nests supported by scanty fibrous stroma. Pleomorphic cells are usually arranged in solid sheets without gland formation showing markedly pleomorphic nuclei and abundant eosinophilic cytoplasm. On the contrary, spindle cells tend to arrange in a storiform pattern and to be more uniform, ovoid to spindle shaped. Furthermore, areas of infiltrating ductal adenocarcinoma at the periphery of the lesions, squamous differentiation, and areas of phagocytic activity are frequently observed[73,81,82]. Although this bizarre morphology, electron microscopy, and above all, immunohistochemistry analysis have demonstrated the epithelial origin of UC neoplastic cells. In particular, similar to PDAC, these cells express CKs such as CK7, CK8, CK18 and CK19. Vimentin, CA 19.9, CEA, and p53 may also be expressed. In addition, similar to PDAC, KRAS point mutations are frequently observed upon molecular analysis[73,82]. Although further studies are still needed, these specific features support the hypothesis that these cells may be the result of a dedifferentiation process of a preceding ductal adenocarcinoma.

On the other end, UC with OCGC is defined by the abundance of non-neoplastic osteoclast-like multinucleated giant cells admixed with a mononuclear histiocytic component and a neoplastic mononuclear cell component[4]. OCGC is frequently located in the junction between necrotic hemorrhagic areas and viable areas of the tumor. Abundant hemosiderin pigment is scattered throughout the tumor; moreover, osteoid formation and foci of *in situ* or invasive adenocarcinoma may also be found. To date, the real origin of these cells is still not clear. In contrast to pleomorphic and spindle cells, OCGC is immunohistochemically negative for CKs, but positive for histiocytic markers, supporting the hypothesis of a histiocytic origin. Indeed, some authors have hypothesized that OCGC may result from the fusion of histiocyte/macrophages chemoattracted to the tumor site by factors released by neoplastic cells, and this may be the reason why phagocytic areas are commonly described[83]. Neoplastic cells may be spindle-shaped or epithelioid and can be very large and pleomorphic; these cells can show keratin positivity and have a high Ki-67 proliferation index.

Imaging

In reported imaging series, on enhanced CT scan, UC is described as a large mass, usually in the pancreatic head, with relative hypodensity compared to normal parenchyma during the pancreatic and portal vein phases, with a peripheral contrast enhancement. Therefore, it may sometimes be difficult to distinguish UC from cystic lesions. Additionally, pancreatic duct dilatation and rare calcifications was observed[84-86].

At MRI, several authors have described UC as a well-defined lesion with low intensity on T1-, T2-, and diffusion-weighted images. This last feature, in particular, may be related to hemosiderin deposits on mononuclear histiocytic and OCGC and may be helpful for the differential diagnosis between UC and cystic lesions, in which hemorrhage and hemosiderin deposits are unusual. Calcifications are also occasionally described[85,86].

Treatment

To date, there is no a standard treatment for UC. For patients with resectable disease, surgery represents the only curative option, with a survival rate that is comparable with that of patients with PDAC who undergo R0 resection. No significant benefits have been reported for neoadjuvant/adjuvant treatments or palliative surgery[74,87].

For advanced disease, only few case reports with various systemic treatment have been reported[80,87]. For example, Wong *et al*[88] treated a patient with GEMOX in combination with radiofrequency ablation of liver metastases, reaching SD with an OS of 15 mo[88]. Gemcitabine-base regimes was also explored by Brunetti *et al*[26], who reported PR with an OS of 14 mo and SD with OS of 8 mo in two patients treated with gemcitabine plus nab-paclitaxel. Furthermore, there are case reports showing benefits of first-line and second-line therapy with FOLFIRINOX[89] and FOLFIRI[90], respectively. Finally, an interesting approach presented by Wakatsuki *et al*[91]. In their case report, paclitaxel as a single agent was selected to treat the patient, using a chemosensitivity test with the adenosine triphosphate assay achieving a complete response after two cycles and a disease-free survival of 23 mo[91].

PANCREATOBLASTOMA

Epidemiology and prognosis

Pancreatoblastoma (PBL) presents more often in children, where it accounts for 25% of all pancreatic tumors[92], whereas it is an extremely rare histotype in adults, accounting for less than 1% of PC. In the few series available in English literature (accounting for < 100 patients in total), PBL typically appears as a large tumor (up to 20 cm), which is more frequently localized in the pancreatic head. It usually presents with nonspecific signs and symptoms such as abdominal pain, abdominal mass, jaundice, weight loss, chronic diarrhea and upper gastrointestinal bleeding[93-96]. The prognosis is poor, with an mOS of 5 mo in patients who cannot undergo surgery. About 40% of patients are metastatic at diagnosis, with the liver being the most common site of secondary involvement; local infiltration of surrounding tissues is also frequent[97]. Of note, even if PBL is considered to be a sporadic tumor, there have been reports of its association with familial adenomatous polyposis syndrome[93,98] and Beckwith-Weidemann syndrome[99].

Pathology and molecular biology

There are two populations of cells with distinctive characteristics: blast-like tumor cells and squamous morules. In particular, blast-like cells are monotonous, round, and small (1.5-2.0 times the size of a red blood cell), with a high nuclear-to-cytoplasmic ratio, scant cytoplasm, and infrequent anisonucleosis. Focal nuclear molding and crushing resembling small-cell carcinoma are seen in all cases. Abnormal mitotic figures were occasionally described in a case with metastatic disease with a poor prognosis (< 10 d). The immunohistochemical staining was positive for trypsin, chymotrypsin, lipase, BCL10 and alpha-fetoprotein. Squamous morules were seen in 75% of patients. They were composed of whorling or streaming epithelioid cells with abundant, dense, granular cytoplasm; syncytial arrangement; low nuclear to cytoplasmic ratio; and elongated nuclei with blunted ends and vesicular chromatin. Morule overexpress estrogen receptor beta and have aberrant nuclear/cytoplasmatic B catenin staining. Aberrant Wnt pathway activation manifest as somatic *CTNNB1* mutations (in 90% of cases) and loss of heterozygosity (LOH) of *APC* (in 10%). Other abnormalities include upregulation of the R-spondin/LGR5/RNF43 module, a progenitor-like pancreatic cell expression profile, and LOH of chromosome 11p. APC/β-catenin pathway alterations are seen in patients with and without familial adenomatous polyposis. Another syndrome seen in childhood PBL is Beckwith-Wiedemann syndrome, which is also associated with chromosome 11p LOH.

Previous data on nine case of pancreatoblastoma showed in 78% of cases strong nuclear and cytoplasmic accumulation of b-catenin protein, 86% had LOH for TH and D11S1984, microsatellite markers near the WT-2 locus on chromosome 11p15.5. There is frequent involvement of the APC/β-catenin pathway, with no evidence for *KRAS* mutations or *TP53* tumor suppressor gene alterations[98,100].

Imaging

On ultrasound, PBL appears as a solid inseparable pancreatic mass, with mixed echotexture[101]. On CT scan, it is usually a large, well-circumscribed, and

heterogeneous mass, with both solid and cystic components[102]. MRI can be used to delineate intratumoral hemorrhage and necrotic areas.

Treatments

Due to the rarity of this histology there is no guideline regarding treatment for this cancer. The only curative therapy remains surgical resection. The role of postoperative chemoradiotherapies is still unclear and there is no consensus on the best chemo regimen neither in adjuvant nor in palliative setting. There are clinical case reports describing the use of regimens containing platinum and/or doxorubicin and fluorouracil (*e.g.*, cisplatin/vincristine/bleomycin, 5-FU/doxorubicin/mitomycin, doxorubicin/carboplatin, cisplatin/doxorubicin), with mixed results[103,104]. Furthermore, there are reports of long-term disease-free survival for patients with liver metastases undergoing surgical resection of primary and metastatic lesions, suggesting a role for aggressive surgical approach[105,106].

ADENOSQUAMOUS CARCINOMA AND SQUAMOUS CELL PANCREATIC CARCINOMA

Epidemiology and prognosis

Adenosquamous pancreatic cancer (ASPC) accounts for about 1%-4% of all pancreatic cancer and it is defined as a mixture of the adenocarcinoma and the squamous cell carcinoma components[107]. Based on data from the National Cancer Database and Surveillance, Epidemiology and End Results database, ASPC tends to be larger, more frequently in body/tail, undifferentiated and in early stage at diagnosis time[108,109]. When compared to the 205328 PDAC present in the NCBD, overall prognosis of the 1745 ASPC is similarly poor, with a mOS of 5.7 mo. In patients who underwent surgery, ASCP had worse OS (14.8 mo *vs* 20.5 mo, $P < 0.001$) than PDAC, unless there was negative lymph node status, R0 surgical resection, and receipt of chemotherapy[109]. In surgical patients retrieved from the Surveillance, Epidemiology and End Results database between 2004 and 2015, mOS was 12 mo, with a median cancer specific survival of 16 mo.

Pure primary pancreatic squamous cell carcinoma is extremely rare and accounts for 0.5% to 5% of all exocrine PC. It is characterized by a worse prognosis than PDAC, with reported overall mOS of about 4-7 mo. Squamous cell carcinoma is more frequently located in the head of the pancreas, commonly presenting with pain, weight loss and jaundice. More than half of patients are diagnosed in stage IV, with liver being the most frequent site of metastasis, and the median age at diagnosis is 69 years[10,110,111].

Pathology and molecular biology

Squamous differentiation in PC usually occurs in association with conventional PDAC, in which a squamous component of at least 30% of the neoplasm should be detected in order to classify it as an adenosquamous carcinoma[4].

On macroscopy, ASPC made of is yellowish-white to gray, firm masses. Common findings are central necrosis and cystic degeneration.

Histologically, the adenocarcinoma component forms glandular structures, while squamous differentiation is detected by sheets of cells with distinct cellular borders, prominent intercellular junctions, dense eosinophilic cytoplasm and keratinization, expressing p63, p40 and low molecular weight cytokeratins[4].

The immunohistochemistry analysis demonstrate loss of p16 protein expression and strong reactivity for p53, with a profile similar to PDAC[4].

Almost all cases harbor *KRAS* mutation at codon 12 and *TP53* mutations. There are reports showing that the adenous and the squamous part of the adenosquamous cancer had similar molecular alterations, suggesting that the two components may results from the same progenitor cancer cell origin[112].

The angiogenic pattern appears to be more active in ASCP than PDAC[113], while there are data showing high PD-L1 expression mostly in the squamous cell carcinoma component of ASPC[114].

Of note, in the integrated evaluation about histopathological pancreatic cancer and whole-genome and deep-exome sequencing of PC, ASCPs were mostly represented in the squamous subtype, that was also an independent poor prognostic factor[115]. Squamous tumors are characterized by alterations in four core gene programs, including gene networks involved in inflammation, hypoxia response, metabolic

reprogramming, TGF-β signaling, MYC pathway activation, autophagy and upregulated expression of TP63ΔN and its target genes.

Imaging

ASPC is a hyper-vascular tumor. On CT scan with contrast enhancement, it appears as a large well defined predominantly solid and lobulated mass with ring enhancement in the peripheral area and central necrosis. Patients with vessel invasion have a poor prognosis[116].

Usually ASPC presents as a large mass in the pancreatic tail; small adenosquamous lesions appear to be more frequently in the head of pancreas. Venous tumor thrombus was seen only in large masses[117].

At CT scan hypervascularity can be observed. Fajardo *et al*[118] reported the use of dynamic CT scan with a bolus injection of intravenous contrast to examine a patient with pancreatic squamous cell carcinoma: the attenuation of this tumor increased from 35 HU to 61 HU[118].

Treatment

As per the other types of PC, surgical resection is the only curative approach for ASPC and SCC , that can significantly improve survival[107,110,119,120]. Patients affected by ASPC or SCC undergoing surgery and post-operative treatment with chemotherapy, radiotherapy or both appear to have a benefit[108,121-123], even though there is no consensus regarding the best regimen to use, that are commonly fluoropyrimidine-based, gemcitabine based or platinum-based. Data from Johns Hopkins Hospital show, in particular, benefit for ASPC patients treated with platinum-based chemotherapy, with a mOS of 19.1 mo[121].

In the metastatic setting, several chemotherapy regimens are reported to be active, including fluoropyrimidine-, gemcitabine- or platinum-based therapy[124-127]. For instance, Brunetti *et al*[26] and De Souza *et al*[128] reported 1 PR each in patients affected by ASPC treated with gemcitabine and GEMOX, respectively, and SD maintained for 10 mo, 9 mo and 8 mo in patients treated with gemcitabine, FOLFOXIRI and cisplatin + gemcitabine, respectively.

CONCLUSION

Despite the increase of retrospective case series and data regarding rare histological subtypes of PC that can help in their diagnosis, there are still many unanswered questions about the management of these cancers, due to the absence of prospective trials or guidelines. For clinicians facing these patients in their real-life routine, the key question is whether they have to be approached and treated in the same way as the more common PDAC or if they need to have a specific strategy. As described above, if taken singularly, these histotypes may differ significantly from PDAC, especially in terms of prognosis. While ASPC has a similar prognosis to PDAC and SCC appears in some reports to have even worse outcomes, the natural history of a patient with PT or ACC can be radically different, considering the unequivocal survival advantage showed in these subtypes[129]. Based on the analysis of 57804 PC patients who underwent surgical resection, the mOS of PDAC and ACC is 20.2 mo (22% 5-year OS) and 67.5 mo (51% 5-year OS), respectively, while the mOS of resected PT is not even reached (97% 5-year OS)[10]. This consideration alone may justify for a more aggressive surgical approach for these tumors than what we are used to consider for PDAC, with special interest to the possible benefit reported for the surgical resection of metastatic disease. On the contrary, the poor prognosis and aggressive behavior of UC, ASPC and SCC must be taken in consideration for the treatment strategy of these tumors.

Finally, for the majority of these subtypes, there are no clear data regarding chemosensitivity and the role of specific chemotherapy regimens both for locoregional and advanced disease. Nevertheless, we tried to summarize the most relevant data that, to the best of our knowledge, can give some inputs for clinical decision-making.

Even if the very low incidence of these malignancies makes it almost impossible to design and run prospective clinical trials, we believe that multi center collaborations are essential, in order to gather as much homogeneous information as possible leading to a more histotype-guided therapeutic approach to these cancers.

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MINIREVIEWS

Gastric neuroendocrine tumor: A practical literature review

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Abstract

Gastric neuroendocrine tumors are gastric neoplasms originating from enterochromaffin type cells and are inserted in a larger group, named gastroenteropancreatic neuroendocrine tumors. They are considered rare and variable in terms of their clinical, morphological and functional characteristics and may be indolent or aggressive. They are classified into types I, II and III, according to their pathophysiology, behavior and treatment. Their diagnosis occurs, in most cases, incidentally during upper digestive endoscopies, presenting as simple gastric polyps. Most cases (type I and type II) are related to hypergastrinemia, can be multiple and are treated by endoscopic resection, whenever possible. The use of somatostatin analogs for tumor control may be one of the options for therapy, in addition to total or subtotal gastrectomy for selected cases. Adjuvant chemotherapy is only reserved for poorly differentiated neuroendocrine carcinomas. Although rare, gastric neuroendocrine tumors have an increasing incidence over the years, therefore deserving more comprehensive studies on its adequate treatment. The present study reviews and updates management recommendations for gastric neuroendocrine tumors.

Key words: Gastric neuroendocrine tumor; Gastroenteropancreatic tumor; Hypergastrinemia; Gastric carcinoid; Endoscopic resection

Core tip: Gastric neuroendocrine tumors are rare lesions that are part of the

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gastroenteropancreatic neuroendocrine tumors group. They are classified into types I, II and III according to their clinical and pathophysiological characteristics. Their diagnosis is usually made incidentally by upper gastrointestinal endoscopy, and most cases are treated by endoscopic resection. Surgical resections, as well as somatostatin analog treatments, are reserved for selected cases. Although rare, gastric neuroendocrine tumors need further research as their incidence has increased over the years.

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INTRODUCTION

Gastric neuroendocrine tumors (G-NETs), once called gastric carcinoids, are neoplasms derived from enterochromaffin-like cells (ECF) of the stomach mucosa and correspond to less than 2% of all gastric neoplasms[1]. They are part of a larger group called gastroenteropancreatic NETs (GEP-NET), which comprise well-differentiated NETs from the gastrointestinal tract. Well-differentiated NETs, together with poorly differentiated neuroendocrine carcinomas (NECs) form the neuroendocrine neoplasms. In immunohistochemistry, like other GEP-NET, G-NETs usually express neuroendocrine markers, such as chromogranin and synaptophysin. They are considered rare and of heterogeneous spectrum with a wide variety of morphological, functional and clinical characteristics[2-4]. Their behavior is generally indolent, although may be highly aggressive[5].

The real prevalence of NETs is unknown due to a worldwide difficulty in standardizing and categorizing the data. Nonetheless, increasing incidence over time is certainly related to a greater access to endoscopic and imaging methods, favoring its diagnosis[1,6-9]. A 2015 multicenter study involving national registries from several countries estimated that the prevalence of G-NET in Europe is 0.32 per 10000 inhabitants, while in the United States it is 0.17 and 0.05 in Japan[10]. Most G-NETs are incidentally diagnosed as simple gastric polyps during endoscopies of the upper gastrointestinal tract, corresponding to 0.6% to 2% of gastric polyp cases[6,9,11-16].

The present review of the English literature presents updated definitions as well as epidemiology, diagnosis and management recommendations for G-NET.

DISCUSSION

In order to standardize the classification of GEP-NETs and facilitate their understanding, the World Health Organization in 2010 divided GEP-NETs (including G-NETs) into three histological grades (G1, G2 and G3) based on the mitotic index (number of mitoses per ten high magnification fields) and/or on the Ki-67 index (mitotic and cellular proliferative index) (Table 1). This division was important due to the clinical and prognostic variability between G1, G2 and G3 groups. G1 and G2 GEP-NETs were considered well differentiated while high-grade NECs (G3) were considered poorly differentiated with significantly more aggressive behavior. In 2019, World Health Organization revised the classification and recognized a new category of high-grade but still well-differentiated GEP-NET (G3 NET-Neuroendocrine Tumors) (Table 2). Unlike G3 NECs, G3 NETs usually have a Ki-67 index below 55% and a prognosis not as poor as G3 NECs[17]. In addition to the grade classification established by the World Health Organization, which is fundamental for all GEP-NETs, well-differentiated G-NETs are also clinically divided into three types according to their pathophysiology and behavior, which influences treatment recommendations (Table 3).

Below we will describe the three types of G-NETs with their clinical characteristics and approach to localized disease.

Type I

Type I tumors correspond to the majority of G-NETs. They constitute about 70%-80%

Table 1 Classification of gastroenteropancreatic neuroendocrine tumors according to the World Health Organization 2010

	Grade I	Grade II	Grade III
Tumor size in cm	≤ 2	> 2	Any
Mitoses/10 HPF	< 2	2–20	> 20
Ki 67 index, %	< 3	3–20	> 20
Differentiation	Well differentiated	Well differentiated	Poorly differentiated

Adapted from[18]. HPF: High-power fields.

Table 2 Classification of gastroenteropancreatic neuroendocrine tumors: Neuroendocrine neoplasms according to the World Health Organization 2019

Terminology	Differentiation	Grade	Mitotic rate	Ki 67 index, %
NET, G1	Well differentiated	Low	< 2	< 3
NET, G2	Well differentiated	Intermediate	2-20	3-20
NET, G3	Well differentiated	High	> 20	> 20
NEC, SCNEC	Poorly differentiated	High	> 20	> 20
NEC, LCNEC	Poorly differentiated	High	> 20	> 20
MiNEN	Well or poorly differentiated	Variable	Variable	Variable

Adapted from[17]. NET: Neuroendocrine tumor; NEC: Neuroendocrine carcinoma; SCNEC: Small cell neuroendocrine carcinoma; LCNEC: Large cell neuroendocrine carcinoma; MiNEN: Mixed neuroendocrine non-neuroendocrine neoplasm.

of the lesions and are associated with chronic autoimmune atrophic gastritis[18-23].

The destruction of parietal cells leads to achlorhydria, which stimulates the production of gastrin. This results in hypergastrinemia as a physiological response to the demand generated by the shortage of HCl. The excess of gastrin generates hypertrophy and hyperplasia of the ECFs, favoring the appearance of innumerable small lesions, which are usually not very aggressive[18,20,22,23]. Serum gastrin is always elevated in type I G-NETs. Vitamin B12 deficiency with or without macrocytic anemia (pernicious or megaloblastic) may be present due to the reduction of the intrinsic factor, with a consequent reduction in the absorption of vitamin B12[18,20,22-24]. Parallel to this, serum antiparietal cell antibodies are positive in 80% of cases[20,24-26].

The diagnosis is made by upper digestive endoscopy with biopsy. There are pale, yellowish and transparent blood vessels that contrast with the smooth and red mucosa of areas not affected by the tumor, presenting as red, small and numerous polyps[11,19,20,22,24-26]. Histological analysis of the gastric mucosa shows atrophy of mucosal cells, hyperplasia of neuroendocrine cells and absence of parietal cells.

For type I G-NETs, treatment is generally more conservative to avoid gastrectomy because they are smaller and more defined lesions. The prognosis is good. The treatment of choice is endoscopic resection for lesions ≥ 0.5 cm and endoscopic observation in smaller ones. In lesions smaller than 2 cm, the risk of metastasis is less than 10%[27]. In general, for lesions smaller than 1 cm, no other complementary imaging exam is necessary. However, for lesions ≥ 1 cm, echo-endoscopy is recommended to identify the depth of tumor invasion in the gastric wall and the possible involvement of regional lymph nodes. Gastrectomy is reserved for submucosa tumors and/or lymph node involvement and/or positive margin in the polypectomy sample[19,22,28]. Patients with small type I G-NETs are managed by regular endoscopic follow-up.

When the lesions are multiple and impossible to resect endoscopically or when there are repeated recurrences after endoscopic treatment, both gastrectomy and prescription of somatostatin analogs can be used to reduce serum gastrin and tumor control[29,30]. Reports of the use of somatostatin analogues in small groups of patients showed that the interruption after 12 mo caused the serum gastrin to rise again without the reappearance of new lesions[11,31,32]. However, data are still insufficient to show the long-term efficacy of pharmacological treatment of localized type I G-NETs[21,22]. More rarely, antrectomy may be indicated in an attempt to reduce

Table 3 Types of gastric neuroendocrine tumors

	Type I	Type II	Type III
Prevalence, %	70-80	5-10	10-20
Background	Chronic atrophic gastritis	Gastrinomas (Zollinger-Ellison syndrome)	Normal mucosa
Other syndromes	Autoimmune polyglandular syndrome	MEN-1 syndrome	
Number of lesions	Multiple	Multiple	Single
Site of tumor	Fundus/body	Fundus/body	Fundus/body
Cell of origin	ECL	ECL	ECL, EC or X cell
Serum gastrin levels	Elevated	Elevated	Normal
Gastric PH	High	Low	Normal
Underlying mucosa	Atrophic	Hypertrophic	Normal
Size of tumors, usual	1-2 cm	1 cm	> 2 cm
Invasion	Rare	More common	Common
Metastases			
Lymph nodes	5%-10%	10%-20% (duodenal tumors)	50%-100%
Liver	2%-5%	10%	22%-75%
Prognosis	Excellent	Very good	Similar to gastric adenocarcinoma

Adapted from[18]. ECL: Enterochromaffin-like; EC: Endocrine.

hypergastrinemia.

Type II

They correspond to 5%-10% of G-NETs. In type II, hypergastrinemia also occurs, but it is due to the presence of Zollinger-Ellison syndrome mostly in the context of MEN-1 syndrome. Therefore, in the suspicion of a type II G-NET, it is recommended to determine the serum concentration of both pituitary and parathyroid hormones as well as serum calcium and gastrin levels to assess the possibility of MEN-1 syndrome. The patient may experience abdominal pain and diarrhea in addition to peptic ulcers. Similar to type I G-NETs, excess gastrin causes hypertrophy and hyperplasia of the ECFs. In these cases, it is also common for lesions to be small and multiple[2,18,33-35].

Upon diagnosis, upper endoscopy reveals normal or hypertrophic gastric mucosa in addition to hypergastrinemia and low pH due to hyperchlorhydria. Unlike type I, type II G-NETs tend to be slightly larger, affect younger patients and have a slightly worse prognosis with the risk of lymph node metastases reaching 30%[27].

In general, the management of type II G-NETs is similar to type I, except for the need to also resect the gastrinoma. Most cases are treated endoscopically with resections. Surgery is rarely necessary. The use of somatostatin analogues is still debated as well as in type 1 G-NETs[20,22].

When confirming the diagnosis, the primary gastrinoma should be located and resected, although it is not always possible to locate it and multiple lesions may exist. For that, we include computed tomography, magnetic resonance imaging, endoscopic ultrasound, scintigraphy with octreotide, selective angiography, positron emission tomography and/or intraoperative ultrasound in the workup. It is also possible to use an anatomical reference known as the gastrinoma triangle composed of the junction of the cystic duct with the common liver, the transition from the second to the third duodenal portion and the pancreatic neck[11,20,35].

Type III

G-NETs of this type are sporadic and not associated with any known clinical condition. They correspond to 10%-15% of all G-NETs. The production of gastrin and HCl is within normal values, except in rare cases where the tumor itself can produce gastrin[36]. They are generally characterized by being single lesions, larger than 1 cm in size and with greater likelihood of evolving to regional and systemic metastases[2,20,33,34]. More than half of patients with type III G-NET are metastatic at

diagnosis, mainly to the liver. In these cases, carcinoid syndrome may be present, which is a paraneoplastic syndrome caused by endogenous secretion of serotonin and kallikrein secondary to carcinoid tumors. It becomes manifest when those vasoactive substances from the tumors enter the systemic circulation escaping hepatic degradation. Clinical components of the typical carcinoid syndrome are flushing, diarrhea and abdominal pain. It occurs more frequently in the context of high-volume hepatic metastases and primary tumors located in the small bowel, although it may happen with G-NETs, when atypical symptoms, such as bronchoconstriction, may be present due to the release of histamine.

Recently, some groups have suggested the existence of a type IV G-NET, which consists of the same characteristics described above for type III but being neuroendocrine carcinomas or mixed neuroendocrine non-neuroendocrine neoplasm. Therefore, they have a more aggressive behavior and even worse prognosis[37]. However, the subclassification of type IV is still not well established.

Type III lesions are also investigated by upper endoscopy with biopsy, which shows a single lesion with normal mucosa. The pH is < 4, which is normal for the gastric pattern[2,18,20,33,34]. In addition to the neoplastic lesion, the adjacent normal mucosa should also be biopsied in order to assess whether there is atrophic gastritis, intestinal metaplasia and ECF hyperplasia, which are not usually present[2,18,19,33,34].

Total or subtotal gastrectomy is performed together with lymphadenectomy, as recommended in gastric adenocarcinomas[22,38]. For patients with any surgical contraindication, endoscopic resection may be an alternative, but the risk of regional lymph node spread is high. When the anatomopathological part of the resection specimen shows a slightly differentiated NEC, adjuvant chemotherapy based on platinum, such as cisplatin and etoposide, is used (similar to small-cell lung carcinomas).

Treatment of metastatic disease

The goal of metastatic G-NET therapy is to control symptoms by reducing circulating hormones (when present) and tumor growth in order to increase quality of life and ensure greater survival[39]. In general, the treatment of well-differentiated metastatic disease (G1, G2 or G3 NET) is usually similar to other NETs, taking into account the patient's performance, available drugs, toxicity profile, the volume and extent of the metastatic disease, the expression of somatostatin receptors in functional images (Octreoscan or ^{68}Ga-Dotatate) and the presence/lack of a functioning syndrome. Surgical resection of metastases, local-regional therapies such as embolization or ablation when there is exclusive liver involvement, somatostatin analogs, target-molecular drugs (everolimus), ^{177}Lu-OctreoTate or even chemotherapy regimens when G3 should be considered when possible[40]. Despite the low response rates, the somatostatin analogue (Octreotide or Lanreotide) is usually the initial treatment of choice because it is well tolerated[41,42]. In the presence of carcinoid syndrome (8% to 35% of G-NETs), the use of the somatostatin analog is mandatory to reduce symptoms and decrease the long-term risks of an uncontrolled carcinoid syndrome. The ideal sequencing for patients with G-NETs, as in other NETs, remains unknown.

In the case of metastatic NEC, the treatment usually follows the protocols of small-cell lung carcinomas, in which the most commonly administered regimen is the combination of cisplatin and etoposide[43]. In these cases, despite good initial response rates, the prognosis is often poor.

CONCLUSION

Although relatively rare, the incidence of G-NETs has increased over time. They comprise a diverse entity of three subtypes with different pathophysiology, prognosis and management. Further studies are needed for further advances in the treatment of G-NETs.

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ORIGINAL ARTICLE

Basic Study

Identification of an immune-related gene-based signature to predict prognosis of patients with gastric cancer

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Author contributions: He J and Niu X conceived and designed the study; Qiu XT and Song YC conducted most of the experiments and data analyses, and wrote the manuscript; Liu J and Wang ZM participated in collecting the data and helped to draft the manuscript; all authors reviewed and approved the manuscript.

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Abstract

BACKGROUND

Gastric cancer (GC) is the most commonly diagnosed malignancy worldwide. Increasing evidence suggests that it is necessary to further explore genetic and immunological characteristics of GC.

AIM

To construct an immune-related gene (IRG) signature for accurately predicting the prognosis of patients with GC.

METHODS

Differentially expressed genes (DEGs) between 375 gastric cancer tissues and 32 normal adjacent tissues were obtained from The Cancer Genome Atlas (TCGA) GDC data portal. Then, differentially expressed IRGs from the ImmPort database were identified for GC. Cox univariate survival analysis was used to screen survival-related IRGs. Differentially expressed survival-related IRGs were considered as hub IRGs. Genetic mutations of hub IRGs were analyzed. Then, hub IRGs were selected to conduct a prognostic signature. Receiver operating characteristic (ROC) curve analysis was used to evaluate the prognostic performance of the signature. The correlation of the signature with clinical features and tumor-infiltrating immune cells was analyzed.

RESULTS

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Among all DEGs, 70 hub IRGs were obtained for GC. The deletions and amplifications were the two most common types of genetic mutations of hub IRGs. A prognostic signature was identified, consisting of ten hub IRGs (including *S100A12, DEFB126, KAL1, APOH, CGB5, GRP, GLP2R, LGR6, PTGER3*, and *CTLA4*). This prognostic signature could accurately distinguish patients into high- and low- risk groups, and overall survival analysis showed that high risk patients had shortened survival time than low risk patients ($P < 0.0001$). The area under curve of the ROC of the signature was 0.761, suggesting that the prognostic signature had a high sensitivity and accuracy. Multivariate regression analysis demonstrated that the prognostic signature could become an independent prognostic predictor for GC after adjustment for other clinical features. Furthermore, we found that the prognostic signature was significantly correlated with macrophage infiltration.

CONCLUSION
Our study proposed an immune-related prognostic signature for GC, which could help develop treatment strategies for patients with GC in the future.

Key words: Gastric cancer; Immune-related genes; Tumor microenvironment; Immune infiltration; Prognosis; Signature

Core tip: Gastric cancer (GC) is the most commonly diagnosed malignancy worldwide. Increasing evidence suggests that it is necessary to further explore genetic and immunological characteristics of GC. Our study identified an immune-related prognostic signature for GC, which could accurately distinguish patients into high- and low- risk groups. High risk patients had a poorer prognosis. Multivariate Cox regression analysis demonstrated that the prognostic signature could independently predict GC prognosis. Furthermore, it was significantly associated with immune cell infiltration (especially macrophages). Therefore, the signature may possess prognostic value as a prediction tool for identification of patients who will benefit from immunotherapy.

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INTRODUCTION

Gastric cancer (GC) is the fourth most commonly diagnosed malignancy and the second leading cause of cancer-related death worldwide[1]. Although the incidence of GC has decreased year by year, the prognosis of GC patients is still not optimistic, especially in China[2]. Yet, its pathogenesis remains unclear, therefore, identification of effective biomarkers and therapeutic targets needs to be addressed.

The tumor microenvironment (TME), especially the immune system, plays a pivotal role in the occurrence and development of GC[3]. Dysfunction of the immune system assists tumor cells to avoid immune surveillance. Immunotherapy such as programmed death-1 (PD-1) blockade has become a promising strategy for GC treatment[4]. However, the clinical outcomes of GC patients are still unsatisfactory, and most of novel immunotherapies are still in the early stages of clinical research[5,6]. The underlying mechanisms of the immune checkpoint blockade response are complex. Thus, deeper genetic and immunological characterization of GC is required to guide clinicians in selecting and determining the best treatment options. The prognosis of GC is closely related to crosstalk between immune cells and tumor cells[7]. Nevertheless, the role of immune-related genes (IRGs) in predicting GC patients' prognosis has not yet been elucidated.

Considering the prognostic potential of IRGs in GC, in this study, we studied

immune-related molecular features. We analyzed IRGs using a large amount of transcription data of GC and explored their potential molecular mechanisms. Based on differentially expressed IRGs, we developed an immune-related prognostic model. Our results will help develop treatment strategies for patients with GC.

MATERIALS AND METHODS

GC datasets

Transcriptome RNA-seq data and corresponding clinical information of GC were retrieved from the Genomic Data Commons (GDC) data portal (https://gdc.xenahubs.net), including 375 GC tissues and 32 normal adjacent tissues[8]. A list of IRGs were downloaded from the Immunology Database and Analysis Portal (ImmPort) database[9]. Furthermore, we derived a list of transcription factors (TFs) from Cistrome database (http://cistrome.org/CistromeCancer/)[10]. Mutation data were obtained from the Broad GDAC Firehose.

Differential expression analysis

Differential expression analysis was performed using the edgeR package (http://bioconductor.org/packages/edgeR/) in R[11]. The raw data were normalized by Trimmed mean of M values (TMM). The genes with |log fold change (FC)| > 1 and false discovery rate (FDR) < 0.01 were considered as differentially expressed genes (DEGs). Differentially expressed IRGs and TFs were extracted from these DEGs.

Functional enrichment analysis

The clusterProfiler package was used to annotate DEGs, including gene ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG)[12]. GO terms include biological process, molecular function, and cellular component. $P < 0.05$ was considered to be significantly enriched.

Cox univariate survival analysis

Cox univariate survival analysis of IRGs was performed using the survival package in R. $P < 0.05$ was set as the screening criterion. Survival-related IRGs were identified. Furthermore, differentially expressed survival-related IRGs were considered as hub IRGs.

Protein-protein interaction network

Hub IRGs were analyzed based on the STRING database (https://string-db.org/). Protein-protein interaction (PPI) network was analyzed using Cytoscape. The interactions among proteins could be involved in the progression of diseases. The nodes with high degree could possess potential as hub genes/proteins.

Molecular characteristics

Mutation data of GC samples from Broad GDAC Firehose website were used to analyze genetic alterations of hub IRGs. The results were visualized into waterfall maps using maftools[13].

Construction of an immune-related prognostic signature

Hub IRGs were analyzed by multivariate regression analysis. Then, hub IRGs were selected to conduct a prognostic signature. The risk score was calculated by the expression levels of hub IRGs and Cox regression coefficient. All patients with GC were divided into a high risk group and low risk group according to the median value of risk score. To verify the prognostic potential, the area under the time-dependent receiver operating characteristic (ROC) curve (AUC) was calculated with the survival ROC package[14]. The correlation between clinical features and the prognostic signature was evaluated. Immune infiltrate levels of GC were obtained from the TIMER database[15]. The tumor-infiltrating immune cells included macrophages, B cells, CD4+ T cells, CD8+ T cells, dendritic cells, and neutrophils. The relationships between immune cells and the prognostic signature were calculated.

RESULTS

Identification of DEGs for GC

Four thousand two hundred and fifty-nine DEGs with |logFC| > 1 and FDR < 0.01 were identified in 375 GC tissues compared to 32 normal adjacent tissues, including 1951 up-regulated and 2356 down-regulated genes, as depicted in volcano plot (Figure 1A). Hierarchical clustering analysis showed that these DEGs can obviously distinguish GC tissue samples from normal adjacent tissue samples (Figure 1B). These DEGs were significantly associated with immune-related biological processes like humoral immune response and acute inflammatory response (Figure 1C). The top ten cellular components and molecular functions enriched by DEGs are depicted in Figure 1D and 1E, respectively. As shown in Figure 1F, the DEGs were mainly enriched in several pathways related with GC, such as neuroactive ligand-receptor interaction, cytokine-cytokine receptor interaction, chemical carcinogenesis and so on. These results suggested that these DEGs might be involved in the pathogenesis of GC.

Identification of differentially expressed IRGs for GC

A list of IRGs were downloaded from the ImmPort database. Differentially expressed IRGs were screened from all DEGs, including 181 up-regulated and 354 down-regulated IRGs. The results are visualized into volcano plot (Figure 2A) and heatmap (Figure 2B). In the biological process results, the differentially expressed IRGs were mainly enriched in immune-related processes such as humoral immune response, phagocytosis, and B cell mediated immunity (Figure 2C). As for CC, the genes were in association with immunoglobulin complex, receptor binding, and circulating immunoglobulin complex (Figure 2D). Intriguingly, these IRGs have the molecular immune-related functions like receptor ligand activity, antigen binding, and cytokine activity (Figure 2E). As expected, these differentially expressed IRGs were mainly enriched in immune-related pathways such as cytokine-cytokine receptor interaction, neuroactive ligand-receptor interaction, and viral protein interaction with cytokine and cytokine receptor (Figure 2F).

Identification of differentially expressed TFs for GC

Sixty-seven TFs were differentially expressed between GC tissues and normal tissues, including 37 up-regulated and 30 down-regulated TFs, as shown in volcano plot and heatmap (Figure 3A and B). We further explored their potential functions. The results showed that these TFs are mainly involved in regionalization, pattern specification process, and gland development (Figure 3C). As expected, these TFs could regulate TF complex, nuclear TF complex, and nuclear chromatin (Figure 3D). Similarly, they have the function of TF activity (Figure 3E). According to KEGG pathway enrichment results, these TFs are involved in several cancers (Figure 3F).

Identification of survival-related IRGs for GC

Cox univariate survival analysis of all IRGs was performed using the survival package in R. The results showed that 183 IRGs were significantly related to overall survival of patients with GC ($P < 0.05$).

Identification of hub IRGs for GC

By intersection of differentially expressed IRGs and survival-related IRGs, 70 hub IRGs were obtained for GC (Figure 4A). The forest plot of hazard ratios (HRs) suggested that except *CTLA4*, *LGR6*, and *KIR2DS4*, other hub IRGs were risk factors for GC (Figure 4B). As shown in PPI network, *IL6*, *F2R*, and *AGT* were the top three hub genes (Figure 4C). GO enrichment analysis results showed that these hub IRGs were enriched in many biological processes like positive regulation of response to external stimulus, muscle cell proliferation, and peptidyl-tyrosine phosphorylation (Figure 4D). In the cellular component results, receptor complex, vacuolar lumen, and secretory granule lumen were mainly enriched (Figure 4E). As for molecular function, the hub IRGs were significantly associated with receptor ligand activity, growth factor activity, and peptide hormone binding (Figure 4F). As shown in Figure 4G, hub IGRs are mainly involved in GC-related pathways such as neuroactive ligand-receptor interaction, Rap1 signaling pathway, PI3K-Akt signaling pathway, and cytokine-cytokine receptor interaction.

Genetic mutations of hub IRGs for GC

We further analyzed the molecular features of hub IRGs for GC. Their genetic alterations were detected. Among 438 samples, 323 had genetic alterations. The results

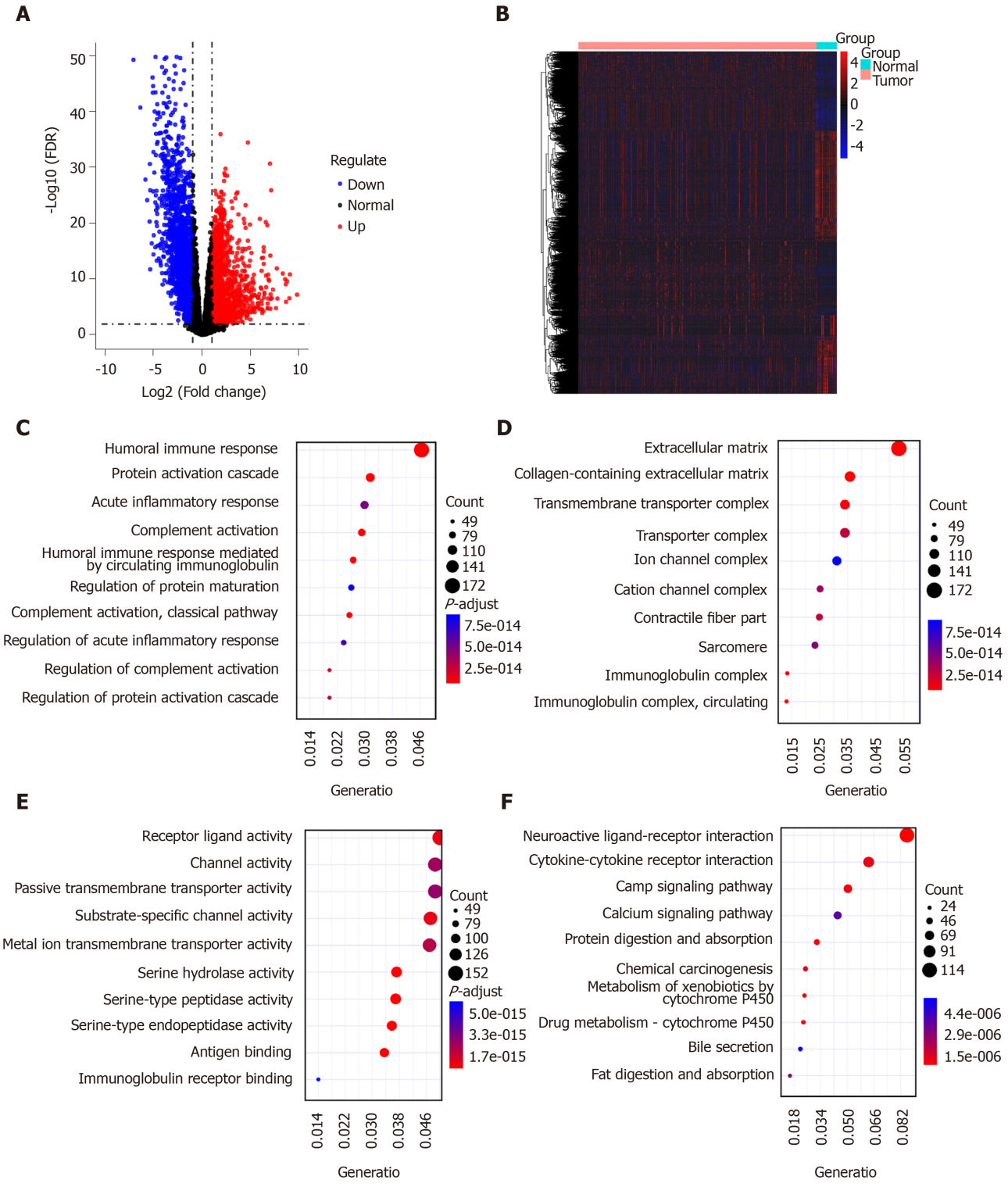


Figure 1 Identification of differentially expressed genes for gastric cancer. A and B: Volcano plot (A) and heatmap (B) showing differentially expressed genes (DEGs) between gastric cancer tissues and normal tissues. Blue represents down-regulation and red represents up-regulation; C-E: Gene Ontology enrichment results of the top ten DEGs including biological processes (C), cellular component (D), and molecular function (E); F: Kyoto Encyclopedia of Genes and Genomes enrichment results of the top ten DEGs.

showed that deletions and amplifications were the two most common types of genetic mutations (Figure 5).

Construction of a prognostic signature for GC

Multivariate Cox regression analysis was performed based on the hub IRGs using the survival package in R. Ten hub IRGs were selected to construct a prognostic signature for GC. As depicted in the forest plot, each hub IRG in the prognostic signature can accurately predict GC patients' prognosis (Figure 6A). Among them, *S100A12* (HR =

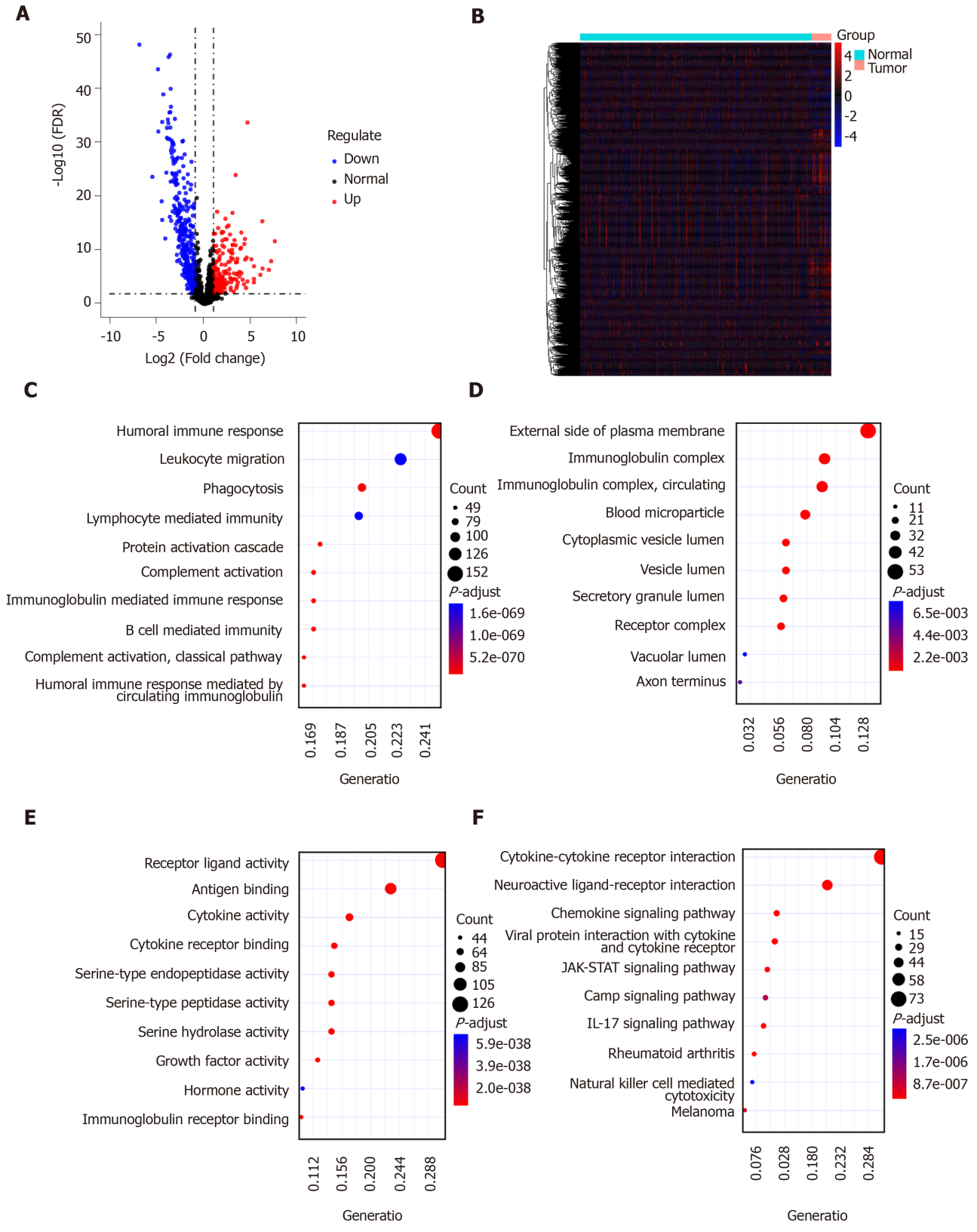


Figure 2 Identification of differentially expressed immune-related genes for gastric cancer. A and B: Volcano plot (A) and heatmap (B) showing differentially expressed immune-related gene (IRGs) between gastric cancer tissues and normal tissues. Blue represents down-regulation and red represents up-regulation; C-E: Gene Ontology enrichment results of the top ten differentially expressed IRGs including biological processes (C), cellular component (D), and molecular function (E); F: Kyoto Encyclopedia of Genes and Genomes enrichment results of the top ten differentially expressed IRGs.

1.28; 95% confidence interval [CI]: 1.1-1.48; *P* = 0.001), *DEFB126* (HR = 1.79; 95%CI: 1.01-3.17; *P* = 0.045), *KAL1* (HR = 1.36; 95%CI: 1.13-1.63; *P* = 0.001), *APOH* (HR = 1.13; 95%CI: 1.01-1.28; *P* = 0.034), *CGB5* (HR = 1.2; 95%CI: 1.04-1.39; *P* = 0.015), *GRP* (HR = 1.33; 95%CI: 1.06-1.67; *P* = 0.014), and *GLP2R* (HR = 1.45; 95%CI: 1.17-1.79; *P* = 0.001)

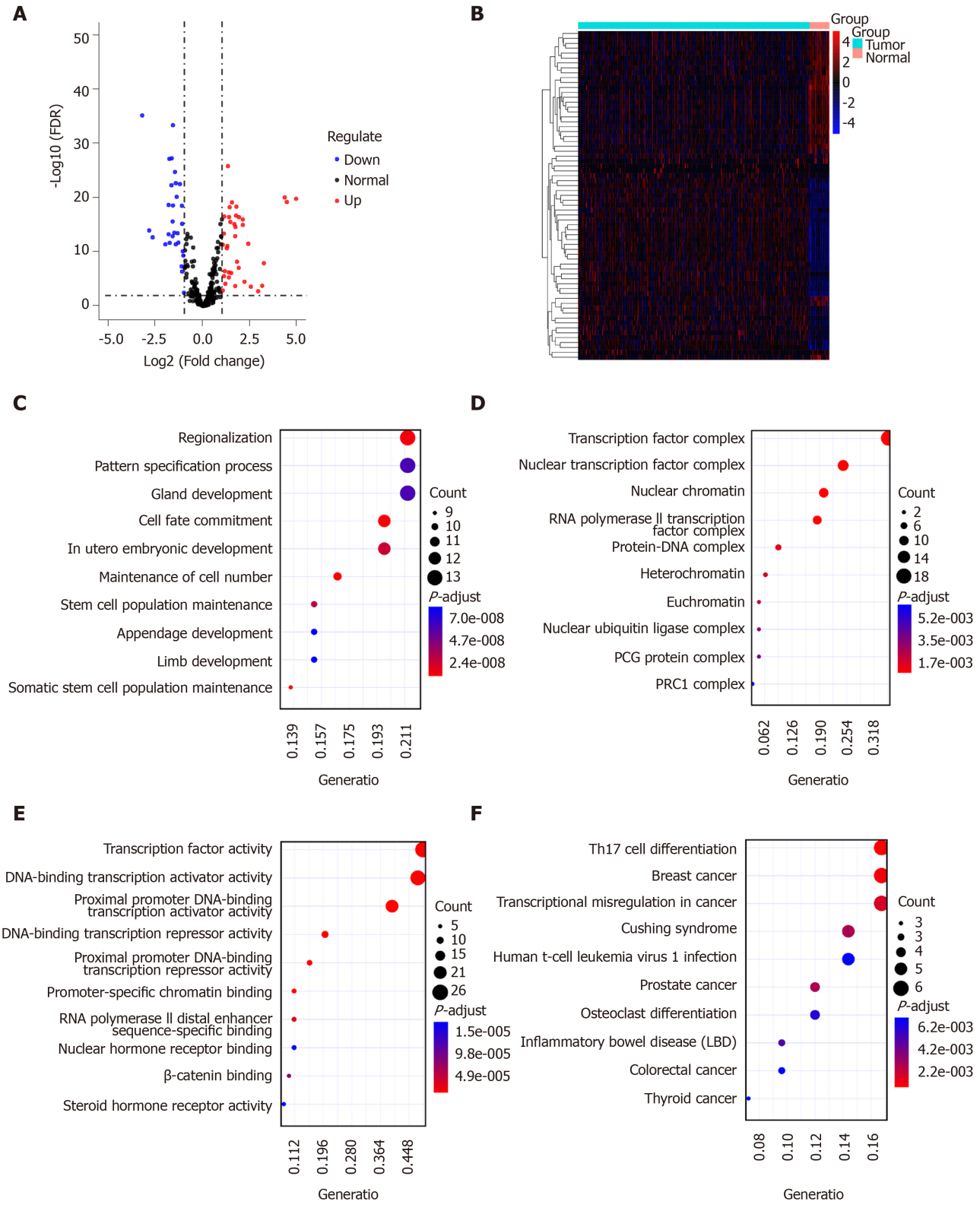
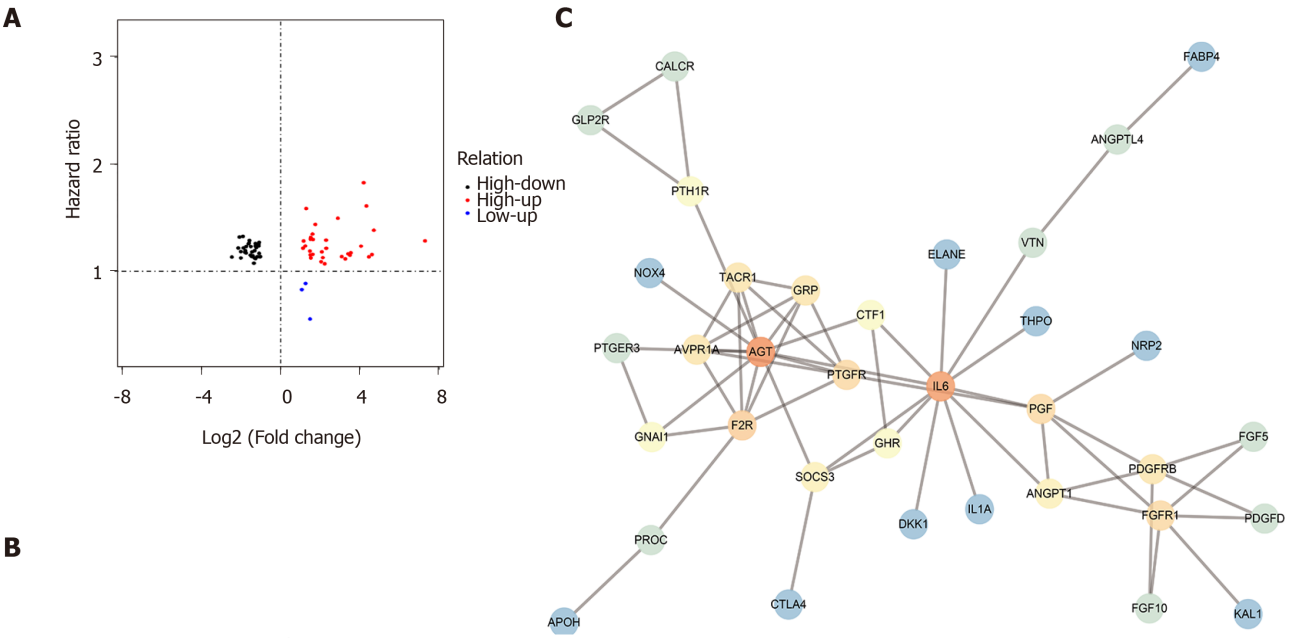


Figure 3 Identification of differentially expressed transcription factors for gastric cancer. A and B: Volcano plot (A) and heatmap (B) showing differentially expressed transcription factors (TFs) between gastric cancer tissues and normal tissues. Blue represents down-regulation and red represents up-regulation; C-E: Gene Ontology enrichment results of the top ten differentially expressed TFs including biological processes (C), cellular component (D), and molecular function (E); F: Kyoto Encyclopedia of Genes and Genomes enrichment results of the top ten differentially expressed TFs.

were risk factors for GC, while *LGR6* (HR = 0.86; 95%CI: 0.78-0.95; *P* = 0.002), *PTGER3* (HR = 0.79; 95%CI: 0.64-0.99; *P* = 0.037), and *CTLA4* (HR = 0.8; 95%CI: 0.66-0.97; *P* = 0.021) were protective factors for GC. Risk score was calculated and the patients were divided into a high risk group and low risk group based on the median value of risk score (Figure 6B, C). Heatmap depicts the expression patterns of the ten hub IRGs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Relation
High-down
High-up
Low-up
C
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GLP2R
PTH1R
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ANGPTL4
VTN
ELANE
NOX4
TACR1
GRP
CTF1
THPO
PTGER3
AVPR1A
AGT
PTGFR
IL6
NRP2
PGF
GNAI1
F2R
GHR
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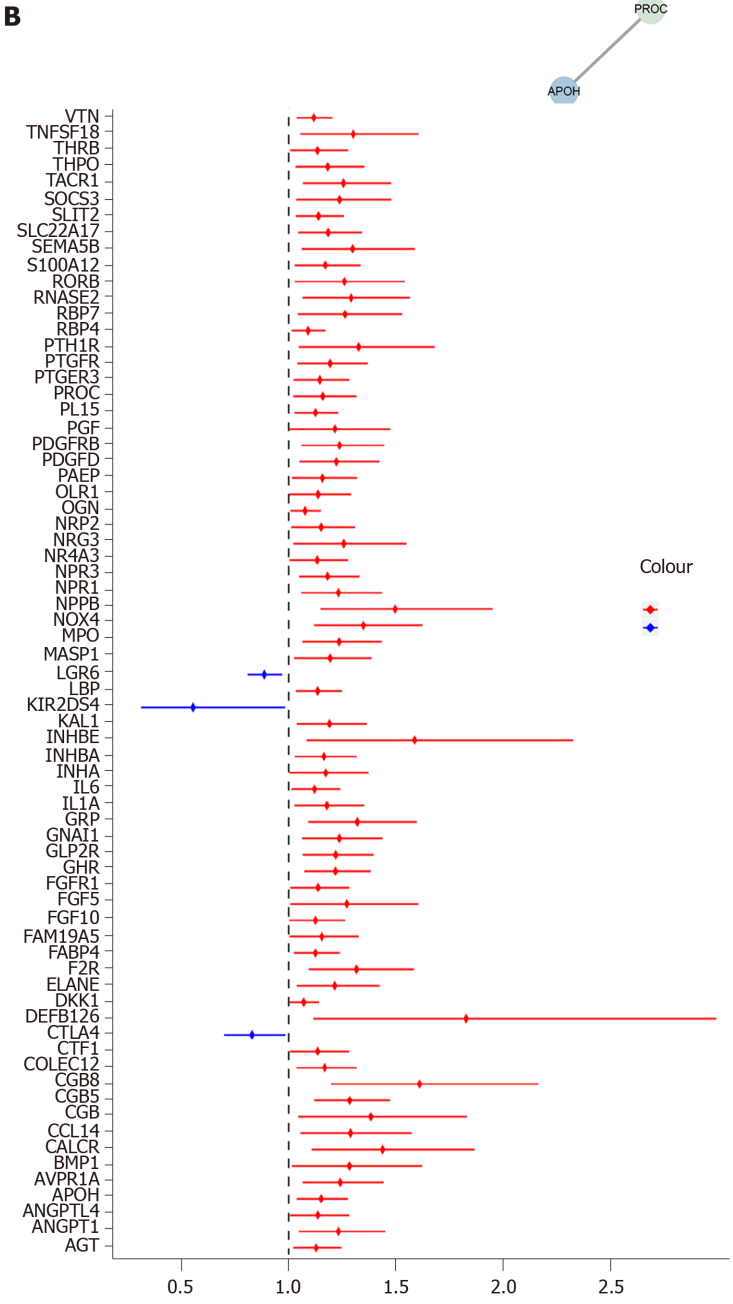
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THRB
THPO
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SLIT2
SLC22A17
SEMA5B
S100A12
RORB
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LBP
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GHR
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COLEC12
CGB8
CGB5
CGB
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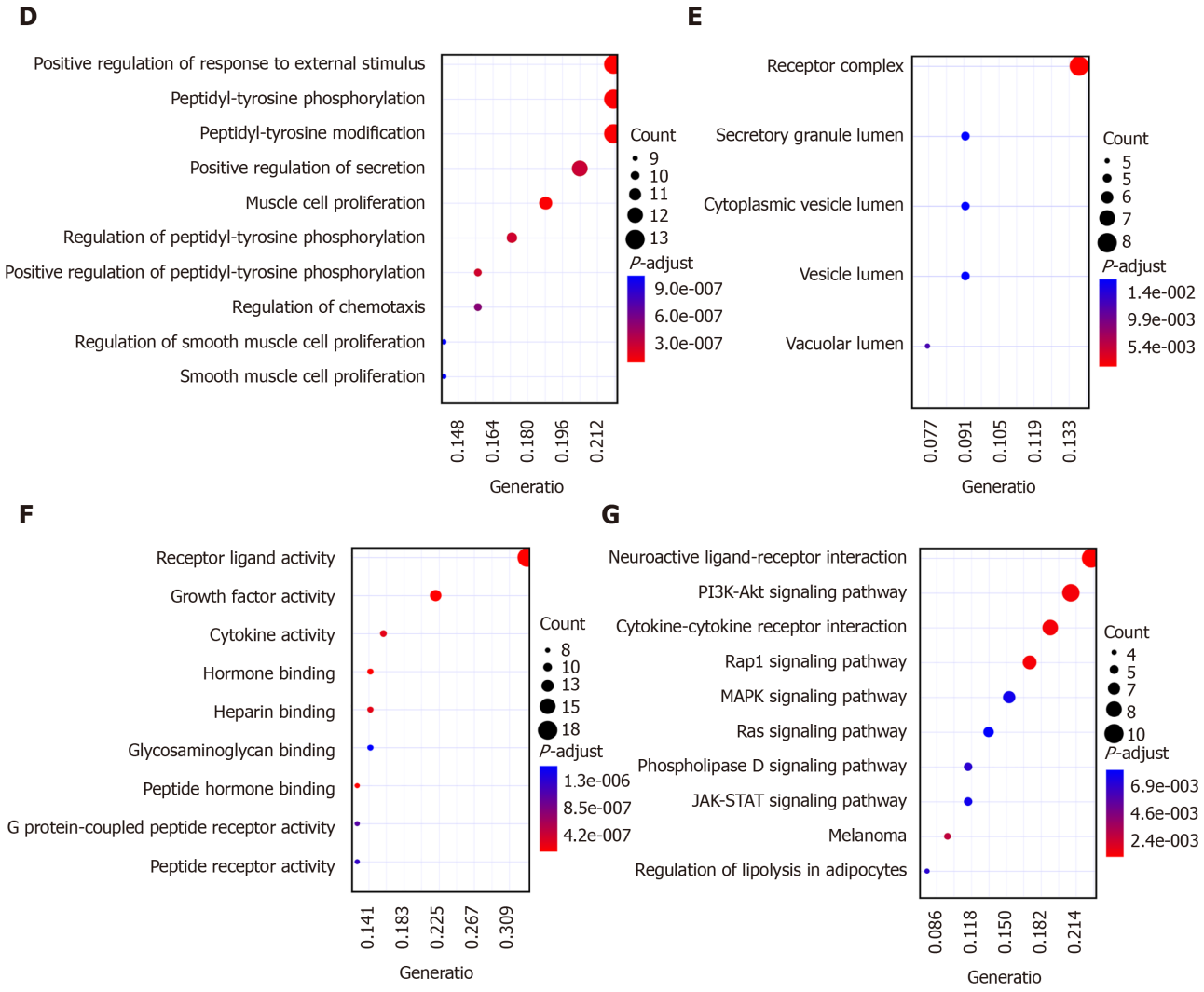


Figure 4 Identification of hub immune-related genes for gastric cancer. A: The intersection of differentially expressed immune-related gene (IRGs) and survival-related IRGs; B: Forest plot showing prognostic value of hub IRGs. The X-axis represents hazard ratio, and the Y-axis represents the differentially expressed hub IRG; C: Protein-protein interaction network of hub IRGs; D-F: Gene Ontology enrichment results of the top ten differentially expressed transcription factors (TFs) including biological processes (D), cellular component (E), and molecular function (F); G: Kyoto Encyclopedia of Genes and Genomes enrichment results of the top ten differentially expressed TFs.

between the high risk group and low risk group (Figure 6D). Overall survival analysis showed that high risk patients had shortened survival time than t low risk patients (Figure 6E; $P < 0.0001$). The AUC was 0.761, suggesting that the prognostic signature had a high sensitivity and accuracy (Figure 6F). Correlation analysis showed that the prognostic signature was not significantly associated with clinical features including age (Figure 7A), gender (Figure 7B), pathologic T (Figure 7C), pathologic N (Figure 7D), pathologic M (Figure 7E), and pathologic stage (Figure 7F). As shown in multivariate regression analysis results, the prognostic signature could become an independent prognostic predictor after adjustment for other factors including age, gender, pathologic T, pathologic N, pathologic M, and pathologic stage (Table 1). Furthermore, we also found a difference in expression patterns of the ten hub IRGs in the prognostic signature between GC tissues and normal tissues, including *APOH* (Figure 8A; $P = 0.006$), *CGB5* (Figure 8B; $P = 0.00097$), *CTLA4* (Figure 8C; $P = 0.00021$), *DEFB126* (Figure 8D; $P = 0.096$), *GLP2R* (Figure 8E; P = 2e-13), *GRP* (Figure 8F; P = 6.4e-09), *KAL1* (Figure 8G; P = 8.3e-12), *LGR6* (Figure 8H; P = 2.9e-06), PTGER3 (Figure 8I; P = 3.7e-10), and *S100A12* (Figure 8J; $P = 0.0003$).

Relationship between immune infiltration and the prognostic signature

To explore whether the hub IRGs in the prognostic signature are involved in the tumor immune microenvironment, the correlation between immune cell infiltration and prognostic signature was analyzed. We found that the prognostic signature was significantly correlated with macrophage infiltration (Figure 9A). However, there was

Table 1 Univariate and multivariate Cox regression analyses of clinical features and risk score for gastric cancer

Variable	Univariate analysis		Multivariate analysis	
	Hazard ratio (95%CI)	*P* value	Hazard ratio (95%CI)	*P* value
Age	1.554 (1.064- 2.268)	0.022424	2 (1.36-2.95)	4.15E-04
Gender	1.274 (0.896- 1.812)	0.177496	1.12 (0.78-1.6)	0.537783
Pathologic T	1.753 (1.156- 2.659)	0.00829	1.39 (0.87-2.21)	0.170758
Pathologic N	1.877 (1.242- 2.835)	0.002784	1.15 (0.66-2.01)	0.625769
Pathologic M	1.939 (1.208- 3.112)	0.006063	1.61 (0.99-2.61)	0.054747
Pathologic stage	1.988 (1.398- 2.827)	1.30E-04	1.49 (0.89-2.47)	0.126572
Risk score	2.718 (2.13-3.47)	< 0.0001	2.74 (2.13-3.52)	< 0.0001

no significant correlation between the prognostic signature and infiltration of other immune cells including B cells (Figure 9B), CD4+ T cells (Figure 9C), CD8+ T cells (Figure 9D), dendritic cells (Figure 9E), and neutrophils (Figure 9F).

DISCUSSION

In this study, we comprehensively analyzed the transcriptome RNA-seq data of GC and identified 4259 DEGs. Among all DEGs, 181 up-regulated and 354 down-regulated DEGs were IRGs. These differentially expressed IRGs were mainly enriched in immune-related processes and pathways such as humoral immune response, phagocytosis, B cell mediated immunity, and cytokine-cytokine receptor interaction. Humoral immune response is in association with the progression of GC[16]. B cells play a role in regulating the immune response in GC. B cell depletion may be a useful strategy to enhance anti-tumor immune response[17,18]. To explore the potential molecular mechanisms of GC, we also analyzed TFs among all DEGs. The results showed that 67 TFs were differentially expressed in GC. Functional enrichment analysis confirmed that these TFs have the function of TF activity. Thus, it is necessary to further explore the potential mechanisms of TFs we identified in the pathogenesis of GC.

One hundred and eighty-three survival-related IRGs were identified for GC by Cox univariate survival analysis. Among them, 70 hub IRGs were differentially expressed and associated with overall survival of GC. PPI network analysis indicated that *IL6*, *F2R*, and *AGT* were the top three hub genes. For example, a previous study has found that IL-6 could become a target to overcome chemotherapy resistance in GC[19]. These hub IGRs are mainly involved in GC-related pathways such as Rap1 signaling pathway[20], PI3K-Akt signaling pathway[21], and cytokine-cytokine receptor interaction[22]. Among 438 samples, 323 had genetic alterations. Deletions and amplifications frequently occurred for all hub IRGs. Genetic alteration could become a promising target for the therapy of GC[23]. In this study, we constructed an immune-related prognostic signature consisting of ten hub IRGs. Each hub IRG could separately predict the overall survival of patients with GC. Among them, a member of the S100 family, S100A12, as a component of ubiquitinylation complex, could be involved in β-catenin degradation[24]. A previous study found that S100A12 could be significantly associated with invasion and metastasis of GC. It has been considered as an independent prognostic factor for GC, which is consistent with our results[25]. Furthermore, CGB5 is correlated with a poor prognosis in patients with advanced GC[26,27]. It has been reported that LGR6 might be involved in the development of GC *via* the PI3K/AKT/mTOR axis[28]. However, the functions of other hub IRGs in GC remain unclear.

Our findings suggested that high risk patients had shortened overall survival time than low risk patients based on the median value of risk score. ROC curve analysis confirmed that the prognostic signature had an excellent performance. Our further analysis showed that the prognostic signature could become an independent prognostic factor after adjusting for other prognostic factors including age, gender, pathologic T, pathologic N, pathologic M, and pathologic stage. Moreover, we found that the prognostic signature was positively correlated with the level of macrophage infiltration. M2 macrophages have been shown to be associated with a poor prognosis

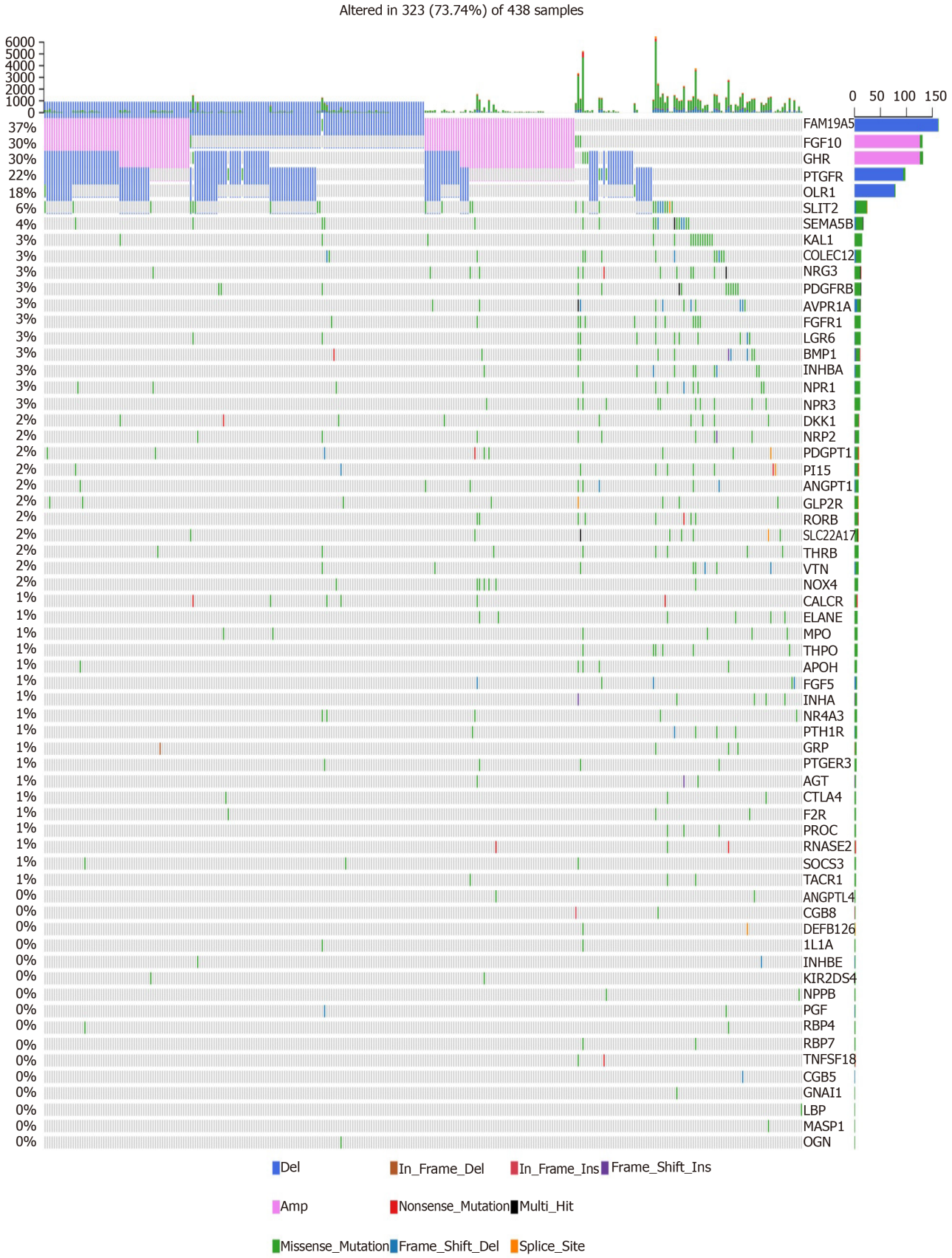
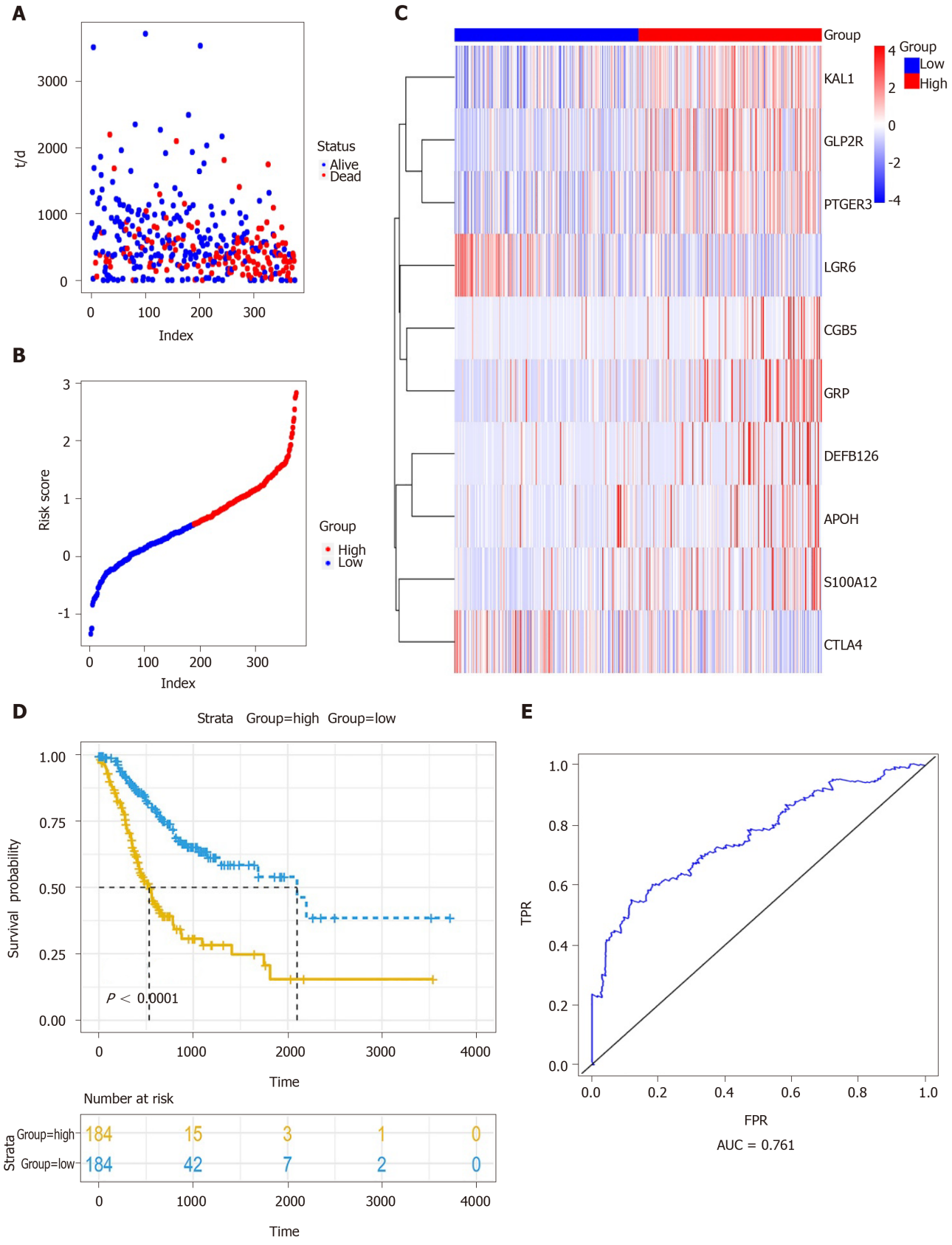


Figure 5 Mutation landscape of hub immune-related genes for gastric cancer. The horizontal axis represents different samples, and the vertical axis represents immune-related gene. Different colors represent different types of variation.

in a variety of cancers, including GC[29-31].

Our research has the following limitations. On the one hand, our prognostic markers were based on gene expression profiles. Due to their shortcomings such as


A
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Low
C
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KAL1
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LGR6
CGB5
GRP
DEFB126
APOH
S100A12
CTLA4
Group
Low
High
D
Strata Group=high Group=low
Survival probability
P < 0.0001
Time
Number at risk
Strata
Group=high 184 15 3 1 0
Group=low 184 42 7 2 0
Time
E
TPR
FPR
AUC = 0.761

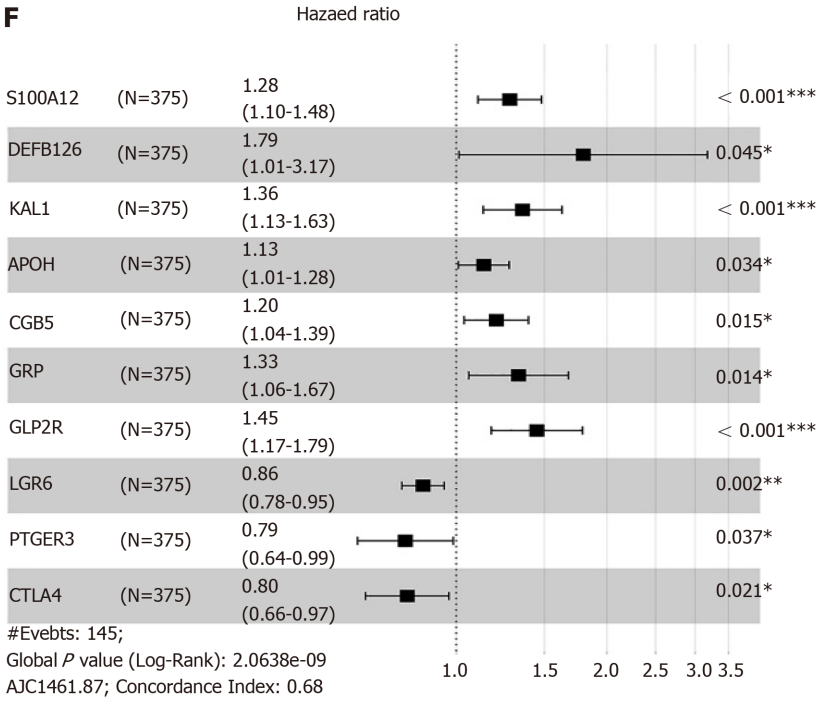


Figure 6 Construction of a prognostic signature for gastric cancer. A: Forest plot showing the prognostic values of ten hub immune-related gene (IRGs) in the prognostic signature; B: Prognostic index rank; C: Survival status of patients in the two groups; D: Heatmap showing the expression patterns of ten hub IRGs; E: Overall survival analysis results; F: The area under the receiver operating characteristic curve.

high price, long translation cycle, and high requirements for bioinformatics, it is difficult to popularize them in routine clinical applications. However, some alternative methods may be worthy of further exploration, such as screening for optimized signatures from prognostic characteristics through immunohistochemistry. On the other hand, the training cohort of the immune signature model we constructed was from retrospective studies. Therefore, the model requires to be validated by more datasets.

In summary, we have identified differentially expressed IRGs in this study, which may provide a promising perspective for the treatment of GC. We also found that the signature is positively correlated with immune cell infiltration (especially macrophages) and inflammatory responses. The immune gene signature could effectively predict GC patients' survival, which may be a useful prediction tool to identify patients who will benefit from immunotherapy.

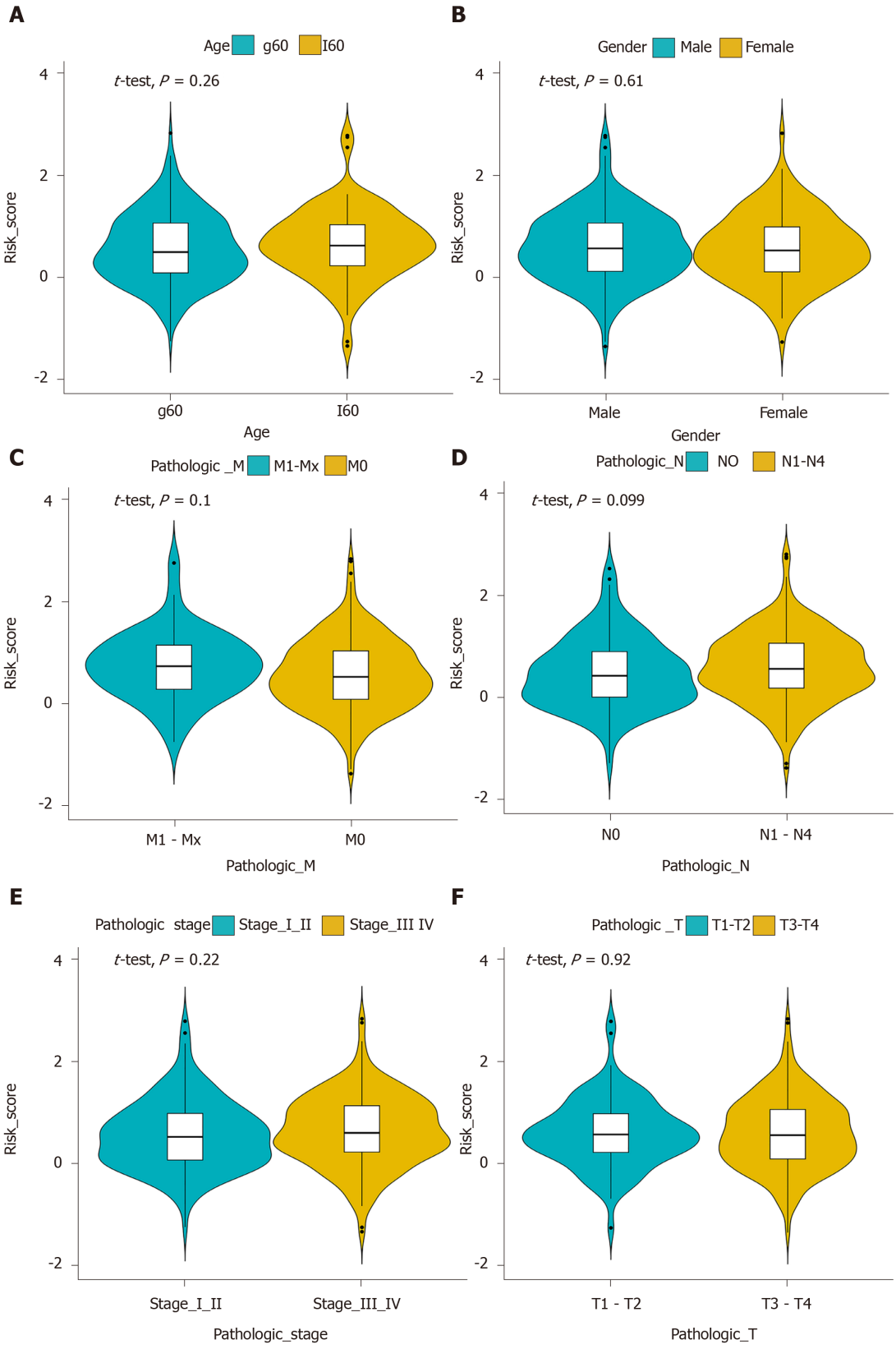
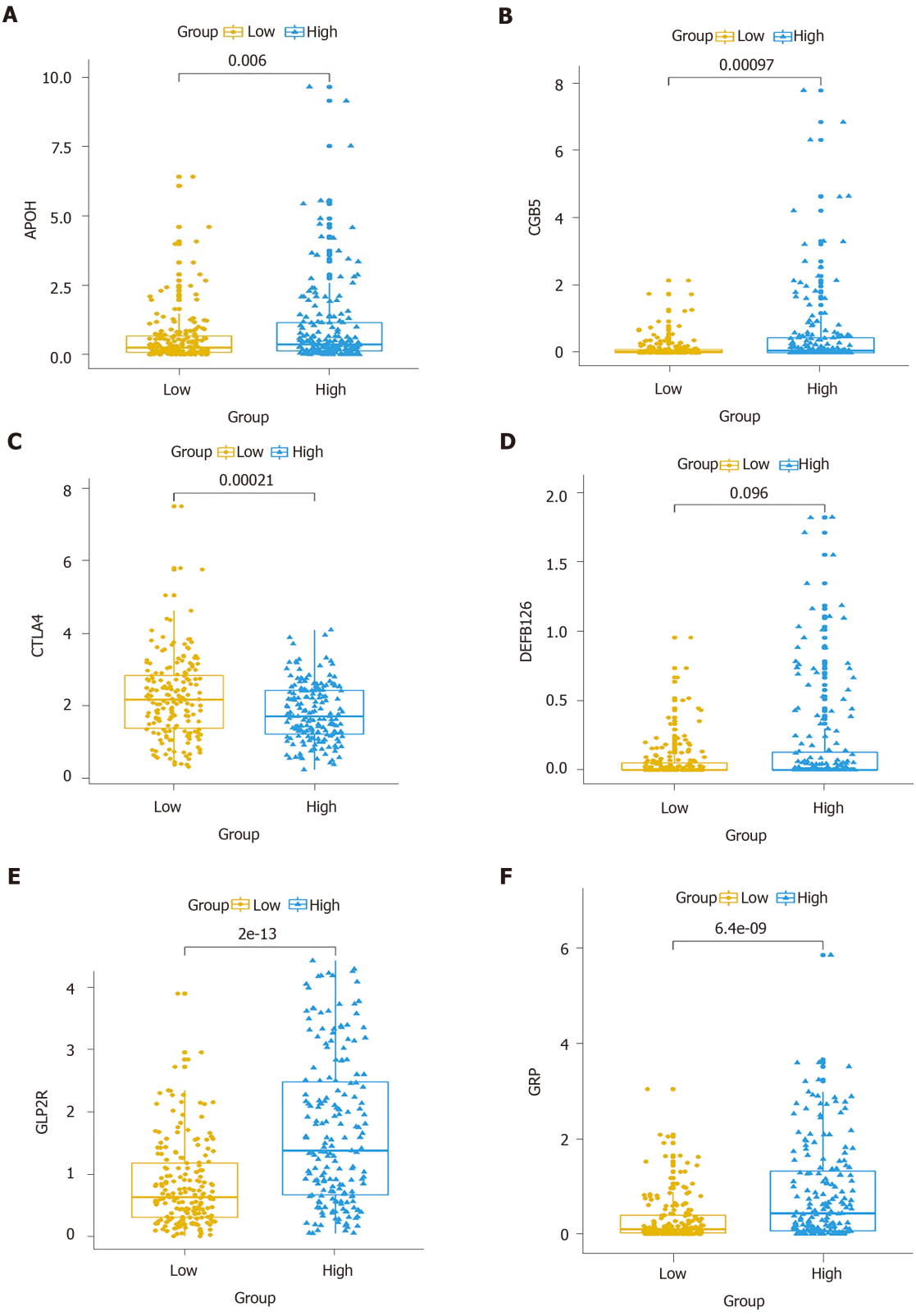


Figure 7 Correlation between clinical features and the prognostic signature for gastric cancer. The clinical features included age (A), gender (B), pathologic T (C), pathologic N (D), pathologic M (E), and pathologic stage (F).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CGB5
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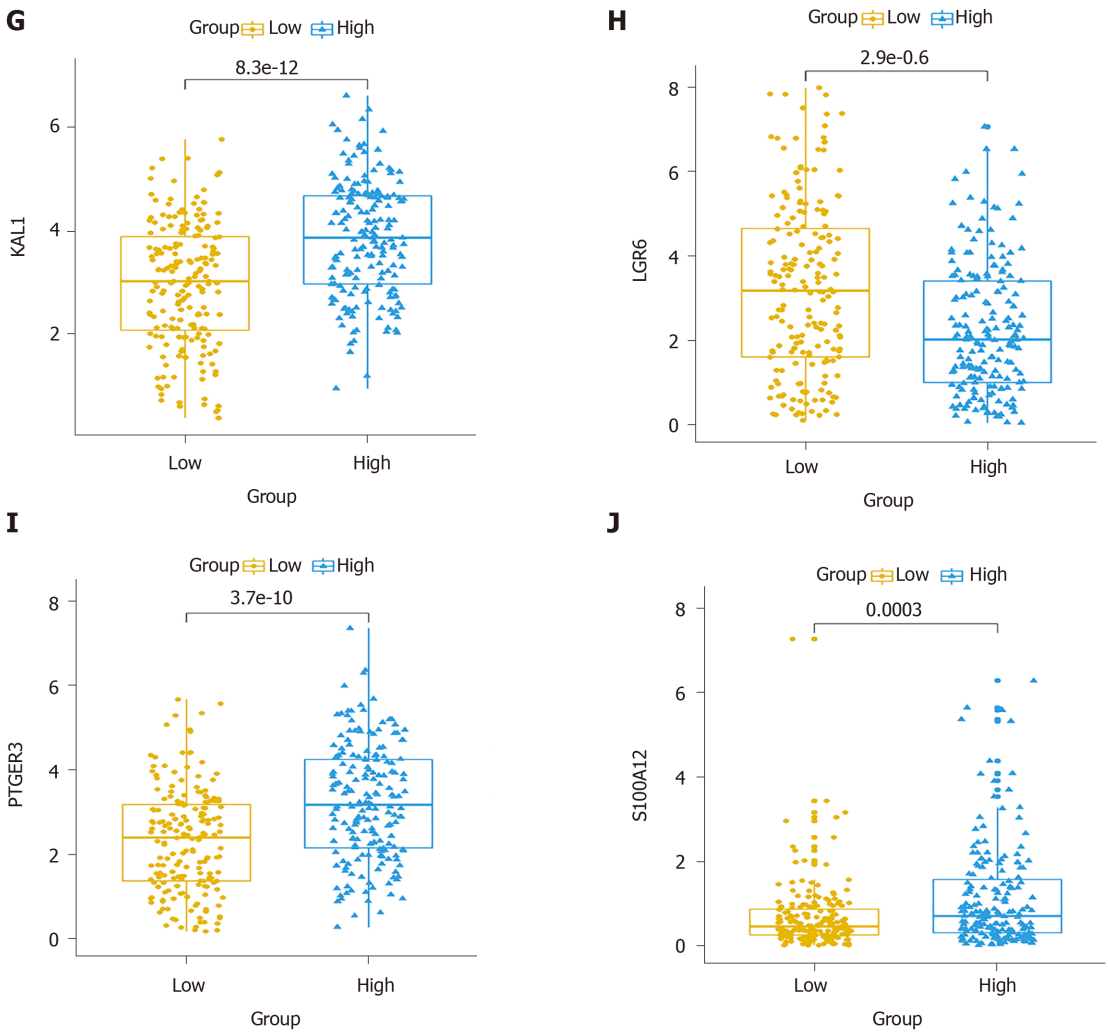
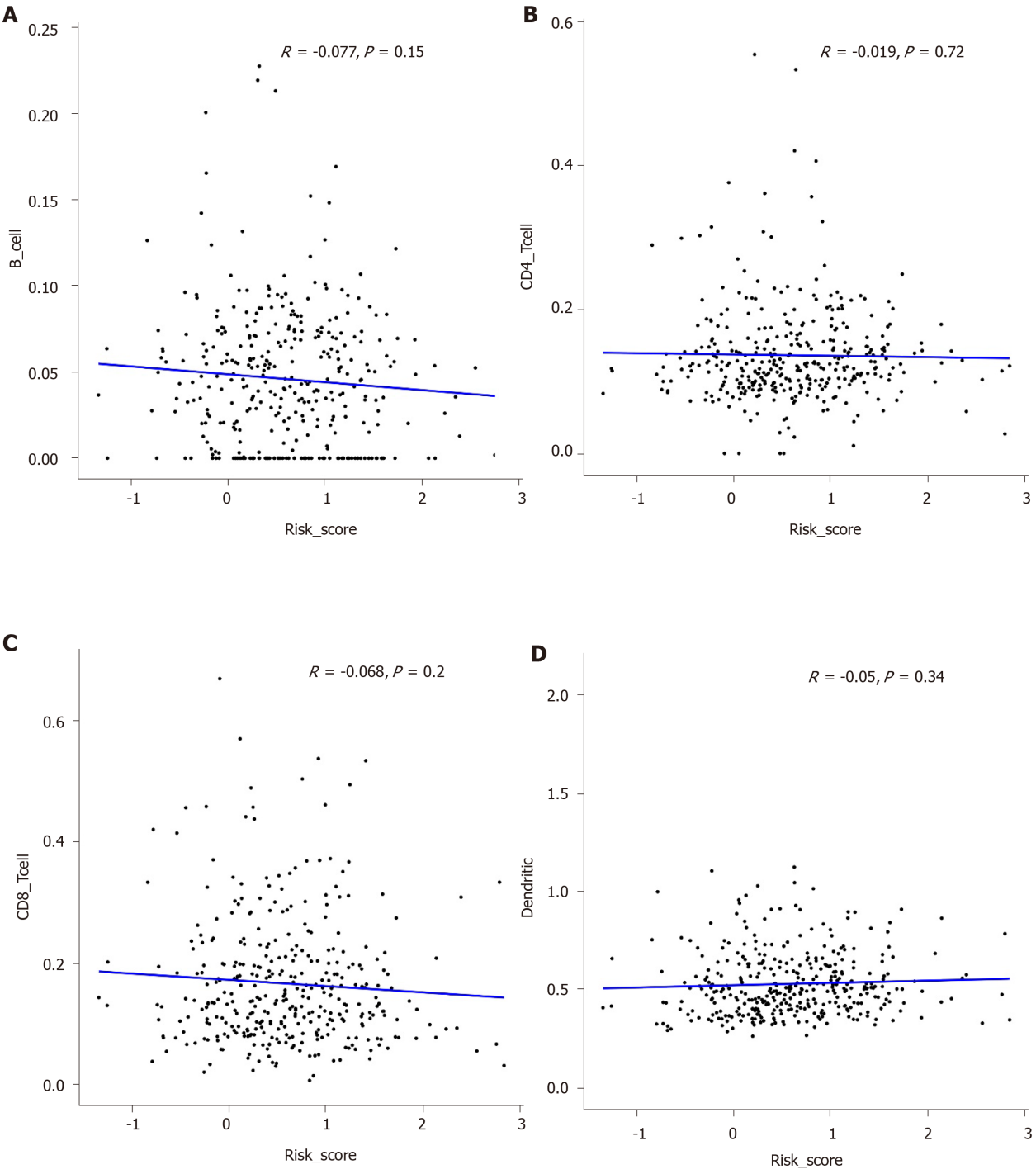


Figure 8 Expression patterns of the ten hub immune-related genes in the prognostic signature between gastric cancer tissues and normal tissues. A: *APOH*; B: *CGB5*; C: *CTLA4*; D: *DEFB126*; E: *GLP2R*; F: *GRP*; G: *KAL1*; H: *LGR6*; I: *PTGER3*; J: *S100A12*.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R = -0.077, P = 0.15
B_cell
Risk_score
B
R = -0.019, P = 0.72
CD4_Tcell
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Risk_score

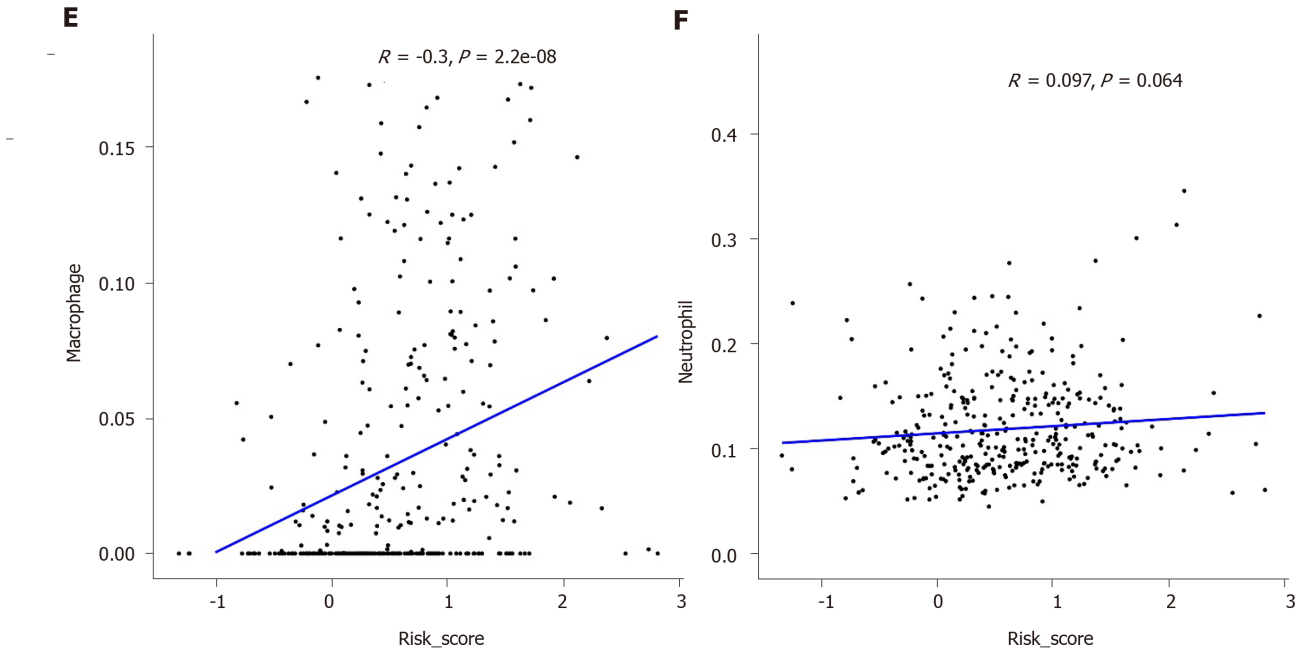

Figure 9 Correlation between the prognostic signature and immune cells including macrophages (A), B cells (B), CD4+ T cells (C), CD8+ T cells (D), dendritic cells (E), and neutrophils (F).

ARTICLE HIGHLIGHTS

Research background

Gastric cancer (GC) is the most commonly diagnosed malignancy worldwide. Increasing evidence suggests that it is necessary to further explore genetic and immunological characteristics of GC.

Research motivation

The prognosis of GC is closely related to the crosstalk between immune cells and tumor cells. Nevertheless, the role of immune-related genes in predicting GC patients' prognosis has not yet been elucidated.

Research objectives

In this study, we aimed to construct an immune-related gene signature for accurately predicting the prognosis of patients with GC.

Research methods

Cox univariate survival analysis was performed to screen survival-related immune-related genes (IRGs). Differentially expressed survival-related IRGs were considered as hub IRGs. Hub IRGs were selected to conduct a prognostic signature. Receiver operating characteristic (ROC) curve analysis was performed to evaluate its prognostic performance. The correlation of the signature with clinical features and tumor-infiltrating immune cells was analyzed.

Research results

Our study constructed a prognostic signature consisting of ten hub IRGs (including *S100A12*, *DEFB126*, *KAL1*, *APOH*, *CGB5*, *GRP*, *GLP2R*, *LGR6*, *PTGER3*, and *CTLA4*), and it could be an independent prognostic predictor for GC. Furthermore, it was significantly associated with immune cell infiltration (especially macrophages).

Research conclusions

We have proposed an immune-related prognostic signature for GC, which may possess prognostic value as a prediction tool for identification of patients who will benefit from immunotherapy.

Research perspectives

The prognostic signature could help develop treatment strategies for patients with GC in the future.

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ORIGINAL ARTICLE

Basic Study

Interleukin-1 receptor antagonist enhances chemosensitivity to fluorouracil in treatment of Kras mutant colon cancer

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Abstract

BACKGROUND

Kras mutant colon cancer shows abnormal activation of the nuclear factor kappa-B (NF-κB) pathway, resulting in the proliferation of tumor cells. Treatment with fluorouracil (5-FU) might not achieve the expected inhibition of proliferation of malignant cells based on the fluorouracil-induced activation of the NF-κB pathway.

AIM

To detect whether interleukin (IL)-1 receptor antagonist (IL-1RA) could increase the chemosensitivity to 5-FU by decreasing the activation of the NF-κB pathway and reducing the proliferation of colon cancer cells.

METHODS

Western blot analysis was performed to detect the persistent activation of the NF-κB pathway in colon cancer cell lines. Reverse transcription-polymerase chain reaction was used to detect the IL-1RA-reduced expression levels of IL-6, IL-8, IL-17, IL-21 and TLR4 in colon cancer cell lines. We used a xenograft nude mouse model to demonstrate the downregulation of the NF-κB pathway by blocking the NF-κB-regulated IL-1α feedforward loop, which could increase the efficacy of chemotherapeutic agents in inhibiting tumor cell growth.

RESULTS

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IL-1 receptor antagonist could decrease the expression of IL-1α and IL-1β and downregulate the activity of the NF-κB pathway in Kras mutant colon cancer cells. Treatment with 5-FU combined with IL-1RA could increase the chemosensitivity of the SW620 cell line, and decreased expression of the TAK1/NF-κB and MEK pathways resulted in limited proliferation in the SW620 cell line.

CONCLUSION
Adjuvant chemotherapy with IL-1RA and 5-FU has a stronger effect than single chemotherapeutic drugs. IL-1RA combined with fluorouracil could be a potential neoadjuvant chemotherapy in the clinic.

Key words: Colon carcinoma; Chemotherapy; Nuclear factor kappa-B; Interleukin-1; Proliferation; Fluorouracil

Core tip: A feedback loop between the upregulated nuclear factor kappa-B (NF-κB) pathway and interleukin (IL)-1 lads to the proliferation of cancer cells. Fluorouracil (5-FU), a chemotherapy drug used to treat colon carcinoma cells, can activate the NF-κB pathway and lead to chemotherapy resistance. IL-1 receptor antagonist combined with 5-FU has a stronger inhibitory effect on the proliferation of colon cancer cells than single 5-FU treatment due to the blockade of IL-1. This report could provide an adjuvant chemotherapy strategy for the clinic and provide a theoretical basis for neoadjuvant chemotherapy.

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INTRODUCTION

Colonicrectal cancer is the primary cause of death among gastrointestinal cancers and the third most common cancer worldwide[1,2]. Nearly 50% of patients with recurrent colon cancer undergo colon cancer procedures and chemotherapy[3], and it has been indicated that the current chemotherapy regimen may not be effective in controlling the recurrence and metastasis of the tumor[4]. Systemic toxicity and drug resistance of tumor cells are two major problems in cancer chemotherapy[5]. Therefore, various studies have explored how to reduce the toxicity of conventional chemotherapeutic drugs and increase the chemical sensitivity to achieve better curative effects of chemotherapy and gain more benefits for patients with colon cancer[6,7].

Fluorouracil (5-fluorouracil, 5-FU) is the first-choice drug for various chemotherapy regimens of colon cancer in recent decades[8]. Even the current classic chemotherapy plans, including the FOLFOX regimen and FOLFIRI regimen, contain 5-FU as a component for colon cancer treatment. This drug can inhibit the synthesis of adenylate synthetase and interfere with the synthesis of DNA in tumor cells. The growth of cells remained at a low level, and cell apoptosis was increased[9]. However, the effect of 5-FU is not ideal due to the chemoresistance in colon carcinoma patients treated with 5-FU[10]. The clinical benefit of colon cancer patients is considered to be limited, especially for those with advanced tumors[11]. Adjuvant chemotherapy has been studied to overcome the chemoresistance to 5-FU in colon cancer.

Kras mutant colon carcinoma shows persistent activation of the nuclear factor kappa-B (NF-κB) pathway, which promotes the proliferation and metastasis of tumor cells[12]. The persistently activated NF-κB pathway could promote chemoresistance to 5-FU in colon cancer treatment[13]. NF-κB is a transcription factor protein that includes five subunits: Rel (cRel), p65 (RelA, NF-κB3), RelB, and p50 (NF-κB1)[14]. The high expression of NF-κB is related to inflammatory factors and is closely related to cell

growth and proliferation[15-17]. In tumor biology, the NF-κB pathway is highly active with high expression in various tumor cells[18]. It was found that 5-FU could increase the phosphorylation of P65 in colon cancer cells, which increased the chemotherapy resistance to 5-FU in clinical treatment[19,20]. However, downregulating the NF-κB pathway increased the chemosensitivity to 5-FU in colon cancer chemotherapy[21].

Our previous studies have shown that NF-κB remains persistently activated in *Kras* mutant pancreatic cancer[22,23], which is closely related to the high expression of interleukin (IL)-1α[24]. IL-1α can increase the activity of the NF-κB pathway by upregulating AP-1 in pancreatic cancer cells[25]. Similarly, the inhibition of NF-κB activity also decreased the expression of IL-1 in pancreatic cancer cells. IL-1 and NF-κB show a cyclic relationship, which leads to persistent activation of NF-κB in tumor cells[26]. In Kras and *p53* mutant mice, we found that the NF-κB activity was downregulated by inhibiting the IL-1 receptor, which could effectively slow tumor growth. Other studies have shown that an NF-kB inhibitor had proapoptotic effects on colon cancer cells following IL-6 stimulation[27]. The aim of this study was to assess whether treatment with 5-FU combined with IL-1 receptor antagonist can increase the chemosensitivity to 5-FU by decreasing the activation of the NF-κB pathway and reducing the proliferation of colon cancer cells. The results obtained will provide a theoretical basis for clinical adjuvant chemotherapy.

MATERIALS AND METHODS

Cell lines, reagents, and animals

The normal epithelial cell line (NCM460 cell line) and the human colon carcinoma cell lines (including COLO205, SW480, HT-29, LoVo, HCT116, DLD1, SW620, LS174T, and SW1116) were purchased from Nanjing Purisi Biotechnology Company (Jiangsu, China). All cell lines were cultured in Dulbecco's modified Eagle's medium (DMEM Caisson Laboratories, Inc.).

TRIzol (American Invitrogen 15596-026); ethanol, chloroform, isopropanol (National Drug Group); cDNA first chain synthesis kit (United States Thermo Fisher K1622); and SYBR Premix Ex Taq II (Japanese TaKaRa RR820A) were used in this study. Primer design was performed by Nanjing Golden Srey Technology Co., Ltd. Substance synthesis and PAGE primer purification were also performed. The drug 5-FU was purchased from Thermo Biocompany. IL-1RA was purchased from Nanjing Purisi Biotechnology Company.

Thirty male athymic nude mice (NCI-nu), which were 4-6 weeks old and weighed approximately 24.9-33.0 g, were purchased from Nanjing Puruisi Biological Company. All mice were housed and treated at Shandong University in accordance with the guidelines of The Animal Care and Use Committee, which provided the license number SYNK (Lu) 2019-0005, and the scope of application: Barrier environment and SPF level (dogs, rabbits, rats, and mice). SW620 colon cancer cells were harvested in PBS with 20% Matrigel (Fisher Scientific). Then, all nude mice were subcapsularly injected with SW620 colon cancer cells (1.0×10^6 cells in 50 μL of PBS) in the subcutaneous tissue of the back. The effect of chemotherapy was observed in 15 nude mice with tumor loads that were euthanized by carbon dioxide inhalation (the flow rate of CO_2 used for euthanasia increased from 0% to 20% of the chamber volume per minute). Lack of breath and discoloration of the eyes were observed in all nude mice. The flow of carbon dioxide was maintained for a minimum of 1 min after respiratory arrest, and the tumor tissues were dissected (cervical dislocation was used for the approved secondary physical method of euthanasia). All mice were evaluated every 3 d to observe tumor growth during the 3-wk treatment. Tumor volume was determined as follows: $V = (\text{length} \times \text{width}^2)/2$. If multiple tumors were present, the final result was the sum of the measured results of each single tumor. The limited diameter of the tumor was 3 cm, which measured a single tumor or the sum of multiple tumors.

The survival time was observed in the other 15 nude mice, which died due to cachexia or overloaded tumors more than 3 cm in diameter. The groups were as follows: Control group (5 nude mice with PBS treatment), 5-FU group (5 nude mice with 5-FU treatment), and 5-FU and IL-1RA group (5 nude mice with 5-FU and IL-1RA treatment). For *in vivo* studies, 1.5 mg of intraperitoneal rhIL-1RA diluted with PBS was used to treat the nude mice (daily, 3 wk), and 20 mg/kg of intraperitoneal 5-FU diluted by PBS was used to treat the nude mice (twice a week, 3 wk).

Western blot assay

Cell lysates were extracted from cells with radioimmunoprecipitation assay protein

lysate buffer. The cellular extracts were boiled for 5 min to denature the protein. A total of 30 μg of protein was loaded into each well and separated on a gel. Then, the protein samples were transferred to a polyvinylidene fluoride (PVDF) membrane for 1 h at 300 mA. The PVDF membrane was blocked with 5% skim milk powder in 0.1% TBST for 1 h and incubated overnight with primary antibodies at 4°C. The primary antibodies against phosphorylated p65, p65, phosphorylated TAK1, TAK1, phosphorylated MEK, and MEK were purchased from Nanjing Puruisi Biotechnology Company and diluted 1:500. The primary antibody against IL-1α was purchased from ABcam Biotechnology Company and diluted 1:200. The secondary antibodies and β-actin antibody were purchased from ABcam Biotechnology Company.

Reverse transcription-polymerase chain reaction

The optical density (OD) values of RNA samples extracted from cells were measured at 260 nm and 280 nm. RNA concentration was calculated as OD_{260} × dilution factor × 0.04 μg/μL. The range of $OD_{260/280}$ was 1.8 to 2.1, indicating a high purity of the extracted RNA. Then, the samples were mixed with nuclease-free enzyme, oligo dT (18), and nuclease-free double-distilled water to the total volume. Mixed RNase inhibitor, reaction buffer, dNTPs, DTT (1 M), reverse transcriptase (AMV), and nuclease-free double-distilled water were added in reverse transcription-polymerase chain reaction (RT-PCR) tubes. After the cDNA reaction, the samples were subjected to amplification. The cycle conditions were as follows: Denaturation for 30 s at 95 °C, annealing for 30 s at 55 °C, and extension for 35 s at 72 °C. A total of 32 cycles were performed. The sequence information is as follows: hIL-1α-F, 3'-TCCCCAGGGACCTCTCTCTA-5' and hIL-1α-R, 3'-GAGGGTTTGCTACAACATGGG-5'; hIL-1β-F, 3'-TCGCCAGTGAAATGATGGCT-5' and hIL-1β-R, 3'-TGGAAGGAGCACTTCATCTG; hIL-6-F, 3'-TCAATATTAGAGTCTCAACCCCCA-5 and hIL-6-R 3'-GAAGGCGCTTGTGGAGAAGG-5; hIL-8-F, 3'-GCTCTGTGTGAAGGTG CAGTT-5' and hIL-8-R, 3'-ACCCAGTTTTCCTTGGGGTC-5'; hIL-17-F, 3'-TGGAATCTCCACCGCAATGA-5' and hIL-17-R, 3'-GCTGGATGGGGACAGAGTTC-5'; hIL-21-F, 3'-ACACAGACTAACATGCCCTTCA-5' and hIL-21-R, 3'-ACCGTGAGTAACTAAGAAGCAA-5'; TLR4-F, 3'-GGTCAGACGGTGATAGCGAG-5' and hTLR4-R, 3'-TTTAGGGCCAAGTCTCCACG-5'; hP65-F, 3'-ACAACAACCCCTTCCAAGAAGA-5' and hP65-R, 3'-TCACTCGGCAGATCTTGTTG-5'.

Gene silencing assay

After being treated with P65-siRNA for 48 h for interference, the SW4690 cell line was assessed for the RNA and protein levels of the target gene by RT-PCR and/or Western blot assays. The P65-siRNA oligo package was purchased from Suzhou Gemma Gene Biotechnology Company. The information of two basic P65-siRNAs is as follows: (1) siRNA1: Sense, 5'-GGCGAGAGGAGCACAGAUACC-3' and antisense, 5'-UAUC UGUGCUCCUCUCGCCUG-3'; and (2) siRNA2: Sense, 5'-CCCACGAGCUU GUAGGAAAGG-3' and antisense, 5'-UUUCCUACAAGCUCGUGGGGG-3'. The sequence of siRNA scramble (GenePharma Company, Shanghai) is: Sense, 5'-UUCUCCGAACGUGUCACGUTT-3' and antisense, 5'–ACGUGACACG UUCGGAGAATT–3'. The information of two basic P65-siRNAs is as follows: Sense: 5'-UUCUCCGAACGUGUCACGUTT-3'; antisense: 5'–ACGUGACACGUUCG GAGAATT–3'.

Cell proliferation assay

For the MTT assay, the cell suspension was seeded in each well of 96-well plates. After 12 h, attached cells were treated with various doses of 5-FU or/and IL-1RA. The cells were incubated in 4.5% CO_2 at 37 °C for 1, 2, 3, 4, and 5 d, 10 μL of MTT solution (5 mg/mL) was added to crystallize the cells for 4 h, and 150 μL of DMSO was added for 10 min to oscillate the cells. The absorbance value was measured at 490 nm. In the colony formation assay, DMEM-diluted cell suspension was inoculated in a 6-well culture dish containing 10 mL of 37 °C incubation medium at a density of 300 cells per well. After 12 h, attached cells were treated with various doses of 5-FU or/and IL-1RA. The cells were incubated in a cell incubator for 2 wk, and then, the colonies were immobilized with formalin (Sigma-Aldrich) within 30 min and stained with crystal violet (Sigma-Aldrich) within 1 h.

Statistical analysis

Commercially available SPSS version 19.0 software (Chicago, IL, United States) and GraphPad Prism software (La Jolla, CA, United States) were used for statistical

analyses. An unpaired *t*-test (one-tailed) was used to analyze the differences between groups. One-way ANOVA was used to analyze the differences among multiple groups. The log-rank test was used to analyze the differences in survival time between groups. The Bonferroni test was used following ANOVA for multiple comparisons. $P < 0.05$ was considered statistically significant.

RESULTS

The Kras gene persistently activates the NF-κB pathway in colon carcinoma cells

The abnormal activity of cancer cells was determined by the NF-κB pathway, which led to the proliferation of malignant cells, and this pathway could be persistently activated by the *Kras* mutant gene targeting TAK1 and AP1[26]. In colon carcinoma cell lines, including COLO205, SW480, HT-29, LoVo, HCT116, DLD1, SW620, LS174T, and SW1116, the activity of the *Kras* gene remained high compared with that in NCM460, a normal epithelial cell line (Figure 1A). *Kras* gene mutation in colon cancer also resulted in excessive proliferation of malignant cells through persistent activation of the NF-κB pathway[28]. The expression of phosphorylated P65 in the COLO205, SW620, and HCT116 cell lines was significantly higher than that in the NCM460 cell line, as shown by Western blot assays (Figure 1B and C). The expression of IL-1α in the COLO205, SW620, and HCT116 cell lines remained at a high level compared with that in the NCM460 cell line (Figure 1D and E).

Interleukins are expressed at high levels in NF-κB-activated colon carcinoma cells

Previous studies focused on IL-6 for growth inhibition of colon cancer cells[29]. In this study, IL-1α and IL-1β were targeted to detect their expression, which remained at a high level in colon carcinoma cells with persistent activation of the NF-κB pathway (Figure 2A and B). The continuous activation of the NF-κB pathway was confirmed to increase the expressions of IL-6, IL-8, IL-17, IL-21, and TLR4 in colon carcinoma cell lines (Figure 2C-G). The activity of the NF-κB pathway was inhibited to observe whether it could decrease IL-1α and IL-1β in the colon carcinoma cell line. The SW620 cells were treated with siRNA to downregulate the activity of the NF-κB pathway. The mRNA level of P65 was decreased by interference with siRNA1 and siRNA2 in the SW620 cell line, as shown by RT-PCR assays, compared with that of the untransfected SW620 cells (Figure 3A and B). The mRNA levels of IL-1α and IL-1β were also significantly decreased with siNF-κB interference in the SW620 cell line (Figure 3C and D). The expression levels of IL-6, IL-8, IL-17, IL-21, and TLR4 were reduced in the colon cancer cell lines after siRNA interference in the NF-κB pathway (Figure 3E-I). The results suggested that IL-1 is closely related to the NF-kappa B pathway in the SW620 cell line of Kras mutant colon carcinoma.

Inhibition of IL-1 reduces the abnormal activation of the NF-κB pathway induced by 5-FU and decreases the high expression of TAK1 and MEK

Abnormal activity of the NF-κB pathway was found in SW620 cells when they were treated with 5-FU chemotherapy. After 5-FU treatment, SW620 cells expressed high levels of phosphorylated TAK1 and phosphorylated MEK, which could explain the unexpected proliferation of drug-resistant malignant cells after chemotherapy (Figure 4A). Western blot assays showed that the expression of phosphorylated TAK1 and phosphorylated MEK was significantly higher in the SW620 cell line treated with 5-FU than in the untreated SW620 cell line (Figure 4B and D). This phenomenon in the SW620 cell line could be inhibited by 5-FU combined with IL-1 receptor antagonist, which decreased the phosphorylation of P65 and had an inhibitory effect on the phosphorylation of TAK1 and MEK. Western blot assays showed that the expression levels of phosphorylated TAK1 and phosphorylated MEK were significantly lower in the 5-FU and IL-1 receptor antagonist treatment groups than in the 5-FU treatment group (Figure 4B-D).

IL-1RA combined with 5-FU inhibits the NF-κB pathway to decrease the proliferation of colon carcinoma cells

As a chemotherapeutic agent for colon cancer, 5-FU is widely used in clinical treatment, but side effects and drug resistance can occur. First, 6.25, 12.5, and 25 mg/mL of 5-FU was used to treat the SW620 and HCT116 cell lines, and the trend of the cell growth curve was observed at 96 h by MTT assays. IL-1RA combined with 5-FU was more effective in inhibiting the proliferation of the SW620 and HCT116 cell

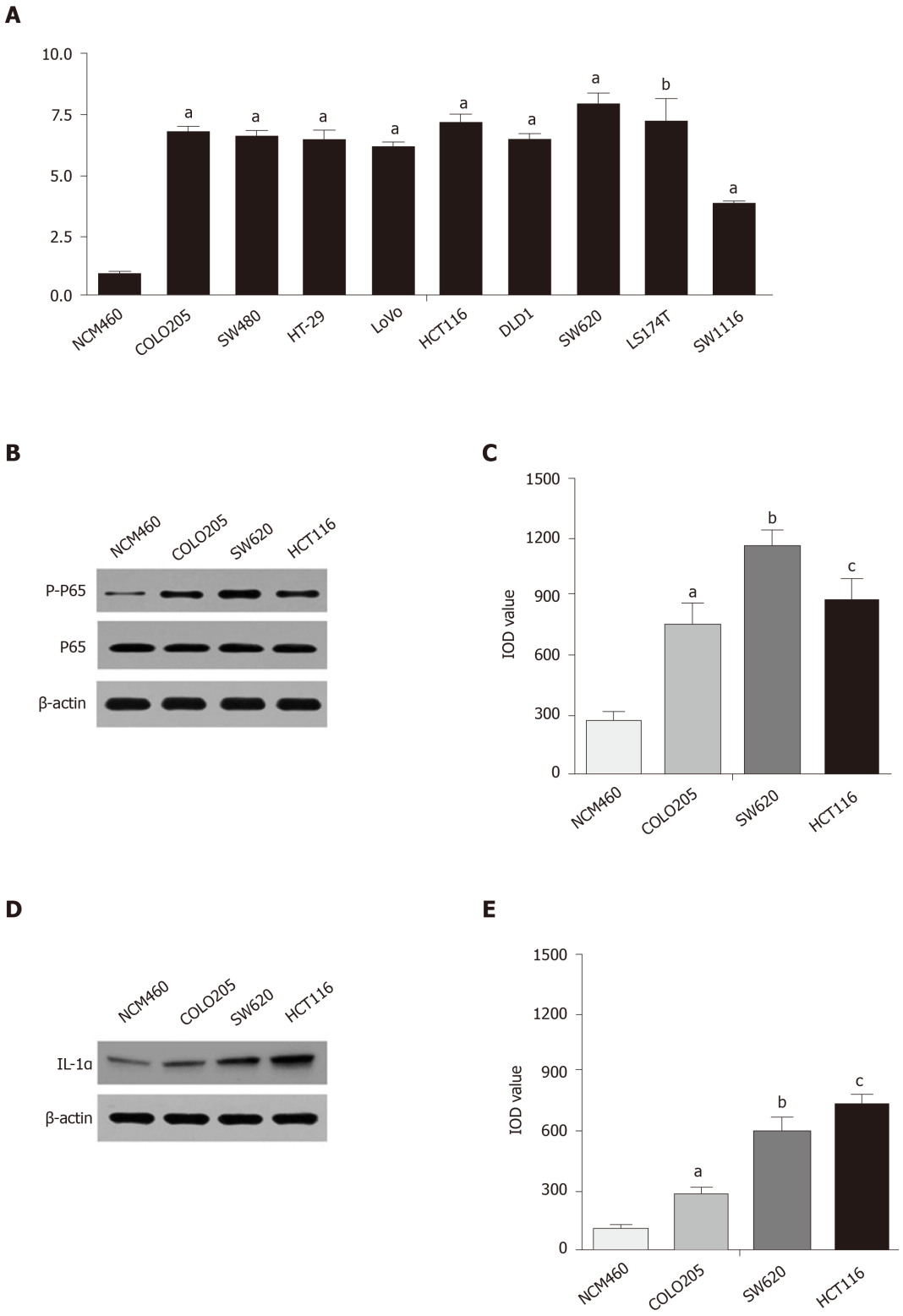


Figure 1 Expression of phosphorylated nuclear factor kappa-B in Kras mutant colon carcinoma cell lines. A: Reverse transcription-polymerase chain reaction assays showed that the expression of the *Kras* gene in the COLO205, SW480, HT-29, LoVo, HCT116, DLD1, SW620, LS174T, and SW1116 cell lines was high. The expression level of Kras in the COLO205 cell line was significantly higher than that in NCM460 ($^{a}P < 0.0001$, $^{b}P = 0.0001$ *vs* NCM460; comparison of multiple groups: $P < 0.0001$); B and C: Western blot assays showed that the expression levels of phosphorylated nuclear factor kappa-B (NF-κB) in the COLO205, SW620, and HCT116 cell lines were increased compared with that of NCM460 (COLO205 *vs* NCM460, $^{a}P = 0.0467$; SW620 *vs* NCM460, $^{b}P = 0.0050$; HCT116 *vs* NCM460, $^{c}P = 0.0177$; comparison of multiple groups: $P = 0.0081$); D and E: Western blot assays showed that the expression of interleukin-1α was higher in the COLO205, SW620, and HCT116 cell lines than the NCM460 cell line (COLO205 *vs* NCM460, $^{a}P = 0.0427$; SW620 *vs* NCM460, $^{b}P = 0.0100$; HCT116 *vs* NCM460, $^{c}P = 0.0024$; comparison of multiple groups: $P = 0.0019$).

lines than 5-FU alone. The results showed that 200 mg/mL of IL-1RA combined with 12.5 mg/mL of 5-FU could significantly inhibit cell growth (Figure 5). IL-1RA

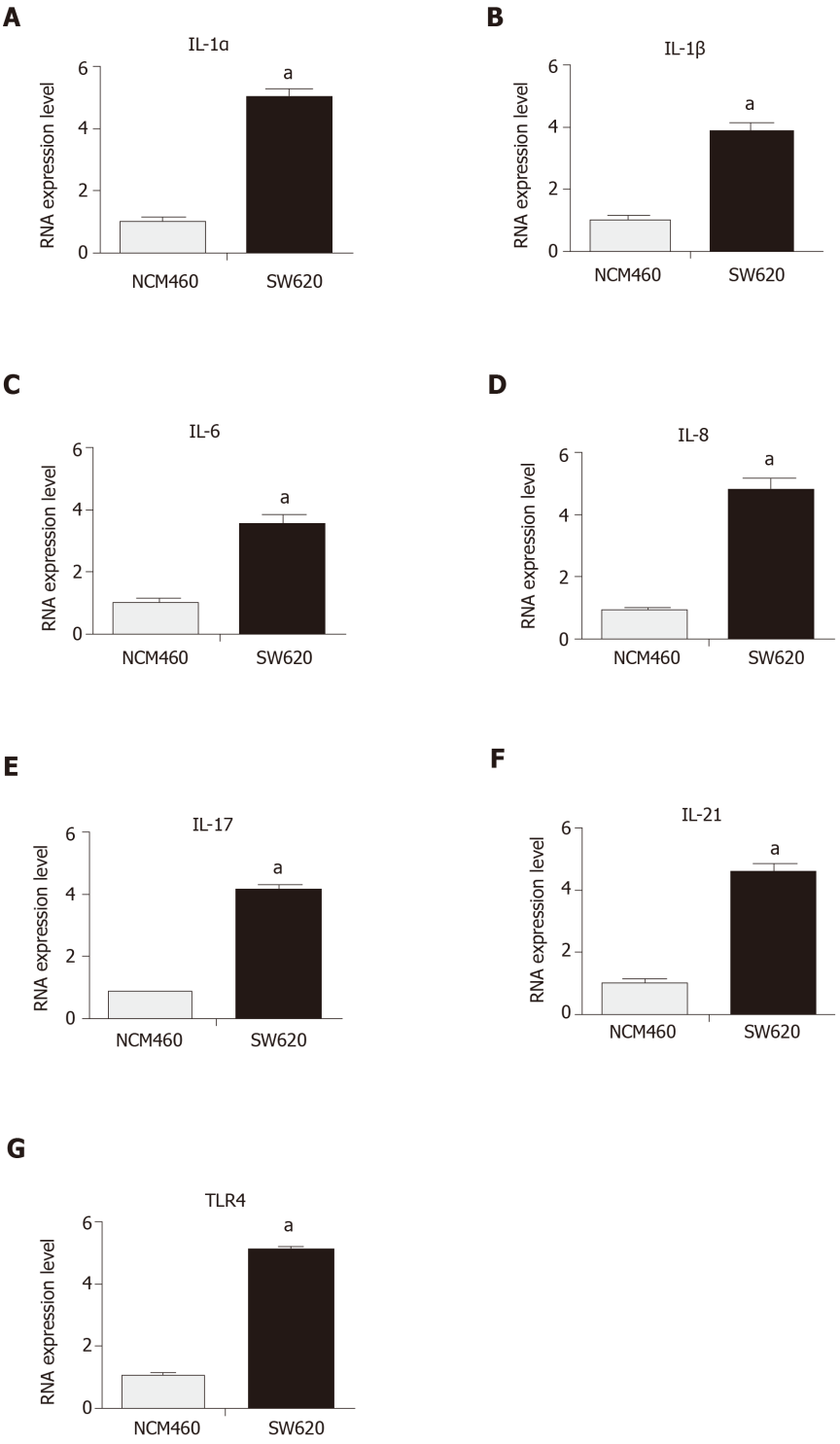


Figure 2 Expression of interleukins in the activated nuclear factor kappa-B pathway in SW620 cells. A and B: Reverse transcription-polymerase chain reaction (RT-PCR) assay showed that the expression of interleukin (IL)-1α and IL-1β was significantly higher in SW620 cells than in NCM460 cells (IL-1α: SW620 *vs* NCM460, $^{a}P = 0.0003$; IL-1β: SW620 *vs* NCM460, $^{a}P = 0.0001$); C-G: RT-PCR assay showed that the expression of IL-6, IL-8, IL-17, IL-21, and TLR4 was significantly higher in SW620 cells than in NCM460 cells (IL-6: SW620 *vs* NCM460, $^{a}P = 0.0005$; IL-8: SW620 *vs* NCM460, $^{a}P = 0.0007$; IL-17: SW620 *vs* NCM460, $^{a}P < 0.0001$; IL-21: SW620 *vs* NCM460, $^{a}P < 0.0001$; TLR4-6 : SW620 *vs* NCM460, $^{a}P < 0.0001$).

combined with 5-FU had a greater inhibitory effect on the monoclonal formation than single treatment. In clonogenicity assays, we used 200 mg/mL of IL-1RA and 6.25 mg/mL of 5-FU to treat the colon cancer cell line for 3 d. We found that the inhibitory effect of 200 mg/mL IL-1RA alone on the colony formation of tumor cells was weak, but IL-1RA combined with 5-FU had an obvious inhibitory effect on colony formation compared with 6.25 mg/mL 5-FU (Figure 6).

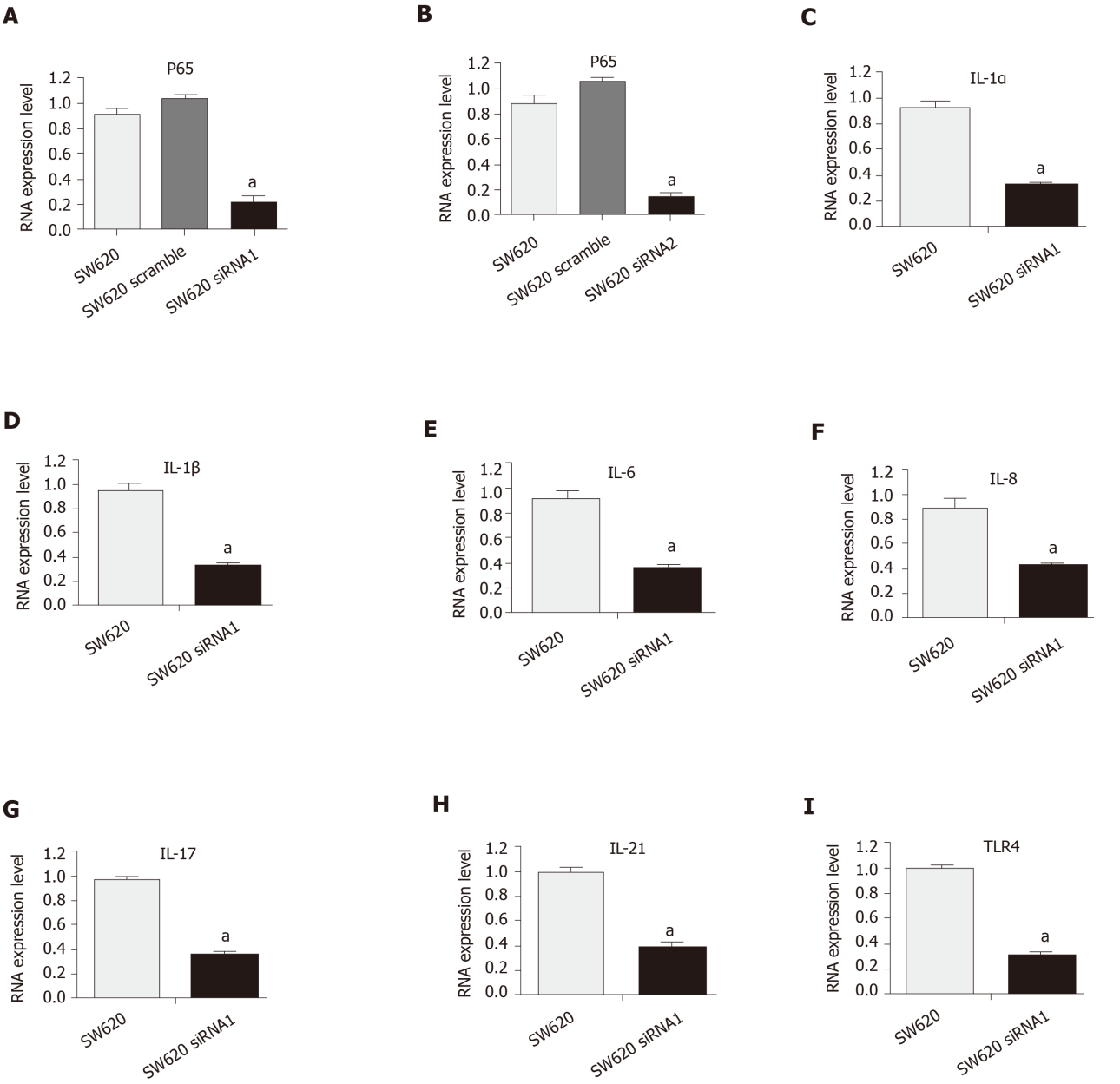


Figure 3 Expression of interleukin-1α, interleukin-1β, and other interleukins in the siRNA-P65 SW620 cell line. A: Reverse transcription-polymerase chain reaction (RT-PCR) assay showed the nuclear factor kappa-B (NF-Kb) pathway was downregulated in the SW620 cell line after siRNA1 interference at 24 h (SW620 siRNA1 *vs* SW620, $^{a}P = 0.0003$); B RT-PCR assay showed the NF-κB pathway was downregulated in the SW620 cell line after siRNA2 treatment at 24 h (SW620 siRNA2 *vs* SW620, $^{a}P = 0.0001$); C: RT-PCR assay showed that the expression of IL-1α was significantly decreased in the SW620 siNF-κB cell line compared with the SW620 non-siNF-κB cell line (SW620 siNF-κB1 *vs* SW620, $^{a}P = 0.0002$); D: RT-PCR assay showed that the expression of IL-1β was significantly decreased in the SW620 siNF-κB cell line compared with the SW620 non-siNF-κB cell line (SW620 siNF-κB1 *vs* SW620, $^{a}P = 0.0012$); E: RT-PCR assay showed that the expression of IL-6 was significantly decreased in the SW620 siNF-κB cell line compared with the SW620 non-siNF-κB cell line (SW620 siNF-κB1 *vs* SW620, $^{a}P = 0.0012$); F: RT-PCR assay showed that the expression of IL-8 was significantly decreased in the SW620 siNF-κB cell line compared with the SW620 non-siNF-κB cell line (SW620 siNF-κB1 *vs* SW620, $^{a}P = 0.0033$); G: RT-PCR assay showed that the expression of IL-17 was significantly decreased in the SW620 siNF-κB cell line compared with the SW620 non-siNF-κB cell line (SW620 siNF-κB1 *vs* SW620, $^{a}P < 0.0001$); H: RT-PCR assay showed that the expression of IL-21 was significantly decreased in the SW620 siNF-κB cell line compared with the SW620 non-siNF-κB cell line (SW620 siNF-κB1 *vs* SW620, $^{a}P = 0.0004$); I: RT-PCR assay showed that the expression of TLR4 was significantly decreased in the SW620 siNF-κB cell line compared with the SW620 non-siNF-κB cell line (SW620 siNF-κB1 *vs* SW620, $^{a}P < 0.0001$).

Effectiveness of the NF-κB signaling blockade through IL1RA in enhancing the chemosensitivity to 5-FU in vivo

We used a xenograft nude mouse model to demonstrate the downregulation of the NF-κB pathway by blocking the NF-κB-regulated IL-1α feedforward loop, which could increase the efficacy of chemotherapeutic agents in inhibiting tumor cell growth. The tumor size of the control group treated with PBS (100 μL/mouse) for 3 wk significantly increased compared to that of the experimental groups (Figure 7A and B).

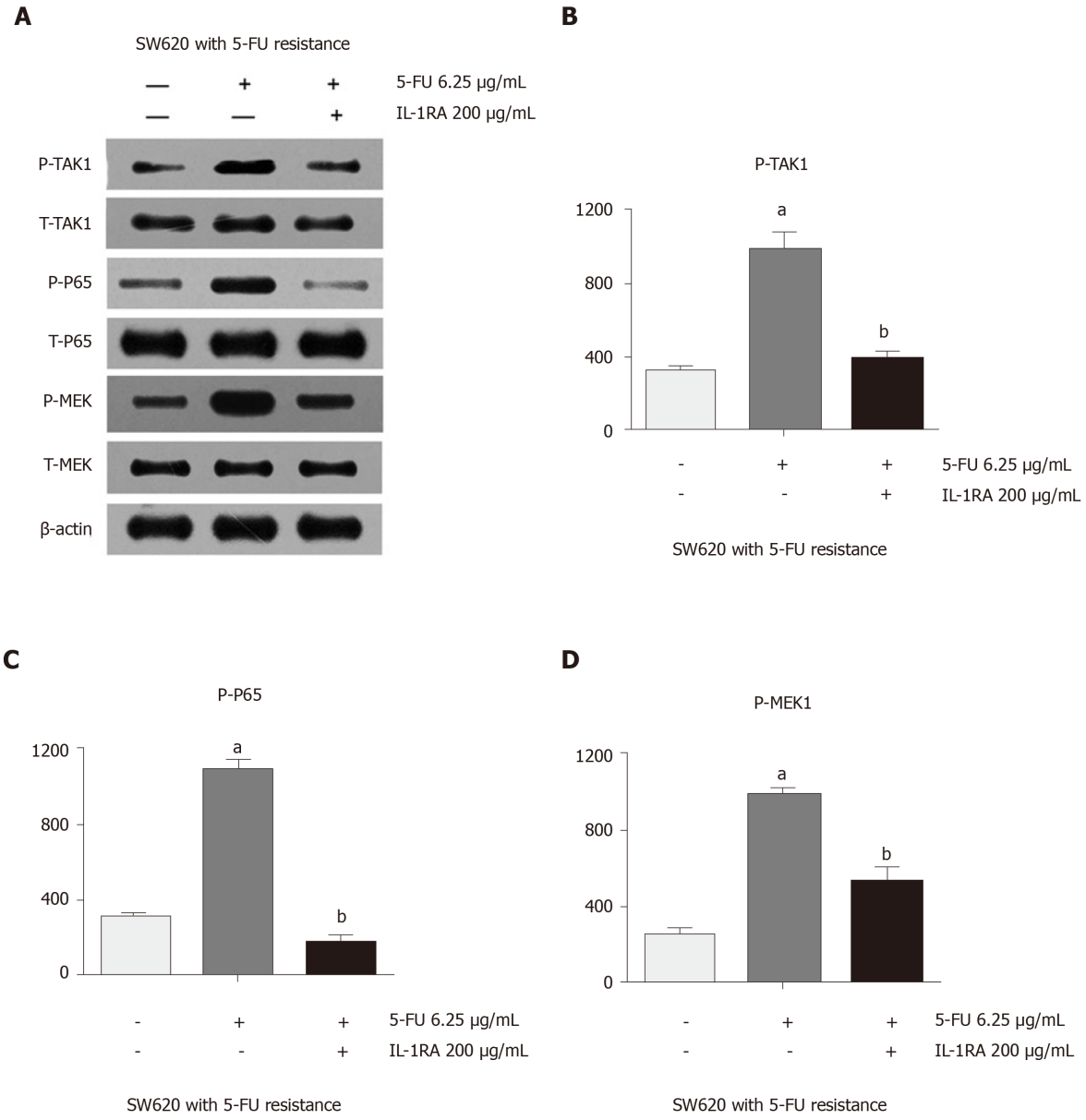
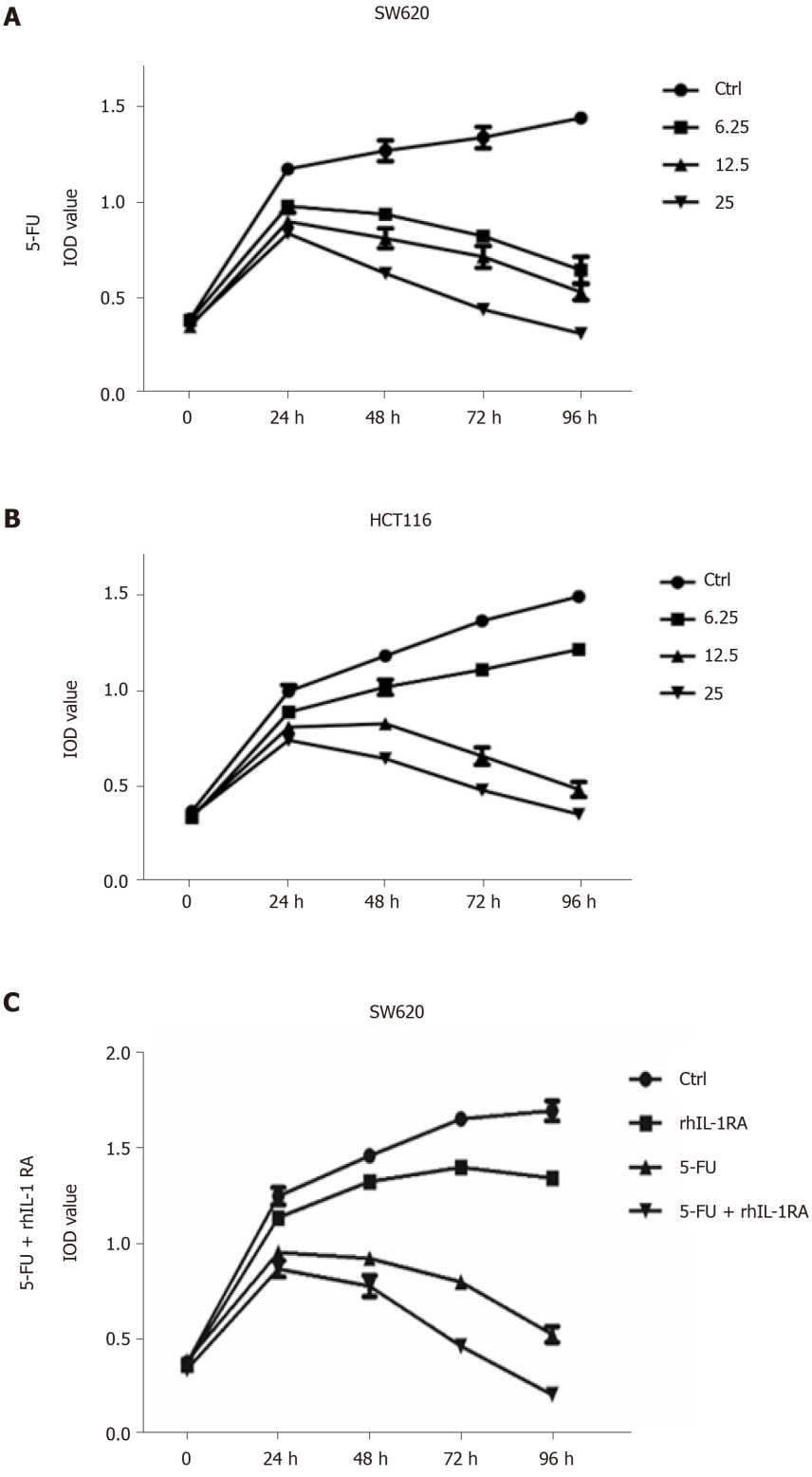


Figure 4 Interleukin-1 RA could counteract the abnormally high expression of P-TAK1, P-P65, and P-MEK caused by fluorouracil in the SW620 cell line. A: Western blot assay showed that the abnormally high expression of P-TAK1, P-P65 and P-MEK caused by fluorouracil (5-FU) was decreased by interleukin (IL)-1 RA treatment in the SW620 cell line; B: The abnormally high expression of P-TAK1 in the SW620 cell line caused by 5-FU was significantly decreased in the 5-FU and IL-1 RA group (SW620: 5-FU *vs* the control, $^{a}P = 0.0199$; 5-FU and IL-1RA *vs* 5-FU, $^{b}P = 0.0269$); C: The abnormally high expression of P-P65 in the SW620 cell line caused by 5-FU was significantly decreased in the 5-FU and IL-1 RA group (SW620: 5-FU *vs* the control, $^{a}P = 0.0048$; IL-1RA *vs* 5-FU, $^{b}P = 0.0040$); D: The abnormally high expression of P-MEK in the SW620 cell line caused by 5-FU was significantly decreased in the 5-FU and IL-1 RA group (SW620: 5-FU *vs* the control, $^{a}P = 0.0019$; IL-1RA *vs* 5-FU, $^{b}P = 0.0201$).

After 3 wk of chemotherapy, the tumor weights of the nude mice treated with 5-FU and IL-1RA were significantly decreased compared with those in the single 5-FU treatment group (Figure 7C). In the 3-wk treatment, IL-1RA combined with 5-FU treatment limited the speed of tumor growth in the nude mice, according to the changes in tumor volume (Figure 7D) and diameter (Figure 7E). The survival time of the xenograft mouse model treated with chemotherapy was significantly longer than that of the control group treated with PBS (100 μL/mouse) (Figure 7F). IL-1RA combined with 5-FU treatment had a greater effect in extending the survival time of the xenograft tumor-bearing nude mice than 5-FU single therapy.

DISCUSSION

Previous studies have found that 30%-40% of colon cancers have *Kras* gene mutations,


A
SW620
5-FU
IOD value
1.5
1.0
0.5
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24 h
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6.25
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25
B
HCT116
IOD value
1.5
1.0
0.5
0.0
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24 h
48 h
72 h
96 h
Ctrl
6.25
12.5
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C
SW620
5-FU + rhIL-1 RA
IOD value
2.0
1.5
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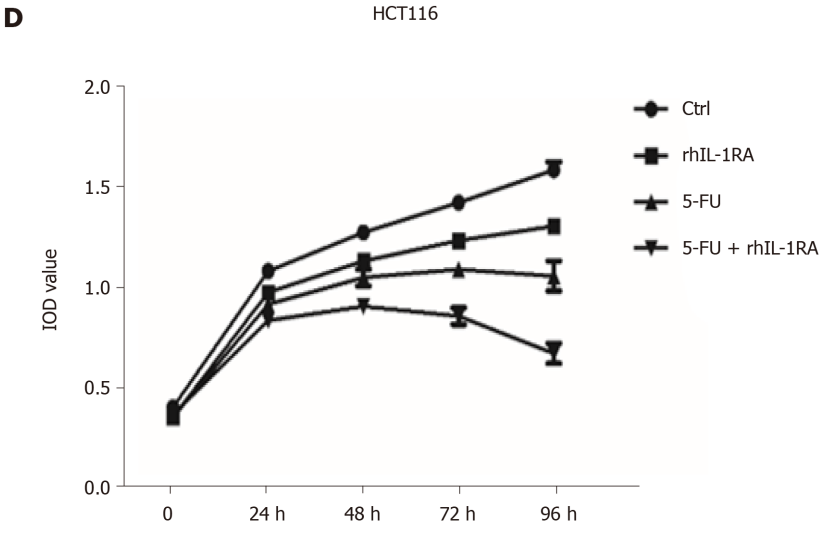

Figure 5 Changes in the growth curves of the SW620 and HCT116 cell lines treated with interleukin-1RA and/or fluorouracil. A and B: MTT assays showed that the cell growth curves of the SW620 and HCT116 colon cancer cell lines treated with fluorouracil (5-FU) at concentrations of 6.25, 12.5, and 25 mg/mL exhibited a downward trend (SW620: 5-FU at 6.25 μg/mL for 96 h *vs* Ctrl, $P < 0.0001$; HCT116: 5-FU at 6.25 μg/mL for 96 h *vs* Ctrl, $P < 0.0001$); C: MTT assays showed that the cell growth curve of the SW620 colon cancer cell lines treated with interleukin (IL)-1RA was significantly different compared with the Ctrl (SW620: IL-1RA at 200 μg/mL for 96 h *vs* Ctrl, $P = 0.0005$). The cell growth curve of the SW620 colon cancer cell lines treated with IL-1RA combined with 5-FU significantly decreased compared to that in the 5-FU group (SW620: 5-FU at 12.5 μg/mL and IL-1RA at 200 μg/mL for 96 h *vs* 5-FU at 12.5 μg/mL, $P = 0.0003$); D: MTT assays showed that the cell growth curve of the HCT116 colon cancer cell lines treated with IL-1RA was significantly different compared with that of the Ctrl (HCT116: IL-1RA at 200 μg/mL for 96 h *vs* Ctrl, $P = 0.0004$). The cell growth curve of the SW620 colon cancer cell lines treated with IL-1RA combined with 5-FU significantly decreased compared to that in the 5-FU group (HCT116: 5-FU at 12.5 μg/mL and IL-1RA at 200 μg/mL for 96 h *vs* 5-FU at 12.5 μg/mL, $P = 0.0027$).

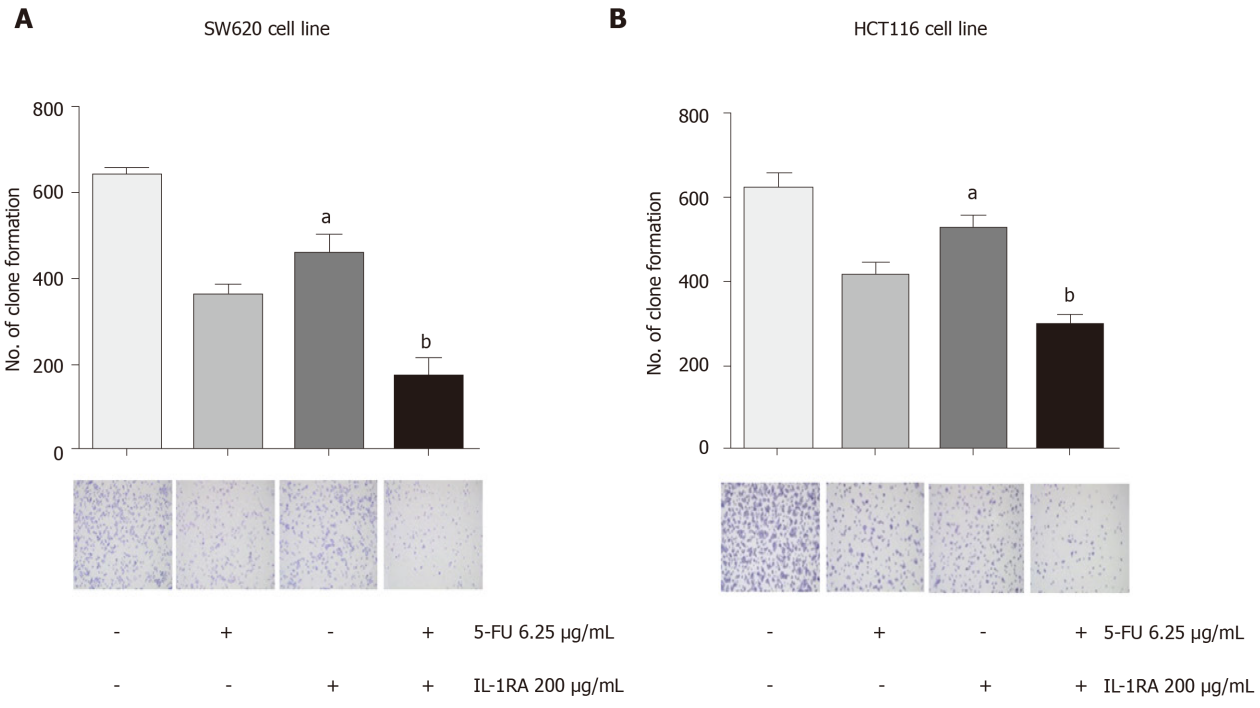

Figure 6 Changes in colony formation in the SW620 and HCT116 cell lines treated with interleukin-1RA and/or fluorouracil. A: Colony formation assay showed that interleukin (IL)-1RA single drug treatment decreased the colony formation of SW620 cells, while this parameter significantly decreased in the SW620 cells treated with 6.25 mg/mL fluorouracil (5-FU) combined with 200 mg/mLIL-1RA compared with the untreated cells (5-FU *vs* Ctrl, $P = 0.0027$; IL-1RA *vs* Ctrl, $^{a}P = 0.0226$; 5-FU and IL-1RA *vs* IL-1RA, $^{b}P = 0.0178$; comparison of multiple groups: $P = 0.0016$); B: Colony formation assay showed that IL-1RA single drug treatment decreased the colony formation of HCT116 cells, while this parameter significantly decreased in the HCT116 cells treated with 6.25 mg/mL 5-FU combined with 200 mg/mLIL-1RA compared with the untreated cells (5-FU *vs* Ctrl, $P = 0.0104$; IL-1RA *vs* Ctrl, $^{a}P = 0.0451$; 5-FU and IL-1RA *vs* IL-1RA, $^{b}P = 0.0063$; comparison of multiple groups: $P = 0.0013$).

which can promote the activity of the NF-κB pathway in carcinoma cells[30]. Currently, only four of the drugs used to treat colorectal cancer are related to genetic mutations,

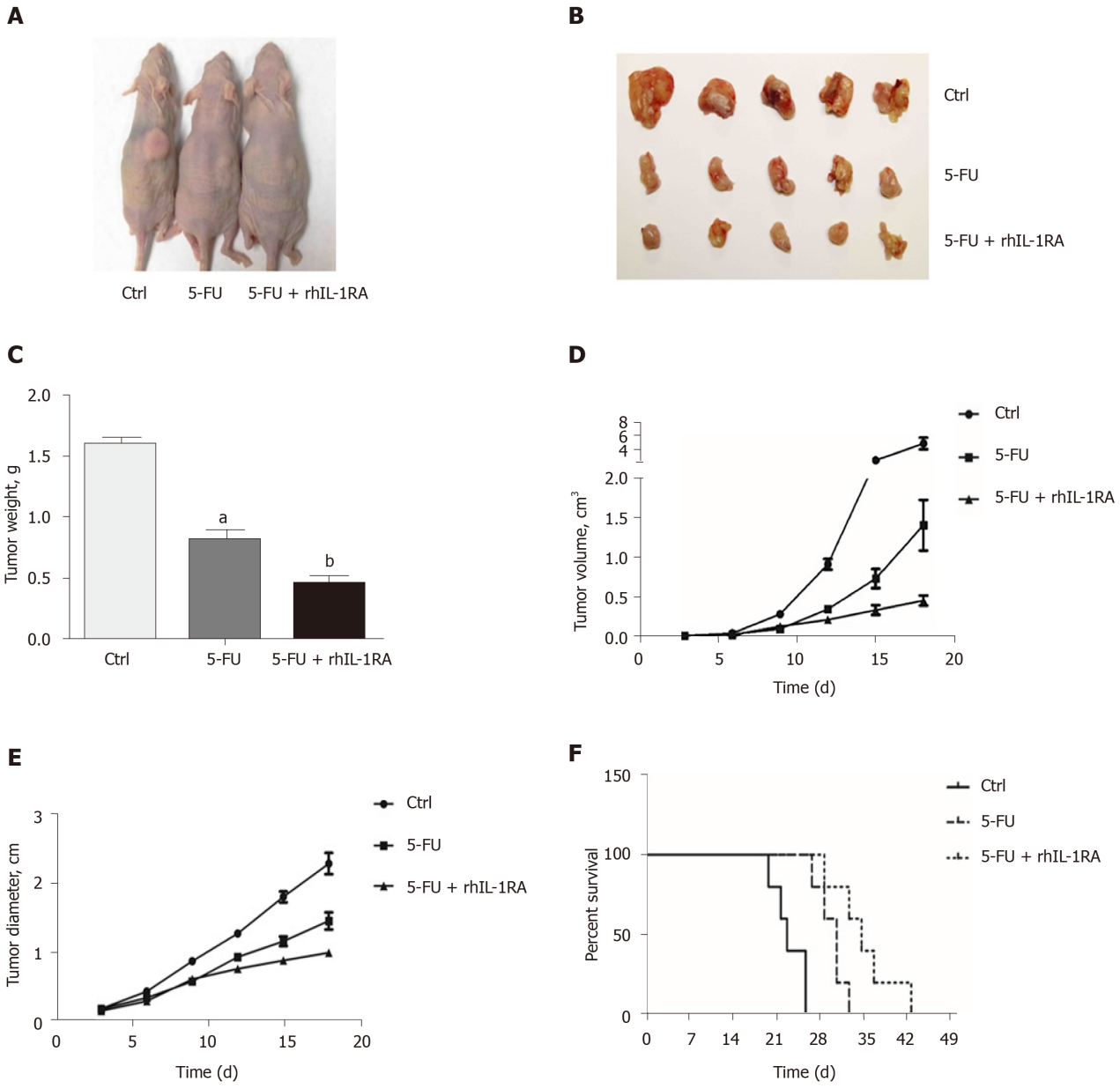

Figure 7 Interleukin-1RA enhances the chemosensitivity to fluorouracil and delays the survival time of tumor-bearing nude mice. A and B: The tumor size of the control group treated with PBS (100 μL/mouse) for 3 wk significantly increased compared to that in the fluorouracil (5-FU) group and the 5-FU and interleukin (IL)-1RA group (dose per nude mouse: 20 mg/kg of 5-FU; 1.5 mg of IL-1RA); C: The tumor weights (g) of the nude mice in the 5-FU and IL-1RA group were decreased compared with those in the 5-FU group (5-FU group *vs* Ctrl: $^{a}P < 0.0001$; 5-FU and IL-1RA group *vs* 5-FU group: $^{b}P = 0.0006$; comparison of multiple groups: $P < 0.0001$); D: The tumor volumes (cm^3) of nude mice in the 5-FU and IL-1RA group were decreased compared with those in the 5-FU group and the control group (18^{th} day: 5-FU and IL-1RA group *vs* Ctrl: $P = 0.0007$; 5-FU and IL-1RA group *vs* 5-FU group: $P = 0.0192$; comparison of multiple groups: $P = 0.0001$); E: The tumor diameter (cm) of the nude mice in the 5-FU and IL-1RA group was decreased compared with that in the 5-FU group and the control group (18^{th} day: t5-FU and IL-1RA group *vs* Ctrl: $P < 0.0001$; 5-FU and IL-1RA group *vs* 5-FU group: $P = 0.0086$; comparison of multiple groups: $P < 0.0001$); F: The survival time (d) of nude mice with xenograft tumors treated with 5-FU and IL-1RA was significantly longer than that of the nude mice with tumors treated with 5-FU (log-rank test, $P = 0.0008$; 5-FU and IL-1RA group *vs* 5-FU group: $P = 0.0413$).

and biomarkers must be detected. The detection-based biomarkers for treatment include EGFR, MSI-H/dMMR, BRAF + MEK, and NTRK fusion targets[31,32]. These patients must have wild-type KRAS/NRAS/BRAF and only left-sided tumors after gene testing. However, the 5-year survival rate of colorectal cancer is only 11%[33]. This study attempted to extend the detection of biomarkers to provide guidance for precise treatment of Kras mutant colon cancer, thus improving the patient survival rate.

IL-1 receptor antagonist targets IL-1 and competitively inhibits the activity of the NF-κB pathway in Kras mutant colorectal cancer, and it was combined with 5-FU as a neoadjuvant chemotherapy in this study. IL-1α and IL-1β, two types of IL-1, were associated with the NF-κB pathway and highly expressed in *Kras* mutant colon cancer cell lines, which suggested that IL-1 was closely related to the NF-κB pathway in colon

carcinoma. The NF-kB pathway plays an essential role in the transcriptional regulation in response to various stimuli[30]. Consistent with other studies, high expression of phosphorylated P65 could activate downstream genes and promote the proliferation of colon cancer cell lines, such as COLO205, SW620, and HCT116[34]. This change persistently activated the NF-κB pathway, which was associated with the mutant *Kras* gene as an oncogene that promotes the proliferation of colon cancer cells[34]. IL-1 and other interleukins, such as IL-6, IL-8, IL-17, and IL-21, and TLR4 are also highly expressed in colon cancer cells and are associated with the NF-κB pathway in a feedback loop[34]. In Kras mutant colon cancer cells, the activation of P65 was inhibited by siRNA, and decreased expression of interleukins was detected. Among the interleukins, IL-1α and IL-1β were sensitive to changes in the NF-κB pathway, and a significant decrease was found in the si-P65 colon cancer cell line.

Inhibition of the interleukins associated with the NF-κB pathway could reduce the proliferation of tumor cells and promote their apoptosis[35,36]. A previous study found that inhibition of IL-6 could decrease the growth of cancer cells and promote cellular apoptosis[36,37]. Furthermore, IL-1 receptor antagonist combined with 5-FU chemotherapeutic drugs may achieve enhanced effects compared to 5-FU alone in treating the colon cancer cell lines SW620 and HCT116. IL-1 RA could counteract the abnormally high expression of P-TAK1, P-P65, and P-MEK caused by 5-FU in the SW620 cell line. The anti-pyrimidine chemotherapeutic drug 5-FU is currently one of the most commonly used drugs in the clinic[38]. Clinical studies have shown that 5-FU has certain therapeutic effects on many kinds of tumors, such as digestive tract tumors, breast cancer, ovarian cancer, chorionic carcinoma, cervical cancer, liver cancer, bladder cancer, skin cancer (local smear), and leukoplakia (local smear), and inhibits the synthesis of DNA[39]. This drug has been clinically used in various chemotherapy regimens for colon cancer. Studies have shown that 5-FU has notable side effects, including bone marrow suppression, gastrointestinal reaction, and hair loss[40]. A previous study found that 5-FU chemotherapy combined with IL-6 inhibitors could better inhibit the growth of colon cancer cells than single treatment[41], and a low dose of 5-FU combined with IL-6 inhibitors can achieve the same effect and reduce the side effects of chemotherapy[42].

In this study, the combination treatment of 5-FU and IL-1 receptor antagonist in SW620 and HCT116 cell lines significantly inhibited the cell proliferation compared with single 5-FU treatment. The activation of TAK1 and high expression of MEK may lead to the proliferation of malignant cells with drug resistance to 5-FU. The chemosensitivity to 5-FU could be enhanced by IL-1 receptor antagonist, which inhibits the expression of phosphorylated P65, TAK1, and MEK in SW620 and HCT116 cell lines. In the clonogenicity assay, IL-1 receptor antagonist combined with 5-FU had a stronger effect in inhibiting cell clone formation than 5-FU alone. Therefore, treatment with IL-1 receptor antagonist combined with 5-FU can reduce the 5-FU dose to achieve an inhibitory effect on the proliferation of colon cancer cells and reduce the side effects of chemotherapeutic drugs. Notably, treatment with IL-1 receptor antagonist alone in this study did not achieve a significant inhibitory effect on the proliferation of Kras mutant colon cancer cells. A low dose of IL-1 receptor antagonist (50 mg/mL, 100 mg/mL) combined with 5-FU did not have a strong inhibitory effect on the proliferation of colon cancer cells.

In summary, the mutant *Kras* gene can promote the activity of the NF-κB pathway in colon carcinoma cells. There is a feedback loop between the upregulated NF-κB pathway and IL-1, which leads to the proliferation of cancer cells. The chemotherapy drug 5-FU can activate the NF-κB pathway and lead to chemotherapy resistance in colon carcinoma cells. IL-1 receptor antagonist combined with 5-FU has a stronger inhibitory effect on the proliferation of colon cancer cells than single drug treatment due to the blockade of IL-1. More experiments are needed to confirm and explore the underlying mechanism to provide a potential adjuvant chemotherapy for the clinic and a theoretical basis for neoadjuvant chemotherapy.

ARTICLE HIGHLIGHTS

Research background

The systemic toxicity and drug resistance of tumor cells are still two major problems in cancer chemotherapy. The chemotherapeutic drug 5-fluorouracil (5-FU) can inhibit the synthesis of adenylate synthetase and interfere with the synthesis of DNA in tumor cells. The effect of 5-FU is not ideal due to the chemoresistance in colon carcinoma patients treated with 5-FU.

Research motivation

Interleukin (IL)-1 and nuclear factor kappa-B (NF-κB) show a cyclic relationship, which leads to persistent activation of NF-κB in tumor cells. In *Kras* and *p53* gene mutant mice, we found that the activity of NF-κB was downregulated by inhibiting the IL-1 receptor, which could effectively slow tumor growth.

Research objectives

The aim of this study was to determine whether treatment with 5-FU combined with IL-1 receptor antagonist can increase the chemosensitivity to 5-FU by decreasing the activation of the NF-κB pathway and reducing the proliferation of colon cancer cells. The results obtained provide a theoretical basis for clinical adjuvant chemotherapy.

Research methods

The expression of phosphorylated P65 in the COLO205, SW620, and HCT116 cell lines was significantly higher than that in the NCM460 cell line, as shown by Western blot assays. We used a xenograft nude mouse model to demonstrate the downregulation of the NF-κB pathway by blocking the NF-κB-regulated IL-1α feedforward loop, which could increase the efficacy of chemotherapeutic agents in inhibiting colon tumor cell growth.

Research results

IL-1RA combined with 5-FU showed stronger inhibition of the proliferation of the SW620 and HCT116 cell lines than 5-FU treatment. IL-1RA combined with 5-FU treatment had a greater effect in extending the survival time of the tumor-bearing nude mice than 5-FU single therapy.

Research conclusions

IL-1 receptor antagonist combined with 5-FU has a stronger inhibitory effect on the proliferation of colon cancer cells than 5-FU alone due to the blockade of IL-1.

Research perspectives

These results could provide an adjuvant chemotherapy strategy for the clinic and provide a theoretical basis for neoadjuvant chemotherapy.

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ORIGINAL ARTICLE

Case Control Study

Clinical and pathological characteristics and prognosis of 132 cases of rectal neuroendocrine tumors

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Abstract

BACKGROUND

Neuroendocrine tumors (NETs) frequently occur in the gastrointestinal tract, lung, and pancreas, and the rectum and appendix are the sites with the highest incidence. Epidemiology statistics show that an estimated 8000 people every year in the United States are diagnosed with NETs occurring in the gastrointestinal tract, including the stomach, intestine, appendix, colon, and rectum. The pathological changes and clinical symptoms of NETs are not specific, and therefore they are frequently misdiagnosed.

AIM

To investigate the clinical symptoms, pathological characteristics, treatment, and prognosis of rectal neuroendocrine tumors (RNETs) by analyzing the clinical and pathological data of 132 RNET cases at our hospital.

METHODS

All RNETs were graded according to Ki-67 positivity and mitotic events. The tumors were staged as clinical stages I, II, III, and IV according to infiltrative depth and tumor size. COX proportional hazard model was used to assess the main risk factors for survival.

RESULTS

These 132 RNETs included 83 cases of G1, 21 cases of G2, and 28 cases of G3 (neuroendocrine carcinoma) disease. Immunohistochemical staining showed that 89.4% of RNETs were positive for synaptophysin and 39.4% positive for chromogranin A. There were 19, 85, 23, and 5 cases of clinical stages I, II, III, and IV, respectively. The median patient age was 52.96 years. The diameter of tumor, depth of invasion, and pathological grade were the main reference factors for the

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treatment of RNETs. The survival rates at 6, 12, 36, and 60 mo after operation were 98.5%, 94.6%, 90.2%, and 85.6%, respectively. Gender, tumor size, tumor grade, lymph node or distant organ metastasis, and radical resection were the main factors associated with prognosis of RNETs. Multivariate analysis showed that tumor size and grade were independent prognostic factors.

CONCLUSION
The clinical symptoms of RNETs are not specific, and they are easy to misdiagnose. Surgery is the main treatment method. The grade and stage of RNETs are the main indices to evaluate prognosis.

Key words: Neuroendocrine tumors; Prognosis; Univariate analysis; Tumor size; Tumor grade; Neuroendocrine carcinoma

Core tip: Tumor size and grade were the most significantly associated factors, and tumor size was the sole factor that was independently related to survival in a multivariate analysis. Patients with tumors larger than 2 cm had a ten-fold higher risk of death. Patients with advanced neuroendocrine carcinomas had a significantly decreased 5-year overall survival compared to patients with grades 1 and 2 disease.

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INTRODUCTION

Neuroendocrine tumors (NETs) frequently occur in the gastrointestinal tract, lung, and pancreas, and the rectum and appendix are the sites with the highest incidence[1,2]. Epidemiology statistics show that an estimated 8000 people every year in the United States are diagnosed with NETs occurring in the gastrointestinal tract, including the stomach, intestine, appendix, colon, and rectum. Most NETs follow a benign course. However, some display malignant characteristics. NETs are believed to arise from various neuroendocrine cells and are graded histologically according to markers of cellular proliferation[3].

Rectal NETs (RNETs) only account for 1% to 2% of rectal tumors, but represent a high proportion of gastrointestinal NETs. Compared to gastrointestinal NETs in other locations, RNETs have a relatively small average volume and unique biological behavior[4,5]. RNETs are more common in patients from 40-60 years of age, most occur as a single tumor, and the clinical symptoms and signs of RNETs are not typically observed in RNETs[6,7]. Although surgical treatment is still the first choice for the treatment of RNETs, with the continuous development of endoscopic technology, as well as endoscopic treatment with less trauma, faster recovery, and decreased cost, more people choose endoscopic treatment[8].

In this study, we retrospectively analyzed the pathological characteristics and prognosis of 132 patients with RNETs at our hospital according to the classification and nomenclature of NETs of the digestive system, and investigated the clinicopathological characteristics and treatment of RNETs.

MATERIALS AND METHODS

Patients

The study was approved by the Ethics Committee of Tianjin Union Medical Center in January 2015. The Ethics Committee approved related screening, treatment, data collection, and follow-up of these patients, and all subjects signed a written informed

consent form. All research was undertaken following the provisions of the Declaration of Helsinki. Patients with the following criteria were included: (1) Confirmed RNETs according to the 2006 European NET Association gastrointestinal NETs grading recommendations and 2010 World Health Organization (WHO) digestive system tumor classification criteria; (2) Complete clinical examination and case data; (3) Age at least 18 years; and (4) Available resected samples.

Pathological grade of RNETs

NETs were graded histologically according to Ki-67 IHC staining and mitosis[9]. The grades were defined as follows: G1, < 2 mitotic events per 10 high power fields (HPFs) and Ki-67 index < 3%; G2, ≥ 2 and ≤ 20 mitotic events per 10 HPFs, and Ki-67 index ≥ 3% and ≤ 20%; G3: > 20 mitotic events per 10 HPFs, and Ki-67 index > 20%. G3 is also sometimes referred to as neuroendocrine carcinoma (NEC).

Clinical stage of RNETs

Currently, there is no staging system for NETs of all locations. Based on anatomical location, RNETs were staged as clinical stages I, II, III, or IV according to infiltrative depth and tumor size as follows[10-13]: Stage I, invasion into the lamina propria or submucosa with the greatest dimension ≤ 2 cm; stage II, invasion into the muscularis propria, or the greatest dimension > 2 cm with invasion of the lamina propria or submucosa, or invasion through the muscularis propria into the subserosal tissue without penetration of the overlying serosa; stage III, invasion into the visceral peritoneum (serosal) or other organs or adjacent structures, or lymph node involvement[10]; and stage IV, distant metastasis.

Immunohistochemical staining and analysis

Resected samples were fixed in 4% formaldehyde solution. After conventional dehydration and paraffin embedding, samples were sectioned at a thickness of 4 μm. Hematoxylin and eosin (HE) staining and immunohistochemical staining were performed. Primary antibodies against synaptophysin (Syn) (Zhongshan Golden Bridge Biotechnology, Beijing, China; ZM-0246, dilution 1:100), chromogranin A (CgA) (Zhongshan Golden Bridge Biotechnology; ZA-0076, dilution 1:100), and Ki-67 (Zhongshan Golden Bridge Biotechnology; ZM-0167, dilution 1:200) were used. Positive and negative controls were used for each antibody. Ten unique fields of each section under a light microscope (400 ×) were selected for image analysis, and the numbers of positive cells and total cells were counted. In this study, immunohistochemical staining for Syn and CgA was used to validate the diagnosis of RNET. Positive expression of Syn and CgA was defined as > 30% of positive cells. The percentage of Ki-67 positive cells was defined as the number of positive cells in 100 tumor cells.

Data collection and follow-up

Patient data, including tumor size, lymph node and distant metastasis, endoscopic morphology characteristics, treatment, pathological diagnosis, postoperative complications, and the survival rate, were retrospectively analyzed. All patients were followed by outpatient visits or telephone, and June 30, 2012 was employed as the end of the follow-up.

Data analysis

SPSS 19.0 statistical software (IBM Corporation, United States) was used for data analyses. Count data are expressed as ratios. COX proportional hazard model was used for analyzing tumor prognostic factors. $P < 0.05$ was considered statistically significant. Survival time of patients with RNETs was analyzed using the Kaplan-Meier method and differences were assessed using the log-rank test.

RESULTS

Clinical characteristics

From December 2005 to May 2012, a total of 132 patients were diagnosed with RNETs at our hospital. The median patient age was 52.96 years. In addition, 62.9% (83) patients were male and 37.1% (49) were female. The main clinical symptoms included anal bulge discomfort, blood in the stool, and bowel habit change. Approximately 1/3 of the patients did not exhibit any symptoms, and RNETs in these patients were

discovered by colorectal cancer screening.

Operation and postoperative grading

One hundred and eighteen (89.4%) tumors occurred mainly in the middle and upper rectum (the distal margin of the tumor was more than 8 cm from the anal margin) and four (3%) occurred near the anal dentate line. All of the tumors were located in the rectum and were 15 cm from the anal verge. Tumor diameter was 0.2-1.0 cm in 60 cases, 1.1-2.0 cm in 45 cases, and > 2.0 cm in 27 cases. Most G1 and G2 RNETs displayed as a protruded mass on colonoscopy (Figure 1A, a and b), and ulcers were detected in some cases, as is common in NECs (Figure 1A, c). There were 19, 85, 23, and 5 cases of clinical stages I, I, III, and IV disease, respectively, according to the 2006 European NET Society of Gastrointestinal NET classification and the 2010 WHO digestive system tumor classification. All 132 patients received surgical treatment, including 113 patients who underwent endoscopic local tumor resection, in whom pathological results confirmed that 12 cases were basal positive. Eight cases of laparotomy and 11 cases of endoscopic surgery were performed. Miles surgery was performed in 4 patients, 14 patients underwent anterior resection, and 1 patient received combined rectal and metastatic liver resection.

In the treatment of RNETs, the diameter of tumor, depth of invasion, and pathological grade were the main factors used for comprehensive evaluation. Ultrasound endoscopy was often used to detect the tumor size and depth of invasion. The tumor less than 1 cm in diameter had a lower probability of metastasis, and most of them were G1/G2 RNETs. Endoscopic resection of tumor was used if the tumor has not invaded the muscularis propria. If the tumor infiltrated into the muscularis propria, local surgical removal was required. For patients with RNETs more than 2 cm in diameter, the probability of distant metastasis was greatly increased. Imaging examination was used to exclude distant metastasis. Presacral resection or total mesorectal excision was feasible for those patients without distant metastasis. For tumors with a diameter of 1-2 cm, local resection of tumor was used for those patients whose tumor did not metastasize and the invasive depth did not reach the muscularis propria. For patients whose tumor invasive depth reached or exceeded the muscularis propria, presacral resection or total mesorectal excision should be used. For NEC patients without distant metastasis, it should be treated as adenocarcinoma regardless of the diameter of the tumor. After surgery, adjuvant radiotherapy and chemotherapy were given according to the pathological stage. For RNET patients with distant metastasis, surgery was only used for relieving local symptoms, such as obstruction and bleeding.

Pathologic and immunohistochemical features

The typical morphological characteristics of RNETs included adenoid, trabecular, and papillary structures. The tumor cells were small and regular based on the HE staining (Figure 1B, a-c). Based on Ki-67 index and mitotic events per 10 HPFs, it was showed that 83 cases were G1 (Figure 1B, d), 21 were G2 (Figure 1B, e), and 28 were NEC (Figure 1B, f). One hundred and eighteen (89.4%) cases were positive for Syn by immunohistochemical staining (Figure 2A and B), and 52 were positive for CgA (Figure 2C and D). Four cases were found to have a single distant organ metastasis, and 1 case had multiple distant organ metastases. Thirteen cases were postoperatively confirmed to have lymph node metastases (Figure 2E), and intravascular tumor thrombus was found in 2 cases (Figure 2F).

Follow-up

The follow-up period of the 132 patients ranged from 3 to 60 mo, and effective follow-up was completed in 102 (77.3%) patients. Thirty patients were lost to follow-up. The survival rates at 6, 12, 36, and 60 mo after operation were 98.5%, 94.6%, 90.2%, and 85.6%, respectively. The 5-year survival rates of patients with NETs and neuroendocrine carcinoma were 96.2% and 25.0%, respectively. Twenty-eight patients with NECs received postoperative adjuvant therapy, 9 received postoperative chemotherapy alone, 4 received radiotherapy alone, and 15 received both radiotherapy and chemotherapy. At least one follow-up was performed in 25 patients with NECs and the follow-up rate was 89.28%. The average follow-up time was 38 mo. As of June 30, 2012, 19 patients died of NECs. Metastasis including 9 cases of liver metastasis occurred in 13 patients.

COX regression analysis

Univariate COX regression analysis showed that gender, tumor size, lymph node

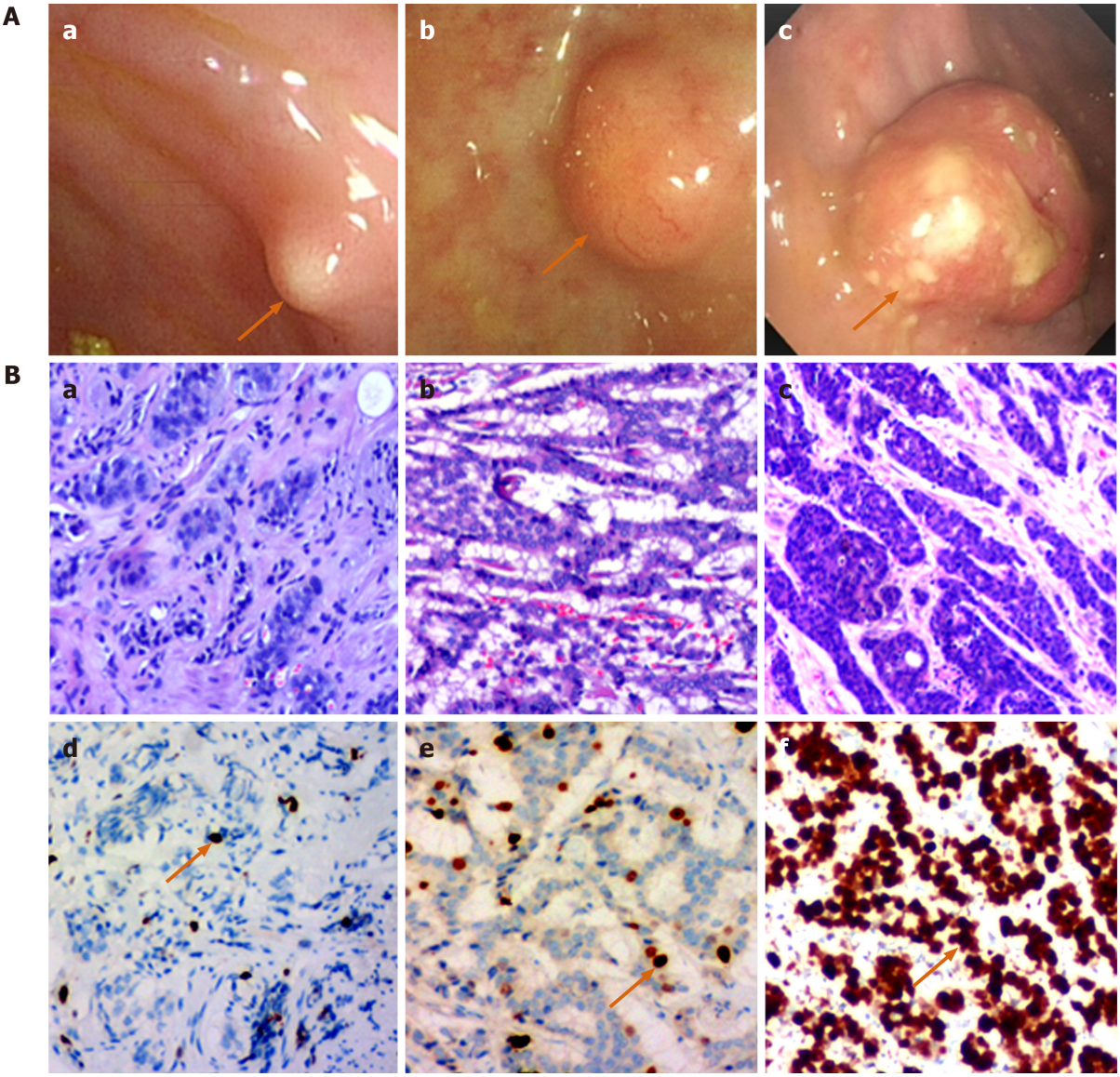

Figure 1 Representative rectal neuroendocrine tumor morphology and histochemistry. A: a. Gross morphology of a G1 rectal neuroendocrine tumor (RNET) (black arrow heads the tumor); b. Gross morphology of a G2 RNET (black arrow heads the tumor); c. Gross morphology of a neuroendocrine carcinoma (black arrow heads the tumor). The diameter of the rectal tumor was approximately 2.0 cm, and it was 11 cm away from the anus. Pathological examination confirmed that it was a neuroendocrine carcinoma. B: a. Hematoxylin and eosin (HE) staining of a G1 RNET (× 100); b. HE staining of a G2 RNET (× 100); c. HE staining of a rectal neuroendocrine carcinoma (× 100); d. Ki-67 staining in a G1 RNET (black arrow heads the positive staining; × 100); e. Ki-67 staining in a G2 RNET (black arrow heads the positive staining; × 100); f. Ki-67 staining in an rectal neuroendocrine carcinoma (black arrow heads the positive staining; × 100).

metastasis, radical resection, and pathological grade influenced the prognosis of RNETs ($P < 0.05$), while age, distant metastasis, and Syn and CgA expression had no influence on the prognosis of RNETs ($P > 0.05$) (Table 1). Of these independent variables, age, gender, tumor size, lymph node metastasis, radical resection, and pathological grade ($P < 0.01$) were considered as potential independent variables that can be used for multivariate COX regression analysis. When controlling for factors such as age and gender, tumor size was an independent factor for prognosis, the risk of death in patients with tumors ≥ 2 cm was 10.173 times that of patients with tumors < 2 cm ($P > 0.05$, Table 2). In addition, Kaplan-Meier survival analysis was used to compare the differences of 5-year survival rate in 132 cases of patients with RNETs, which showed that gender, tumor size, lymph node metastasis, radical excision, and pathological grade had statistical significance. Male patients ($\chi^2 = 4.327$, $P = 0.038$), tumors with a diameter more than 2 cm ($\chi^2 = 64.98$, $P = 0.000$), positive lymph node metastasis ($\chi^2 = 22.37$, $P = 0.000$), non-radical excision ($\chi^2 = 25.89$, $P = 0.000$), and patients with NECs ($\chi^2 = 71.79$, $P = 0.000$) were associated with poor 5-year survival (Table 3).

Table 1 Single factor COX regression analysis

		B	SE	Wald	df	*P* value	RR	95%CI	
Age (yr)	< 55	0.847	0.458	3.415	1	0.065	2.332	0.950	5.723
	≥ 55								
Gender	Male	0.850	0.429	3.918	1	0.048	2.340	1.008	5.429
	Female								
Tumor size	< 2 cm	3.177	0.748	18.046	1	0.000	23.984	5.537	103.895
	≥ 2cm								
Lymph node metastasis	No	2.290	0.480	22.743	1	0.000	9.878	3.854	25.320
	Yes								
Distant metastasis	No	0.806	0.552	2.129	1	0.144	2.239	0.758	6.608
	Yes								
Radical excision	No	-1.970	0.424	21.612	1	0.000	0.139	0.061	0.320
	Yes								
Pathological grade	G1 + G2	2.648	0.630	17.657	1	0.000	14.121	4.107	48.552
	NEC								
Syn	Negative	-0.295	0.547	0.291	1	0.589	0.744	0.255	2.175
	Positive								
CgA	Negative	-0.049	0.456	0.012	1	0.914	0.952	0.389	2.326
	Positive								

Syn: Synaptophysin; CgA: Chromogranin A; NEC: Neuroendocrine carcinoma.

Table 2 Multivariate COX regression analysis

	B	SE	Wald	df	*P* value	RR	95%CI	
Gender	0.331	0.476	0.481	1	0.488	1.392	0.547	3.541
Tumor size	2.320	1.151	4.065	1	0.044	10.173	1.067	97.012
Lymph node metastasis	0.613	0.628	0.954	1	0.329	1.846	0.539	6.320
Radical excision	-0.581	0.488	1.416	1	0.234	0.559	0.215	1.456
Pathological grade	-0.048	1.009	0.002	1	0.962	0.953	0.132	6.889

DISCUSSION

NETs occur in various parts of the body, and the rectum is a high incidence area of NETs. The incidence of RNETs has risen in recent years[13,14]. The incidence of rectal NETs (carcinoid tumors) in an Asian population, including Chinese patients, is 4.99 times that of non-Asian populations[15-17]. In our study, the occurrence of RNETs was higher in men than in women. Moreover, RNETs are more common in patients 40-60 years old, and the median age of our study participants was 52.96 years old, which is consistent with literature reports[18].

The pathological changes and clinical symptoms of NETs are not specific, and therefore they are frequently misdiagnosed. However, despite the heterogeneity of NECs, advanced tumors are often accompanied by metastasis and high mortality[12,19,20].

In our study, there was a significant difference in prognosis in patients with NETs and NECs. Pathological morphological observation and immunohistochemical staining are the most accurate methods to identify RNETs. The typical morphological characteristics of G1 and G2 NETs in the gastrointestinal tract include adenoid, trabecular, and papillary structures, and the tumor cells are small and regular. Eosinophilic granules can be seen in the cytoplasm. Most tumor cell nuclei are round

Table 3 Difference in 5-year survival rate in 132 patients with neuroendocrine tumors by age, gender, tumor size, lymph node metastasis, distant metastasis, radical excision, pathological grade, and synaptophysin and chromogranin A expression

		n	5-yr survival rate	χ^2	*P* value
Age (yr)	< 55	73	83.6	0.554	0.457
	≥ 55	59	88.1		
Gender	Male	83	80.7	4.327	0.038
	Female	49	93.9		
Tumor size	< 2 cm	105	98.1	64.98	0.000
	≥ 2cm	27	37		
Lymph node metastasis	No	111	91.9	22.37	0.000
	Yes	21	52.4		
Distant metastasis	No	127	86.6	2.765	0.096
	Yes	5	60		
Radical excision	No	16	43.8	25.89	0.000
	Yes	116	91.4		
Pathological grade	G1 + G2	104	99	71.79	0.000
	NEC	28	35.7		
Syn	Negative	14	78.6	0.629	0.428
	Positive	118	86.4		
CgA	Negative	80	83.8	0.568	0.451
	Positive	52	88.5		

Syn: Synaptophysin; CgA: Chromogranin A; NEC: Neuroendocrine carcinoma.

and of similar size, and mitotic events are rare. Rectal adenocarcinoma and RNETs are not always easy to distinguish, and accurate diagnosis depends on the detection of tumor markers, such as CgA and Syn, by IHC. In our study, 89.4% and 39.4% of tumors were positive for Syn and CgA, respectively.

Surgical resection is the main treatment option for RNETs, although the postoperative long-term prognosis is different between different grades and stages. The European Society for Medical Oncology guidelines state that surgery is the first treatment choice for gastrointestinal NETs[21]. Five-year survival rates of patients with RNETs can reach 80%-100%[19,22,23]. In our study, the 5-year survival rate was 96.2%, which is consistent with previous studies. The pathological grade of RNETs was based on the cell proliferation index (Ki-67 IHC staining) and the number of mitotic events per 10 HPFs, and the stage was based on infiltrative depth and tumor size. We did not observe any significant differences in survival between patients with stage I disease receiving local therapy and those who underwent radical surgery. Therefore, for patients with stage I RNETs, we recommend less invasive local surgery for treatment. For patients with stage II RNETs, due to the invasive depth of the tumor, there is a potential risk of lymph node metastasis. Therefore, we suggest curative resection. For patients with stages III and IV disease, due to the existence of obvious lymph node metastasis and distant organ metastasis, radical surgery might be the best choice.

Early NETs could obtain good benefit through endoscopic resection[24]. Another study performed in Chinese patients found that endoscopic submucosal resection and surgical treatment achieved satisfactory results and good prognosis in patients with RNETs. However, other reports indicate that mucosal stripping has a higher rate of complete resection than mucosal resection in early RNETs. The selection of operation mainly depends on the size of the tumor. Endoscopic resection can be performed in mucosal or submucosal layer NETs when the maximum diameter is ≤ 1 cm, and transanal resection, low rectal anterior resection, and mesorectal excision should be performed in patients with metastasis to the broad base or myometrial invasion or in patients with lymph node metastasis when the maximum tumor diameter is > 2 cm[25].

The prognosis of NETs is associated with many factors. The single factor analysis in

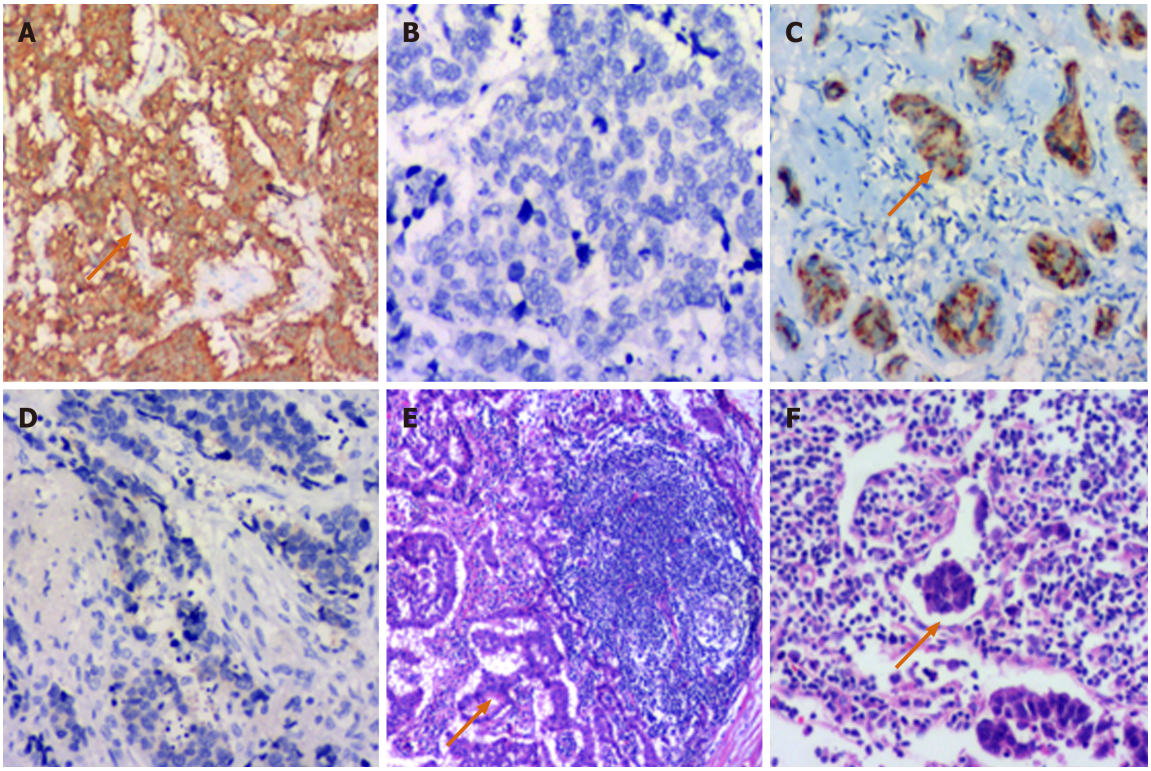

Figure 2 Immunohistochemical features. A: Positive synaptophysin immunohistochemical staining in a G2 rectal neuroendocrine tumor (RNET) (black arrow heads the positive staining; × 100). B: Negative synaptophysin immunohistochemical staining in an RNEC (× 100); C: Positive chromogranin A immunohistochemical staining in a G1 RNET (black arrow heads the positive staining; × 100); D: Negative chromogranin A immunohistochemical staining in an RNEC (× 100); E: Lymph node metastasis of a G2 RNET (black arrow shows the tumor tissue; hematoxylin and eosin staining, × 100); F: Black arrow shows a thrombus in the lymph vessel (hematoxylin and eosin staining, × 100).

our study showed that the prognosis of RNETs was correlated with tumor size, tumor stage, lymph node and vascular metastasis, and the degree of radical resection. A multivariate analysis showed that tumor size was an independent prognostic factor for RNETs. Zhang *et al*[26] found that the prognosis of colorectal NETs was closely related to WHO classification. They indicated that metastasis and the overall survival rate were statistically different in differently graded groups. Shields's study found that tumor size was an independent risk factor affecting lymph node metastasis of colorectal NETs and was closely related to the prognosis of colorectal NETs. Another Chinese study reached a similar conclusion, but it also concluded that the focal depth of invasion and lymphatic invasion were important prognostic factors for colorectal NETs. Consistent with their conclusion, for the 12 patients who received endoscopic partial resection with positive basement in our study, metastasis occurred in 3 patients, and 2 patients died from the disease.

In conclusion, different grades and stages of RNETs have obviously different prognoses. The main treatment option is surgical excision. Determining the proper surgical methods is based on the size of the primary tumor. Endoscopic therapy can be used at early stages, and the operative method for late stage RNETs is similar to other malignant tumors. There are many factors influencing the long-term prognosis of RNETs. Early detection and radical surgery are still the best choices for the treatment of RNETs.

ARTICLE HIGHLIGHTS

Research background

Epidemiology statistics show that an estimated 8000 people every year in the United States are diagnosed with NETs occurring in the gastrointestinal tract, including the stomach, intestine, appendix, colon, and rectum. The pathological changes and clinical symptoms of NETs are not specific, and therefore they are frequently misdiagnosed.

Research motivation
The aim of this study was to investigate the clinical symptoms, pathological characteristics, treatment, and prognosis of rectal NETs (RNETs).

Research objectives
To analyze the clinical and pathological data of 132 RNET cases at our hospital.

Research methods
All RNETs were graded according to Ki-67 positivity and mitotic events. The tumors were staged as clinical stages I, II, III, and IV according to infiltrative depth and tumor size. COX proportional hazard model was used to assess the main risk factors for survival.

Research results
These 132 RNETs included 83 cases of G1, 21 cases of G2, and 28 cases of G3 (neuroendocrine carcinoma) disease. Immunohistochemical staining showed that 89.4% of RNETs were positive for synaptophysin and 39.4% positive for chromogranin A. There were 19, 85, 23, and 5 cases of clinical stages I, II, III, and IV, respectively. The median patient age was 52.96 years. The diameter of tumor, depth of invasion, and pathological grade were the main reference factors for the treatment of RNETs. The survival rates at 6, 12, 36, and 60 mo after operation were 98.5%, 94.6%, 90.2%, and 85.6%, respectively. Gender, tumor size, tumor grade, lymph node or distant organ metastasis, and radical resection were the main factors associated with prognosis of RNETs. Multivariate analysis showed that tumor size and grade were independent prognostic factors.

Research conclusions
Different grades and stages of RNETs have obviously different prognoses. The main treatment option is surgical excision. Determining the proper surgical methods is based on the size of the primary tumor. Early detection and radical surgery are still the best choices for the treatment of RNETs. Gender, tumor size, tumor grade, lymph node or distant organ metastasis, and radical resection of RNETs are the main indices to evaluate prognosis.

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ORIGINAL ARTICLE

Retrospective Cohort Study

Comparison of hyperthermic intraperitoneal chemotherapy regimens for treatment of peritoneal-metastasized colorectal cancer

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Abstract

BACKGROUND

Cytoreductive surgery (CRS) in combination with hyperthermic intraperitoneal chemotherapy (HIPEC) improves patient survival in colorectal cancer (CRC) with peritoneal carcinomatosis (PC). Commonly used cytotoxic agents include mitomycin C (MMC) and oxaliplatin. Studies have reported varying results, and the evidence for the choice of the HIPEC agent and uniform procedure protocols is limited.

AIM

To evaluate therapeutic benefits and complications of CRS + MMC *vs* oxaliplatin HIPEC in patients with peritoneal metastasized CRC as well as prognostic factors.

METHODS

One hundred and two consecutive patients who had undergone CRS and HIPEC for CRC PC between 2007 and 2019 at the Medical Center of the University Freiburg regarding interdisciplinary cancer conference decision were retrospectively analysed. Oxaliplatin and MMC were used in 68 and 34 patients, respectively. Each patient's demographics and tumour characteristics, operative details, postoperative complications and survival were noted. Complications were stratified and graded using Clavien/Dindo analysis. Prognostic outcome factors were identified using univariate and multivariate analysis of survival.

RESULTS

The two groups did not differ significantly regarding baseline characteristics. We found no difference in median overall survival between MMC and oxaliplatin HIPEC. Regarding postoperative complications, patients treated with oxaliplatin

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HIPEC suffered increased complications (66.2% *vs* 35.3%; $P = 0.003$), particularly intestinal atony, intraabdominal infections and urinary tract infection, and had a prolonged intensive care unit stay compared to the MMC group (7.2 d *vs* 4.4 d; $P = 0.035$). Regarding univariate analysis of survival, we found primary tumour factors, nodal positivity and resection margins to be of prognostic value as well as peritoneal cancer index (PCI)-score and the completeness of cytoreduction regarding peritoneal carcinomatosis. Multivariate analysis of survival confirmed primary distant metastasis and primary tumour resection status to have a significant impact on survival and likewise peritoneal cancer index-scoring regarding peritoneal carcinomatosis.

CONCLUSION

In this single-institution retrospective review of patients undergoing CRS with either oxaliplatin or MMC HIPEC, overall survival was not different, though oxaliplatin was associated with a higher postoperative complication rate, indicating treatment favourably with MMC. Further studies comparing HIPEC regimens would improve evidence-based decision-making.

Key words: Colorectal cancer; Peritoneal carcinomatosis; Cytoreductive surgery; Hyperthermic intraperitoneal chemotherapy; Chemotherapy; Mitomycin

Core tip: We evaluated the therapeutic efficiency of cytoreductive surgery in combination with two different hyperthermic intraperitoneal chemotherapy (HIPEC) regimens, comparing mitomycin C HIPEC *vs* oxaliplatin HIPEC. We therefore retrospectively evaluated 102 patients undergoing cytoreductive surgery and HIPEC and statistically analysed demographics, perioperative complication and survival outcome. We found no difference in median overall survival between mitomycin C and oxaliplatin HIPEC. Regarding postoperative complications, patients treated with oxaliplatin HIPEC suffered an increased complication rate. Regarding multivariate analysis of survival, primary distant metastasis and primary tumour resection seem to have a significant impact on survival and likewise peritoneal cancer index-scoring regarding peritoneal carcinomatosis. Further prospective studies comparing HIPEC regimens would improve therapeutic decision-making.

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INTRODUCTION

Among patients with resected colorectal cancer (CRC), approximately 50% develop distant metastases either synchronously or metachronously. Most common locations are liver (35%-55%), lungs (10%-20%) and peritoneal carcinomatosis (PC) (10%-25%)[1]. In the past, the median overall survival (OS) of patients diagnosed with PC of CRC origin was 4-7 mo, for patients undergoing palliative surgery or 5-fluorouracil (5-FU)-based systemic chemotherapy[2-4]. Improvement in systemic chemotherapy, using chemotherapeutic agents such as oxaliplatin and irinotecan, along with anti-angiogenesis molecular targeting agents cetuximab and bevacizumab, led to an increased OS of about 12 mo[5].

The introduction of multimodal treatment strategies including systemic chemotherapy and cytoreductive surgery (CRS) plus hyperthermic intraperitoneal chemotherapy (HIPEC) showed promising progress in long-term survival. The HIPEC procedure is intended to destroy any remaining tumour cells after surgical tumour debulking by local administration of chemotherapy to the peritoneal cavity for homogeneous drug distribution and enhanced cytotoxicity induced by heat[6]. Depending on the extent of intraabdominal tumour load, remarkable survival benefits

have been reported compared to systemic chemotherapy with 5-FU/leucovorin alone in a randomized controlled trial[7]. Median OS of selected patients with CRC PC improved to 21-63 mo with a 5-year survival rate up to approximately 58%[8]. The most frequently used cytotoxic drugs for HIPEC in CRC are mitomycin C (MMC) and oxaliplatin combined with systemic 5-FU and leucovorin[9].

Initially, HIPEC regimen was most commonly conducted with MMC but subsequently the addition of oxaliplatin became the standard systemic treatment in CRC[10-12]. This brought about a change of regimen for HIPEC with MMC being only used as salvage treatment[13]. The combination of cisplatin and MMC is also frequently used and seems to be a valid HIPEC protocol in peritoneal metastases of CR origin. Recent studies evaluating this protocol demonstrated prolonged survival with limited toxicity[14,15].

Upfront CRS with HIPEC (CRS-HIPEC) is currently the standard treatment for colorectal peritoneal metastases in eligible patients due to the proven superiority to palliative chemotherapy alone[16,17]. Nevertheless, therapeutic efficacy of this treatment strategy for CRC PC patients remains controversial due to contradicting evidence, especially regarding the value of HIPEC.

The first formal randomized controlled trial for CRC assessing the benefit of a 30 min oxaliplatin-based HIPEC added to surgery failed to show survival improvement[18]. Leung *et al*[19] demonstrated that patients with CRC treated with oxaliplatin HIPEC had better OS than those receiving MMC-based HIPEC (median survival: 56 mo *vs* 26 mo, respectively). In contrast, Prada-Villaverde *et al*[20] reported that HIPEC with MMC may be superior to oxaliplatin-based HIPEC when patients have favourable histology or a low burden of PC (median survival: 54.3 mo *vs* 30.4 mo, respectively). At present there is no prospective study that compares these two HIPEC regimens for treatment of peritoneal metastasized CRC. Thus, a reassessment of HIPEC and the need for structured treatment protocols should be addressed. In this retrospective clinical analysis, we evaluated the outcome of patients undergoing CRS HIPEC at the university medical centre of Freiburg.

MATERIALS AND METHODS

This study evaluated the outcome of 102 consecutive patients with PC of colorectal origin, who underwent CRS and HIPEC between January 2007 and March 2019 at the Medical Center of the University Freiburg (MCUF). Patients receiving HIPEC with either palliative or CRS were included.

Patients with appendiceal tumours/pseudomyxoma peritonei and PC of other origin (non-colorectal) were excluded as well as patients who were planned for HIPEC but had not received HIPEC treatment due to surgeon's intraoperative decision. HIPEC regimens were chosen regarding current available data with MMC or oxaliplatin.

From 2007 until 2014, MMC was used, and from 2014 to 2018 it changed to oxaliplatin. Analogous to PRODIGE7 trial, HIPECs since 2018 were conducted with MMC.

Informed consent was obtained from all patients before their inclusion in the cancer registry. The study was approved by the Medical Ethics Committee of the University of Freiburg (EK-FR 4/20). The analysed data was extracted from the anaesthetic protocols and the electronic health records.

Pretherapeutic work-up

Preoperative work-up started in the outpatient setting of MCUF. Previous oncological therapies and comorbidities were recorded, and pulmonary and cardiac check-ups were routinely performed in high-risk patients. Pretherapeutic diagnostics included thoraco-abdominal computerized tomography in all patients and endoscopy or diagnostic laparoscopy with biopsies when appropriate. All patients were discussed in our interdisciplinary cancer conference, and decision for CRS with HIPEC was made if a complete resection seemed achievable. Extensive liver metastases as well as extra abdominal or retroperitoneal metastases were seen as contraindication for surgical intervention.

Depending on the treating physician's protocol and interdisciplinary consensus as well as timing of diagnosis and previous chemotherapy courses, perioperative systemic therapy consisted of either neoadjuvant and adjuvant cycles of capecitabine with oxaliplatin, neoadjuvant and adjuvant cycles of 5-FU/leucovorin with oxaliplatin, or neoadjuvant cycles of 5-FU/leucovorin with irinotecan followed by capecitabine or

adjuvant cycles of fluoropyrimidine monotherapy.

For patients with intestinal obstruction, palliative resections and palliative HIPEC were considered according to interdisciplinary cancer conference decision.

Surgical therapy

The operative procedure was chosen according to the extent and location of the primary tumour and the peritoneal metastases. After explorative midline laparotomy, the complete abdominal cavity was inspected to assess the extent of peritoneal carcinomatosis, defined by the peritoneal cancer index (PCI). According to Sugarbaker's original work, the PCI system divides the abdomen and the pelvis into 13 regions. The lesions are graded according to size (0 through 3) in each abdominopelvic region and are added as a numerical score[21].

Afterwards, the Sugarbaker protocol (Sugarbaker *et al*[6], 1995) was adhered, which assessed tumour resection and resection of visceral organs and peritoneum. Here, resections were classified and subdivided into large intestine, small intestine, liver, diaphragm, omentum and peritoneum.

The Completeness of Cytoreduction (CC) Score, which quantifies the completion of CRS, was assessed after resection. Before closure of the abdominal cavity at least four 24CH silicon-drainages and a temperature probe for the HIPEC were placed.

HIPEC

Simultaneous application of cytotoxic drugs both intraperitoneal and intravenously (i.v.) was used when performing an oxaliplatin based HIPEC with 5-FU + leukovorin i.v. (bidirectional HIPEC). Cytotoxic drugs were prepared by our clinic pharmacy using saline solution as carrier solution in a 50 mL syringe. Dosage level was 30 mg/m² body surface for MMC, 300 mg/m² for oxaliplatin, 400 mg/m² for 5-FU and 20 mg/m² for leukovorin.

The chemo infusion was performed in a closed abdominal system using an extra corporal roller pump system with heat exchanger. Three silicon-drainages were used as fluid inlets and one as outlet. After establishing a stable circulation of saline solution, the cytotoxic drug was added. The degree of hyperthermia ranged between 39 °C to 43 °C using 42 °C as target level. The intraperitoneal circulating time of oxaliplatin was 30 min, respectively 90 min for MMC. After completing the circulation time, the roller pump was used to aspirate the intraabdominal fluids. Silicon drainages were left in the early postoperative setting to allow drainage of remaining accumulated fluids. All patients were transferred postoperatively to the intensive care unit (ICU) for further monitoring.

Follow-up

Perioperative complications were recorded up to 90 d after surgery and were graded according to Clavien/Dindo-Classification[22]. Grade 1 complications (minor deviation) were not recorded. Discharged patients were followed up at least once in the surgical outpatient department and referred back either to the oncology department or to a resident oncologist for further follow-up. The survival data were systematically obtained from the cancer registry of the MCUF Cancer Center. Data regarding postoperative chemotherapy were directly obtained from the resident oncologist or general physician.

Statistical analysis

The results of our study were gained by retrospective analysis of our prospective CRC databases. SPSS 22 for Windows™ was used for statistical analysis (SPSS, Armonk, NY, United States). Categorical variables were given in absolute and relative frequencies; differences were evaluated by Fisher's exact test. Quantitative values were expressed as mean ± standard deviation and medians with range, as appropriate, and differences were measured using the Kruskal-Wallis test. A Mann-Whitney-U-test was added to compare groups. Survival was univariately analysed by the Kaplan-Meier method with a log-rank test for the comparison of subgroups. Multivariate survival analysis was performed by the Cox proportional hazard model (forward selection strategy using a likelihood ratio statistic) including the report of relative risks and their 95%-confidential intervals. A P value < 0.05 was considered statistically significant.

RESULTS

Demographics

From January 2007 to March 2019, 102 patients underwent CRS-HIPEC or palliative resections and HIPEC. The cohort contained 60 male patients and 42 female patients. Sixty-eight patients were treated with oxaliplatin/5-FU HIPEC and 34 patients with MMC HIPEC. Three patients in the MMC-group received early postoperative intraperitoneal chemotherapy during the first 48 hours after CRS.

The groups were balanced regarding baseline characteristics, besides a higher American Society of Anesthesiologists (ASA) (P = 0.002) score and a higher rate of T4b (P = 0.027) tumours in the Oxaliplatin group. Median PCI-score was not statistically different across groups but was lower by trend in the Oxaliplatin group [8 (range 0-30) *vs* 12 (range 0-39) in the MMC-group; P = 0.312].

Palliative resections without cytoreduction were performed in one patient receiving oxaliplatin/5-FU HIPEC and in two patients treated with MMC-HIPEC (Table 1).

We had a loss to follow-up rate of 3.9 % (four patients). All of them were treated with MMC-HIPEC.

Perioperative results

There was no difference in the overall length of hospital stay [11.4 d (4-35)] for MMC *vs* 12.4 (2-46) for oxaliplatin; however, the oxaliplatin based HIPEC showed a significantly longer ICU stay [7.2 d (2-50) *vs* 4.4 d (2-9); P = 0.035].

Our data showed a total complication rate of 56%, with a statistically significant higher complication rate associated with oxaliplatin compared to MMC: 35% *vs* 66% (P = 0.003).

In further subgroup analysis we found an increased rate of intestinal atony (9% *vs* 29%; P = 0.015), abdominal infections (3% *vs* 21%; P = 0.013) and urinary tract infections (0% *vs* 9%; P = 0.034) for oxaliplatin HIPEC. The severity of complications, stratified according to the Clavien-Dindo classification, was also higher in the Oxaliplatin group (P = 0.029).

No patients died perioperatively, and 11 patients died during the first 90 d after surgery due to oncological or other medical reasons (Table 2).

Analysis of survival

Mean follow-up was 23.3 mo. There was no statistically significant difference recording median OS (P = 0.139). We performed a univariate survival analysis to compare potential prognostic factors. No differences in survival rates were found comparing sex, age, body mass index (BMI) and ASA-scoring (Table 3). Likewise, primary tumour location (colon *vs* rectum) did not affect survival rate in our cohort (P = 1.0). Our data showed no difference in median survival when comparing primary T-stage (49 mo for T1-3 *vs* 30 mo for T4a *vs* not reached for T4b) but a significant influence of primary nodal stage (88 mo for N0 *vs* 51 mo for N1 *vs* 30 mo for N2a and 18 mo for N2b; P = 0.013). Likewise, according to our data, synchronous diagnosis of the PC or other distant metastasis was associated with a worse median survival (57 mo for M0 *vs* 35 mo for M+; P = 0.046). Furthermore, tumour grading and primary resection level also affected median survival (Figure 1).

In addition, lower PCI-score and a CC0- resection were associated with higher median survival. Patients undergoing a simultaneous liver metastasis resection during CRS had a worse survival prognosis (51 mo *vs* 27 mo for liver metastasis resection; P = 0.024).

To analyse further survival outcome factors, we performed multivariate analysis (Cox regression) with forward selection strategy using a likelihood ratio statistic. Synchronous distant metastasis (P = 0.029) and primary tumour resection status (P = 0.016) were confirmed to have a significant impact on survival as well as PCI-scoring regarding PC (P = 0.001). After carrying out a separate multivariate analysis, adapting the cut-off P value for inclusion to include HIPEC regimen into the analysis, HIPEC regimen failed to prove significance regarding OS at a P value of 0.144 (Figure 2).

DISCUSSION

With varying evidence for the therapeutic value of CRS-HIPEC in metastatic colon cancer, attention has refocused upon standardization and optimization of this procedure. However, there is a severe lack of evidence regarding comparison of survival benefits for the most commonly utilized chemotherapeutic agents for HIPEC

Table 1 Patients, tumours and treatment, *n* (%)

	All, *n* = 102	MMC, *n* = 34	Oxaliplatin/5-FU, *n* = 68	*P* value[1]
Male sex	60 (59)	24 (40)	36 (60)	0.135
Age in yr, median (range)	57.2 (23-80)	56.3 (23-73)	57.7 (40-80)	0.884
BMI in kg/m²	25.3 (15.9-39.6)	25.5 (19.1-33.6)	25.2 (15.9-39.6)	0.266
ASA-score				0.002[b]
1-2	49 (48)	24 (71)	25 (37)	
3-4	53 (52)	10 (29)	43 (63)	
Tumour location				1.000
Colon	91 (89)	30 (88)	61 (90)	
Rectum	11 (11)	4 (12)	7 (10)	
Surgical approach				0.257
Complete cytoreduction	99 (97)	32 (94)	67 (99)	
Palliative resection	3 (3)	2 (6)	1 (2)	
Resection				
Peritoneum	81 (80)	29 (85)	52 (77)	0.437
Omentum	66 (65)	26 (77)	40 (59)	0.123
Colon/rectum	55 (54)	18 (53)	37 (54)	1.000
Small intestine	49 (48)	15 (44)	34 (50)	0.675
Liver	42 (41)	13 (38)	29 (43)	0.831
Diaphragm	16 (16)	9 (27)	7 (10)	0.045[a]
Other	63 (64)	21 (68)	42 (62)	0.655
Pretherapeutic T stage				0.027[a]
T1	2 (2)	0	2 (3)	
T2	2 (2)	0	2 (3)	
T3	34 (34)	13 (41)	21 (31)	
T4a	40 (40)	17 (53)	23 (34)	
T4b	22 (22)	2 (6)	20 (30)	
Pretherapeutic N stage				1.000
N0	26 (26)	8 (25)	18 (27)	
N+	73 (74)	24 (75)	49 (73)	
Pretherapeutic M stage				1.000
M0	36 (37)	12 (35)	24 (38)	
M+	62 (63)	22 (65)	40 (63)	
Tumour grading				1.000
G1	0	0	0	
G2	59 (63)	22 (65)	37 (63)	
G3	34 (37)	12 (35)	22 (37)	
PCI score (0-39)	9.4 (0-39)	12.0 (0-39)	8.1 (0-30)	0.312
Postop CC-level				0.350
CC0	89 (87)	28 (82)	61 (90)	
CC1	8 (8)	3 (9)	5 (7)	

CC2/3	5 (5)	3 (9)	2 (3)	
Mucinous cells	21 (21)	6 (18)	15 (22)	0.796

[1]Fisher's exact test.
[a]$P < 0.05$.
[b]$P < 0.01$. 5-FU: 5-Fluorouracil; ASA: American Society of Anesthesiologists; BMI: Body mass index; MMC: Mitomycin C; PCI: Peritoneal cancer index.

oxaliplatin and MMC. This study is one of a few to focus on prognostic factors and treatment strategies after the development of peritoneal metastasis. Furthermore, the two most commonly used cytotoxic agents were compared regarding survival benefits and outcome rates.

Oxaliplatin and MMC, both interfering with DNA and DNA-synthesis, can reach high intraperitoneal drug concentrations during HIPEC with simultaneous limited systemic absorption[23,24]. Furthermore, they have elevated cytotoxicity under hyperthermia with a concordant tissue penetration depth of 2 mm[9]. The most commonly used intraperitoneal dose for oxaliplatin is 460 mg/m² with a perfusion time limited to 30 min. In contrast, the recommended intraperitoneal dose for MMC is 35 mg/m² with a prolonged perfusion duration of 90 min[9,25,26]. With the objective of potentiating the oxaliplatin activity, patients in the Oxaliplatin group received intravenous 5-FU and folinic acid approximately 1 hour before starting intraperitoneal HIPEC circulation.

Our study shows a 3-year-survival rate of 43% after CRS/HIPEC for peritoneal metastasized CRC. We could not show any statistically significant survival benefit comparing HIPEC regimens with oxaliplatin/5-FU *vs* MMC. Nevertheless, a statistical trend towards the oxaliplatin/5-FU group was noticed (Figure 2; median survival 30 mo for MMC *vs* not reached for oxaliplatin/5-FU). In our cohort, MMC group had a trend towards a higher PCI-scoring and a smaller number of CC-0 resections, which could possibly be responsible for the observed trend towards a prolonged survival in the Oxaliplatin group as well as differences in systemic preoperative treatments regarding multi-agent and targeted systemic therapy and surgical approach.

Regarding PRODIGE 7 trial, subgroup analysis showed a significant survival benefit for CRS + oxaliplatin HIPEC *vs* CRS for a subgroup with PCI 10-15[15]. Thus, there is a need of further studies, stratifying patients by PCI and prospectively examining the relative therapeutic effectiveness of MMC and oxaliplatin.

On the other hand, our study demonstrates significant differences between the two regimes regarding postoperative morbidity and complication rates. In our collective, patients treated with oxaliplatin/5-FU suffered increased rates of postoperative complications, especially intraperitoneal infections, urinary tract infections and intestinal atony.

Postoperative morbidity has to be taken into account when selecting an appropriate cytotoxic agent. Oxaliplatin has been suggested to cause higher morbidity rates with Grade II and III complication compared to MMC[27], as confirmed in this study. Reported complications in oxaliplatin trials include fistula formation, pneumonia or intraabdominal abscess formation[28]. The PRODIGE 7 trial likewise reported enhanced complication rates for CRS + oxaliplatin HIPEC *vs* CRS. A similar study design focusing on hematologic changes after CRS and HIPEC with either MMC or oxaliplatin was not able to show an increased complication rate after oxaliplatin HIPEC but a different complication scheme[29]. Contrary to this study, our analysis focuses on surgical complications in the postoperative phase. Therefore, the difference in the results can be explained.

Increased postoperative complication rates, especially severe complications (grade IIIb and IV according to Clavien-Dindo analysis), were also associated with prolonged ICU stay for the Oxaliplatin group compared to MMC (7.2 d *vs* 4.4 d; $P = 0.035$), which adds to evidence supporting MC for CRS-HIPEC.

Furthermore, we were able to identify different primary tumour factors affecting OS in this collective of peritoneal metastasized CRC. Interestingly, clinical factors such as age, sex, BMI or even ASA-scoring at CRS-HIPEC operation time have no influence on OS. Literature describes poorly differentiated carcinoma, venous invasion, lymphatic invasion, T4 disease, lymph node metastasis, malignant bowel obstruction and adjuvant chemotherapy as having negative impact on OS[30].

Even though primary T-stage and tumour location (colon/rectum) had no influence on survival outcome, primary nodal positivity and poor differentiation grade seem to affect tumour recurrence and lower survival rates in our patients with peritoneal carcinomatosis. This agrees with numerous other studies[31-33].

Table 2 Impact of hyperthermic intraperitoneal chemotherapy regimen on perioperative outcome, *n* (%)

Parameter	Total, *n* = 102	Mitomycin, *n* = 34	Oxaliplatin/5-FU, *n* = 68	*P* value[1]
Median operative time in min	379 (95-774)	410 (95-774)	363 (96-722)	0.260
Median blood substitution in mL	105 (0-1800)	185 (0 -1800)	66 (0-1200)	0.068
Hospitalization in d	12 (2-46)	11,4 (4-35)	12,4 (2-46)	0.315
ICU stay in d	6.3 (2-50)	4.4 (2-9)	7.2 (2-50)	0.035[a]
In-hospital mortality				
Rate of complications	57 (56)	12 (35)	45 (66)	0.003[b]
Cardio-pulmonary morbidity				
Pneumonia	5 (5)	2 (6)	3 (4)	0.542
Re-intubation	2 (2)	0	2 (3)	0.442
Pulmonary embolism/thrombosis	2 (2)	0	2 (3)	0.442
Hematoma	2 (2)	1 (3)	1 (2)	0.558
Postoperative haemorrhage	4 (4)	1 (3)	3 (4)	0.593
Surgical morbidity				
Intestinal atony	23 (23)	3 (9)	20 (30)	0.015[a]
Wound infection	15 (15)	5 (15)	10 (15)	0.608
Abdominal abscess	13 (13)	5 (15)	8 (12)	0.448
Abdominal infection	15 (15)	1 (3)	14 (21)	0.013[a]
Burst abdomen	8 (8)	1 (3)	7 (10)	0.184
Peritonitis	6 (6)	0	6 (9)	0.081
Sepsis	6 (6)	0	6 (9)	0.081
Renal complications				
Urinary retention	4 (4)	0	4 (6)	0.192
Renal failure	7 (7)	2 (6)	5 (7)	0.344
Urinary tract infections	8 (8)	0	8 (12)	0.034[a]
Severity of complications[b]				0.029[a]
Grade 0/I	45 (44)	22 (65)	23 (34)	
Grade II	23 (23)	3 (9)	20 (30)	
Grade IIIa	16 (16)	5 (15)	11 (16)	
Grade IIIb	10 (10)	3 (9)	7 (10)	
Grade IV	8 (8)	1 (3)	7 (10)	
Grade V (in-hospital mortality)	0	0	0	
Mortality				0.139
30 d	5 (5)	4 (12)	1 (1)	
90 d	11 (10)	5 (15)	6 (9)	

[1]Mann-Whitney *U* test/Fishers exact test.
[a]$P < 0.05$.
[b]$P < 0.01$. 5-FU: 5-Fluorouracil; ICU: Intensive care unit.

In our cohorts, 21% of tumours (18% in the MMC group and 22% in the Oxaliplatin group) were mucinous carcinoma. Regarding univariate analysis, we found no survival benefits for mucinous carcinoma *vs* adenocarcinoma. Our cohort contains no patients with adenosquamous or squamous carcinoma. As both groups contain a similar percentage of mucinous carcinoma, we expect no selection bias due to this

Table 3 Impact of other prognostic factors on overall survival

Predictor	*n*	Median survival in mo	*P* value[1]
Sex			0.884
Male	60	49	
Female	42	57	
Age			0.147
< 50 yr	27	38	
≥ 50 yr	75	49	
Preoperative BMI			0.423
< 18.5	4	4	
18.5-25	49	53	
25-30	35	51	
> 30	14	49	
ASA score			0.457
1-2	49	49	
3-4	53	57	
Primary tumour location			0.620
Colon	91	49	
Rectum	11	23	
Primary T-stage			0.669
T1-3	38	49	
T4a	40	30	
T4b	22	Not reached	
Primary nodal stage			0.013[a]
N0	26	88	
N1	31	51	
N2a	19	30	
N2b	23	18	
Primary distant metastasis			0.046[a]
M0	36	57	
M+	62	35	
Primary tumour grading			0.010[a]
G2	59	51	
G3	34	29	
Primary tumour resection			0.035[a]
R0	76	51	
R1	20	30	
R2	4	16	
Cytoreduction level			< 0.001[b]
CC0	89	49	
CC1	10	12	
CC2-3 + palliative resections	3	3	

PCI-score			< 0.001[b]
< 10	56	51	
10-20	29	27	
20-30	11	10	
> 30	5	9	
Operation extent			
Partial colectomy	55	53	0.189
No colon resection	47	31	
Small bowel resection	49	30	0.355
No small bowel resection	53	51	
Liver metastasis resection	42	27	0.024[a]
No liver resection	60	51	
HIPEC regimen			0.139
MMC	34	30	
Oxaliplatin/5-FU	68	Not reached	

[1]Univariate analysis by Kaplan-Meier method with log-rank test for the comparison of subgroups.
[a]$P < 0.05$.
[b]$P < 0.01$. 5-FU: 5-Fluorouracil; ASA: American Society of Anesthesiologists; BMI: Body mass index; HIPEC: Hyperthermic intraperitoneal chemotherapy; MMC: Mitomycin C; PCI: Peritoneal cancer index.

histopathological criterion.

We also found R1-resections of primary tumours to be a prognostic factor after peritoneal metastasis, as well as synchronous metastatic spread. Two studies[34,35] analysed the prognostic influence of disease-free resection margins on survival and also found this to have independent prognostic value. These results are useful to identify optimal subgroups for high risk of recurrent PC.

An important prognostic factor of survival is the concept of tumour burden correlated with PCI-scoring. Oncologic results seem to be significantly better when PCI is < 10[36] or ≤ 13[37]. However, PCI ≥ 20 is associated with decreased survival according to many different studies[38-40]. This agrees with our results from univariate and multivariate analysis of survival. Patients with distant metastasis, especially liver metastasis, were included in this analysis. Current literature suggests that patients with distant metastasis amendable to resection should not be excluded from CRS and HIPEC[38,41]. Concordant to the literature, univariate analysis of survival of our data shows a significant reduced survival for patients undergoing liver resections during CRS and HIPEC (27 mo *vs* 51 mo without liver resection; $P = 0.024$).

There are several limitations in this study that should be considered. Mainly, the retrospective non-randomized study design lowers comparability between the groups. Furthermore, the retrospective database lacks complete information regarding Tumour Node Metastasis staging, preoperative treatments especially chemotherapy as well as varying follow-up duration. The patients were treated over a time period of 10 years with changes in perioperative management and systemic chemotherapy. Different surgeons performed HIPECs at the university hospital of Freiburg. Therefore, an individual learning curve cannot be assessed. Nevertheless, the learning curve of the complete surgical department could influence postoperative outcome depending on operation timing.

For this special collective of patients with PC based on a colorectal primary tumour, several outcome predictors were identified. We were also able to show comparable outcome results for CRS/HIPEC with oxaliplatin and MMC. Nevertheless, increased complication rates for oxaliplatin were demonstrated, which, according to the literature, significantly affects OS[42] indicating that patients should be treated favourably with MMC-HIPEC. As we could not show any survival benefit for patients treated with MMC or oxaliplatin HIPEC, it remains to be determined whether there is enough evidence for HIPEC. However, the importance of complete cytoreduction has been established, which has been broadly discussed in the literature and is consistent with our data.

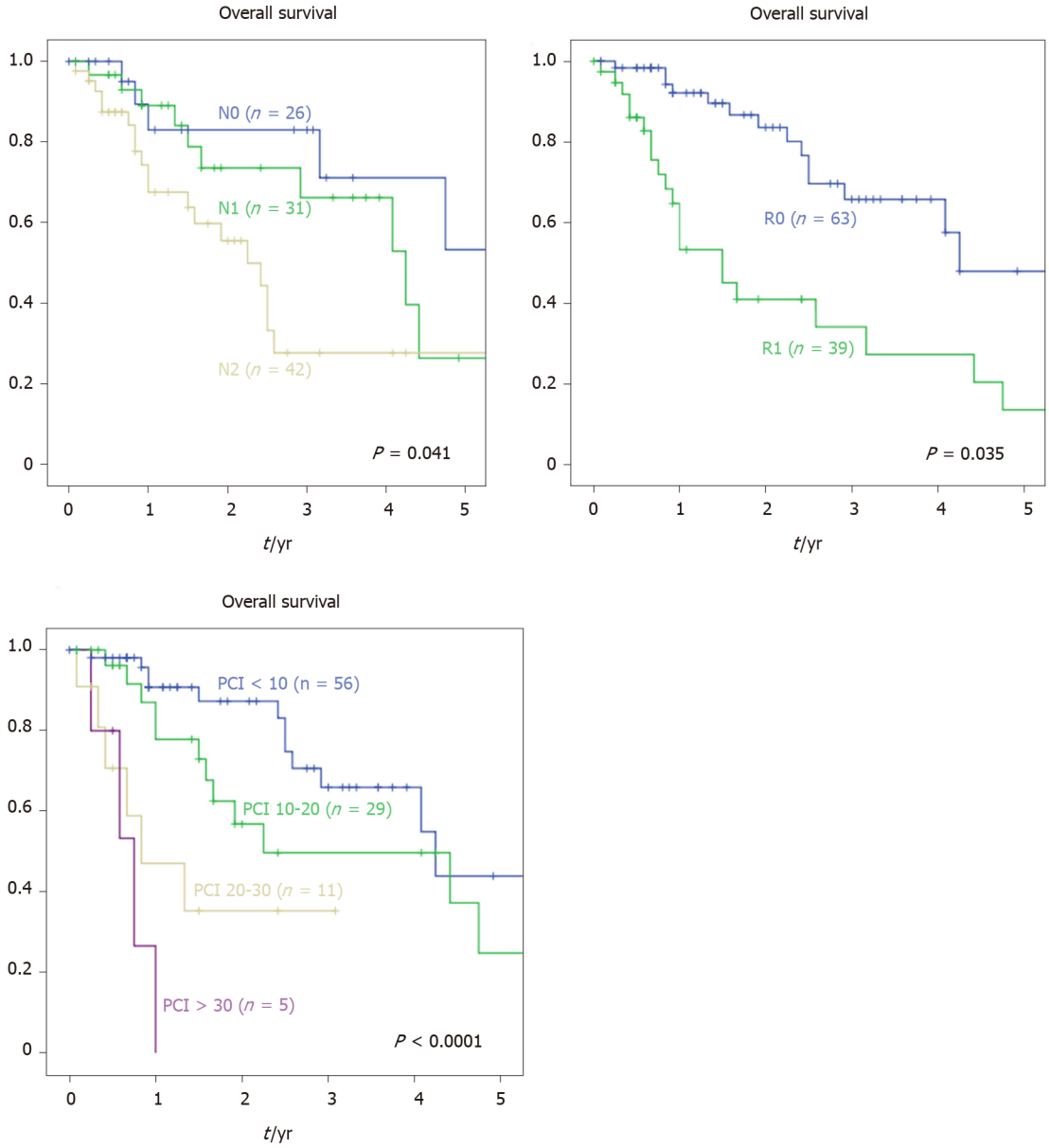

Figure 1 Kaplan-Maier: 5-year overall survival after cytoreductive surgery + hyperthermic intraperitoneal chemotherapy depending on different outcome factors. Univariate analysis of survival of patients with peritoneal metastasized colorectal cancer dependent on primary tumour nodal status, resection status and peritoneal cancer index scoring system.

Further studies, in particular a phase III clinical trial comparing both HIPEC regimens, would improve evidence-based decision-making.

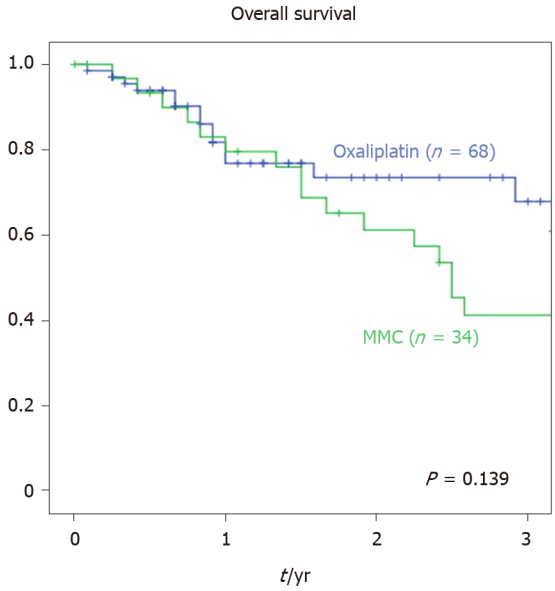

Figure 2 Kaplan-Maier: 3-year overall survival cytoreductive surgery + hyperthermic intraperitoneal chemotherapy. Kaplan-Maier analysis of 3-year overall survival of patients with peritoneal metastasized colorectal cancer being treated with cytoreductive surgery and oxaliplatin or mitomycin C-hyperthermic intraperitoneal chemotherapy.

ARTICLE HIGHLIGHTS

Research background

Cytoreductive Surgery (CRS) in combination with hyperthermic intraperitoneal chemotherapy (HIPEC) improves patient survival in colorectal cancer (CRC) with peritoneal carcinomatosis (PC). Commonly used cytotoxic agents nowadays include mitomycin C (MMC) and oxaliplatin. Evidence for the choice of the HIPEC agent and uniform procedure protocols is scarce, with studies reporting varying results.

Research motivation

There's a severe lack of evidence regarding comparison of survival benefits for most commonly utilized chemotherapeutic agents for HIPEC oxaliplatin and MMC. At present there is no prospective study that compares these two HIPEC regimens for treatment of peritoneal metastasized CRC, thus leading to the reassessment of HIPEC and the need for structured treatment protocols. In this retrospective clinical analysis, we evaluated the outcome of patients undergoing CRS HIPEC at the university medical centre of Freiburg. Furthermore, this study is one of a few to focus on prognostic factors and treatment strategies after the development of peritoneal metastasis.

Research objectives

The aim of the study was to evaluate therapeutic benefits and operative and postoperative complications of CRS + MMC *vs* oxaliplatin HIPEC in patients with peritoneal metastasized CRC as well as prognostic factors for overall survival (OS).

Research methods

One hundred two patients who had undergone CRS and HIPEC for CRC PC between 2007 and 2019 at the Medical Center of the University Freiburg regarding interdisciplinary cancer conference decision were retrospectively analysed. Oxaliplatin and MMC were used in 68 and 34 patients, respectively. Each patient's demographics and tumour characteristics, operative details, postoperative complications and survival were noted and compared. Complications were stratified and graded using Clavien/Dindo analysis. Prognostic outcome factors were identified using univariate and multivariate analysis of survival.

Research results

The two groups did not differ significantly regarding baseline characteristics. We found no difference in median OS. Patients treated with oxaliplatin HIPEC suffered

increased postoperative complications (66.2% *vs* 35.3%; P = 0.003), particularly intestinal atony, intraabdominal infections and urinary tract infections, and had a prolonged intensive care unit (ICU) stay compared to the MMC group (7.2 d *vs* 4.4 d; P = 0.035). Regarding univariate analysis of survival, we found primary tumour factors, nodal positivity and resection margins to be of prognostic value as well as PC index (PCI)-score and the completeness of cytoreduction regarding peritoneal carcinomatosis. Multivariate analysis of survival confirmed primary distant metastasis and primary tumour resection status to have a significant impact on survival and likewise PCI-scoring regarding peritoneal carcinomatosis.

Research conclusions

We could not show any survival advantage for neither HIPEC regimens. Oxaliplatin showed an increased complication rate. Increased postoperative complication rates, especially severe complications (grade IIIb and IV according to Clavien-Dindo analysis), were also associated with prolonged ICU stay for the Oxaliplatin group compared to MMC (7.2 d *vs* 4.4 d; P = 0.035), which improves evidence to choose MMC for CRS-HIPEC.

Primary distant metastasis and primary tumour resection seem to have a significant impact on survival and likewise PCI-scoring regarding peritoneal carcinomatosis.

Research perspectives

For this special collective of patients with PC based on a colorectal primary tumour, several outcome predictors could be identified. We were also able to show comparable outcome results for CRS/HIPEC with oxaliplatin and MMC. Nevertheless, increased complication rates for oxaliplatin were demonstrated, which, according to literature, significantly affects OS, indicating that patients should be treated favourably with MMC-HIPEC. Further studies, in particular a phase III clinical trial comparing both HIPEC regimens would improve evidence-based decision-making.

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ORIGINAL ARTICLE

Retrospective Study

Endoscopic mucosal resection *vs* endoscopic submucosal dissection for superficial non-ampullary duodenal tumors

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Abstract

BACKGROUND

Institutional review board statement: This study was conducted according to the ethical principles of the Declaration of Helsinki, and each institution's review board and ethical committee approved the study's protocol.

Informed consent statement: Written informed consent for performing endoscopic resection was obtained from each patient before treatment in accordance with the protocol at each institution. Consent for using the data in this study was waived because of the retrospective nature of the study.

Conflict-of-interest statement: We have no financial relationships to disclose.

Data sharing statement: The datasets used and/or analyzed during the current study are available from corresponding author on reasonable request.

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The selection of endoscopic treatments for superficial non-ampullary duodenal epithelial tumors (SNADETs) is controversial.

AIM
To compare the efficacy and safety of endoscopic mucosal resection (EMR) and endoscopic submucosal dissection (ESD) for SNADETs.

METHODS
We retrospectively analyzed the data of patients with SNADETs from a database of endoscopic treatment for SNADETs, which included eight hospitals in Fukuoka, Japan, between April 2001 and October 2017. A total of 142 patients with SNADETs treated with EMR or ESD were analyzed. Propensity score matching was performed to adjust for the differences in the patient characteristics between the two groups. We analyzed the treatment outcomes, including the rates of *en bloc*/complete resection, procedure time, adverse event rate, hospital stay, and local or metastatic recurrence.

RESULTS
Twenty-eight pairs of patients were created. The characteristics of patients between the two groups were similar after matching. The EMR group had a significantly shorter procedure time and hospital stay than those of the ESD group [median procedure time (interquartile range): 6 (3-10.75) min *vs* 87.5 (68.5-136.5) min, $P < 0.001$, hospital stay: 8 (6-10.75) d *vs* 11 (8.25-14.75) d, $P = 0.006$]. Other outcomes were not significantly different between the two groups (*en bloc* resection rate: 82.1% *vs* 92.9%, $P = 0.42$; complete resection rate: 71.4% *vs* 89.3%, $P = 0.18$; and adverse event rate: 3.6% *vs* 17.9%, $P = 0.19$, local recurrence rate: 3.6% *vs* 0%, $P = 1$; metastatic recurrence rate: 0% in both). Only one patient in the ESD group underwent emergency surgery owing to intraoperative perforation.

CONCLUSION
EMR has significantly shorter procedure time and hospital stay than ESD, and provides acceptable curability and safety compared to ESD. Accordingly, EMR for SNADETs is associated with lower medical costs.

Key words: Endoscopic mucosal resection; Endoscopic submucosal dissection; Superficial non-ampullary duodenal epithelial tumor; Short-term; Outcome; Propensity score matching

Core tip: The standard treatment for superficial non-ampullary duodenal epithelial tumors (SNADETs) is controversial. We conducted a multi-center retrospective study, which aimed to compare the treatment outcomes of endoscopic mucosal resection (EMR) with those of endoscopic submucosal dissection for SNADETs by propensity score matching analysis. Twenty-eight patients were matched in each group. EMR achieved shorter procedure time and hospital stay than endoscopic submucosal dissection without any significant differences in curability and safety. Therefore, EMR for SNADETs has an advantage in total medical costs of endoscopic treatment.

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INTRODUCTION

Considering the quality of life of patients, endoscopic resection was accepted as an alternative local treatment, instead of invasive surgery for gastrointestinal neoplasms,

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including those in the stomach, esophagus, colon, and rectum[1-3]. Endoscopic mucosal resection (EMR) - an original endoscopic treatment — is a simple and safe endoscopic resection technique, but it is associated with curability issues, especially for gastric neoplasms[4]. Therefore, endoscopic submucosal dissection (ESD) was invented to overcome this problem in patients with gastric neoplasms; ESD resulted in a higher rate of *en bloc* resection and resulted in precise pathological diagnoses[5]. However, ESD was time-consuming, more difficult to perform, and resulted in a higher rate of adverse events, including perforation and bleeding[6-8].

Endoscopic treatments, instead of pancreaticoduodenectomy, have been subsequently used as local treatments for superficial non-ampullary duodenal epithelial tumors (SNADETs), with a high rate of perioperative complications[9,10]. However, the standard procedure for endoscopic resection remains controversial. In addition, there are limited data regarding the comparison between the two procedures of ESD and EMR[9,11-13]. No randomized-controlled trial till date has compared ESD and EMR owing to various reasons, including patient recruitment, especially the limited number of endoscopic resections performed for SNADETs in each institution. Moreover, confounding bias was noted in previous observational studies, which might have affected the treatment outcomes. Propensity score matching is used to compensate for such biases[14,15]. Accordingly, we conducted a multi-center retrospective study, using propensity score matching to adjust for the differences in the baseline characteristics between patients who underwent EMR and those who underwent ESD. The specific objectives of this study were to compare the treatment outcomes of patients who underwent endoscopic resection and to compare the rates of adverse events in patients who underwent EMR and those who underwent ESD.

MATERIALS AND METHODS

Study design and ethics

The current multi-center, retrospective study was conducted at eight centers, including Kyushu University, Aso Iizuka Hospital, Saiseikai Fukuoka General Hospital, Kitakyushu Municipal Medical Center, Kyushu Rosai Hospital, National Hospital Organization Kyushu Medical Center, National Hospital Organization Fukuokahigashi Medical Center, and Harasanshin Hospital. The study protocol was performed in accordance with the 2008 revision of the Declaration of Helsinki and was approved by the Institutional Review Board of all eight centers. We reviewed the medical data, including patient characteristics and clinical outcomes, from the EMR/ESD database, endoscopic reports, and medical records at each center. A new database of endoscopic treatment for SNADETs was prepared for this study. Written informed consent for performing endoscopic resection was obtained from each patient before treatment in accordance with the protocol at each institution. However, the need for consent for using the data in this study was waived because of the retrospective nature of the study.

Patients

We identified a total of 200 consecutive patients who underwent endoscopic resection for SNADETs in all the centers between April 2001 and October 2017. Subsequently, 58 patients were excluded because of the following reasons: Non-neoplasms in 29 patients, neuro-endocrine tumors in 12 patients, lesions treated with polypectomy in 6 patients, and lesions treated *via* laparoscopic-endoscopic cooperative surgery in 11 patients. The remaining 142 patients with SNADETs were included in the current study. EMR or ESD was performed for each included patient.

Indications for endoscopic resection

The following indications were used for performing endoscopic resection: (1) Histological diagnosis of adenoma or adenocarcinoma on endoscopic biopsy; and (2) Endoscopic suspicion of adenoma or adenocarcinoma without endoscopic biopsy. Endoscopic diagnoses were made *via* routine endoscopy, magnifying endoscopy, and chromoendoscopy with indigo carmine. If neoplasms were strongly suspected, endoscopic resection was considered as a treatment option without the need for biopsy, because the scar made by biopsy might affect the success of endoscopic resection[16,17].

Procedure for endoscopic resection

Endoscopic resection was performed with the patient under intravenous sedation or general anesthesia. A standard single-channel endoscope (GIF-Q260J; Olympus Optical, Tokyo, Japan or EG-L600WR7; Fujifilm, Tokyo, Japan) was used for endoscopic resection. VIO 300D or ICC200 (ERBE Elektromedizin, GmbH, Tübingen, Germany) was used as an electrical power unit. All patients treated *via* EMR or ESD were admitted to one of the treating institutions. On day 2 or 3 after endoscopic resection, patients were started on a liquid diet, and patients with an uneventful postoperative course were discharged from the hospital after endoscopic resection. All the endoscopists were experts with an experience of at least 50 EMR and ESD procedures each.

Procedure for EMR

The procedure for EMR has been previously described in detail[9,13]. In brief, the procedure for EMR involves a submucosal injection, followed by mucosal resection using an electrocautery snare (Figure 1A and B). Normal saline or 10% glycerin solution (Glyceol; Taiyo Pharma., Tokyo, Japan) was submucosally injected with a small amount of indigo carmine dye to lift up the lesion[18-20]. Various snares were used according to the tumor size at the endoscopists' discretion. EMR was performed as described above, with no modifications. If *en bloc* resection was not achieved during the initial EMR procedure, additional snaring or coagulation was performed using hemostatic forceps or argon plasma coagulation for the residual portion of the lesion. When additional snaring or coagulation was performed after initial EMR, it was considered piecemeal resection.

Procedure for ESD

The procedure for ESD has been previously described in detail[9,13,21]. In brief, circumferential marking dots were placed by using the tip of an endo-knife. Sodium hyaluronate (MucoUp 0.4%; Boston Scientific Japan Co., Tokyo, Japan) was submucosally injected with a small amount of indigo carmine dye to achieve adequate and sustained submucosal lifting[18-20]. A circumferential mucosal incision was made around the marking dots, and the submucosal layer was dissected by using the endo-knife (Figure 2A and B). The endoscopic techniques performed, and the type of endo-knives used, including the needle-type knife, insulated tip knife, and scissor-type knife, were at the endoscopists' discretion. In some cases, dental floss clip traction was used to achieve good visualization and reduce the difficulty in dissection. In other cases, snaring was performed during submucosal dissection. The use of traction and the choice of the snaring method were at the endoscopists' discretion. Bleeding during the procedure was stopped *via* coagulation with the endo-knife itself or by using hemostatic forceps. When additional snaring or coagulation was performed after resection of the main lesion *via* ESD, it was considered piecemeal resection.

Mucosal defects in most cases, including those with intraoperative perforations, were closed after endoscopic resection, including EMR and ESD, *via* clip closure or tissue shielding methods to prevent delayed bleeding or perforation (Figure 1C and D, Figure 2C and D).

Histopathological evaluation

After removal, EMR/ESD specimens were fixed in 10% formalin. The specimens were embedded in 10% paraffin, sectioned at 2-mm intervals, and stained with hematoxylin and eosin. The pathological diagnoses and evaluation of curability were made by expert gastrointestinal pathologists in each institution. The following valuables were assessed for each tumor: macroscopic type, tumor size, depth of invasion, degree of differentiation, lymphatic invasion, venous invasion, and ulceration (scarring).

Clinical outcomes

We analyzed the short-term outcomes of endoscopic resection, such as the rates of *en bloc* resection and complete resection, procedure time, and incidence of adverse events, including delayed bleeding and intraprocedural or delayed perforation. In addition, we analyzed the local and metastatic recurrences during the follow-up period after endoscopic treatment. The procedure time was defined as the time from the start of mucosal injection to the completion of tumor resection. *En bloc* resection was defined as resection in a single piece in contrast to piecemeal resection. Complete resection was defined as *en bloc* resection with horizontal and vertical margins that were free of the tumor. Intraprocedural perforation was identified as a visible break in the duodenal wall confirmed *via* endoscopy during endoscopic resection. Delayed perforation was

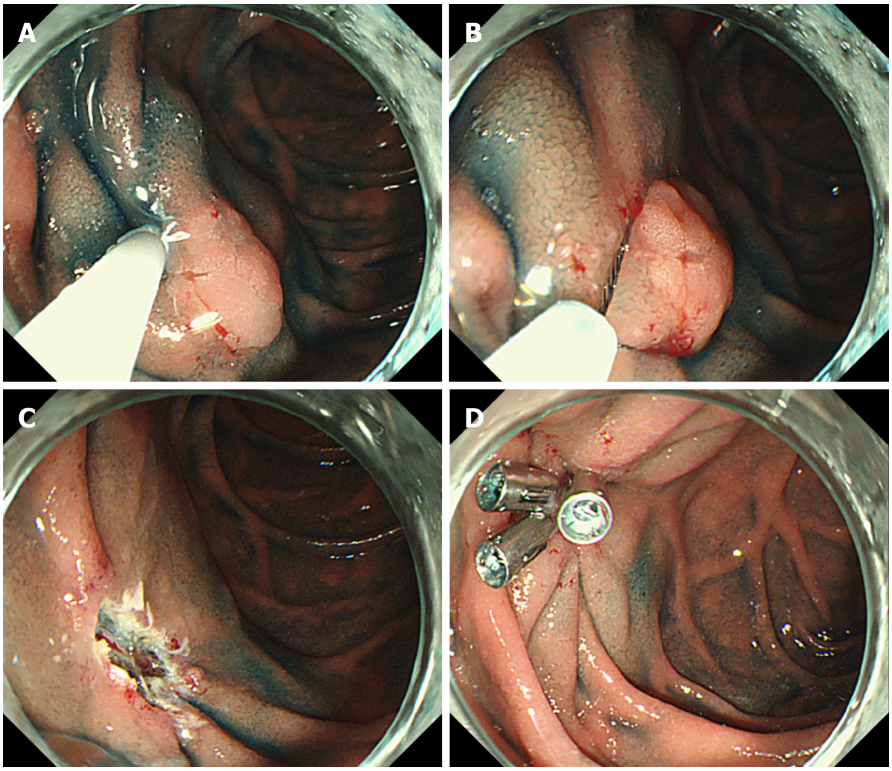

Figure 1 Procedure for endoscopic mucosal resection. A: Injection into the submucosal layer; B: Snaring of the lesion; C: Mucosal defect after endoscopic submucosal dissection; and D: Clip closure of mucosal defect.

diagnosed as the presence of free air confirmed on radiography or computed tomography scans after endoscopic resection without intraprocedural perforation. Delayed bleeding was defined as the clinical evidence of bleeding after endoscopic resection that required endoscopic hemostasis or transfusion. Local recurrence was defined as tumor relapse from the treatment scar, which was diagnosed by endoscopy or biopsy during the follow-up period. Metastatic recurrence was defined as tumor relapse in the lymph nodes and/or other organs, which was diagnosed by computed tomography during the follow-up period.

Statistical analysis

The sample size could not be calculated because this was a retrospective study. Furthermore, this was not a randomized-controlled study with confounding differences between the two groups. Therefore, propensity score matching was adopted to compensate for the confounding biases that might have influenced the treatment outcomes[22,23]. Logistic regression analysis was performed considering the endoscopic procedures (EMR *vs* ESD), and the propensity score was analyzed for the following factors: Age (years), sex (man/woman), tumor location (blubs/second or third portion), tumor morphology (protruded/others), tumor size (mm, ≥ 11 mm/ < 11 mm), tumor depth (mucosa/submucosa), and histology (adenoma/adenocarcinoma). This model yielded an area under the receiver operating characteristics curve of 0.86, which indicated a good predictive power. The propensity score for ESD was calculated using logistic regression analysis, which indicated the possibility that a patient would undergo ESD. After estimating the propensity scores, patients in the ESD group were matched to patients in the EMR group. The matching algorithm used calipers with a width equal to 0.2 of the standard deviation of the log of the propensity score without replacement. The effect of the matching was evaluated in terms of the absolute standardized difference.

Categorical variables were presented as the number and percentage. Continuous variables that were distributed abnormally were presented as the median and interquartile range. The differences in the baseline clinicopathological characteristics and treatment outcomes of this study were compared between the two groups by using Fisher's exact test for categorical data or Mann-Whitney *U* test for continuous data that were not distributed normally. *P* values < 0.05 were statistically significant for all tests. All statistical data analyses were performed using JMP software (version

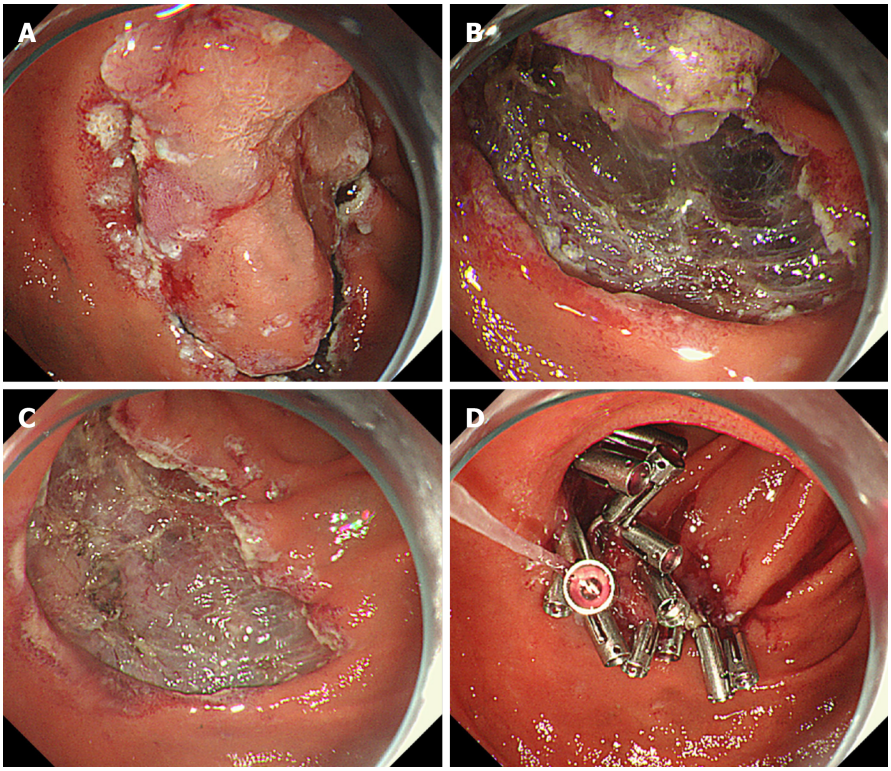

Figure 2 Procedure for endoscopic submucosal dissection. A: Circumferential mucosal incision; B: Dissection of the submucosal layer; C: Mucosal defect after endoscopic submucosal dissection; D: Clip closure of mucosal defect.

14.0.0, SAS Institute, Cary, NC, United States).

RESULTS

Baseline characteristics before propensity score matching

EMR was performed in 87 patients and ESD in 55. Figure 3 shows the flowchart of patient enrollment. The baseline clinicopathological characteristics of the enrolled patients are shown Table 1. The EMR group included significantly fewer women than the ESD group. In addition, the median tumor size was significantly smaller in the EMR group than in the ESD group [7.0 (interquartile range: 5-10) mm *vs* 15 (10.5-20) mm, $P < 0.001$]. The rate of tumors > 11 mm was significantly lower in the EMR group than in the ESD group (18.4% *vs* 74.5%, $P < 0.001$). The rate of adenocarcinoma was significantly lower in the EMR group than in the ESD group (17.2% *vs* 43.6%, $P = 0.001$). There were no significant differences in the other factors between the two groups.

Treatment outcomes before propensity score matching

The treatment outcomes before propensity score matching are shown in Supplemental Table 1. In the EMR group, the median procedure time was 5 (3.5-10) min and the rates of *en bloc* and complete resection were 87.4% and 71.3%, respectively. The rate of adverse events in the EMR group was 4.6% (observed in 4 of 87 patients); delayed bleeding occurred in 4.6% of the patients (4/87), and neither intraoperative nor delayed perforation was observed in any patient. The median hospital stay in the EMR group was 7.0 (6-9) d. In contrast, in the ESD group, the median procedure time was 90 (67-134.5) min and the rates of *en bloc* and complete resection were 94.5% and 83.6%, respectively. The rate of adverse events in the ESD group was 18.2% (observed in 10 of 55 patients); delayed bleeding occurred in 1.8% of the patients (1/55), intraoperative perforation in 12.7% (7/55), and delayed perforation in 3.6% (2/55). The median hospital stay in the ESD group was 11 (9-14) d. In fact, only one patient with intraoperative perforation in the ESD group required emergency surgery immediately after ESD. Nevertheless, none of the patients in either group died due to adverse events.

Table 1 Comparison of baseline clinicopathological characteristics between the endoscopic mucosal resection and endoscopic submucosal dissection groups

	All, *n* = 142	EMR group, *n* = 87	ESD group, *n* = 55	*P* value
Age, yr				
Median (IQR)	63.5 (57-71.75)	62 (57-70)	66 (59-73.5)	0.15
Sex, *n* (%)				
Male	79 (55.6)	56 (64.4)	23 (41.8)	0.01
Female	63 (44.4)	31 (35.6)	32 (58.2)	
Tumor location, *n* (%)				
Bulbs	32 (22.5)	17 (19.5)	15 (27.3)	0.31
Second portion or later	110 (77.5)	70 (80.5)	40 (72.7)	
Morphology, *n* (%)				
Flat or depressed	28 (19.7)	17 (19.5)	11 (20.0)	1
Protruded	114 (80.3)	70 (80.5)	44 (80.0)	
Tumor size, mm				
Median (IQR)	8 (5.25-15)	7 (5-10)	15 (10.5-20)	< 0.001
< 11 mm	85 (59.9)	71 (81.6)	14 (25.5)	< 0.001
≥ 11 mm	57 (40.1)	16 (18.4)	41 (74.5)	
Tumor depth, *n* (%)				
Mucosa	139 (97.9)	86 (98.9)	53 (96.4)	0.56
Submucosa	3 (2.1)	1 (1.1)	2 (3.6)	
Histology, *n* (%)				
Adenoma	103 (72.5)	72 (82.8)	31 (56.4)	0.001
Adenocarcinoma	39 (27.5)	15 (17.2)	24 (43.6)	

P values were calculated using the Fisher exact test for categorical data and the Mann-Whitney *U* test for continuous data. EMR: Endoscopic mucosal resection; ESD: Endoscopic submucosal dissection; IQR: Interquartile range.

Follow-up duration and 1-year follow-up rate were not significantly different between the two groups: Median follow-up duration, 24.5 (15-53.75) mo; 1-year follow-up rate, 81.0% (115/142). Three cases of local recurrence occurred in EMR, which were successfully managed by salvage endoscopic treatment. No metastatic recurrence occurred in either groups.

Baseline characteristics after propensity score matching

Twenty-eight patients in the EMR group were matched with 28 patients in the ESD group by using propensity score matching. The matching factors between both the groups, which are shown in Table 2, were quite similar without any significant differences. All the absolute standardized differences ranged within $1.96(2/n)^{1/2}$, which indicated well-balanced characteristics[22]. The median tumor size was 11 (6.25-15) mm in the EMR group and 10.5 (8-13) mm in the ESD group ($P = 0.90$).

Treatment outcomes after propensity score matching

The treatment outcomes of patients in the EMR and ESD groups after propensity score matching are summarized in Table 3. The procedure time was significantly shorter in the EMR group than in the ESD group [6 (3-10.75) min *vs* 87.5 (68.5-136.5) min, $P < 0.001$). Furthermore, the median hospital stay was significantly shorter in the EMR group than in the ESD group [8 (6-10.75) d *vs* 11 (8.25-14.75) d, $P = 0.006$]. There were no significant differences in *en bloc* resection and curative resection rates between both groups (*en bloc* resection rate: 82.1% *vs* 92.9%, $P = 0.42$; complete resection rate: 71.4% *vs* 89.3%, $P = 0.18$). There was also no significant difference in the rate of adverse events between both groups (3.6% *vs* 17.9%, $P = 0.19$). Delayed bleeding in the EMR

Table 2 Comparison of clinicopathological characteristics of the endoscopic mucosal resection and endoscopic submucosal dissection groups after propensity score matching

	EMR group, *n* = 28	ESD group, *n* = 28	*P* value	ASD
Variable matching between groups				
Age, yr; ≥ 65/< 65	8/20	6/22	0.76	0.17
Sex; male/female	17/11	16/12	1	0.073
Tumor location; bulbs/others	8/20	6/22	0.67	0.17
Morphology; protruded/flat or depressed	22/6	23/5	1	0.090
Histology; adenocarcinoma/adenoma	8/20	7/21	1	0.081
Tumor depth; mucosa/submucosa	27/1	27/1	1	0
Tumor size; median (IQR)	11 (6.25-15)	10.5 (8-13)	0.90	0.026
Tumor size; ≥ 11 mm/< 11 mm	14/14	14/14	1	0

P values were calculated using the Fisher exact test for categorical data and the Mann-Whitney *U* test for continuous data. EMR: Endoscopic mucosal resection; ESD: Endoscopic submucosal dissection; ASD: Absolute standardized difference; IQR: Interquartile range.

Table 3 Comparison of treatment outcomes between the endoscopic mucosal resection and endoscopic submucosal dissection groups after propensity score matching

	EMR group, *n* = 28	ESD group, *n* = 28	*P* value
Procedure time, min			
Median (IQR)	6 (3-10.75)	87.5 (68.5-136.5)	< 0.001
En bloc resection, *n* (%)	23 (82.1)	26 (92.9)	0.42
Complete resection, *n* (%)	20 (71.4)	25 (89.3)	0.18
Closure of mucosal defects, *n* (%)	24 (85.7)	27 (96.4)	0.35
Adverse events, *n* (%)	1 (3.6)	5 (17.9)	0.19
Intraoperative perforation, *n* (%)	0 (0)	3 (10.7)	0.24
Delayed perforation, *n* (%)	0 (0)	1 (3.6)	1
Delayed bleeding, *n* (%)	1 (3.6)	0 (0)	1
Emergency surgery, *n* (%)	0 (0)	1 (3.6)	1
Hospital stay, d			
Median (IQR)	8 (6-10.75)	11 (8.25-14.75)	0.006
Follow-up duration, mo	23 (11-35.5)	24 (9.75-57.5)	0.831
Median (IQR)			
One-year follow-up, *n* (%)	21 (75)	20 (71.4)	1
Local recurrence, *n* (%)	1 (3.6)	0 (0)	1
Metastatic recurrence, *n* (%)	0 (0)	0 (0)	-

P values were calculated using Fisher's exact test for categorical data and Mann-Whitney *U* test for continuous data. EMR: Endoscopic mucosal resection; ESD: Endoscopic submucosal dissection; IQR: Interquartile range.

group was successfully managed using a conservative approach without surgery. Only one patient with intraoperative perforation in the ESD group required emergency surgery immediately after ESD. None of the patients in either group died due to adverse events. As for recurrence events, only one local recurrence was observed in the EMR group, and no metastatic recurrence was seen during the follow-up period.

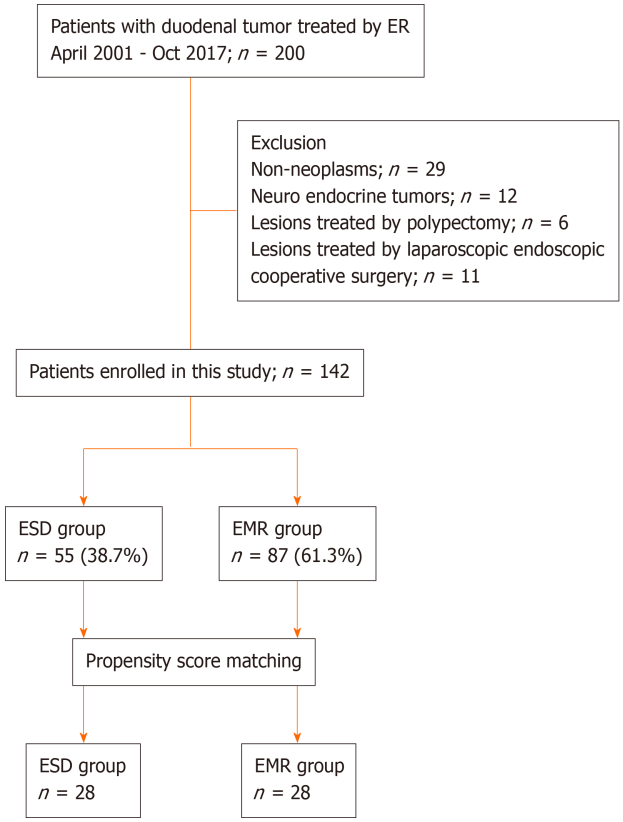

Figure 3 Flowchart showing patient enrollment in the current study. ESD: Endoscopic submucosal dissection; EMR: Endoscopic mucosal resection.

DISCUSSION

To the best of our knowledge, the current study is the first to compare the efficacy and safety of EMR with those of ESD for SNADETs using propensity score matching. Although ESD tended to result in a higher complete resection rate than did EMR, ESD was a significantly longer procedure and required longer hospital stay with a tendency of having a higher adverse event rate. In fact, one patient in the ESD group required emergency surgery for a perforation. Local recurrent lesions in EMR were successfully treated by endoscopic resection. Therefore, although ESD was more effective than EMR, all SNADETs cannot be treated with ESD because of the possible risk of adverse events and higher cost of hospitalization.

ESD for duodenal tumors achieved higher curability rates with a higher adverse event risk than EMR[11-13]. However, these previous studies, as well as the current study, were retrospective studies and not randomized-controlled trials. Therefore, there were some biases owing to the difference in the background characteristics of each group. Some factors are associated with the outcomes of endoscopic resection for SNADETs. For example, the tumor size was associated with the rate of adverse events after endoscopic resection and the *en bloc* resection rate[24,25]. In addition, the presence of a tumor in the distal part of the second portion, especially distal to the ampulla of Vater, was associated with the occurrence of delayed perforation after endoscopic resection[26,27]. Therefore, we performed propensity score matching in the current study instead of a randomized-controlled trial. All such factors that were associated with the treatment outcomes were included as covariates; this contributed to the reduction of bias. Accordingly, the factors were quite similar between both groups after propensity score matching. Therefore, the current clinical study had fewer biases than previous studies.

Previous reports suggested that duodenal tumors < 20 mm in size should be treated with EMR and not ESD[11]. However, approximately 60% of duodenal tumors with a diameter of 11-20 mm were treated with ESD in a recent large-scale case series[28]. Accordingly, the criteria for selecting the treatment method for SNADETs < 20 mm are still controversial, and more studies are required to compare the treatment outcomes between EMR and ESD. In the current study, most lesions were < 20 mm, with more than half of the included lesions being 11-20 mm in size. Therefore, we believe that the results of the current study can be used to standardize the treatment method for

SNADETs, especially for small lesions.

ESD resulted in an extremely high curability rate in the current study; the *en bloc* resection rate was > 90%, and the complete resection rate also reached approximately 90%. These outcomes are similar to or better than those of previous studies[28-31]. In addition, although the curability of EMR in the current study seemed to be lower than that of ESD, the difference was not significant. In previous studies, piecemeal resection was required during EMR for lesions that were > 10-15 mm in diameter. In fact, the *en bloc* resection rate exceeded 80%, and the complete resection rate was approximately 70% in the current study, both of which are higher than those reported in previous studies[32]. The advancements in the endoscopic devices and the electrosurgical power unit, as well as advancements in the skill of the endoscopists, might have contributed to the better treatment outcomes. During follow-up, three local recurrences before matching (one local recurrence after matching) were observed only in the EMR group, although no recurrence was observed in the ESD group. All recurrent lesions were attributed to the piecemeal resection but could be managed by salvage endoscopic treatment. Furthermore, no metastatic lesion was observed in either group during the follow-up period. The high rate of *en bloc* resection in the EMR group might contribute to the comparably low rate of local recurrence as that in ESD group. Accordingly, the curative potential of EMR in the current study seems to be acceptable, even though the follow-up duration was short.

Duodenal ESD is difficult to perform because the duodenum has a very thin wall (< 2 mm thickness) with limited space surrounding the duodenum, and therefore, the maneuverability around the space is limited, possibly resulting in a higher risk of perforation than ESD for lesions in the rest of the gastrointestinal tract[10,33]. Considering the safety of ESD and EMR in the current study, the adverse event rates after EMR and ESD were not significantly different. After matching, adverse events occurred in only 1 patient who underwent EMR, whereas adverse events were observed in 5 patients who underwent ESD, which were quite low compared with those obtained in previous studies[29-31]. Especially, no delayed bleeding occurred in ESD after matching. This result might be owing to the closure of the mucosal defect after ESD. In fact, in the current study, closure of the perforation site and prophylactic endoscopic closure of the mucosal defect were performed. In a previous study, prophylactic endoscopic closure contributed to the prevention of delayed bleeding[34]. Furthermore, complete closure of the mucosal defects after duodenal ESD reduced the risk of delayed adverse events[35]. The mucosal defect was closed in almost all patients who underwent ESD (96.4%, 27/28), which might have contributed to the low rate of delayed adverse events. However, 1 patient who underwent ESD could not be managed conservatively, and, therefore, required emergency surgery.

The time taken for the procedure and the hospital stay were significantly shorter in patients who underwent EMR than in those who underwent ESD. The results of the current study showed that a shorter procedure time for EMR than for ESD reduces the cost of medical staff, including the operator for the endoscopic procedure, assistant for manipulating the device, and assistant for monitoring patients. In addition, the endo-knife used during ESD with hemostatic forceps is much more expensive than the snare used during EMR. Moreover, the low rate of adverse events might result in shorter hospitalization, thereby contributing to the cost of hospital stay. A previous study also showed that patients who underwent ESD had lower medical costs than those who underwent surgery, although the data were of patients with early gastric cancer[36]. Thus, EMR will contribute to a reduction in the total medical cost of endoscopic resection, compared with ESD.

The current study had some limitations. First, this was a retrospective study and did not include a randomized population. Although propensity score matching reduced the confounding biases, not all biases, such as the endoscopists' preference of EMR or ESD, could be eliminated. There was a possibility of selection bias because lesions that could be easily snared were selected for EMR. Second, lesions treated with EMR tended to include adenomas, mucosal lesions, and small lesions. These rates among two groups were similar after matching, but the comparison of treatment outcomes was limited primarily to such lesions. Therefore, it is questionable whether these findings could be generalized to adenocarcinomas, submucosal invasive lesions, or large lesions. Third, the sample size was relatively small owing to propensity score matching, even though this was a multi-center study. Therefore, the differences in the effectiveness and safety between EMR and ESD are unclear for SNADETs. A prospective study with a larger randomized population is expected to be conducted in the future. Fourth, the follow-up period in this study was insufficient to evaluate long-term outcomes. Median follow-up duration was 24.5 (15-53.75) mo, and the 1-year follow up rate was 81.0% (115/142). Longer follow-up will be required to evaluate the

accurate curative potential of endoscopic resection. Fifth, new advanced treatment methods, including underwater EMR, cold polypectomy, and laparoscopic-endoscopic cooperative surgery, have been used as local treatments for SNADETs, in addition to conventional EMR or ESD[37-39]. Such methods were not performed for treating SNADETs in the current study period or patients who underwent these procedures were excluded from this study. Accordingly, the treatment outcomes should be compared between conventional EMR and ESD and such new procedures in future studies.

In conclusion, the results of our study demonstrated that EMR required a significantly shorter procedure time and hospital stay than did ESD, with comparable curative potential and a lower risk of adverse events. Therefore, EMR should preferably be selected as a local treatment for SNADETs, especially for adenomas, mucosal lesions, and small lesions.

ARTICLE HIGHLIGHTS

Research background

Endoscopic treatments have been used as local treatments for superficial non-ampullary duodenal epithelial tumors (SNADETs) instead of surgery.

Research motivation

It remains to be determined whether endoscopic mucosal resection (EMR) or endoscopic submucosal dissection (ESD) is more appropriate for treating SNADETs.

Research objectives

The aim of this multi-center retrospective study was to compare the treatment outcomes of EMR and ESD for SNADETs.

Research methods

Patients with SNADETs treated by EMR or ESD at eight institutions between April 2001 and October 2017. Patients were categorized into an EMR group or an ESD group. Propensity score matching analysis was conducted to compensate for confounding differences between the two groups that may affect the outcomes. After matching, the treatment outcomes were compared between the two groups.

Research results

A total of 152 patients were included and 28 pairs were matched. The EMR group had significantly shorter procedure time and hospital stay than the ESD group. The rates of *en bloc* resection, complete resection, and adverse events were not significantly different between the two groups.

Research conclusions

EMR provides acceptable efficacy and safety with a significantly shorter procedure time and hospital stay than ESD. Additionally, EMR for SNADETs has an advantage in total medical costs of endoscopic treatment.

Research perspectives

This was a retrospective study with a relatively small sample size and follow-up duration. Therefore, further large-scale, randomized, prospective studies are needed.

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ORIGINAL ARTICLE

Retrospective Study

Accurate ultrasonography-based portal pressure assessment in patients with hepatocellular carcinoma

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Abstract

BACKGROUND

Portal pressure is of great significance in the treatment of hepatocellular carcinoma (HCC), but direct measurement is complicated and costly; thus, non-invasive measurement methods are urgently needed.

AIM

To investigate whether ultrasonography (US)-based portal pressure assessment could replace invasive transjugular measurement.

METHODS

A cohort of 102 patients with HCC was selected (mean age: 54 ± 13 years, male/female: 65/37). Pre-operative US parameters were assessed by two independent investigators, and multivariate logistic analysis and linear regression analysis were conducted to develop a predictive formula for the portal pressure gradient (PPG). The estimated PPG predictors were compared with the transjugular PPG measurements. Validation was conducted on another cohort of 20 non-surgical patients.

RESULTS

The mean PPG was 17.32 ± 1.97 mmHg. Univariate analysis identified the association of the following four parameters with PPG: Spleen volume, portal vein diameter, portal vein velocity (PVV), and portal blood flow (PBF). Multiple linear regression analysis was performed, and the predictive formula using the PVV and PBF was as follows: PPG score = 19.336 - 0.312 × PVV (cm/s) + 0.001 × PBF (mL/min). The PPG score was confirmed to have good accuracy with an area

available database in hospital.

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under the curve (AUC) of 0.75 (0.68-0.81) in training patients. The formula was also accurate in the validation patients with an AUC of 0.820 (0.53-0.83).

CONCLUSION
The formula based on ultrasonographic Doppler flow parameters shows a significant correlation with invasive PPG and, if further confirmed by prospective validation, may replace the invasive transjugular assessment.

Key words: Portal pressure gradient; Hepatic vein pressure gradient; Hepatocellular carcinoma; Transjugular; Portal pressure; Portal vein pressure

Core tip: The direct measurement of portal pressure is complicated; therefore, non-invasive measurement methods are urgently needed to guide the treatment of hepatocellular carcinoma. The combined measurements of portal vein velocity and portal blood flow could be clinically and economically useful in estimating portal pressure gradient.

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INTRODUCTION

Hepatocellular carcinoma (HCC) is a significant public health problem worldwide and is currently the main event leading to death in patients with cirrhosis[1]. The current treatment modalities for HCC include liver resection (LR) and liver transplantation. Portal pressure accurately predicts the risk of peri-operative morbidity and mortality[1,2]. The European Association for the Study of the Liver and American Association for the Study of Liver Diseases guidelines for the management of HCC consider a hepatic venous pressure gradient (HVPG) ≥ 10 mmHg to be a contraindication for LR[3,4].

Portal pressure gradient (PPG), ranges between 1 mmHg and 5 mmHg in normal conditions, which represents the hepatic perfusion pressure of portal blood[5]. HVPG measurement has the advantages of simple measurement techniques and low risk, which is widely used to estimate PPG and is regarded as the gold standard for the diagnosis of portal hypertension. Based on HVPG, clinically significant portal hypertension (CSPH) is defined as an HVPG of at least 10 mmHg[6-8]. The limitations of HVPG measurement are that it is invasive and impractical for routine clinical practice. Many non-invasive portal pressure assessment techniques have been introduced in recent years[9-13]. Doppler sonography offers real-time observation of blood flow with qualitative and quantitative assessments, and the application of microbubble-based contrast agents has improved the detectability of peripheral blood flow. In addition, elastography of the liver and spleen covers a wider field beyond the original purpose of fibrosis assessment. These developments enhance the practical use of ultrasonography (US) in the evaluation of portal hemodynamic abnormalities[12,14]. However, none of these methods have gained extensive clinical acceptance, as a consequence of small sample size, lack of external validation, and/or their low accuracy in the prediction of CSPH.

The aim of this study was to clarify whether simple, non-invasive US parameters correlate with the invasive transjugular PPG measurement and to develop a formula to estimate PPG.

MATERIALS AND METHODS

The present study was based on a retrospective analysis of prospectively collected data in our department. This study was compliant with the Health Insurance

Portability and Accountability Act. Due to the retrospective nature of the study, informed consent was waived. This study was approved by the hospital ethics committee.

Inclusion and exclusion criteria

All consecutive patients who underwent transjugular PPG measurement from January 2016 to June 2018 were included.

The inclusion criteria were as follows: (1) Patients aged 18-70 years; (2) Patients who were diagnosed with HCC; (3) Patients who underwent transjugular portal pressure measurement, abdominal computed tomography (CT) angiography, and Doppler US; (4) Patients received no treatment for HCC at the time of PPG measurement, and underwent US examination at the same time as PPG measurement; and (5) Patients with a follow-up period of minimum 12 mo.

The exclusion criteria were: (1) Patients with portal vein thrombosis or hepatic vein thrombosis; (2) Those with massive ascites in which accurate measurements by Doppler US were not possible; and (3) Pregnant or lactating women.

Clinical assessment

Baseline demographic, clinical, and laboratory characteristics were retrieved from clinical records. All patients underwent hematological tests including complete blood counts, routine coagulation examination, and kidney and liver function tests at admission. Details pertaining to the use of alcohol and hepatotoxic drugs were recorded. Patient sera were tested for hepatitis B surface antigen and antibody to hepatitis C virus. Other appropriate tests for determining etiology were also performed, if required. The Child-Pugh and Model for End-stage Liver Disease (MELD) scores were calculated on the basis of clinical data. The severity of liver disease at inclusion and during follow-up was assessed by the Child–Pugh grade and MELD score. The ALBI grade was calculated using the following equation: Linear predictor = ($\log_{10}$ bilirubin μmol/L × 0.66) + (albumin g/L × -0.085).

Ultrasound examination

US was performed before the hemodynamic investigation in patients fasted for 8 h. US examination was performed using a 3.5-MHz sector transducer (iU22 Ultrasound System; Philips Healthcare, Reedsville, PA, United States). The diameter of the portal vein was measured using B-mode US. In each patient, all measurements were carried out on a longitudinal section of the vessel and were repeated by one radiologist who had no knowledge of the hemodynamic values. These measurements included the diameter of the portal vein and portal blood velocity. All measurements were performed in triplicate and then averaged.

The portal blood flow was calculated as portal vein velocity (PVV, cm/s) × portal vein cross-sectional area × 0.57, and the congestion index (CI) of the portal vein was calculated as previously reported[15]: The "congestion index" is used to mean the ratio between the cross-sectional area (cm^2) and the blood flow velocity (cm/s) of the portal vein, as determined by a duplex Doppler system.

Transjugular PPG and HVPG measurements

Transjugular PPG and HVPG measurements were performed under general anesthesia in the angiography suite by an experienced radiologist. Pressure measurements were conducted using a balloon catheter (Edwards Lifesciences, Irvine, CA, United States) with a pressure transducer at the tip. A zero measurement with the transducer open to air was needed before transjugular catheterization. All measurements were performed in triplicate and then averaged.

Transjugular PPG measurement

Using an established technique to measure PPG[16], the portal vein was punctured with a modified transjugular liver biopsy needle under ultrasonographic and radiological guidance, and was aimed at the right portal vein branch 1-3 cm above the portal vein bifurcation. After successful puncture, the portal vein was catheterized using a 5F catheter, and baseline measurements of portal venous pressure, inferior vena cava pressure, and the PPG were obtained.

Transjugular HVPG measurement

Transjugular HVPG measurement was conducted according to the standard protocol[17]. The free HVPG was measured in the right hepatic vein (approximately 1-3 cm from the IVC). Then, as the balloon was inflated for total occlusion of the right

hepatic vein, the wedged hepatic venous pressure was measured. Continuous recording was necessary until the pressure reached a plateau. HVPG was calculated by subtracting the free venous hepatic pressure from the wedged hepatic pressure.

CT-based HVPG

The CT-based portal pressure score was calculated as follows: 17.37-4.91 × ln (liver-to-spleen volume ratio) + 3.8 (if perihepatic ascites is present)[18].

Statistical analysis

Quantitative variables are expressed as the mean ± SD and qualitative data are expressed as percentages. The independent *t* test or analysis of variance was applied for comparisons of normally distributed variables. For non-normally distributed data, the Kruskal-Wallis test or Wilcoxon's rank-sum (Mann-Whitney) test was used to analyze the statistical significance of intergroup differences. Pearson's correlation for normally distributed variables and Spearman's rank-correlation coefficient for non-normally distributed data were used, as appropriate. Linear regression analyses were performed according to the least-squares method. Spearman correlation coefficient analysis (R^2 value) and the Bland-Altman plot were used to assess the correlation and the agreement between transjugular PPG and HVPG, and between estimated PPG and transjugular PPG, respectively. The proposed PPG predictive models were subsequently tested on a validation cohort, which included 20 patients (none of these patients underwent surgery or transplantation). The performance of the estimated PPG in predicting transjugular PPG was assessed using receiver operator characteristic curves and the area under the curve (AUC) was calculated. Two-sided $P < 0.05$ was considered statistically significant. Statistical analyses were performed with the SPSS 20.0 package (SPSS, Chicago, IL, United States) and Graphpad Prism 8.0 (Graphpad Software Inc., United States).

RESULTS

Demographics

A total of 102 patients with HCC were included, and their demographics and clinicopathological parameters are shown in Table 1. The baseline liver function of these patients was as follows: Alanine aminotransferase, 24.4 ± 18.0 IU/L; aspartate aminotransferase, 35.0 ± 24.4 IU/L; and total bilirubin, 2.20 ± 3.61 mg/dL. No complications during the measurement of direct PPG were recorded in the present series.

US Doppler parameters

Doppler liver and abdominal vascular scans were performed for all patients. These US Doppler parameters are summarized in Table 2. The preoperative US Doppler parameters were as follows: Portal vein diameter, 1.20 cm ± 0.37 cm; portal vein velocity, 25.1 cm/s ± 11.4 cm/s; portal blood flow, 1729.9 mL/min ± 1003.1 mL/min; and CI, 0.11 ± 0.07.

Correlation between HVPG and PPG

HVPG was 17.07 ± 4.78 mmHg and PPG was 17.32 ± 1.97 mmHg. The paired *t* test showed no significant difference between HVPG and PPG (Figure 1A). Correlation analysis showed that the correlation coefficient between HVPG and PPG was 0.51, and the R^2 was 0.46 ($P = 0.13$, Figure 1B). The Bland-Altman plot showed a difference between HVPG and PPG (Figure 1C). These results indicated that the PPG had a good correlation with HVPG.

Development of a predictive formula of PPG

Table 3 shows the correlations between the PPG and other comparable parameters. The correlation analysis identified four variables as significantly negatively correlated with PPG: SV, PVD, PVV, and PBF ($P < 0.05$). Other parameters were not correlated with the PPG in these patients.

The four selected US parameters were examined for correlations with PPG using multiple linear regression analysis by the stepwise method (Table 4). Based on this result, the following regression equation was established: PPG score = 19.336 - 0.312 × PVV (cm/s) + 0.001 × PBF (mL/min).

Table 1 Characteristics of the included patients, *n* = 102

Index		Index	
Age (yr)	54 ± 13	Globulin (g/dL)	30.5 ± 8.9
Gender (male/female)	65/37	Albumin (g/dL)	34.9 ± 5.9
Etiology, *n*	102	Total protein (g/dL)	65.5 ± 10.0
Virus	48	ALP (U/L)	120.6 ± 86.7
Alcohol	20	GGT (U/L)	67.7 ± 82.3
Cryptogenic	5	BUN (mmol/L)	6.8 ± 5.4
Multifactorial	20	Creatinine (μmol/L)	88.7 ± 138.6
Others	9	LDH (UL)	194.3 ± 59.8
GB history, *n* (%)	76 (74.51)	K (mmol/L)	4.0 ± 0.7
Refractory ascites, *n* (%)	78 (76.47)	Na (mmol/L)	138.4 ± 14.5
Encephalopathy, *n* (%)	4 (3.92)	Cl (mmol/L)	106.4 ± 5.1
Red blood cells (10^{12}/L)	3.3 ± 1.6	Ca (mmol/L)	2.15 ± 0.15
Hemoglobin (g/L)	91.6 ± 25.3	Blood ammonia (μmol/L)	51.2 ± 30.0
White blood cells (10^{12}/L)	4.1 ± 4.0	FIB (n/L)	2.3 ± 1.5
Platelet count (10^{9}/L)	106.7 ± 95.7	APTT (s)	34.5 ± 6.3
ALT (U/L)	24.4 ± 18.0	TT (s)	17.7 ± 5.4
AST (U/L)	35.0 ± 24.4	D dimer level (μg/L)	809 ± 1009
TBIL (mg/dL)	2.20 ± 3.61	Child–Pugh class, *n* (A/B/C)	26/58/18
DBIL (mg/dL)	1.41 ± 3.14	ALBI score	-2.06 ± 0.47
IBIL (mg/dL)	0.67 ± 0.43	MELD score	7.66 ± 5.46
PT(s)	14.6 ± 3.6	HVPG (mmHg)	17.07 ± 4.78
PT (%)	61.1 ± 16.6	PVP (mmHg)	34.40 ± 5.95
INR	1.4 ± 0.3	PPG (mmHg)	17.32 ± 1.97

GB: Gastrointestinal bleeding; AST: Aspartate aminotransferase; ALT: Alanine aminotransferase; PT: Prothrombin time; TBIL: Total bilirubin; INR: International normalized ratio; ALP: Alkaline phosphatase; GGT: Glutamyl transpeptidase; BUN: Blood urea nitrogen; LDH: Lactate dehydrogenase; APTT: Activated partial thromboplastin time; TT: Thrombin time; HVPG: Hepatic venous pressure gradient; PVP: Portal vein pressure; PPG: Portal pressure gradient.

Correlation between estimated PPG score and actual PPG

The mean estimated PPG using the predictive formula was 17.16 ± 1.92 mmHg (11.51-21.14 mmHg). There was a statistically significant correlation between the PPG score and PPG in overall participants (n = 102, R = 0.884, P < 0.001, Figure 2A). A similar result was achieved using the Bland-Altman plot (Figure 2B). The proposed PPG score was applied to the training patients, which confirmed its good accuracy with an AUC of 0.75 (0.68-0.81).

Validation of the model for prediction of PPG

In addition, 20 patients were enrolled as the validation cohort, which included 12 with hepatic virus infection, 6 with alcoholic liver diseases, and 1 each with non-alcoholic liver disease and primary biliary cholangitis. The proposed PPG score was applied to the validation group and the results confirmed its good accuracy with an AUC of 0.68 (0.53-0.83, Figure 3A).

Comparison between HVPG- and CT-based HVPG scores

The CT-based HVPG score was applied to estimate HVPG, which confirmed its good accuracy with an AUC of 0.63 (0.55-0.71, Figure 3B). Compared with the estimated PPG formula proposed in this study, the power of the test was equivalent, but the ultrasound data in this study were relatively easy to obtain and there was no radiation

Table 2 Results of ultrasonography examination

Parameter	Result
Portal vein diameter (cm)	1.20 ± 0.37
Portal vein velocity (cm/s)	25.1 ± 11.4
Portal blood flow (mL/min)	1729.9 ± 1003.1
Congestion index	0.11 ± 0.07
IVC diameter (cm)	8.7 ± 2.9
IVC blood velocity (cm/s)	62.2 ± 31.0
Spleen vein diameter (cm)	1.12 ± 0.23
Spleen vein velocity (cm/s)	11.51 ± 3.23

IVC: Inferior vena cava.

Table 3 Correlations between portal pressure gradient and clinicopathologic parameters and parameters of Doppler ultrasound

Index	Correlation with PPG (γ)	*P* value
Age (yr)	0.345	0.632
Peri-hepatic ascites (yes *vs* no)	0.753	0.233
Platelet count (× 10^9/L)	-0.341	0.061
Total bilirubin (mg/dL)	-0.231	0.487
Serum albumin (g/dL)	0.542	0.683
AST (IU/L)	0.452	0.712
ALT (IU/L)	0.028	0.652
NH_3 (μg/dL)	0.126	0.515
MELD score	0.025	0.523
Portal vein diameter (cm)	0.102	0.019
Portal vein velocity (cm/s)	-0.321	0.034
Portal blood flow (mL/min)	-0.032	0.048
PV-CI	0.285	0.021
IVC diameter (cm)	0.129	0.496
IVC blood velocity (cm/s)	0.163	0.389
Spleen vein diameter (cm)	0.142	0.248
Spleen vein velocity (cm/s)	-0.062	0.654

AST: Aspartate aminotransferase; ALT: Alanine aminotransferase; MELD: Model for End-stage Liver Disease; PVV: Portal vein velocity; PV-CI: Portal vein congestion index.

damage during CT examination.

DISCUSSION

Currently, the golden standard for measuring portal hypertension and its severity is usually HVPG measurement[19,20]. Measuring this gradient is safe and relatively simple to perform, but it is invasive and costly. In this study, the PVV and PBF showed independent positive correlations with the PPG. Thus, we developed an US-based estimated PPG formula and further validated its performance in the non-invasive diagnosis of portal pressure in patients with HCC. As expected, the estimated PPG

Table 4 Multiple linear regression stepwise method output using ultrasonography Doppler data for correlations with portal pressure gradient

Model	Unstandardized coefficients		Standardized coefficient	*T* value	*P* value	95% Confidence Interval
	β	Standard error	β			
1 (Constant)	19.432	2.785		8.538	0.000	15.133-21.482
SV (cm^3)	-0.212	0.214	-0.265	-1.432	0.654	-0.378-0.431
PVD (cm)	0.322	0.254	0.331	0.085	0.723	0.134-0.564
PVV (cm/s)	-0.323	0.187	-0.353	-1.572	0.157	-0.623-0.113
PBF (mL/min)	0.001	0.056	0.274	1.431	0.197	0.023-0.422
2 (Constant)	19.345	2.634		8.634	0.000	15.268-21.372
PVD (cm)	0.312	0.262	0.232	0.079	0.654	0.211-0.592
PVV (cm/s)	-0.343	0.232	-0.412	-1.548	0.132	-0.451-0.065
PBF (mL/min)	0.001	0.067	0.283			
3 (Constant)	19.336	2.543		8.634	0.000	16.235-22.354
PVV (cm/s)	-0.312	0.134	-0.532	-2.645	0.032	-0.454-0.001
PBF (mL/min)	0.001	0.078	0.276	2,143	0.025	0.034-0.462

SV: Spleen volume; PVD: Portal vein diameter; PVV: Portal vein velocity; PBF: Portal vein flow.

showed significant agreement with invasive PPG measurement.

Hepatic hemodynamic changes in patients with portal hypertension are often complicated. As a non-invasive method for assessing portal hypertension, Doppler US is economical, simple, and easy to repeat. Its development prospects are considerable. It is expected to become one of the development directions in the non-invasive diagnosis of portal hypertension. Some Doppler parameters have been proposed as candidate surrogates of the HVPG[21,22]. However, in validation studies, none of these parameters have proved to be accurate. A possible reason for this is that Doppler measurements can be influenced by many factors, such as respiration and vasoactive drugs, as well as by inter-observer and inter-equipment variability. However, measuring liver stiffness by ultrasound and dynamically detecting hemodynamic parameters can be used as non-invasive indicators for evaluating portal pressure and the presence or absence of portal hypertension[14]. Indeed, portal vein hemodynamics are predictive markers and lower velocity in the portal trunk in compensated cirrhosis is an indicator of decompensation[23]. As with any other vascular system, portal pressure is the product of two independent factors, namely, resistance to blood flow and amount of flow, as stated by Ohm's law: Pressure = Resistance × Flow[24]. Liver stiffness measurement accurately reflects liver fibrosis in chronic liver diseases. However, the exact HVPG value cannot be reliably estimated by LSM (correlation *R* ranges from 0.59 to 0.70)[25].

In the present study, the combined measurements of the PVV and PBF were clinically and economically useful in distinguishing those patients who truly required further assessment for portal hypertension using more invasive and expensive procedures such as PPG determination. By comparing the calculated PPG with the actual PPG, a strong correlation was observed even though both the calculated PPG and the actual PPG were not always the same in each patient, and the calculated PPG was extremely accurate in the prediction of PPG (AUC = 0.75) in the training cohort. During the validation study, based on a cohort of 20 patients, the calculated score was slightly lower, but still showed good accuracy with an AUC of 0.68. In another study, the diagnostic accuracy of HVPG reached 0.83, but the non-invasive HVPG interpretation is relatively time-consuming (approximately 2.5 h per case)[26]. The formula can save time in each patient and may be used as a preliminary choice before the virtual evaluation of HVPG. However, based on the research conditions of this study, there may be the following restrictions when using this formula. The sample of this study is mainly the Chinese population. The cause of cirrhosis is mainly viral cirrhosis, which is different from the alcoholic cirrhosis in Western countries. When using this formula, we should consider the differences caused by different etiology.

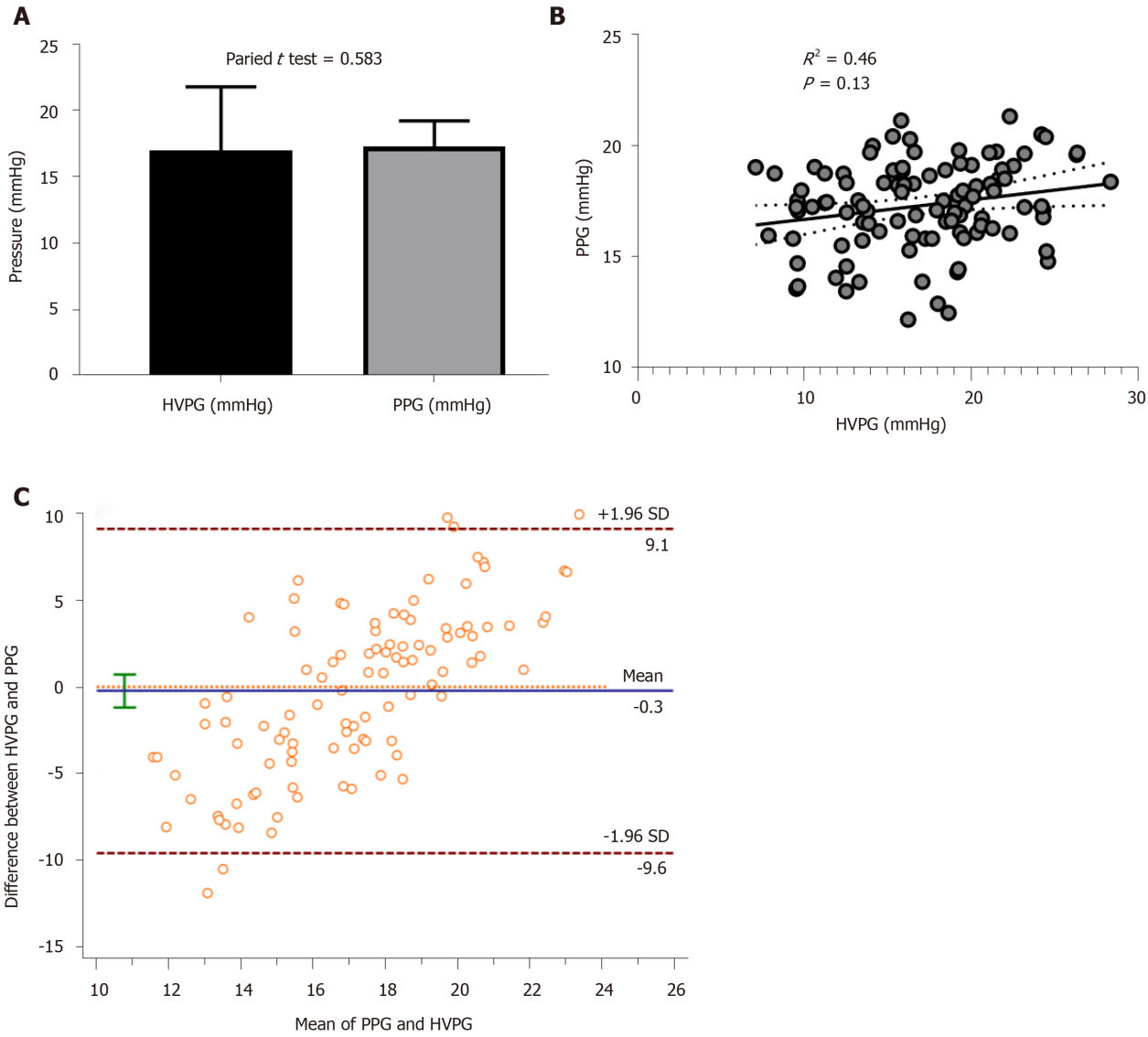

Figure 1 Correlation between portal pressure gradient and hepatic venous pressure gradient in the overall group. A: Paired *t*-test showed that there was no significant difference between hepatic venous pressure gradient (HVPG) and portal pressure gradient (PPG); B: Scatterplot shows agreement between PPG and HVPG; C: Bland-Altman plot shows the difference between PPG and HVPG.

There are several limitations to this study. Due to the limited sample size in this study, the detection index was also small, which affected the accuracy of the results to some extent. In future studies, prospective studies with a large sample size are required to increase the test indicators and identify indicators that can objectively and accurately reflect PPG. Despite the very good accuracies of the proposed model including PVV and PBF, a larger sample size may further improve the study power. A further external validation appears mandatory prior to potential wider clinical use.

In conclusion, PVV and PBF are independently and positively correlated with PPG, suggesting the usefulness of these parameters as non-invasive predictors of PPG. Monitoring of PVV and PBF may be clinically useful for the early detection and management of portal hypertension to distinguish those patients who require further invasive and expensive procedures such as PPG determination.

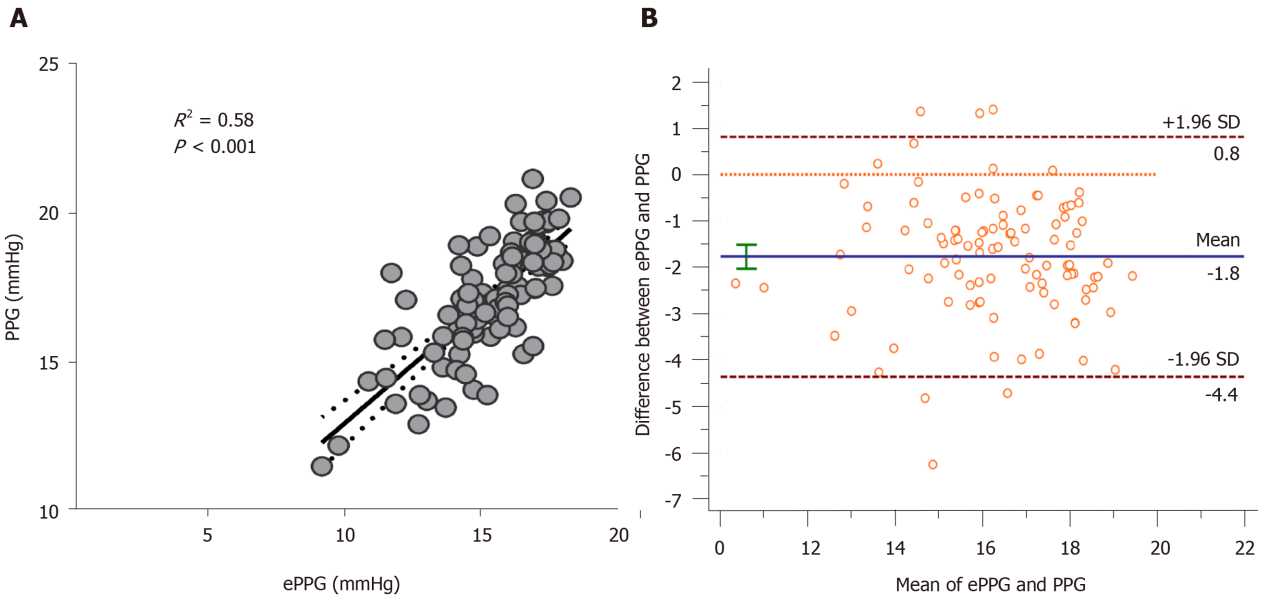

Figure 2 Correlation between portal pressure gradient and estimated portal pressure gradient in the overall group. A: Scatterplot shows agreement between portal pressure gradient (PPG) and estimated PPG (ePPG); B: Bland-Altman plot shows the difference between PPG and ePPG.

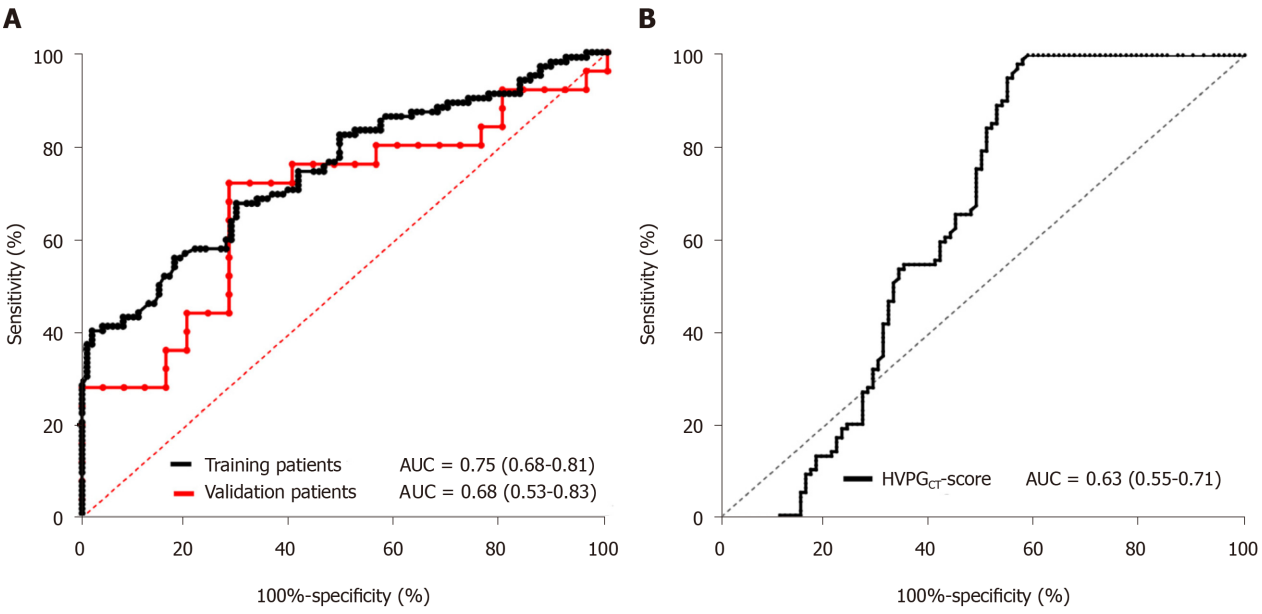

Figure 3 Diagnostic performance of estimated portal pressure gradient for portal pressure gradient. A: Receiver operating characteristic curves of estimated portal pressure gradient (PPG) for predicting PPG in the training and validation cohorts (n = 102 and n = 20, respectively); B: Receiver operating characteristic curves of the HVPG$_{CT}$ score. AUC: Area under curve; HVPG$_{CT}$ score: CT-based portal pressure score.

ARTICLE HIGHLIGHTS

Research background

Portal pressure accurately predicts the risk of peri-operative morbidity and mortality in liver carcinoma. The limitations of HVPG measurement are that it is invasive and impractical for routine clinical practice. Thus, non-invasive measurement methods are urgently needed.

Research motivation

Doppler sonography offers real-time observation of blood flow with qualitative and quantitative assessments, and the application of microbubble-based contrast agents has improved the detectability of peripheral blood flow. The aim of this study was to clarify whether simple, non-invasive US parameters correlate with the invasive

transjugular PPG measurement and to develop a formula to estimate PPG.

Research objectives

To investigate whether ultrasonography (US)-based portal pressure assessment could replace invasive transjugular measurement.

Research methods

A cohort of 102 patients with HCC was selected (mean age: 54 ± 13 years, male/female: 65/37). Pre-operative US parameters were assessed by two independent investigators, and multivariate logistic analysis and linear regression analysis were conducted to develop a predictive formula for the portal pressure gradient (PPG). The estimated PPG predictors were compared with the transjugular PPG measurements. Validation was conducted on another cohort of 20 non-surgical patients.

Research results

The mean PPG was 17.32 ± 1.97 mmHg. Univariate analysis identified the association of the following four parameters with PPG: Spleen volume, portal vein diameter, portal vein velocity (PVV), and portal blood flow (PBF). Multiple linear regression analysis was performed, and the predictive formula using the PVV and PBF was as follows: PPG score = 19.336-0.312 x PVV (cm/s) + 0.001x PBF (mL/min). The PPG score was confirmed to have good accuracy with an area under the curve (AUC) of 0.75 (0.68-0.81) in training patients. The formula was also accurate in the validation patients with an AUC of 0.820 (0.53–0.83).

Research conclusions

The formula based on ultrasonographic Doppler flow parameters shows a significant correlation with invasive PPG and, if further confirmed by prospective validation, may replace the invasive transjugular assessment.

Research perspectives

The formula for the prediction of PPG should be verified on a larger and external validation cohort for widespread acceptance.

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