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FIELD OF VISION

SARS-COV-2 infection (coronavirus disease 2019) for the gastrointestinal consultant

Kaveh Hajifathalian, Srihari Mahadev, Robert E Schwartz, Shawn Shah, Kartik Sampath, Felice Schnoll-Sussman, Robert S Brown Jr, David Carr-Locke, David E Cohen, Reem Z Sharaiha

ORCID number: Kaveh Hajifathalian (0000-0002-3931-2576); Srihari Mahadev (0000-0002-4123-3922); Robert E Schwartz (0000-0002-8846-0428); Shawn Shah (0000-0002-4504-2154); Kartik Sampath (0000-0001-9804-4519); Felice Schnoll-Sussman (0000-0001-6892-5905); Robert S Brown Jr (0000-0002-8220-065X); David Carr-Locke (0000-0003-1931-714X); David E Cohen (0000-0001-9827-6926); Reem Z Sharaiha (0000-0002-3235-6904).

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Kaveh Hajifathalian, Srihari Mahadev, Robert E Schwartz, Shawn Shah, Kartik Sampath, Felice Schnoll-Sussman, Robert S Brown Jr, David Carr-Locke, David E Cohen, Reem Z Sharaiha, Division of Gastroenterology and Hepatology, Weill Cornell Medicine, New York-Presbyterian Hospital, New York, NY 10021, United States

Corresponding author: Reem Z Sharaiha, MD, Associate Professor, Division of Gastroenterology and Hepatology, Weill Cornell Medicine, New York-Presbyterian Hospital, 1283 York Avenue, 9th floor, New York, NY 10021, United States. rzs9001@med.cornell.edu

Abstract

The current pandemic due to the severe acute respiratory syndrome coronavirus 2 has caused an extreme burden for health care systems globally, and the number of cases is expected to continue to increase, at least in the immediate future. The virus is estimated to have infected more than 1.5 million individuals. The available reports suggest that gastrointestinal (GI) involvement in coronavirus disease 2019 (COVID-19) is common and in some cases the GI symptoms may precede the respiratory symptoms. In addition to direct effects of severe acute respiratory syndrome coronavirus 2, the infected patients remain at risk for the complications commonly managed by gastroenterology and hepatology consultants. The most commonly reported GI manifestation of COVID-19 is diarrhea, which is reported in a third to up to more than half of the patients. Mild to moderate elevation of the liver enzymes are also common, although no case of acute liver failure has been reported so far. Many of the medications used for treatment of COVID-19 can also be associated with GI symptoms or liver injury and can be included in the differential diagnosis in these patients. Although the diagnosis of the infection is currently based on RNA analysis in respiratory samples, the available literature on fecal shedding of this virus suggests that fecal RNA testing might prove to be a useful diagnostic test. It is reasonable to delay all non-urgent endoscopic procedures during the peak of the pandemic and use additional protective equipment such as N95 respirators during endoscopy while most patients can be considered high risk for having been exposed to the virus.

Key words: SARS-CoV-2; COVID-19; Gastroenterology; Hepatology; Liver

Core tip: The coronavirus disease 2019 (COVID-19) has become the first pandemic of

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the 21st century, engaging the health care providers in almost all countries around the world. Similar to previous coronavirus infections such as severe acute respiratory syndrome coronavirus, the COVID-19 is associated with a high prevalence of gastrointestinal (GI) and liver manifestations and abnormalities. Here we present a comprehensive summary of the available evidence on GI involvement of COVID-19 and its implications for the GI consultants.

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INTRODUCTION

Since its emergence in December 2019, the coronavirus disease 2019 (COVID-19) has spread to over 146 countries and has been declared a pandemic by the World Health Organization (WHO). The virus that causes COVID-19, designated the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), is estimated to have infected more than one million individuals and has resulted in more than 59000 deaths to date[1]. Community transmission of the virus has now been confirmed across all inhabited continents and emergency measures have been taken to further curb the transmission of the disease.

SARS-CoV-2 is a positive-sense single-stranded RNA betacoronavirus sharing sequence homology with other pathogenic coronaviruses including Middle East respiratory syndrome coronavirus (MERS-CoV, 2012), and severe acute respiratory syndrome coronavirus (SARS-Cov-1, 2003). The virus is thought to have emerged from animal reservoirs, namely horseshoe bats. The COVID-19 infection varies widely in severity, but primarily manifests as pneumonia. The median age of COVID-19 patients is reported to be in the fifth decade of life, with male predominance, less than 1% of cases occurring in patients younger than 10 to 15 years of age, and higher risk of severe disease in elderly or those with underlying medical comorbidities[2]. SARS-CoV-2 is thought to be transmitted mainly through contact with respiratory droplets and potentially airborne route; however presence of the virus in the stool of infected patients has suggested the fecal-oral route as a possible mode of transmission[3,4].

The first case of COVID-19 was detected in Wuhan, Hubei Province, China, in early December 2019, and the first cohort of patients have been linked to a local live animal market, suggesting the emergence of this virus from animal reservoir. Unlike MERS-CoV and SARS-CoV where widespread international transmission was limited by high case fatality rates and more limited person-to-person transmission (in some instances happening to a large extent in health-care and research facilities), SARS-CoV-2 has shown a relatively lower case fatality rate and easy person-to-person transmission, potentially even during the asymptomatic phase of the disease, leading to a rapid global spread and causing the first known coronavirus pandemic[5].

Although the available information on transmission, pathogenesis, clinical presentations, and management of COVID-19 is limited by the novelty of this new pandemic, it is still important to review the available literature in preparation for an ongoing increase in the number of infected patients over the next several weeks, and potentially months. Here we present a narrative review of the available literature regarding the involvement of the liver and digestive system in patients infected with SARS-CoV-2 infection.

LITERATURE SEARCH

The MEDLINE database was searched through PubMed using a search query constructed with the following medical subject heading (MeSH) terms: ((("severe acute respiratory syndrome coronavirus 2" [Supplementary Concept]) OR "COVID-19"[Supplementary Concept]) OR "coronavirus 2019"[Title/Abstract] OR "sars-cov-2"[Title/Abstract]) AND ("Gastroenterology"[Mesh])) as well as with addition of different keywords to increase the sensitivity and specificity of the search (*e.g.*, "Signs

and Symptoms, Digestive"[Mesh] OR "GI" OR "gastro*" OR "liver" OR "hepat*" OR "digestive" OR "diarrhea" OR "nause*" OR "vomit*" OR "abdomen*" OR "bowel" OR "colon" OR "bile" OR "bilia*" OR "dyspep*" OR "stomach" OR "gastr*" OR "esophag*" OR "duoden*" OR "jejun*" OR "ile*" OR "transamin*"). A similar search was performed in Google Scholar engine. The reference list of the papers, Websites of leading gastroenterology and hepatology journals, as well as WHO, and Center for Disease Control and Prevention publications were reviewed manually by the authors. The full texts of articles were reviewed by the authors to extract the relevant information and was constructed into a narrative review. Literature review was limited to the sources available in English. Fifty-five studies were selected for full-text review by the authors.

RESULTS

Clinical manifestation and complications

Pneumonia is the most common serious clinical manifestation of COVID-19, with fever, fatigue, myalgia, and dry cough being the most common features[6]. Other common symptoms include the anorexia, headache, dyspnea, as well as sore throat and rhinorrhea. Gastrointestinal (GI) symptoms present less commonly, and include diarrhea, liver test abnormalities, nausea, and abdominal pain.

Diarrhea

In a report of 41 patients from Wuhan hospitalized with COVID-19, diarrhea was present in 3% of cases[7]. Interestingly, none of the patients with severe disease needing intensive care unit (ICU) care had diarrhea and all the cases of diarrhea happened in patient with less severe disease in this study. A study of 18 COVID-19 cases in Singapore similarly reported diarrhea in 25% of patients who did not need supplemental oxygen, but none of the cases who required oxygen supplementation[8]. However, a second report from 138 patients hospitalized with COVID-19 in Wuhan showed a higher prevalence of diarrhea with 10% of patients having diarrhea, and a higher prevalence of diarrhea among ICU patients (17%) compared to non-ICU patients (8%, difference not statistically significant)[6]. Authors reported nausea in 10% of cases, vomiting in 4%, and abdominal pain in 2% of cases. Interestingly, ICU patients were significantly more likely to have abdominal pain compared to non-ICU patients (8% *vs* 0%, $P = 0.02$).

Liver test abnormalities

The same report of 41 patients mentioned above showed elevated levels of aspartate aminotransferase (AST) in 37% of patients, including 62% of ICU patients and 25% of non-ICU patients. The study of 138 patients hospitalized in Wuhan showed that although 3% of cases had pre-existing chronic liver disease, none of these needed ICU care in this cohort[6]. Mean AST and alanine aminotransferase (ALT) were both mildly elevated in ICU patients (52 and 35 U/L, respectively), but not in non-ICU patients (29 and 23 U/L), with the AST/ALT ratio of 1.5. A meta-analysis of 10 studies on COVID-19 reported the prevalence of aminotransferase elevation to be between 17% and 37% of patients from different studies[9]. A study on 1099 patients with COVID-19 in China reported an 11% prevalence of elevated total bilirubin (> 1 mg/dL) with both elevated aminotransferase levels and total bilirubin being more common among patients who experienced a composite outcome of ICU admission, mechanical ventilation or death[2]. Elevation of alakaline phosphatase does not seem to be common in patients with COVID-19 and has been reported to happen in 1%-2% of cases[10]. Neither of these studies report acute fulminant liver injury or acute liver failure as a complication of COVID-19. However, given that up to a third of ICU cases can be complicated by shock, it is expected to see varying degrees of ischemic liver injury in these patients. One study has found that an ALT level of > 40 IU/L is associated with inpatient mortality[11], and another study has shown that elevated AST and bilirubin levels can be associated with higher risk of progression to respiratory failure and death[12]. Although it is unclear from the available evidence whether elevation of liver enzymes is an "independent" predictor of poor prognosis, these abnormalities (similar to other indicators of end-organ damage) are encountered more frequently in patients with severe disease and need for ICU care and mechanical ventilation.

Summary of GI manifestations

Considering these findings, it seems that diarrhea is the most common GI manifestation of SARS-CoV-2, with nausea and vomiting, abdominal pain, and mild elevation of AST and ALT as the other presentations (Table 1). Previous studies have shown a higher prevalence of diarrhea (20% to 26%) and other GI symptoms in

patients with SARS-CoV and MERS-CoV[13,14], suggesting a different tropism compared to SARS-CoV-2, although there might be significant variability in the definition used and reporting of GI symptoms for COVID-19 from different hospitals[2,15]. The latest available evidence from a paper focusing on digestive system symptoms in 204 COVID-19 cases from Hubei, China, reports diarrhea in up to 29% of cases, but vomiting and abdominal pain in only 0.8% and 0.4%, respectively, again showing some degree of variability in GI presentations of the disease[16]. Interestingly, in our experience at our hospital in New York we have observed that mild diarrhea can be present in a much larger proportion of patients, reaching a prevalence of more than 50% in patients admitted with COVID-19, suggesting a possible different clinical manifestation in the North American population. Finally, there is now at least one report of bloody inflammatory diarrhea possibly caused by SARS-CoV-2 virus associated colitis suggesting that this virus can cause a wider variety of luminal presentations as currently reported[17]. Importantly, 7 patients in the above-mentioned study (3%) presented only with the digestive complaints mentioned above, and without any respiratory symptoms, in addition to 20% of cases who presented with a combination of respiratory and GI symptoms. Interestingly, patients presenting with GI symptoms had a longer time from the onset of disease to hospital admission compared with patients without digestive symptoms (9.0 d *vs* 7.3 d, $P = 0.02$), and GI symptoms were observed to increase with severity and duration of COVID-19[16]. A separate study has also suggested that while COVID-19 shows a male predominance, GI symptoms of the disease are more likely to be present in female patients[18]. These findings suggest that while a small group of patients might present initially with only GI symptoms, most of cases develop these symptoms later on during the course of their disease.

There might be a potential explanation for relatively high prevalence of diarrhea and risk of small bowel involvement with SARS-CoV-2 compared with other GI symptoms, as both SARS-CoV and SARS-CoV-2 are thought to have a high affinity for angiotensin-converting enzyme 2 (ACE2) receptor potentially permitting virus entry into cells, and ileal epithelial cells have a significantly high ACE2 expression, while cholangiocytes and esophageal epithelial cells also express this receptor as a potential target for the virus[3,19,20]. An available report of elevated gamma-glutamyl transferase, a diagnostic biomarker for cholangiocyte injury, in patients with COVID-19 (up to half of cases in a cohort from China) provides further evidence regarding cholangiocyte injury[10]. Although ACE2 expression in hepatocytes is relatively lower than cholangiocytes, it is worth noting that previous autopsy and liver biopsy studies from SARS-CoV patients have found viral particles in hepatic parenchyma as well as eosinophilic bodies and ballooning, suggesting hepatocyte injury[21,22]. It should be noted that the observed GI manifestations including elevated liver enzymes can be confounded by adverse reactions due to different pharmacotherapy agents in COVID-19 patients (discussed below), as well as associated ischemia and hypoxia in severe cases.

Pharmacotherapy

Thus far there is no proven specific treatment for COVID-19, and the mainstay of management remains to be supportive care. However, the available pre-clinical evidence shows *in-vitro* efficacy of both chloroquine and hydroxychloroquine against SARS-CoV-2 infection potentially through increasing endosomial pH and interfering with the glycosylation of cellular receptor of SARS-CoV[23,24]. This has led to clinical use of these drugs in COVID-19, while the results of ongoing clinical trials are pending. It is important to note that although chloroquine and hydroxychloroquine rarely result in clinically significant acute liver injury (except in patients with porphyria cutanea tarda), they should be used with caution in patients with hepatic impairment, or those taking concurrent hepatotoxic medications[25]. Other experimental agents include Lopinavir-Ritonavir, Remdesivir, Favipiravir, Tocilizumab, Sarilumab, and Siltuximab, all with unproven efficacy. Use of Lopinavir-Ritonavir can be associated with GI adverse reactions such as diarrhea, nausea and vomiting, abdominal pain, and increased serum aminotransferase, amylase and lipase levels, as well as risk of hepatitis and exacerbating underlying chronic liver disease, for example in patients with chronic viral hepatitis. For example, Four out of five patients treated with Lopinavir-Ritonavir in an abovementioned study from Singapore developed Nausea, vomiting, and/or diarrhea, and three developed abnormal liver tests[8]. However, after publication of a trial failing to show a significant benefit for Lopinavir-Ritonavir, its use has declined for the treatment of COVID-19[26]. Similarly, Tocilizumab and similar medications such as Sarilumab can be associated with increased aminotransferase levels as well as risk of acute liver injury and failure. The full extent of GI adverse events of the antiviral treatments for COVID-19, such as Remdesivir and Favipiravir, is not yet clear; however, the existing

Table 1 Gastrointestinal manifestations of coronavirus disease 2019

Manifestation	Reported prevalence	
Luminal		
Diarrhea	Very common	10%-29%, potentially 50% in North America
Abdominal pain	Common	1%-29%
Nausea and vomiting	Common	1%-29%
Hemorrhagic enterocolitis	Rare	Case report
Liver		
Acute liver failure	So far not reported	
Mild to moderately elevated AST and ALT	Very Common	17%-62%
Elevated bilirubin	Uncommon	Up to 11%
Elevated alkaline phosphatase	Uncommon	< 5%
Elevated GGT	Common	Up to 54%

GGT: Gamma-glutamyl transferase; ALT: Alanine aminotransferase; AST: Aspartate aminotransferase.

reports suggest nausea and vomiting and varying degrees of liver test abnormalities as potential side effects. It is reasonable to obtain baseline liver function tests before using the abovementioned pharmacologic agents for management of COVID-19 and continue to monitor them during treatment. Regarding immunosuppressive medication used in patients with inflammatory bowel disease and autoimmune hepatitis, the current guidance from a multi-society task force recommends continuation of medication given that risk of disease flare and associated complications currently outweighs the risk of contracting COVID-19[27]. These patients, as well as patients with chronic liver disease and cirrhosis, should be counseled to remain cautious and follow guidelines for at-risk group with optimal hand hygiene and social isolation to minimize their risk during the pandemic. As an example, a report from China has suggested that a simple intervention by sending messages to patients with cirrhosis regarding precautions to take against COVID-19 can significantly decrease the risk of contracting the disease[28].

Testing and fecal shedding

Current guidelines from the WHO and Center for Disease Control and Prevention recommend nasopharyngeal swabs for testing for SARS-CoV-2, with additional oropharyngeal swabs as an option[29]. The presence of SARS-Cov-2 is then verified using reverse-transcription polymerase chain reaction. A positive result confirms the presence of SARS-CoV-2, but due to the potential for false negative results, the WHO recommends re-sampling and repeat testing in case of negative results with clinical suspicion for COVID-19[30]. As mentioned earlier, SARS-CoV-2 RNA is present in patients' stool. A study of 292 cases from China reported the presence of viral RNA in stool to be persistent in 17% of convalescent cases, with 78% of cases having longer duration for stool specimens staying positive for viral RNA compared to viral RNA from throat swabs, with a median delay of 2.0 d[4]. A separate study on 74 patients with confirmed COVID-19 and both fecal and respiratory sample testing reported that while viral RNA is not positive in all cases (it was negative in 45% of cases), the fecal RNA remains positive during convalescence and for a longer period compared to respiratory samples (mean 28 d *vs* 17 d after first symptoms) and can stay positive for up to 5 wk after the initial presentation[31]. Although stool samples are not currently used for diagnosis of COVID-19, these findings suggest a potential role for stool samples to be used both for diagnosis and for evaluation of risk of transmission and need for isolation during convalescence, as well as a potential risk for fecal-oral transmission of this disease. Interestingly, there is now a report of a patient with COVID-19 and positive fecal viral test but with several negative pharyngeal and sputum polymerase chain reaction tests over time, suggesting that fecal testing can potentially play a role in the diagnosis of COVID-19[32]. Fecal microbiata transplant donors are a special group with potential for widespread transmission of the disease, and testing for viral RNA in their stool samples should be seriously considered, especially if they have a history of typical COVID-19 symptoms over the past 4 to 6 wk[33]. Finally, it is reasonable to consider the donors and recipients of liver transplantation as a special population and recommend universal testing for SARS-CoV-2 before liver transplantation[34]. As mentioned above, given reports of a small minority of patients initially presenting exclusively with GI symptoms, it is important

for GI consultants to remain vigilant and include COVID-19 in their differential diagnosis even in the absence of respiratory symptoms, especially in febrile patients.

Endoscopy during SARS-CoV-2 pandemic

A multi-society guideline published on March 15, 2020 by the AASLD, ACG, AGA and ASGE has recommended postponing non-urgent endoscopic procedures during the pandemic[27]. Examples of these procedures include screening and surveillance endoscopic procedures in asymptomatic patients (such as colon cancer screening or Barrett's esophagus surveillance), esophageal pH testing, motility studies (such as esophageal and anorectal manometry), and diagnostic procedures where results are not urgently needed (such as endoscopic ultrasound exam for pancreatic cyst with intermediate risk of malignancy). Naturally, endoscopists will have to continue to perform procedures for urgent cases such as food impactions or severe dysphagia, GI bleeding, cholangitis or acute biliary obstruction, or time-sensitive endoscopic examinations such as evaluation of malignancies and endoscopic or echoendoscopic staging. Multiple guidelines published by different GI and endoscopic societies provides further details regarding endoscopy during the COVID-19 pandemic[35-37]. Patients should be screened for presence of fever and clinical symptoms compatible with COVID-19 according to institutional protocols prior to admission to endoscopy suite, and the number of people present in the endoscopy suite should be minimized to decrease the risk of exposure and transmission and usage of personal protective equipment (PPE). Given the presence of viral DNA in pharyngeal and GI secretion, risk of aerosolization should be minimized by efficient intubation (when needed) with experienced anesthesiology providers, and minimizing the length of the endoscopic procedures and use of CO_2[38]. In addition to standard PPE including disposable hairnet, gowns, gloves, surgical mask and face shield, providers should consider using N95 respirators (or equivalent, such as FFP2 or FFP3) while providing care for all patients during the COVID-19 pandemic. While some authors have suggested using N95 respirators only for confirmed or high risk cases and during upper endoscopy in intermediate risk patients, it should be noted that near universal spread of the virus across communities will qualify almost all patients as "high-risk" according to the current guidelines with a need for using N95 respirators for all endoscopic procedures[37,39]. Finally, it is reasonable to change gastroenterology and hepatology clinic visits to telehealth care using phone calls and video visits (according to availability and institutional protocols) in patients who do not have an absolute need for physical examination.

DISCUSSION

The current pandemic due to SARS-CoV-2 virus has caused an extreme burden for health care systems globally, and the number of cases is expected to continue to increase, at least in the immediate future. Familiarity of health care providers with this virus and its clinical manifestations can significantly help with efficient and timely management of patients with COVID-19. The review of the available literature with a focus on GI manifestations of COVID-19 is presented here. The available reports suggest that GI involvement in COVID-19 is less common compared with previous Coronavirus outbreaks, namely SARS-CoV, and MERS-CoV. Nonetheless, a significant proportion of patients present with GI symptoms and signs in addition to the cardinal manifestations of lower respiratory tract involvement and pneumonia. In some cases the GI symptoms may precede any respiratory symptoms[16]. The most common luminal manifestation of the disease is diarrhea reported in up to 17% of the cases, but the available literature is limited regarding the severity of diarrhea.

The high expression of ACE2 in the ileum suggests it as a potential target of the virus in the GI tract. Additionally, mild to moderate elevation of aminotransferases has been reported in 20% to 50% of the cases. Although acute liver failure has not been reported as a direct consequence of severe COVID-19, the extent of abnormal liver tests seems to be associated with disease severity and worse outcomes. It is important to note that medications used for management of COVID-19, such as chloroquine or Lopinavir-Ritonavir can be associated with varying degrees of liver test abnormalities and GI adverse reactions. It is important to obtain a baseline evaluation of patients' liver function before initiation of treatment and continue to monitor liver function tests during the treatment. It is also important to check patients for presence of chronic viral hepatitis (HBV, and HCV), as well as risk factors for chronic liver disease especially alcohol use before starting the treatment with these agents. In addition to direct effects of SARS-CoV-2 and similar to any other patients with severe illness, these patients remain at risk for the complications commonly

managed by gastroenterology and hepatology consultants such as C. difficile infection in context of frequent antibiotic use, and ischemic liver injury or cholestasis of critical illness. As detailed above, delaying endoscopic procedures in non-urgent cases, and strict adherence to hand hygiene, contact precautions, and correct use of PPE will help minimize the risk of exposure and transmission during COVID-19 pandemic and conserve health-care resources.

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OPINION REVIEW

Optimized timing of using infliximab in perianal fistulizing Crohn's disease

Xue-Liang Sun, Shi-Yi Chen, Shan-Shan Tao, Li-Chao Qiao, Hong-Jin Chen, Bo-Lin Yang

ORCID number: Xue-Liang Sun (0000-0001-8520-6937); Shi-Yi Chen (0000-0003-1714-9867); Shan-Shan Tao (0000-0002-8815-7146); Li-Chao Qiao (0000-0002-0292-2936); Hong-Jin Chen (0000-0003-2222-3582); Bo-Lin Yang (0000-0002-2474-4085).

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Xue-Liang Sun, Shi-Yi Chen, Shan-Shan Tao, Li-Chao Qiao, Hong-Jin Chen, Bo-Lin Yang, First Clinical Medical College, the Affiliated Hospital of Nanjing University of Chinese Medicine, Nanjing 210029, Jiangsu Province, China

Xue-Liang Sun, Department of Colorectal Surgery, Suzhou TCM Hospital Affiliated to Nanjing University of Chinese Medicine, Suzhou 215000, Jiangsu Province, China

Bo-Lin Yang, Department of Colorectal Surgery, The Affiliated Hospital of Nanjing University of Chinese Medicine, Nanjing 210029, Jiangsu Province, China

Corresponding author: Bo-Lin Yang, MD, Chief Doctor, Professor, Surgeon, Department of Colorectal Surgery, The Affiliated Hospital of Nanjing University of Chinese Medicine, 155 Hanzhong Road, Nanjing 210029, Jiangsu Province, China. yfy0051@njucm.edu.cn

Abstract

Infliximab (IFX), as a drug of first-line therapy, can alter the natural progression of Crohn's disease (CD), promote mucosal healing and reduce complications, hospitalizations, and the incidence of surgery. Perianal fistulas are responsible for the refractoriness of CD and represent a more aggressive disease. IFX has been demonstrated as the most effective drug for the treatment of perianal fistulizing CD. Unfortunately, a significant proportion of patients only partially respond to IFX, and optimization of the therapeutic strategy may increase clinical remission. There is a significant association between serum drug concentrations and the rates of fistula healing. Higher IFX levels during induction are associated with a complete fistula response in these patients. Given the apparent relapse of perianal fistulizing CD, maintenance therapy with IFX over a longer period seems to be more beneficial. It appears that patients without deep remission are at an increased risk of relapse after stopping anti-tumor necrosis factor agents. Thus, only patients in prolonged clinical remission should be considered for withdrawal of IFX treatment when biomarker and endoscopic remission is demonstrated, especially when the hyperintense signals of fistulas on T2-weighed images have disappeared on magnetic resonance imaging. Fundamentally, the optimal timing of IFX use is highly individualized and should be determined by a multidisciplinary team.

Key words: Infliximab; Crohn's disease; Perianal fistula; Optimization; Trough level; Deep remission

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Core tip: The long-term outcomes of infliximab in the treatment of perianal fistulizing Crohn's disease are unfavorable, due to loss of response. The optimization of the therapeutic strategy may increase clinical remission. Higher infliximab concentrations during induction are associated with a complete fistula response. Only patients in prolonged clinical remission should be considered for withdrawal of infliximab when biomarker, endoscopic and radiological remission is demonstrated. Fundamentally, the optimal timing of infliximab use is highly individualized and should be determined by a multidisciplinary team.

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INTRODUCTION

Crohn's disease (CD) is a chronic, disabling and idiopathic inflammatory bowel disease that can involve any element of the gastrointestinal tract. Perianal fistulas are a common extraintestinal manifestation of CD, presenting in approximately 40% of patients before or at the time of diagnosis and in 24% after diagnosis[1]. The median interval between CD diagnosis and the first perianal fistula is 2.9-4.4 years[1,2]. Perianal fistulas correlated with CD are indicative of severe disease and a more aggressive course. The natural progression of perianal fistulizing CD (PFCD) is characterized by alternation of remission and relapse periods. The recurrence rate is up to 80% after a median follow-up of 10 years[2]. Repeated perianal symptoms, such as persistent purulent discharge, pain, and fecal incontinence, can cause fatigue, anxiety, and depression, which can be debilitating and negatively impact patients' quality of life. As the disease progresses, fecal diversion may be necessary to achieve clinical remission in the advanced period. However, it is a fatal procedure because the success rate of restoring bowel continuity is only 16.6%[3]. Ultimately, proctectomy is performed in 41.6% of patients suffering from fecal diversion failure[3].

Emerging biologic agents have revolutionized the medical treatment of PFCD and achieved more promising outcomes than immunomodulators[4]. In the biological era, the treatment goal has changed from symptom relief to complete fistula healing, while also preventing relapse. Fistulizing CD was, together with steroid dependence or resistance, the first indication for biological therapy, after surgical drainage of any sepsis[5]. Infliximab (IFX) is the first anti-tumor necrosis factor (TNF) agent for the treatment of PFCD. The ACCENT trial showed that 68% of patients with fistulizing CD achieved symptom improvement following IFX monotherapy, whereas the closure rate of fistulas was only 36% at 54 wk[6,7]. This finding indicated that a substantial proportion of patients partially responded to IFX. Surgical interventions appear to be indispensable in assisting IFX to alter the natural course of PFCD, because the probability of perianal surgery does not significantly decrease after the emergence of biologic agents[8]. Anatomically, CD-related perianal fistulas can be categorized into two types: Simple and complex[9]. Fistulotomy achieves excellent outcomes in the treatment of symptomatic simple fistulas, with a recurrence rate of 3.4% during a mean follow-up of 1.6 years[10]. However, complex fistulas that are associated with an increased risk of fecal incontinence make up a larger proportion in PFCD. Although sphincter-preserving procedures, such as loose-seton and ligation of the intersphincteric fistula tract (LIFT), show promising outcomes in the treatment of PFCD, they might be restricted by concomitant proctitis in the early stage of the disease[11,12]. The optimal timing of IFX combined with perianal surgery is unclear due to a debate on the relationship between proctitis and surgical outcomes. Poor prognosis obliges the optimization of IFX therapy to induce a more complete response, alter the natural course of PFCD, and reduce complications, hospitalizations, and the incidence of major abdominal surgery.

To date, there is absence of a consensus on the optimal timing of IFX use. The purpose of this review is to examine the present state of knowledge regarding the use of IFX in PFCD patients in order to contribute to the better management of PFCD.

WHEN TO INITIATE THE PRESCRIPTION OF IFX

Owing to the clinical application of biologics, the healing rate of PFCD has improved. The capability of anti-TNF agents to modify the natural course of PFCD has been validated. The cumulative incidence of PFCD at 10 years has decreased from 24% in the prebiologic era to 12% in the biologic era; similarly the probability of proctectomy has decreased from 24% to 13%[1]. An increasing proportion of CD patients switch to biologics. Although IFX has been recommended as the first-line therapy for PFCD by current European Crohn's and Colitis Organisation consensus, there is still a divergence in the "top-down" strategy due to the hidden perils of overtreatment and severe adverse events[13].

Colombel *et al*[14] demonstrated that patients treated with IFX alone showed a higher healing rate of intestinal mucosa than those treated with azathioprine monotherapy. A real-life study showed that IFX as the first-line therapy was mainly applied in patients with risk factors, higher disease activity and lower quality of life scores[15]. PFCD patients who have a poorer prognosis may benefit from the early introduction of IFX. IFX immediately works to treat PFCD after its first infusion, while the effect-beginning time of adalimumab is over 4 wk after injection[16]. Moreover, the response rate of fistulizing CD to IFX is negatively related to the disease duration[17]. The "step-up" approach may potentially increase the loss of response due to a prolonged disease course and disease progression. Conversely, IFX used as the initial medication can rapidly induce clinical remission and prevent disease progression. Regarding adverse events, infection is the most common, accounting for 53.7% of CD patients treated with IFX[18]. However, the incidence rate of serious infections is only 2.15%[18]. Mortality and malignancy rates are similar between IFX-treated patients and patients with other treatments. Nonserious cerebrovascular accidents and pulmonary embolisms occur in less than 0.1% of the IFX-treated patients[18]. In light of the above evidence, a "top-down" strategy is better for the treatment of PFCD.

TIMING TO COMBINE IFX WITH DEFINITIVE SURGERY

It is well known that surgical intervention is necessary for the drainage of septic complications before the initiation of IFX therapy. However, whether definitive surgery is needed is controversial since IFX can induce fistula closure in approximately 60% of PFCD patients[19]. Despite clinical closure, most fistula tracts can be visualized on pelvic magnetic resonance imaging (MRI). Perianal surgery can improve fistula response to IFX and promote deep remission. It is reported that IFX combined with surgery can improve clinical efficacy compared to monotherapy[20].

Active proctitis negatively affects the outcomes of PFCD, which determines the timing of IFX combined with surgery. Conventionally, surgical procedures are only considered after the elimination of proctitis by prior IFX therapy[21]. In a small sample size study, definitive surgeries, such as fistulotomy and advancement flap, were performed after proctitis was well controlled, with a median interval of 9 wk between the first infusion of IFX and surgery [22]. The healing rate of perianal fistulas was 80% with a median follow-up of 17.5 mo. Nonetheless, the failure of fistula closure may increase in patients with a partial response or primary nonresponse to IFX due to the increased aggression and complexity of perianal fistulas.

The authors performed loose-seton with the eradication of the internal opening within 1 wk before the first infusion of IFX. The clinical healing rate of perianal fistulas was 96.4% after a median follow-up of 26.4 mo[11]. Another study showed that proctitis was detected in 62.5% of patients who achieved improvement of PFCD following definitive surgery[23]. In a prospective study including 15 patients with PFCD, the healing rate of perianal fistulas following LIFT was 67%, with a follow-up duration of 12 mo and without fecal incontinence (Figure 1)[12]. This is comparable with the success rates in cryptoglandular anal fistulas[24,25]. In the cohort, 9 patients had active proctitis, but this finding was not closely related to LIFT failure. Pretreatment with biologic therapy did not improve the outcomes of LIFT[12,26]. A recent multicenter retrospective study demonstrated that multimodal treatment at the diagnosis of PFCD could reduce the probability of repeat surgery and proctectomy[27].

In addition, the issue of wound healing can be addressed by amelioration of immune inflammation, as the median response time of PFCD to IFX is only 9 d[28]. Early combination therapy without waiting for the disappearance of proctitis is viable and is of great importance, as it can alter the natural course of PFCD as soon as possible and improve the patients' quality of life.

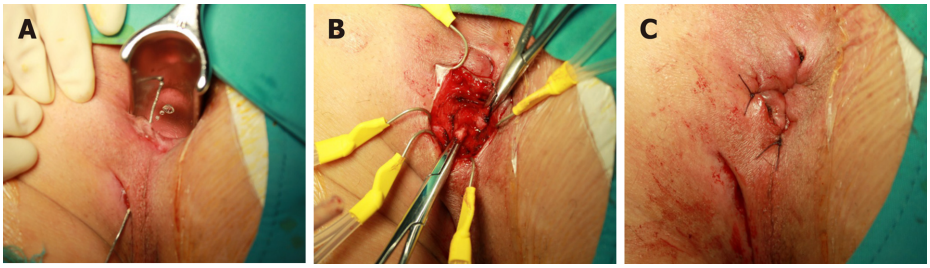

Figure 1 Ligation procedure of the intersphincteric fistula tract for Crohn's disease-related perianal fistula. A: Identification of the fistula tract with a probe; B: Ligation of the intersphincteric tract; and C: Suture of the intersphincteric incision following curetting the remnant tract.

TIMING TO MONITOR AND OPTIMIZE IFX THERAPY

Perianal lesions predict an increased risk of loss of response. Better outcomes are associated with response monitoring and the timely optimization of the therapy regimen during the induction and maintenance of IFX. Nevertheless, the coexistence of luminal and perianal diseases makes the process monitoring and optimization complex and difficult.

Pelvic MRI

It is inadequate to assess the response of PFCD according to clinical symptoms, especially in terms of discriminating between a closed and healed fistula. The inaccurate assessment of the fistula healing process might misguide the adjustment of the IFX therapy regimen, resulting in worse therapeutic efficacy. Pelvic MRI has been suggested as the gold standard for the assessment of the anatomy and activity of CD-related perianal fistulas. Fistula healing is characterized by the disappearance of hyperintense signals on T2-weighted images and the absence of contrast enhancement after gadolinium injection on T1-weighted fat-suppression images (Figure 2)[29]. After anti-TNF therapy, healed fistulas confirmed by MRI account for 50%-61.5% of closed fistulas[30-32]. Persistent tracts indicate a large probability of recurrence and a prolonged duration of maintenance therapy.

Proctitis increases the risk for PFCD occurrence and recurrence. The formation of perianal fistulas occurs in 92% of CD patients with rectal involvement[33]. The absence or disappearance of rectal involvement plays a pivotal role in the deep remission of PFCD, which is defined as clinical remission associated with absence of anal canal ulcers and healing on MRI[31]. In the majority of studies, thickening of the rectal wall was considered an indicator of proctitis[31,32]. In addition, a recent study demonstrated that the size of the mesorectal lymph nodes, mural fat and creeping fat were also relevant to the evaluation of proctitis by pelvic MRI[34].

Changes in the signal intensity and morphology of fistulas and the rectum can indicate the healing or worsening of PFCD. Scheduled pelvic MRI examinations can provide objective evidence for the assessment of treatment efficacy and the optimization or modification of the therapeutic strategy. The monitoring timing varies. The probability of clinical remission is 5 times greater in PFCD patients with a clinical response to anti-TNF agents within 6 wk than in those responding longer than 6 wk[35]. Hence, the sixth week within induction period is a critical time point to evaluate the response of perianal fistulas and proctitis by pelvic MRI. Features of proctitis on MRI are significantly correlated with those on endoscopy during the maintenance therapy period[34]. Given that pelvic MRI is noninvasive and does not have radiation risk, it could be used to dynamically monitor the healing of PFCD at intervals of 8 wk, acquiring accurate information and providing personalized treatment. Radiological healing might lag behind clinical remission by 12 months, suggesting that MRI monitoring should be carried out for at least 1 year[35,36]. Prolonged treatment is often needed to observe the eradication of fistula tracts on MRI.

Trough levels of IFX

When the loss of response is observed on pelvic MRI, clinicians should check the serum trough levels of IFX. The exact mechanism of the loss of response is unclear, but may be associated with drug metabolism or the formation of antidrug antibodies. After exposure, specific antibodies secreted by clonally expanded lymphocytes form immune complexes with IFX. This process is also termed immunogenicity and may cause excessive drug clearance *via* the reticuloendothelial system. The levels of antibodies to IFX have been shown to be higher in patients with a loss of response

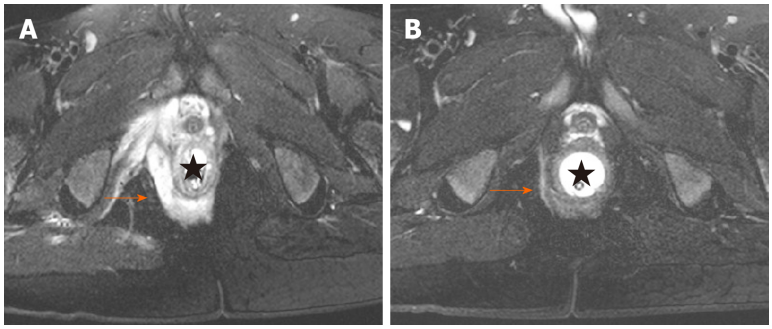

Figure 2 Deep remission of Crohn's disease-related perianal fistula on magnetic resonance imaging. A: Hyperintense signal on T2-weighted fat-suppression imaging showing an active suprasphincteric fistula; B: Disappearance of hyperintense signal on T2-weighted fat-suppression imaging displaying deep remission of the fistula.

than in those who maintained remission[37].

Increasing evidence suggests that low serum trough IFX levels are related to a lack or loss of response[38]. Although a cut-off level of 5.0 μg/mL is recommended as the target concentration for healing the intestinal mucosa, a specific level related to the complete response of PFCD has not been identified[39]. In a recent retrospective cross-sectional study including 29 PFCD patients receiving IFX, higher than 7.1 μg/mL was identified as the optimal threshold value for fistula healing (77.8% sensitivity and 100% specificity)[40]. The median trough concentrations in patients with healed fistulas were significantly higher than those without healed fistulas (8.1 μg/mL *vs* 3.2 μg/mL). Fistula healing was positively related with trough IFX levels. Another similar study with a larger sample size indicated that trough IFX levels above 10.1 μg/mL at 4 wk might provide better outcomes for PFCD[41]. Davidov *et al*[42] demonstrated that trough IFX levels of 9.25 μg/mL at week 2 (89% sensitivity and 90% specificity) and 7.25 μg/mL at week 6 (80% sensitivity and 83% specificity) were the best response predictors of perianal CD. The inconsistency of outcomes may be caused by the various assays and different testing time. Further studies are required to determine the optimal measurement time of drug concentrations and the target IFX levels for fistula healing. More attention should be paid in the induction phase, where multiple factors, such as tissue IFX levels, low albumin, and protein loss, affect the serum drug concentrations.

Therapeutic regimen optimization

As mentioned above, adequate drug concentration is a crucial part of a treat-to-target strategy. The aim of therapeutic regimen optimization is to achieve a steady-state range of serum drug concentrations. Since a higher trough IFX level is necessary for fistula healing than that for mucosal healing, dose escalation should be primarily considered for PFCD patients who do not achieve a response or deep remission prior to switching therapy. Additionally, low drug concentrations can stimulate the germination of immunogenicity, which may be mitigated by early dose optimization. Preexisting antidrug antibodies may be spontaneously degraded in a portion of patients with the continuation of IFX treatment, which also supports the consideration of dose escalation following a loss of response[43]. A dose increase and/or a reduction in the infusion interval are mainly used for increasing serum IFX levels. After dose escalation, 84.8% and 62.3% of CD patients achieved a response, respectively, during the induction and maintenance periods[44]. In terms of safety, trough IFX levels above 7 μg/mL can provide better outcomes for CD patients without increasing the risk of infection[45].

At 54 wk after IFX treatment, antidrug antibodies that were responsible for a loss of response are detected in 62.1% of CD patients[46]. IFX combined with azathioprine is recommended to reduce immunogenicity and mitigate the development of antidrug antibodies. Concomitant therapy can increase serum trough levels of IFX and prolong the duration of fistula closure in CD patients[47,48]. However, early immunosuppressive administration has no effect in increasing clinical remission[49,50]. Furthermore, concomitant therapy does not show better efficacy than IFX monotherapy among CD patients with similar serum IFX levels[51]. Optimized IFX monotherapy leads to similar clinical efficacy as combination therapy[52]. As dose escalation is limited by the increased risk of serious adverse events and increases the consumption of IFX, azathioprine as an adjunct plays a role of dose-sparing by improving the pharmacokinetic features of IFX.

The positive rates of antibodies to IFX were 1.6% at 2 wk, 3.3% at 6 wk, and 17.2% at 14 wk[46]. This discrepancy suggests that a drug concentration below 7 μg/mL at 14 wk is an independent predictive factor for long-term nonresponse. Hence, dose escalation or the addition of immunomodulators within 14 wk can increase the clinical response and remission by elevating serum IFX levels. In addition, the benefits of concomitant therapy should be weighed against the increased risk of serious and opportunistic infections[53].

After IFX failure, it may be beneficial to switch to other biologic agents. Adalimumab (ADA) is another effective anti-TNF agent for the treatment of PFCD, which can maintain remission in 41% of patients naïve to anti-TNF drugs at 12 mo[54]. Moreover, ADA, as a second-line therapy, induced complete response in 50% of PFCD patients refractory to IFX[55]. Previous administration of IFX does not affect the efficacy of ADA induction of fistula closure[56]. Although certolizumab pegol, vedolizumab, and ustekinumab show potential benefits for PFCD patients who failed in IFX or ADA therapy, the dedicated efficacy needs further investigation with large sample size studies[57-59].

TIMING TO WITHDRAW IFX

IFX withdrawal is an important question faced by patients and clinicians after disease remission, due to safety and cost-effectiveness concerns. It is well known that the cessation of IFX therapy after sustained clinical remission is responsible for the recurrence of CD. It has been shown that 29.4%-49.3% of patients with remission experienced relapse within 1-4 years after stopping anti-TNF therapy[60-62]. Overall, approximately 20% of patients never received retreatment with a biologic within a long-term follow-up[63,64]. Fortunately, clinical response can be successfully induced by retreatment with the same anti-TNF agents, primarily IFX, in 80%-94% of cases[60-62]. The high rate of secondary remission may counterbalance the high rate of relapse after withdrawal, suggesting that the discontinuation of IFX therapy and the establishment of a cyclic therapeutic strategy consisting of drug discontinuation and retreatment may be possible[65].

Currently, the decision to withdraw IFX treatment is based on the guidelines for luminal CD because of the absence of dedicated guidelines for PFCD[66]. Heterogeneity of disease phenotype and the absence of controlled trials make it difficult to draw decisive conclusions. Deep remission, defined as clinical remission associated with endoscopic and radiological remission, seems to be the criterion for IFX withdrawal. However, the outcomes are unfavorable, with a relapse rate of approximately 55%[35,63]. The risk factors for relapse after withdrawal included ileal localization at diagnosis, a persistent external opening, prior dose optimization, anemia and a white blood cell count above 5×10^9/L at the time of withdrawal[63,64]. Despite the elimination of risk factors, the optimal timing for withdrawal after deep remission is still unknown, which may affect disease progression. Given that after withdrawal, the relapse of disease is apparent while the clinical benefits, such as a reduction in infection or cancer risk, are theoretical because of the absence of controlled studies, maintenance IFX therapy over a longer period may be more beneficial for PFCD patients. IFX discontinuation as a part of a cyclic therapeutic strategy may be implemented in strictly selected patients. The definitive interruption time should be clarified in future studies.

CONCLUSION

In general, no single treatment can successfully manage PFCD. Although IFX has been recommended as a first-line therapy, early combination with definitive surgery may rapidly lead to clinical remission. Monitoring drug concentrations plays a pivotal role in the optimization of the therapeutic regimen. Scheduled MRI scans can dynamically monitor remission of the internal tract in order to immediately adjust the treatment strategy (Figure 3). IFX withdrawal seems to be possible in the setting of deep remission but is not recommended. The optimal timing of IFX use is highly individualized and should be determined by a multidisciplinary team composed of gastroenterologists, colorectal surgeons, and radiologists.

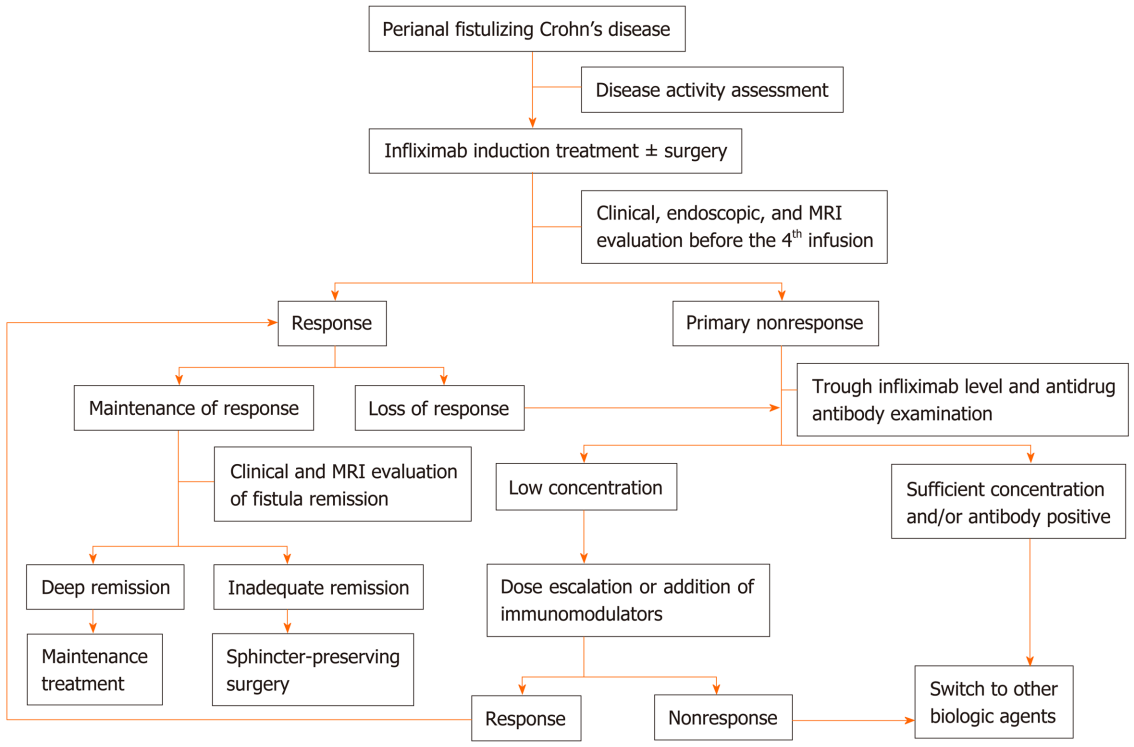

Figure 3 Therapeutic strategy of perianal fistulizing Crohn's disease. MRI: Magnetic resonance imaging.

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REVIEW

Intestinal epithelial barrier and neuromuscular compartment in health and disease

Vanessa D'Antongiovanni, Carolina Pellegrini, Matteo Fornai, Rocchina Colucci, Corrado Blandizzi, Luca Antonioli, Nunzia Bernardini

ORCID number: Vanessa D'Antongiovanni (0000-0002-3303-0674); Carolina Pellegrini (0000-0003-2644-5430); Matteo Fornai (0000-0002-0812-3959); Rocchina Colucci (0000-0002-2593-5457); Corrado Blandizzi (0000-0001-9927-5386); Luca Antonioli (0000-0002-0246-4242); Nunzia Bernardini (0000-0001-9061-9402).

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Vanessa D'Antongiovanni, Matteo Fornai, Corrado Blandizzi, Luca Antonioli, Nunzia Bernardini, Department of Clinical and Experimental Medicine, University of Pisa, Pisa 56126, Italy

Carolina Pellegrini, Department of Pharmacy, University of Pisa, Pisa 56126, Italy

Rocchina Colucci, Department of Pharmaceutical and Pharmacological Sciences, University of Padova, Padova 35131, Italy

Corresponding author: Matteo Fornai, PharmD, PhD, Assistant Professor, Unit of Pharmacology and Pharmacovigilance, Department of Clinical and Experimental Medicine, University of Pisa, Via Roma 55, Pisa 56126, Italy. matteo.fornai@unipi.it

Abstract

A number of digestive and extra-digestive disorders, including inflammatory bowel diseases, irritable bowel syndrome, intestinal infections, metabolic syndrome and neuropsychiatric disorders, share a set of clinical features at gastrointestinal level, such as infrequent bowel movements, abdominal distension, constipation and secretory dysfunctions. Several lines of evidence indicate that morphological and molecular changes in intestinal epithelial barrier and enteric neuromuscular compartment contribute to alterations of both bowel motor and secretory functions in digestive and extra-digestive diseases. The present review has been conceived to provide a comprehensive and critical overview of the available knowledge on the morphological and molecular changes occurring in intestinal epithelial barrier and enteric neuromuscular compartment in both digestive and extra-digestive diseases. In addition, our intent was to highlight whether these morphological and molecular alterations could represent a common path (or share some common features) driving the pathophysiology of bowel motor dysfunctions and related symptoms associated with digestive and extra-digestive disorders. This assessment might help to identify novel targets of potential usefulness to develop original pharmacological approaches for the therapeutic management of such disturbances.

Key words: Digestive disease; Enteric nervous system; Intestinal epithelial barrier; Intestinal motility; Metabolic disorders; Neuropsychiatric disorders

Core tip: Current evidence suggests that impairments of intestinal epithelial barrier and

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enteric neuromuscular compartment might represent a common condition underlying the onset/progression of bowel functional disturbances in both digestive and extra-digestive diseases. In this review, we summarize the impact of morphological and molecular alterations occurring in intestinal epithelial barrier and enteric neuromuscular compartment on bowel motor and secretory functions in digestive and extra-digestive diseases. This assessment, beyond to provide insight on the pathophysiology of bowel motor dysfunctions, could pave the way to the identification of novel therapeutic targets for the management of bowel dysfunctions associated with digestive and extra-digestive disorders.

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INTRODUCTION

A number of digestive and extra-digestive disorders, such as inflammatory bowel diseases (IBDs), irritable bowel syndrome (IBS), intestinal infections, metabolic syndrome and neuropsychiatric disorders, share a set of clinical features at gastrointestinal (GI) level. Digestive functional disturbances, such as infrequent bowel movements, abdominal distension, constipation and secretory dysfunctions, are often complained by patients affected by the above diseases, undermining their quality of life and contributing relevantly to morbidity[1-4].

Several lines of evidence indicate that morphological and molecular changes in intestinal epithelial barrier (IEB) and enteric neuromuscular compartment can be associated with both digestive and extra-digestive diseases. For instance, both IBD and obese patients are characterized by an impairment of IEB and remodeling of enteric neuromuscular compartment, which appear to contribute to alterations of both intestinal motor and secretory functions[5,6]. In parallel, the same or similar morphofunctional GI alterations characterize different neuropsychiatric disorders, such as Parkinson's disease (PD), Alzheimer's disease (AD), multiple sclerosis (MS), amyotrophic lateral sclerosis (ALS), autism spectrum disorder (ASD) and depression[7-9].

Based on this background, the present review has been conceived to provide a comprehensive and critical overview of available knowledge on the morphological and molecular changes occurring in IEB and enteric neuromuscular compartment in both digestive and extra-digestive diseases. In addition, our intent was to highlight whether these alterations could represent a common path (or share some common features) driving the pathophysiology of bowel motor dysfunctions and related symptoms associated with digestive and extra-digestive disorders. This assessment might help to identify novel targets of potential usefulness to develop novel pharmacological approaches for the therapeutic management of such disturbances.

MORPHOLOGY AND FUNCTION OF IEB AND NEUROMUSCULAR COMPARTMENT UNDER PHYSIOLOGICAL CONDITIONS

A dynamic interplay, occurring between IEB, enteric immune system and neuromuscular compartment, contributes relevantly to the maintenance of gut homeostasis[10]. The IEB represents the main physical barrier between the lumen and tissue compartments[11]. The luminal surface of intestinal mucosa is covered by a hydrated gel, consisting mainly of mucins secreted by goblet cells[11]. The outer mucus layer provides a habitat for commensal microorganisms, while the inner mucus layer acts as a physical barrier preventing the penetration of microorganisms and other noxious agents into bowel tissues[11] (Figure 1). Under physiological conditions, there is an equilibrium between the mucus secretion rate and its erosion, due to the movement of luminal contents, ensuring a stable thicknesses of the mucus layer.

Below the mucus layer, the IEB, an epithelial cell monolayer arranged into finger-

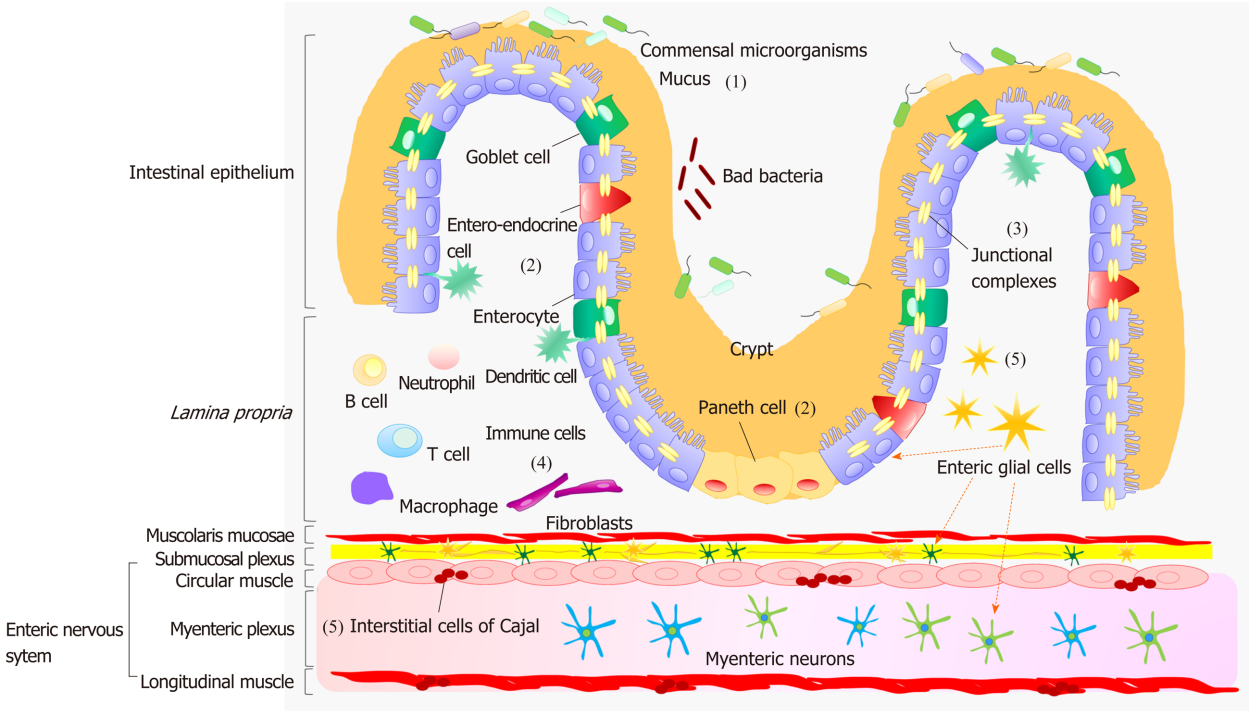

Figure 1 Diagram showing the morphology of intestinal epithelial barrier and neuromuscular compartment. (1) The intestinal mucosa is covered by a hydrated gel, consisting mainly of mucins secreted by goblet cells The outer mucus layer provides a habitat for commensal microorganisms, while the inner mucus layer acts as a physical barrier preventing the penetration of microorganisms and other noxious agents into bowel tissues; (2) The epithelium includes: enterocytes that act as a selective physical barrier and regulatenutrient absorption, goblet cells, entero-endocrine cells that release intestinal hormones or peptides, and Paneth cells that regulate microbial populations and protect neighboring stem cells; (3) Junctional complexes confer mechanical strength to the intestinal epithelial barrier and regulate paracellular permeability; (4) The *lamina propria*, besides containing a number of innate and adaptive immune cells that respond to the insults with the secretion of inflammatory mediators, such as prostaglandins, histamine, and cytokines, is characterized by an intricate network of fibroblasts playing a key role in the proliferation of intestinal epithelium; and (5) Enteric glial cells, a cellular component of the enteric nervous system, are associated with both submucosal and myenteric neurons and are located also in proximity of epithelial cells. They coordinate signal propagation from and to myenteric neurons and epithelial cells, thus regulating bowel motility as well as the secretory and absorptive functions of enteric epithelium; interstitial cells of Cajal are the source of the electrical slow waves responsible for the transmission of excitation to the neighboring smooth muscle cells.

like protrusions (villi) and invaginations (crypts), forms a selective physical barrier[11]. The villi provide an efficient surface for nutrient absorption, while stem cells, located at the basis of crypts, give rise to several types of epithelial cells: Enterocytes, goblet cells, entero-endocrine cells and Paneth cells[11] (Figure 1). Enterocytes are the major cell type in intestinal epithelium. Beyond their critical role as selective physical barrier, they tightly regulates the nutrient absorption (*e.g.*, ions, water, sugar, peptides, and lipids) as well as the secretion of immunoglobulins. In parallel, the entero-endocrine cells release intestinal hormones or peptides into bloodstream upon stimulation, to activate nervous responses. Finally, Paneth cells, located at the base of small intestinal crypts, regulate microbial populations and protect neighboring stem cells, through the secretion of antimicrobial peptides[11].

The IEB holds three fundamental functions: (1) It acts as a physical barrier, preventing the passage of harmful intraluminal entities; (2) It operates as a selective filter, allowing the passage of nutrients and water; and (3) It has secretory functions, such as the release of mucus and immunoglobulins[11].

The efficiency of IEB depends on the maintenance of its integrity, ensured by three junctional complexes that join adjacent epithelial cells and include tight junctions (TJs), adherent junctions and desmosomes[11] (Figure 1). TJs, the most apical intercellular junctions, consist of trans-membrane proteins, such as claudins, occludin and tri-cellulin, which are anchored to the actin cytoskeleton *via* a cytoplasmic plaque including the zona occludens (ZO-1, ZO-2 and ZO-3)[11]. Adherent junctions, located just beneath TJs, share a common structural organization with the junctional complex mentioned above. Desmosomes are located along the lateral membranes beneath adherent junctions. The main tasks of such junctional complexes are to confer mechanical strength to the IEB and regulate paracellular permeability[11].

With regard for the enteric immune system, several review articles have provided a thorough overviews about the intricate networks occurring among the immune cells, resident both in the *lamina propria* and Peyer's patches, and the mucosal and neuromuscular compartment[10] (Figure 1).

The enteric nervous system (ENS) holds a pivotal role in shaping the majority of GI functions[12]. This nervous network is arranged into two plexuses: The submucosal plexus (or Meissner's plexus), located in the submucosa, and the myenteric plexus (or Auerbach's plexus), located between the circular and longitudinal muscle layer[12] (Figure 1). The neurons of submucosal plexus, besides contributing to the motor control of smooth muscles, regulate secretive and absorptive functions, whereas those of the myenteric plexus are involved mainly in the initiation and control of gut motor activity[12]. The ENS, beyond the regulation of GI motor functions, contributes to the control of key functions involved in the maintenance of IEB homeostasis, including paracellular or transcellular permeability, epithelial cell proliferation and TJ expression; it regulates also several mucosal functions, independently of cerebral inputs[13].

Among the cellular components of ENS, there is increasing evidence highlighting a pivotal involvement of enteric glial cells (EGCs), interstitial cells of Cajal (ICC) and smooth muscle cells in the regulation of gut homeostasis. EGCs are associated with both submucosal and myenteric neurons and are located also in proximity to epithelial cells[12]. They coordinate signal propagation from and to myenteric neurons and epithelial cells, thus taking a significant part to the control of bowel motility as well as the secretory and absorptive functions of the enteric epithelium[14,15] (Figure 1). A crucial role in the control of the motor functions of enteric smooth myocytes is played by the ICC, located in the tunica muscularis[12]. These cells generate spontaneous and rhythmic electrical activity, on the basis of which they are considered as pacemakers for gut motility[12] (Figure 1). The muscular compartment consists of two layers of smooth muscle cells: The circular one, where fibers are oriented along the transversal axis and generate forward transit with relatively little mixing, and the longitudinal muscle layer, equipped with fibers oriented along the longitudinal axis, that, beyond the maintenance of intestinal muscle tone, contributes to shorten the lumen and support the propulsion[12] (Figure 1). The outer surface of the muscular layer is covered by the adventitia, which secretes lubricating fluids to reduce friction generated by muscle movements[12].

Overall, the structural and functional integrity of IEB and neuromuscular compartment are essential to ensure an adequate implementation of digestive motor and secretory functions. In particular, a proper interplay between IEB and ENS gives rise to a dynamic network aimed at coordinating the GI physiology and preserving the integrity of gut microenvironment.

MORPHOLOGICAL FEATURES OF IEB AND NEUROMUSCULAR COMPARTMENT IN DIGESTIVE DISEASES

IBDs

IBDs, comprising mainly ulcerative colitis (UC) and Crohn's disease (CD), are chronic intestinal inflammatory disorders, characterized clinically by abdominal pain, diarrhea or constipation, and weight loss[1]. Anatomically, UC is restricted to the rectum, colon and caecum, while CD can affect the entire GI tract, although it commonly affects the terminal ileum and colon[1]. Currently, the etiology of IBDs has not been completely elucidated. Intensive research efforts have been focused on the characterization of the role of IEB and enteric neuromuscular compartment in the onset of IBDs and related digestive disturbances.

Several studies have documented a defective mucus layer in IBD patients. In particular, the histological analysis of UC colonic biopsies has shown a depletion of goblet cells, a reduced mucin glycosylation, and a decrease in mucin (MUC)-2 biosynthesis and secretion[16-19]. By contrast, CD patients display an abnormal glycosylation and mucin hyperproduction accompanied by goblet cell hyperplasia[17] (Table 1). Such alterations can increase the epithelial permeability to luminal bacteria and microbial products, which, upon interaction with immune cells, trigger and maintain the inflammatory response[18-20].

A common feature of IBD patients is the increase in paracellular permeability due to TJ abnormalities that, besides altering the transport of solutes and water and causing leak flux diarrhea, allow the tissue penetration of large molecules and luminal pathogens, triggering innate immune responses[5,21,22]. In this regard, IBD patients have been found to display an increased expression of claudin-2 and claudin-18 as well as a decreased expression and tissue redistribution of occludin, along with an increased serum ZO-1 concentration[5,23-26] (Table 1).

IBD patients are commonly affected by GI motility disorders[27,28]. Indeed, changes in small bowel transit have been reported in both UC and CD patients[27]. Consistent

Table 1 Summary of current human and experimental data on molecular, morphological and functional changes in intestinal epithelial barrier and neuromuscular compartment in digestive disorders

Digestive disorder	Morphofunctional changes in intestinal epithelial barrier	Morphofunctional changes in enteric neuromuscular compartment	Notes	Ref.
Human investigations				
IBD	Altered composition of mucus layer	↓ Myenteric neurons (b)	(a) UC ↓ claudin-1 and -4; CD ↓ claudin-3, -5 and -8	[5,16-19,23-26,29-36]
	Abnormal glycosylation of mucins	↑ SP release (c)	(b) Another study reported an increment of the enteric neuron number	
	↑ Paracellular and transcellular permeability	↑ NK-1 and NK-2 receptors		
	↑ Claudin-2 and claudin-18 (a)	Altered morphology of ICC	(c) Other authors reported a significant reduction of both AChE activity and ACh release in IBD patients suffering from moderate-severe disease, as compared with healthy controls or IBD patients with low disease severity	
	↓ Occludin and ZO-1	Functional alterations of EGCs		
IBS	↑ Mucus secretion	↓ Thickness of muscle layer	(d) Positive correlation between increased intestinal permeability and visceral pain	[51,54-63]
	↑ Paracellular permeability (d)	↑ Entero-endocrine cell activity		
	↓ Occludin and ZO-1	↑ SP release (f)		
	Altered expression of claudins (e)	Altered circulating levels of 5-HT	(e) IBS-D: ↓ claudin-1 and claudin-4, resulting in diarrhea; IBS-C: ↑ claudin-1, claudin-3 and claudin-4, resulting in constipation	
		Altered number and morphology of ICC	(f) Positive correlation between increased SP release and pain scores	
		↑ EGC density		
Intestinal infections	Altered composition of mucus layer	↓ Circulating levels of 5-HT		[72,74,75,76,78,79]
	↓ Goblet cell number	↑ SP release		
	↑ Paracellular permeability altered TJs			
	↑ Epithelial apoptosis			
Diverticulosis and diverticulitis	↑ Mucosal folds	Altered smooth muscle cells	(g) A more recent study did not observe alterations of ENS	[77,80-83]
	Mucosal ulcerations	Altered serotonergic system		
	Crypt distortion	↑ Tachykinergic contractile activity		
		↓ Cholinergic pathway activity		
		↓ ICC number		
		↓ EGC density (g)		
Experimental models				
IBD	Altered composition of mucus layer	↓ Myenteric neurons		[37-50]
	↓ Goblet cell number	Altered morphology of ICC		
	↑ Paracellular and transcellular permeability	↓ EGC density		
	↑ Claudin-1 and claudin-2			
	↓ Occludin and ZO-1			
IBS	↑ Mucus secretion	↓ Thickness of muscle layer	(h) Positive correlation between increased intestinal permeability and visceral pain	[63,65-68,70]
	↑ Paracellular permeability (h)	Altered number of ICC		
	↓ Occludin and ZO-1	↑ SP release		

		↓ Circulating levels of 5-HT	
		↑ EGC density	
Intestinal infections	↑ MUC1 expression	↑ SP release	[84-87]
	↓ MUC2 expression		
	↑ Paracellular permeability		
	Altered TJs		

↑: Increase; ↓: Decrease; 5-HT: Serotonin; Ach: Acetylcholine; AChE: Acetylcholinesterase; CD: Crohn's disease; EGCs: Enteric glial cells; ENS: Enteric nervous system; IBD: Inflammatory bowel disease; IBS: Irritable bowel syndrome; IBS-C: IBS with constipation; IBS-D: IBS with diarrhea; ICC: Interstitial cells of Cajal; MUC: Mucin; NK: Neurokinin; SP: Substance P; TJ: Tight junction; UC: Ulcerative colitis; ZO-1: Zonulin-1.

with these clinical findings, several lines of evidence indicate the occurrence of neuroplastic changes in the neuromuscular compartment and suggest that these are critical steps in contributing to the alterations of digestive motility in the presence of IBDs. In particular, several studies have described a reduction of myenteric neurons[29], mainly in UC than CD tissues[30], likely resulting from increased apoptotic processes, not restricted to specific neural populations[31]. IBD patients display also subtle changes in the expression of enteric neurotransmitters or their receptors. For instance, high levels of substance P (SP) and upregulation of NK-1 and NK-2 receptors have been observed in the colon and rectum of IBD patients[32-34]. Other human studies reported morphological abnormalities of ICC and EGCs, that could participate to the initiation/maintenance of IBDs and their associated symptoms[28,29,35]. In support of this view, histological examinations of UC and CD bowel biopsies pointed out an increase in glial fibrillary acidic protein (GFAP), S100 calcium-binding protein B (S100B), and glial cell line-derived neurotrophic factor (GDNF) in the inflamed area, suggesting that EGCs were activated during the inflammatory processes[36] (Table 1).

The mechanisms underlying pathological interplays among immune/inflammatory processes, IEB, neuromuscular compartment and bowel motor dysfunctions in IBDs remain to be elucidated. In this respect, interesting evidence comes from studies on IBD animal models. *Il10-/-* mice (lacking the expression of IL-10 and developing colitis spontaneously), as well as colitis induced by dextran sodium sulfate (DSS) or dinitrobenzene sulfonic acid (DNBS) display a significant loss of goblet cells and alterations of mucus layer composition, implying a dysfunction in the mucus barrier permeability[18,37-39]. In addition, mouse with DSS colitis showed a reduced expression of occludin and ZO-1 as well as an increase of claudin-1 and claudin-2, along with a marked increase in apoptotic death of epithelial cells[40,41] (Table 1). Of note, the reduction of ZO-1 expression was found to precede the onset of intestinal inflammation, suggesting that the ZO-1 alteration was not a consequence of the inflammatory process, but rather an early event, prodromal to the onset of colitis[40]. In support to this view, studies conducted in *Il10-/-* mice, beyond showing alterations of villus and crypt architecture, displayed an increment of intestinal permeability, that occurred as a primary defect, before the onset of mucosal inflammation, suggesting a disruption of IEB[42,43].

The occurrence of ENS abnormalities, including axonal hypertrophy, a decrease in the number of enteric neurons and morphological alterations of ICC, has been described also in animal models of IBD[44-48]. Brown *et al*[49] reported that the activation of EGCs in the context of neuroinflammation induce enteric neuronal death in DNBS-treated mice, suggesting that glial response to inflammatory mediators might contribute to the development of bowel motor abnormalities. Currently, only one pre-clinical study, conducted in rats with 2,4,6-trinitrobenzene sulfonic acid (TNBS) colitis, reported a loss of EGCs following bowel inflammation, demonstrating that colitis can affect differently the EGCs in the submucosal and myenteric plexus[50] (Table 1). Of note, at present studies on histological alterations of EGC markers such as GFAP, S100B and GDNF in animal tissues of IBDs are lacking. Therefore, further investigations should be implemented to help better clarifying putative correlations among the morphofunctional alterations of EGCs, bowel inflammation and motor dysfunctions in IBDs.

IBS

IBS is a frequent disorder affecting up to 15%-25% of the adult population[2]. IBS patients are classified into subtypes by predominant stool pattern: IBS with diarrhea (IBS-D); constipation (IBS-C); mixed (IBS-M); and unsubtyped IBS (IBS-U)[2]. Among the patients complaining of constipation, 11% have functional slow transit constipation (STC); such patients differ from IBS-C due to the absence of abdominal pain. Emerging evidence suggests that, beyond psychosocial factors and low-grade

intestinal inflammation, alterations of IEB and enteric neuromuscular compartment could contribute to IBS onset, development and related symptoms.

Human studies have reported a status of exuberant mucin secretion by goblet cells along with an increased paracellular permeability due to TJ abnormalities in IBS patients[51]. The increment of IEB permeability is thought to represent an important step in the sequence of events leading to the onset of low-grade intestinal inflammation and disturbed bowel functions[52,53]. The integrity of IEB in IBS patients has been investigated by evaluating the urinary excretion of oral probes, such as ^{13}C mannitol[54]. This approach has allowed to document an increase in the intestinal permeability of IBS patients, likely reflecting alterations of TJs occurring during the acute phase of the disorder[54]. Histological examinations of colonic biopsies showed an abnormal cellular distribution of claudins as well as a reduced expression of ZO-1 and occludin in all IBS subtypes as compared to healthy controls[51,55,56] (Table 1). Currently there is no evidence regarding changes in IEB in STC patients.

As far as the neuromuscular compartment is concerned, several alterations have been described in patients, suggesting their contribution to the pathophysiology of IBS symptoms, such as bowel dysmotility. However, no predominant patterns of motor activity have emerged as markers for IBS. In this context, translational evidence highlighted a hypertrophy of the muscle layer, mainly in IBS-D patients, and alterations of the number and size of ICC both in IBS and STC patients[57-60]. Cheng *et al*[51] reported an abnormal density of entero-endocrine cells in rectal biopsies of IBS patients, along with a strong secretory status, suggesting that the endocrine system may play an important role in the pathophysiology of IBS. Other studies observed an increase in circulating serotonin levels in IBS-D patients, contrary to IBS-C, characterized by reduced levels of circulating serotonin[61,62]. These findings suggest that serotonin, beyond regulating gut motility, plays an important role in immune activation and inflammation, thus contributing to the pathophysiology of IBS. Currently, only few studies have taken into consideration the morphology of EGCs in IBS. For instance, Wang *et al*[63] observed an increment of EGCs in the colonic mucosa of IBS patients (Table 1). By contrast, STC patients displayed a significant decrease in EGCs in both the myenteric and submucosal plexus[64]. At present, there is no evidence to explain the relationship between the altered number of EGCs and bowel motor dysfunctions in IBS and STC patients. Therefore further studies are needed.

Consistently with human findings, an increment of mucus secretion and hyperplasia of goblet cells has been observed in IBS animal models[65]. In addition, in an IBS-D rat model induced by acetic acid, a significant reduction of ZO-1 and occludin expression has been shown[66]. These findings suggest that morphological alterations of mucus layer and TJ proteins could contribute to the increased sensitivity to visceral pain and other aspects of IBS symptoms[65,67] (Table 1).

The occurrence of ENS abnormalities has been described also in IBS animal models. Indeed, similarly to patients, murine models of IBS showed a significant reduction of the total thickness of muscle layer and alterations of ICC[65,68]. Likewise, Wang *et al*[69] showed a significant reduction of ICC number in a rat model of STC. Thus, current data from human and pre-clinical studies indicate that changes in ICC numbers are closely associated with alterations of intestinal motor patterns in both IBS and STC[57,68,70]. Of interest, similarly to IBS patients, Wang *et al*[63] reported an increase in the number of EGCs, observing a positive correlation between changes in EGCs and abdominal pain (Table 1).

Other digestive disorders

For a variety of digestive disorders, such as intestinal infections and diverticular disease (including diverticulosis and diverticulitis), the pathogenesis remains unclear and several hypotheses have been formulated. Nevertheless, alterations of IEB and enteric neuromuscular compartment have been described as common features likely involved in the pathogenesis and progression of these diseases.

In intestinal infections, the presence of pathogens in the intestine can induce pathological alterations of the mucus layer and IEB, resulting in the onset of inflammatory responses within the gut wall[71]. Indeed, infectious agents may damage the intestinal mucosa by a direct interaction with mucins or the release of toxins[72,73]. In this regard, human studies have documented a depletion of goblet cells and an altered composition of mucus, resulting in an enhanced interaction between harmful intraluminal entities and enteric epithelium, exacerbating intestinal inflammation[72,74]. On the other hand, infectious agents have developed mechanisms that target the host's TJs. Clinical data from norovirus-infected patients showed a flattening of epithelium and a severe loss of villi as well as a reduction of TJ expression and an increment of epithelial apoptosis[75,76] (Table 1).

When considering the morphofunctional alterations of the mucus layer and IEB occurring in diverticular disease, a limited number of clinical data are currently

available. For instance, a recent study showed a prominent mucosal folding with crypt distortion, mucosal ulcerations and infiltration of inflammatory cells in patients with diverticulitis[77] (Table 1).

With regard for the neuromuscular compartment, structural and functional abnormalities have been observed, either in patients with intestinal infections and subjects affected by diverticular disease. A common feature in such disorders is the alteration of enteric neurotransmitters. Clinical evidence in *Giardia duodenalis*-infected patients showed a reduction of circulating serotonin and a decreased number of serotonin-containing enterochromaffin cells in the duodenal mucosa[78]. Other authors reported an increment of SP levels in the gut of patients infected with *Cryptosporidium*[79] (Table 1). Similarly to intestinal infections, patients with diverticular disease displayed alterations of the serotonergic system[80] and an increment of tachykinergic motor activity as well as a reduction of cholinergic motility[81]. Other authors reported an altered expression patterns of important molecular factors involved in the regulation of smooth muscle cells contractility at level of the *tunica muscularis*[82]. In addition, Wedel *et al*[83] observed a thickening of muscle layers, along with a reduced number of EGCs and ICC (Table 1).

Consistently with human findings, pre-clinical studies in mice infected with *Citrobacter rodentium* or *Campylobacter jejuni*, beyond showing a depletion of MUC2, displayed an increment of MUC1 secretion[84]. Such an increase, observed both in human and pre-clinical studies, highlights a mechanism of host defense aimed at trapping parasites in the mucus, thereby favoring their expulsion. On the other hand, Elmi *et al*[85] reported an increment of IEB permeability due to TJ alterations in mice infected with *Campylobacter jejuni*, *Escherichia coli* and *Citrobacter rodentium*, that contributed to promote bacterial invasion into host cells and the development of inflammatory process (Table 1).

When considering the morphofunctional alterations of neuromuscular compartment in animal models of intestinal infections, some authors reported a significant increase in SP levels in *Cryptosporidium*-infected macaque or rats infected with *Trichinella spiralis*, suggesting a relationship between the SP content and inflammation associated with pathogen invasion as well as a positive correlation between SP levels and the severity of diarrhea[86,87] (Table 1). Current animal models of diverticular disease, based on low-fiber diets, have generated very inconsistent results and/or a significant impairment of the systemic health status[88]. Thus, at present, pre-clinical studies on the histological alterations of IEB and ENS in models of diverticular disease are strongly needed.

MORPHOLOGICAL FEATURES OF IEB AND NEUROMUSCULAR COMPARTMENT IN EXTRA-DIGESTIVE DISEASES

Metabolic disorders (obesity and diabetes)

Patients with metabolic disorders, including obesity and type 2 diabetes mellitus, often experience GI dysfunctions, such as impaired gastric emptying, infrequent bowel movements and constipation[3]. In this setting, several lines of evidence support the contention that a chronic low-grade systemic inflammatory condition, besides interfering with the metabolic processes, could contribute to alterations of IEB and enteric neuromuscular compartment, which, in turn, could lead to the onset of bowel motor abnormalities.

A recent study showed that obese patients display an increase in IEB permeability, along with a decreased expression of occludin and tri-cellulin as well as an increase in circulating lipopolysaccharide (LPS), an indirect index of intestinal permeability, and ZO-1 levels[6] (Table 2). However, despite these interesting observations, human studies, showing a correlation between altered IEB, changes in the enteric neuromuscular compartment and intestinal motor dysfunctions, are currently lacking. In this respect, pioneering evidence, supporting the relevance of IEB alterations in the pathophysiology of bowel dysmotility in metabolic disorders, comes from pre-clinical studies. For instance, mice with high fat diet (HFD)-induced obesity displayed a decrease in ZO-1, occludin and claudin expression, as well as an increase in circulating LPS levels[89-91]. Likewise, leptin-deficient mice (genetic model of obesity) showed an increased IEB permeability along with morphological changes in villi/crypt length and decreased expression of TJ- and mucus-related genes, that could contribute to the alterations of intestinal motility[92] (Table 2).

Of note, pre-clinical studies have shown that obese mice are characterized by a remarkable morphofunctional rearrangement of the ENS, such as a decrease in the density of nitrergic and VIPergic neurons and an altered intestinal smooth muscle cell

Table 2 Summary of current human and experimental data on molecular, morphological and functional changes in intestinal epithelial barrier and neuromuscular compartment in metabolic disorders

Metabolic disorder	Morphofunctional changes in intestinal epithelial barrier	Morphofunctional changes in enteric neuromuscular compartment	Ref.
Human investigations			
Obesity	↑ Circulating LPS	NA	[6]
	↓ Occludin and tri-cellulin immunopositivity		
	↑ ZO-1		
Diabetes	↑ Intestinal permeability (urinary excretion of lactulose)	NA	[6]
Experimental models			
HFD-induced obese mice	↓ ZO-1, occludin and claudins	↓ Nitrergic and VIPergic neurons Altered smooth muscle cell excitability	[89-91,93,94,96,97]
	↑ Circulating LPS	↓ Enteric inhibitory neurotransmission	
		↑ Enteric excitatory tachykininergic neurotransmission	
		↑ SP immunopositivity	
		↑ A_{2B} adenosine receptor expression	
Lep ob/ob mice	↑ Intestinal permeability	NA	[92]
	Alterations of villi/crypt length		
	↓ TJs and mucus-related genes		
Ob/ob mice	↑ Paracellular permeability	↓ Intestinal motor activity	[95]
	Altered TJs	↓ ACh receptors	
		Delayed intestinal transit rate	

↑: Increase; ↓: Decrease; A_{2B}: Adenosine 2B receptor; Ach: Acetylcholine; HFD: High-fat diet; Lep: Leptin; LPS: Lipopolysaccharide; NA: Not available; Ob/ob: Obese mice; SP: Substance P; TJ: Tight junction; ZO-1: Zonulin-1.

excitability, with consequent impairment of enteric inhibitory neurotransmission[93,94]. In addition, Schacht *et al*[95] showed that ob/ob mice (a genetic model of diabetes) displayed a decrease in the intestinal transit rate, likely resulting from a loss of acetylcholine receptors in muscle layers and an impaired intestinal motor activity (Table 2). These findings support the view that alterations of the enteric neuromuscular compartment could contribute to bowel dysmotility in metabolic disorders. Consistently with this hypothesis, a recent study showed that HFD mice displayed a marked enhancement of enteric excitatory tachykininergic neurotransmission along with an increase in SP immunoreactivity that contributes to colonic dysmotility[96]. In addition, these authors demonstrated that an increase in colonic adenosine A_{2B} receptor expression modulated the activity of excitatory tachykininergic nerves, participating to the enteric dysmotility associated with obesity[97] (Table 2).

Neuropsychiatric disorders

Patients with neuropsychiatric diseases, including PD, AD, ALS, MS, ASD and depression, are often characterized by functional digestive disturbances, including infrequent bowel movements, abdominal distension and constipation[4]. Several lines of evidence suggest that changes in gut microbiota composition, impairments of IEB, intestinal inflammation and rearrangements of the enteric neuromuscular compartment contribute to these bowel motor dysfunctions[4]. In this section, we summarize the most prominent data about the morphofunctional changes in IEB and neuromuscular compartment in the most common central nervous system (CNS) disorders.

Patients with early PD display an increase in IEB permeability, which correlates with staining of intestinal mucosa for *Escherichia coli*, tissue oxidative stress and enteric α-synuclein accumulation[98]. Clairembault *et al*[99] reported an alteration of occludin expression in colonic biopsies from PD patients, although the paracellular and transcellular permeability did not differ among PD patients and controls. Others observed an increase in IEB permeability and decreased colonic ZO-1 expression in

PD patients with severe intestinal symptoms, thus supporting the view that morphofunctional alterations of IEB could contribute to bowel motor dysfunctions in PD[7]. Of note, changes in intestinal permeability have been documented also in patients with MS and ASD, and in all these settings the respective patterns appear to correlate with the disability status[8,9] (Table 3). Nevertheless, current evidence doesn't allow to establish a clear casual link between IEB alterations and bowel motor dysfunctions in CNS disorders.

Besides IEB alterations, several evidence suggest that patients with CNS diseases display alterations of enteric neuromuscular compartment, that could contribute to bowel dysmotility. A recent study has reported an increment of EGCs in colonic biopsies from PD patients[100]. Wunsch *et al*[101] described the presence of ENS nerve fiber disintegration and EGC activation in MS patients. Others reported an increased α-synuclein as well as β-amyloid (Aβ) protein, β-amyloid protein precursor (AβPP) and phosphorylated Tau (p-Tau) immunoreactivity in colonic myenteric and submucosal neurons from PD and AD patients, respectively, suggesting that morphological changes in ENS and protein accumulation in enteric neurons could contribute to bowel motor dysfunctions in CNS diseases[98,102] (Table 3).

However, current human studies don't allow to establish a clear casual link among changes in IEB, alterations of neuromuscular compartment and bowel motor dysfunctions in CNS disorders. In this regard, research efforts have been made in pre-clinical models of neurological disorders. Wu *et al*[103] showed an increase in circulating LPS levels, a decrease in ZO-1 and E-cadherin expression, and an abnormal increase in the number of Paneth cells in ALS mice. Other studies observed the concomitance of abnormal intestinal permeability, enteric α-synuclein accumulation and delayed bowel transit in mice with PD induced by LPS and rotenone[7,104]. Recent pioneering studies in different animal models of PD highlighted relevant rearrangements in the chemical coding of both enteric inhibitory and excitatory neurons, along with impairments of ileum and colonic motor activity, which likely contribute to the decrease in small intestinal and colonic transit rate as well as the efficiency of peristaltic reflex[105-107]. Of note, alterations of enteric neurochemical coding, characterized by a decrease in neuronal nitric oxide synthase (nNOS) and choline acetyltransferase (ChAT), age-related loss of myenteric neurons, EGC activation, intestinal smooth muscle cell atrophy and altered bowel motility have been observed in several animal models of CNS diseases, including AD, MS and ALS[4] (Table 3).

CONCLUSION

Current data from human and pre-clinical studies suggest that impairments of IEB and enteric neuromuscular compartment might represent a common condition underlying the onset/progression of bowel functional disturbances in both digestive and extra-digestive diseases. Indeed, even though each disease displays different clinical and neuropathological features, patients with IBD, IBS, intestinal infections, diverticular disease as well as metabolic and CNS disorders are characterized by significant molecular and morphofunctional alterations of IEB, ENS and intestinal muscular layers. In particular, changes in TJ protein expression and distribution as well as morphofunctional alterations of EGCs represent a common feature of such disorders, that could contribute to the pathophysiology of bowel motor disturbances. However, the molecular mechanisms underlying the interplays between IEB and enteric neuromuscular compartment as well as their role in the pathophysiology of bowel dysmotility in digestive and extra-digestive disorders remain to be elucidated.

Another important aspect of the current evidence from the literature is that changes in gut microbiota composition could also promote the development of functional bowel disorders[108,109]. Indeed, a number of exhaustive review articles have widely described changes of intestinal microbiota in patients with digestive and neuropsychiatric disorders[110-113]. However, human studies do not allow to establish a causal role between gut dysbiosis and bowel functional disturbances in digestive and extra-digestive diseases. Therefore, an integrated overview about the relationship between alterations in gut microbiota composition and bowel functional disturbances associated with digestive and extra-digestive diseases is missing and requires investigations.

In conclusion, based on current knowledge, some important issues remain to be addressed: (1) What is the role of IEB in bowel motor dysfunctions associated with digestive and extra-digestive diseases? (2) What are the molecular mechanisms underlying the interplay between IEB and enteric neuromuscular compartment in the onset of bowel motor abnormalities associated with digestive and extra-digestive

Table 3 Summary of current human and experimental data on molecular, morphological and functional changes in intestinal epithelial barrier and neuromuscular compartment in central nervous system disorders

Central nervous system disorder	Morphofunctional changes in intestinal epithelial barrier	Morphofunctional changes in enteric neuromuscular compartment	Ref.
Human investigations			
PD	↑ Intestinal permeability	↑ EGC density	[7,98-100]
	↓ Occludin and ZO-1 expression	α-syn accumulation in myenteric neurons	
AD	NA	↑ Aβ, AβPP and p-Tau immunoreactivity in colonic myenteric and submucosal neurons	[102]
MS	↑ Intestinal permeability (urinary mannitol concentration)	ENS fiber disgregation	[8,101]
		EGC activation	
ASD	Altered intestinal permeability	NA	[9]
Experimental models			
Rotenone-induced central dopaminergic neurodegeneration	↑ Intestinal permeability	α-syn accumulation in myenteric neurons	[7,104]
		Delayed bowel transit	
LPS-induced central dopaminergic neurodegeneration	↑ intestinal permeability (lactulose/mannitol ratio and sucralose levels)	α-syn accumulation in myenteric neurons	[7,104]
		Delayed bowel transit	
6-OHDA-induced nigrostriatal neurodegeneration	NA	Impairment of colonic cholinergic and tachykininergic motor activity	[105-106]
Tg A53T mice (genetic model of PD)	NA	Impairment of colonic cholinergic motor activity	[107]
		α-syn accumulation in myenteric and submucosal neurons	
APP/PS1 mouse (genetic model of AD)	NA	↑ Aβ protein precursor, Aβ	[4]
		Protein and p-Tau	
		↓ nNOS and ChAT	
		EGC activation	
Tg CRND8 mice (genetic models of AD)	NA	↑ Aβ protein precursor in myenteric neurons	[4]
		Enteric glial activation (GFAP, nestin)	
		Enteric neuronal loss	
		Smooth muscle cell atrophy	
EAE (animal model of MS)	Abnormal intestinal permeability (plasma Na-F and FITC levels)	Crypt depth and thickness of submucosal and muscular layers	[4]
	↓ ZO-1 expression	Enteric glial activation	
		Neuronal loss	
		Abnormal GI motility	
G93A mice (genetic model of ALS)	↑ Circulating LPS	NA	[4,103]
	↓ ZO-1 and E-cadherin expression		
	↑ Paneth cells number		

↑: Increase; ↓: Decrease; 6-OHDA: 6-hydroxydopamine; α-syn: α-synuclein; Aβ: Amyloid β; AβPP: β-amyloid protein precursor; AD: Alzheimer's disease; ALS: Amyotrophic lateral sclerosis; ASD: Autism spectrum disorder; ChAT: Choline acetyltransferase; EGC: Enteric glial cell; ENS: Enteric nervous system; FITC: Fluorescein isothiocyanate; GFAP: Glial fibrillary acidic protein; GI: Gastrointestinal; LPS: Lipopolysaccharide; nNOS: Neuronal nitric oxide synthase; MS: Multiple sclerosis; NA: Not available; PD: Parkinson's disease; p-Tau: Phosphorylated Tau; ZO-1: Zonulin.

diseases? (3) Can diet influence the alterations of IEB and enteric neuromuscular compartment in digestive and extra-digestive diseases? And (4) What is the impact of gut dysbiosis in bowel motor dysfunctions associated with digestive and extra-digestive diseases?

To address these points, research efforts should be made to characterize simultaneously the alterations of IEB and neuromuscular compartment, regarded as an integrated network, in animal models and patients. Understanding these aspects could pave the way to the identification of novel therapeutic targets and the development of novel pharmacological entities for the management of bowel

dysfunctions associated with digestive and extra-digestive disorders. Indeed, at present, there is a lack of therapeutic interventions able to restore IEB integrity and dysfunctions of the enteric neuromuscular compartment. A limited number of clinical studies have reported some benefits in terms of improvement of IEB integrity and restoration of ENS functions, following the administration of probiotics and prebiotics. However, clinical results remain patchy due to heterogenitcity of study protocols, related mainly to the selection of study population, sample size, dosage, formulation and bacterial strains used, as well as the duration of therapy and outcome measures. Therefore, intensive research efforts are needed to deepen the beneficial effects of probiotics and prebiotics observed in clinical studies. Moreover, further research in this area is necessary to identify novel therapeutic targets suitable for strengthening IEB and to treat or prevent GI disorders.

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REVIEW

Gastrointestinal cancer stem cells as targets for innovative immunotherapy

Mihaela Chivu-Economescu, Laura G Necula, Lilia Matei, Denisa Laura Dragu, Ana I Neagu, Irina Alexiu, Coralia Bleotu, Carmen Cristina Diaconu

ORCID number: Mihaela Chivu-Economescu (0000-0001-7512-4700); Laura G Necula (0000-0001-6531-7939); Lilia Matei (0000-0003-4479-655X); Denisa Laura Dragu (0000-0002-5868-2636); Ana I Neagu (0000-0001-6126-8973); Irina Alexiu (0000-0003-4953-7522); Coralia Bleotu (0000-0002-9031-338X); Carmen Cristina Diaconu (0000-0002-2259-1425).

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Mihaela Chivu-Economescu, Laura G Necula, Lilia Matei, Denisa Laura Dragu, Ana I Neagu, Irina Alexiu, Coralia Bleotu, Carmen Cristina Diaconu, Department of Cellular and Molecular Pathology, Stefan S. Nicolau Institute of Virology, Bucharest 030304, Romania

Laura G Necula, Nicolae Cajal Institute, Titu Maiorescu University, Bucharest 040441, Romania

Corresponding author: Mihaela Chivu-Economescu, PhD, Senior Researcher, Department of Cellular and Molecular Pathology, Stefan S Nicolau Institute of Virology, 285 Mihai Bravu Ave, Bucharest 030304, Romania. mihaela.economescu@virology.ro

Abstract

The role of cancer stem cells in gastrointestinal cancer-associated death has been widely recognized. Gastrointestinal cancer stem cells (GCSCs) are considered to be responsible for tumor initiation, growth, resistance to cytotoxic therapies, recurrence and metastasis due to their unique properties. These properties make the current therapeutic trials against GCSCs ineffective. Moreover, recent studies have shown that targeting stem cell surface markers or stemness associated pathways might have an additional off-target effect on the immune system. Recent advances in oncology and precision medicine have opened alternative therapeutic strategies in the form of cancer immunotherapy. This approach differs from classical anti-cancer therapy through its mechanism of action involving the activation and use of a functional immune system against tumor cells, instead of aiming physically destruction of cancer cells through radio- or chemotherapy. New immunological approaches for GCSCs targeting involve the use of different immune cells and various immune mechanisms like targeting specific surface antigens, using innate immune cells like the natural killer and T cells, T-cell chimeric antigen receptor technology, dendritic cell vaccine, or immune checkpoint inhibitors. In this respect, better understandings of immune regulatory mechanisms that govern anti-tumor response bring new hope in obtaining long-term remission for cancer therapy.

Key words: Immunotherapy; Gastrointestinal cancer; Cancer stem cells; CAR-T; Dendritic cells vaccines; Immune checkpoints inhibitors

Core tip: Cancer immunotherapy has emerged in recent years as an alternative to

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classical anti-tumor therapy. It involves the activation of the host immune system in the fight against tumor cells, including cancer stem cells, which are responsible for tumor maintenance, relapse, and metastasis. Here we discuss the different forms of immunotherapy for gastrointestinal cancer stem cells targeting such as using monoclonal antibodies against surface antigens, generation of effector natural killer cells and T cells genetic engineered to target tumor antigens, dendritic vaccines, and immune checkpoints inhibitors.

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INTRODUCTION

Gastrointestinal cancers include several malignancies of the gastrointestinal tract and accessory organs such as stomach, liver and intrahepatic bile duct, gallbladder and pancreas. All of them have epithelial cell origin, and combined accounts for 4974672 estimated new cases, representing 28% of all cancer incidence in 2018. According to GLOBOCAN 2018, all together gastrointestinal cancer are responsible for over 3.5 million deaths which correspond to 37% deaths of total human cancers[1]. Thus the need to understand the molecular background of gastrointestinal cancer, as well as mechanisms involved in occurrences and tumor maintenance, are of tremendous importance.

In the last years, the hypothesis that cancer appears from a population of stem cells has gained widespread support. Cancer stem cells (CSCs) are a distinct subpopulation within the tumor with unique properties. There are two theories about how tumors appear. Stochastic theory, involving the occurrence of unpredictable genetic and epigenetic changes during tumor growth[2,3] and hierarchical theory, which supports the idea of a subpopulation of cells, that have an intrinsic ability to initiate self-regeneration and tumor growth[4-6]. CSCs have been discovered in a wide field of tumors, including gastrointestinal cancer. These cells are at the origin of tumorigenesis and also, are responsible for tumor maintenance due to resistance towards standard oncology treatments, relapse, and metastasis[7]. More and more evidence is constantly accumulating that mechanisms of resistance of gastrointestinal cancer stem cells (GCSC) to conventional therapy are epithelial mesenchymal transition (EMT), drug efflux proteins, and upregulation of aldehyde dehydrogenase (ALDH) activity. Therefore, CSC raised an important challenge regarding the efficacy of current cancer treatment due to these special properties. In this way, more effective therapies that target the GCSCs subpopulation are needed, instead of addressing the entire tumor population. New immunological approaches involve the use of various immune mechanisms like targeting specific surface antigens or immune checkpoints on GCSC surface or using innate immune cells like the natural killer (NK) and T cells, T cell chimeric antigen receptor technology and dendritic cell vaccine (Figure 1).

TARGETING GCSC MARKERS

To target GCSCs markers and track the effect of anti-tumor therapies, it is necessary to identify them. Commonly, the surface antigens such as CD24, CD44, EpCAM, CD133, alone or in combinations, are among the most used markers for the identification of GCSC[8]. Combination of CD44, CD90, CD133 and ALDH1 was commonly used for esophageal tumor type[9,10], CD44 and ALDH1 for gastric tumor type[8,11], CD24, CD44, CD133, EpCAM and ALDH for pancreatic tumor type[12-14], CD44, CD90, CD133 and EpCAM for liver tumor type[15-18], CD24, CD44, CD49f, CD133, EpCAM and ALDH1 for colorectal tumor type[15,19,20] (Table 1). Several other molecules, such as CD90, Musashi-1, LINGO2, oval cell marker OV6[8,21] have been reported as potential surface markers for GCSCs. Identification of specific antigens on the CSC surface may provide more targets for immunotherapy (Table 1).

These markers are relatively conserved across to the broad spectrum of solid cancers and also are common with normal stem cells[22]. Monoclonal antibodies,

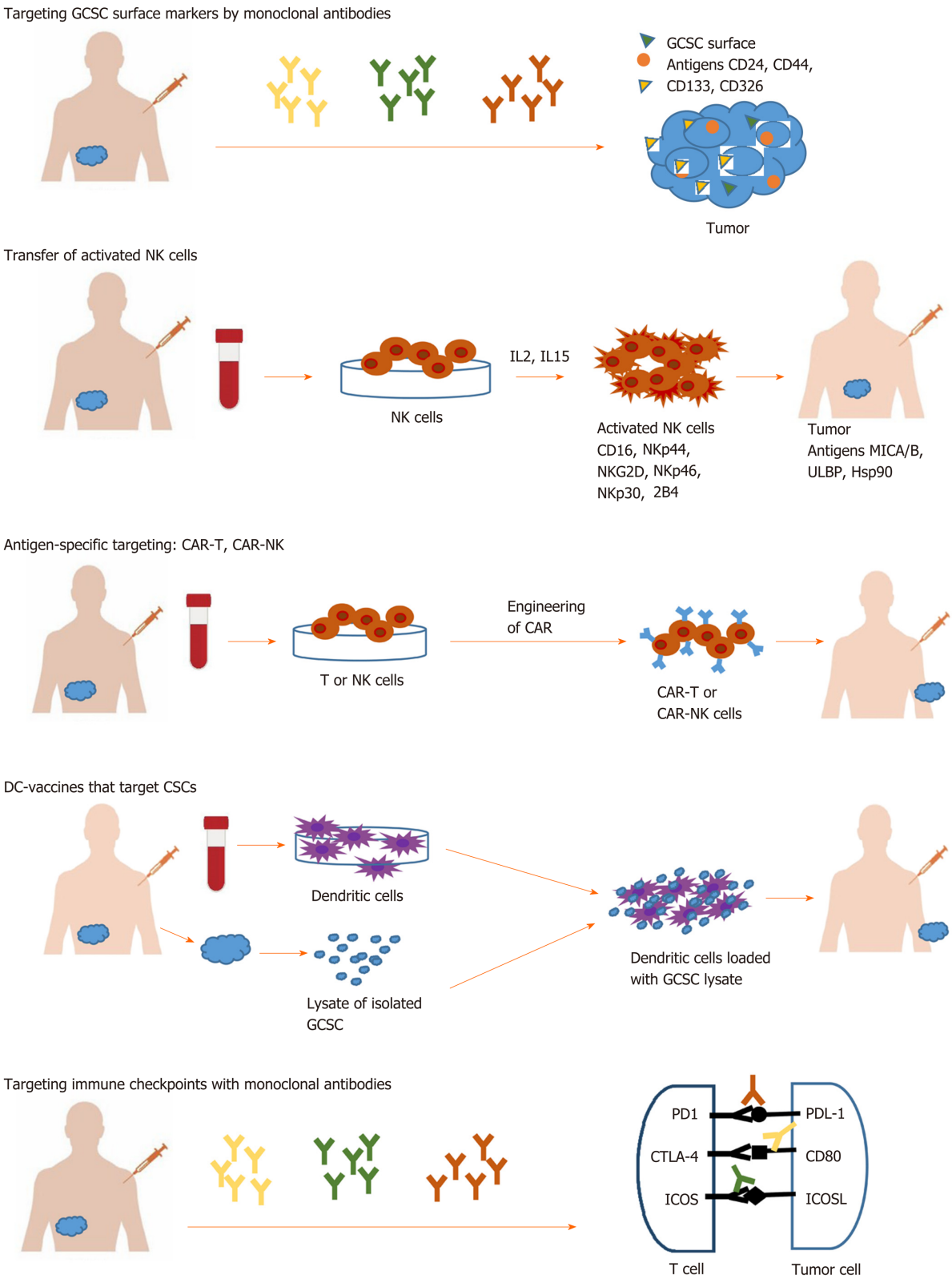

Figure 1 Immunological approaches for gastrointestinal cancer stem cell targeting. GCSC: Gastrointestinal cancer stem cell; NK: Natural killer; CSC: Cancer stem cell; CAR: Chimeric antigen receptor; DC: Dendritic cell.

chimeric, humanized or fully human antibodies that are able to target specific markers were developed for the treatment of major malignant diseases, including gastrointestinal cancers (Table 2).

CD44 is a transmembrane receptor commonly expressed on solid tumor CSC surface. Targeting CD44 with RG7356, a recombinant anti-CD44 monoclonal specific antibody, blocks the binding of CD44 to hyaluronic acid (HA) and inhibits cell

Table 1 Gastrointestinal cancer stem cells markers

Tumor type	Cancer stem cell phenotype	Ref.
Esophageal	CD44+, CD90, CD133+, ALDH1+	[10,11]
Gastric	CD44+, ALDH1+	[9,12]
Pancreatic	CD24+, CD44+, CD133+, EpCAM+, ALDH+	[13-15]
Liver	CD44+, CD90+, CD133+, EpCAM+	[16-19]
Colorectal	CD24+, CD44+, CD49f, CD133+, EpCAM+, ALDH1	[16,20,21]

adhesion, leading to tumor growth inhibition, and activates macrophages in preclinical models[23-25]. However, only a modest clinical efficacy was observed in patients with metastatic or locally advanced CD44-expressing solid malignancies (including breast cancer, melanoma, renal and lung cancer) treated with RG7356 (phase I clinical trial)[26].

CD24 is a highly glycosylated protein localized in the membrane of many type of CSCs. Some CD24 monoclonal antibodies (mAb), such as SWA11and G7 mAbs, were developed for therapeutic purpose, demonstrating a good efficiency in human cancer xenograft models. A potent anti-tumor activity of SWA11 mAb[27] has been demonstrated in human colorectal cancer models, reducing tumor cell proliferation and angiogenesis[28]. Other anti-CD24 monoclonal antibody, G7 mAb was used in liver cancer xenograft models[29,30] demonstrating specificity for tumor tissue and efficacy in suppressing tumor growth, as single-agent treatment or in combination with doxorubicin[31], or cetuximab, a chimerical monoclonal anti-EGFR antibody[31].

CD326 (EpCAM) is a transmembrane glycoprotein mediating intercellular cell-adhesion in epithelial tissues being involved in cell signaling, proliferation, differentiation, invasion, metastasis, and chemo-/radioresistance. EpCAM is a target for immunotherapeutic strategies in epithelial-derived neoplasms of colon, stomach, pancreas *etc*.[32,33]. The antibody Catumaxomab (Removab®) targeting EpCAM was used in clinical trials on patients with ovarian, gastric, colon, breast cancers and malignant ascites (NCT00836654) delaying deterioration in quality of life for a prolonged survival period[34]. Moreover, one phase III clinical trial (NCT00822809) demonstrated that intraperitoneal infusion of catumaxomab activates immune cells such as NK cells, macrophages and T cells in ascites, and favors CD8+T cell accumulation into the peritoneal cavity showing a clinical benefits in treatment of malignant ascites associated with EpCAM positive carcinomas[33,35].

However, precise targeting with monoclonal antibodies seems difficult, since most GCSCs are identified based on a combination of surface markers, often unspecific[36]. Since none of these markers is unique for GCSCs, additionally features of stem cells are such as ALDH activity, side population phenotype, the expression of pluripotency genes (OCT4, SOX2, and NANOG), and aberrant expression of ABC transporters[37] are analyzed in order to permit a more reliable identification and targeting of the cancer cells with stem properties[38,39].

Another widely used strategy against GCSC involved targeting of key signaling pathways like Notch, Hedgehog, Wnt, and IL6. However, accumulating evidence pointed out that the inhibitors used to target self-renewal pathways might have off-target effects on immune cells, impairing T cell proliferation, function, and cytokine production[22,40-43].

At this point, scientists consider that cancer immunotherapy represents a new approach, different from the conventional one that uses chemo and radiotherapy.

IMMUNOLOGICAL APPROACHES FOR GCSC TARGETING

Cancer immunotherapy differs from classical anti-cancer therapy through its mechanism of action involving the activation and use of a functional immune system against tumor cells, instead of aiming physically destruction of cancer cells through radio- or chemotherapy[44]. The immune system is an active part of the tumor microenvironment (TME). Here GCSCs co-exist with other cellular components like stromal cells, endothelial cells, immune cells such as dendritic cells (DC), NK cells, T cells, tumor-associated macrophages, regulatory T cells, tumor-infiltrating lymphocytes, and myeloid derived suppressor cells. There are several mechanisms used by GCSC in their interaction with TME to escape from tumor killer cells like NK and T cells. One of these refers to having a low expression of MHC class I surface molecules. Another one is represented by the crosstalk between GCSC and the other

Table 2 Targeting gastrointestinal cancer stem cell surface markers by monoclonal antibodies

Targeting approach	Cancer model	Effects	Ref.
RG7576 mAb against CD44	Solid tumors	Inhibited tumor growth and induced activation of macrophages	[23-26]
SWA11 against CD24	Colorectal cancer	Reduced tumor cell proliferation and angiogenesis	[27,28]
G7 mAbs against CD24	Liver cancer	Suppressed tumor growth	[29-31]
Catumaxomab (Removab®) mAb targeting EpCAM (CD326)	Gastric, colon cancers, pancreas	Activated immune cells (NK cells, macrophages, and T cells); prolonged survival period	[32-35]

NK: Natural killer; mAb: Monoclonal antibodies.

components of TME, these interactions being mediated by cytokines and chemokines that eventually suppress antitumor immunity. Moreover, recently, co-inhibitory molecules and immune checkpoint ligands such as programmed death-ligand (PD-L) 1 and PD-L2 were identified to be overexpressed on GCSC surface. Due to PD1/PD-L1 (L2) axis, GCSC can easily escape from immune cell action. Understanding the TME and the dynamic cross talk between GCSCs and the TME is equally important to initiate an efficient anti-tumor therapy without impairing the anti-tumor immune response.

Immunological approaches for GCSC targeting involve different immune cells and various immune mechanisms like using innate immune cells such as NK and T cells, using antigen-specific targeting by T cell chimeric antigen receptor (CAR) technology, DC vaccine, or immune checkpoints therapy[45,46]. The targeting strategies against GCSC are listed in Table 3.

NK transfer in cancer immunotherapy

NK cells, the third largest population of immune cells after B and T lymphocytes, serve the innate immunity, usually defending the human organism against infections. NK are good candidates for immunotherapy since they trigger special attacks on cancer cells that express ligands that couples activating receptors on NK cells. This action is mediated through a group of activating receptors containing CD16, NKG2D, NKp30, NKp44, NKp46, 2B4 and DNAM-1 with PVR and NECTIN-2[47-50]. The major activating ligands for NK cells are MICA/B, ULBP and Hsp90 usualy overexpressed on tumor cells[51]. For tumor eradication is necessary total destruction of CSCs. Different studies showed that there are CSCs that express ligands that can be recognized by NK cells and, consequently can be killed[52-54], and certain CSCs which do not show detectable ligands for NK and escape cytotoxicity[55]. An *in vitro* study conducted by Rong *et al*[56] showed that cytokine-induced killer cells, which are NK lymphocytes characterized by the co-expression of CD3 and CD56 surface antigens, killed CSCs in hepatocellular carcinoma via interaction of their membrane receptor NKG2D with stress-inducible molecules, MIC A/B and ULBPs, on target cells. *In vivo*, cytokine-induced killer infusion significantly delayed tumor growth. Similarly, Ames *et al*[57] demonstrated that activated NK cells are capable of preferentially killing tumor cells with a CSC phenotype identified by multiple markers ($CD24^+/CD44^+$, $CD133^+$, and aldehyde dehydrogenase^{bright}) from a wide variety of human cancer cell lines, including pancreatic cell lines like PANC-1 and BXPC3. The mechanism of action implicated an NKG2D-dependent NK activation via MICA/B, Fas, and DR5 ligands expressed on GCSCs. Also, Yin *et al*[58] showed that cells with stem cell phenotype can be more easily killed by NK cells activated by IL2 and IL15. Taken together, these preliminary studies provide evidence that activated NK cells can have translational potential as NK immunotherapy against GCSC phenotype, and other CSC from solid malignancies.

Antigen-specific targeting by T cells, CAR-T, CAR-NK

The next step in cancer therapy resided in the generation of effector T cells and NK cells genetic engineered to produce an artificial T cell receptor, named CAR that gives T cells both the ability to target a specific protein/tumor antigen and to be consequently activated. CAR-T cells against GCSC antigens have been developed and evaluated in several gastrointestinal cancer models (Table 3).

Miyamoto *et al*[59] used CAR-T-based immunotherapies against colorectal CSCs based on the ASB4 gene that was identified as being expressed on colorectal CSC, but not on non-CSC cells or normal cells/tissue. ASB4 as tumor-associated antigen was used to generate specific cytotoxic T lymphocytes (CTL) *in vitro*, that were able to

Table 3 Targeting gastrointestinal cancer stem cell by natural killer cells, chimeric antigen receptor expressed on T cells and dendritic cells based vaccines

Targeting approach	Cancer model	Ref.
CIK cells *via* NKG2D ligands expressed on CSC	Hepatocellular carcinoma	[56]
NK cells *via* NKG2D ligands expressed on CSC	Pancreatic cancer	[57]
CAR-T for CSC antigen ASB4	Colon cancer	[59]
CAR-T for EGFR and CAR-T for CSC antigen CD133	Cholangiocarcinoma	[60]
CAR-T for CSC antigen CD24	Pancreatic adenocarcinoma	[61]
DC loaded with Panc-1 CSC lysate	Pancreatic cancer	[62]
DC loaded with total mRNA from gastric CSC	Gastric cancer	[63]

CIK: Cytokine-induced killer; CSC: Cancer stem cells; NK: Natural killer; CAR-T: Chimeric antigen receptor expressed on T cells; EGFR: Epithelial growth factor; DC: Dendritic cells.

infiltrate implanted colorectal tumors in a mouse model, preventing tumor growth. Another clinical trial was developed by Feng *et al*[60] that used CAR-T cells targeting epidermal growth factor receptor (EGFR) and GCSC surface antigen CD133, respectively. The patient received successive infusions of CAR-T cells for the treatment of unresectable/metastatic cholangiocarcinoma. The results showed a partial response of 8.5 mo from CAR-T for EGFR and 4.5-mo-lasting from the CAR-T for CD133 treatment, however, both therapies induced acute toxicities. Maliar *et al*[61] developed CAR-T for CSC antigen CD24 and evaluated it in mice with pancreatic adenocarcinoma xenografts. The results showed that more than 50% of the animals remained tumor-free after 16 wk.

As concluding remarks, treatment of solid tumors using CAR-T cells induced less favorable results then hematological malignancies, mainly due to short efficacy and off-target toxicity. Davenport *et al*[62] reported in 2018 that responses triggered by CAR-T cytotoxic cells are fast but short, being followed by a rapid loss after 20 h in cytotoxic activity against tumor. The main concern remains on the severe toxicity associated with CAR-T therapy like cytokine release syndrome, which can trigger organ damage and death, neurologic toxicity, insertional oncogenesis, graft versus-host disease, off-target antigen recognition[63]. Two trails using CAR-T cells engineered against ERBB2 or CEACAM5 used for the treatment of gastrointestinal cancers reported poor efficacy and caused acute pulmonary toxicity due to antigen expression on lung epithelium. This resulted in the death of one patient within 5d post-transfer of the cellular product due to multiple organ failure[64,65].

In order to avoid CAR-T therapy accompanied toxicity is essential to choose accurate target antigens and improve tumor discrimination[66,67]. Also, there are some studies on introducing a suicidal gene that can induce apoptosis of T cells to prevent over activation and critical off-tumor cytotoxicity. Such genes are the thymidine kinase gene of herpes simplex virus and inducible caspase 9. If the latter seems successful, the first strategy, related to the thymidine kinase gene of herpes simplex virus seems to raise immunogenicity problems and it will not be used in the clinic[67-69].

Recently, the knowledge of producing CAR-T has been transferred to CAR-NK cells. CAR-NK use seems to be safer in the clinic, as NK cells do not initiate similar toxicity[70]. Between the advantages: CAR-NK cells are able to retain the expression of their activating receptors, showing longer efficacy, and appear safer in terms of cytokine release syndrome and neurotoxicity due to a different pattern of cytokines/chemokines released after activation[71,72]. However, there are also major drawbacks like the poor ability of NK to reach tumor tissue due to TME and some changes that may intervene in the expression of activating receptors/ligands. For example, the level of NKG2D ligand is increased in the early stages of colorectal cancer, but it decreases during tumor progression[73].

There are not ongoing clinical trials for GCSC or other solid CSC. The only clinical trial for solid cancers is a phase I study that use anti-GD2 CAR-T for sarcoma and neuroblastoma patients (NCT02107963), without published results. There are however 30 clinical trials that are recruiting patients with solid cancers including liver, stomach and colorectal for CAR-T therapies using PD-1, CTLA4, EGFR or NKG2D-ligand. Also, there are three trials for solid cancers that are recruiting for CAR-NK therapies using antigens like MUC3, ROBO1, and NKG2D-ligand. The creation of NKG2D/NKG2DL-based multi-functional fusion proteins is becoming one of the most promising strategies in immunotherapy for cancer. Activated CAR-NKG2D receptor on the T or NK cell surface can bind to its respective NKG2D ligand

expressed in tumor cells, enabling immune cells to kill tumor cells.

DC-vaccines that target GCSC

Dendritic cells are crucial players in immune responses, and their ability to control the activation, proliferation and differentiation of specific T cell subsets make them strategic tools for cancer vaccines that target CSCs. A great advantage of DC-vaccines might be the potential capacity to induce immunological memory, eliminating existing CSCs and, at the same time, offering long-term protection against new arising CSCs[74].

Most clinical trials using DC-vaccines are based on DC loaded with lysates of isolated CSCs. Yin *et al*[75] used such DC loaded with pancreatic Panc-1 CSC lysate and observed that modified DC induced proliferation of T cell lymphocytes during co-culture. This approach has at least a few disadvantages like the lack of reliable surface makers that may be used for CSCs isolation, unknowing which neoantigens in the lysate elicit an immune response, and the variability of the number of immunogenic neoantigens on different types of tumors[76,77].

ALDH, a marker frequently used for CSC identification was used in obtaining DC vaccines that significantly inhibited tumor growth, reduced development of pulmonary metastases and prolonged survival. Direct targeting of CSCs was confirmed by the specific binding of IgG produced by ALDH^{high} CSC-DC vaccine primed B cells to ALDH^{high} CSCs, lysing these target CSCs in the presence of complement[78]. This promising approach was reported so far for squamous cell cancer and metastatic melanoma, however, ALDH is a highly expressed marker on various GCSC (Table 1), so we can hypothesize similar positive results in gastrointestinal cancers.

In spite of the promising results, DC-based vaccination strategies need to be improved[78]. A more efficient strategy to eradicate CSCs might be to load autologous DCs with peptides, proteins or even mRNA rather than tumor lysate, this way controlling more accurately the generated immune response. In this regard, Bagheri V *et al*[79] loaded DC with total mRNA from gastric CSC expressing CD44, CD54, and EpCAM markers. These DC were able to induce IFN-γ gene expression in T-lymphocytes after a 12-d co-culture.

Recent studies proposed loading DCs with transcription factors as NANOG, OKT4a, SOX2, c-MYC, and KLF4, which also transform somatic cells into stem cells (iPS). Targeting CSCs unique proteins might be one of the best ways to destroy CSCs. Since the expression of NANOG is low or absent in normal cells and CSCs re-express it, makes NANOG an ideal therapeutic target. DCs loaded with NANOG peptides will be able to generate immunological memory after vaccination and would help the immune system to manage CSC plasticity[22].

In order to maximize the response rates to vaccines, if NANOG peptides cannot be presented by a patient HLA class I molecules, peptides against other stem cell transcription factor OKT4 or SOX2 may be used[76]. Combining DC vaccination against CSCs with other therapies, as immune checkpoint inhibitors, for example, might overcome immunosuppressive mechanisms in cancer and avoid bone marrow damaging by the chemotherapeutics.

IMMUNE CHECKPOINTS

It is considered that CSCs escape immune surveillance due to their immune suppressive profiles based on the expression of co-inhibitory molecules, immune checkpoints ligands and cytokines, drug-resistance and ability to activate an EMT programme[80]. Based on these properties, CSC not only escapes immune surveillance but also directly inhibits T and NK cells anti-tumor activity *via* modulating immune checkpoints.

Several immune checkpoints have been stated during last years with either co-stimulatory activity on immune cells such as CD28/CD80 (CD86), ICOS (CD278)/ICOSL, CD27/CD70, GITR/GITRL, or co-inhibitory like PD-1/PDL-1 (PD-L2), BTLA/HVEM, CTLA4/CD80 (CD86), B7H3, B7H4, B7H5/HVEM, LAG3/MHC II, TIM3/GAL9, TIGIT/Nectin-2, or IDO. Many of them are highly expressed on various CSCs, but the type of molecule seems to vary with tumor type and localization.

From these, PD-L1 (also known as CD274 or B7H1) and B7H3 have been identified as promoters of CSC-like phenotype, EMT, tumor cell proliferation, metastasis and resistance to therapy[81-83].

PD-L1 is one of the most studied immune checkpoints. The interaction between PD-L1/PD-L2 and PD-1 aids CSCs in escaping from the killing through inhibiting tumor-

reactive T cells by binding to its PD-1 receptor. Moreover, PD-L1 is also expressed by tumor-associated myeloid-derived suppressor cells, contributing to T cells blocking and immune deficiency in TME[84]. Hsu *et al*[85] established that PD-L1 high expression in CSCs is due to EMT and to EMT/β-catenin/STT3/PD-L1 signaling axis. Moreover, PD-L1 expression could be enhanced via PI3K/AKT and RAS/MAPK pathways. All these major pathways could be activated by OCT4 and SOX2, key regulatory genes involved in CSC self-renewal and function[86]. The final effect of PD-L1 overexpression on CSC will be an increase in cancer invasion and proliferation via EMT.

This hypothesis was sustained by several experiments on GCSC. Yang *et al*[87] detected PD-L1 overexpression on gastric CSCs, defined as Lgr5+/CD326+/CD45−, were enhanced *in vitro* tumor-promoting capacity of GCSCs by colony-forming assay, and induces their proliferation. In reverse, knockdown of PD-L1 expression in gastric cancer cells significantly suppressed proliferation and invasion *in vitro*[88], and tumor growth in nude mice[89].

An increased level of PD-L1 was observed in esophageal and colorectal CD133+ GCSCs with EMT phenotype. The authors showed by manipulating PD-L1 expression, that higher PD-L1 expression promoted cell proliferation, migration and EMT phenotype. The EMT mechanism could help GCSC escape immune attack during metastasis[90].

The assessment of PD-L1 level on biopsies could bring useful information for establishing therapies regimen. The dynamic change of PD-L1 expression may indicate the response to therapy and have predictive significance on progression free survival. This could be monitored with the help of circulating tumor cells, which may act as substitute for tissue biopsies, and have great utility in real-time cancer management[91].

The expression of these molecules with an immunosuppressive effect on the GCSC surface may be a major problem as cytotoxic T lymphocytes therapies become less effective. However, is an indicator that GCSC resistant to classical anti-tumor therapy could be targets for immune checkpoints inhibitors.

Targeting immune checkpoints with monoclonal antibodies has become a custom treatment since early studies have shown their ability to improve tumor infiltration of $CD8^+$ and $CD4^+$ T cells and inhibit tumorigenesis.

Antibodies blocking PD-L1, PD-1 or CTLA-4 were developed and tested in clinical trials for their cancer therapeutic potential. 2014 was the year of nivolumab and pembrolizumab approval, both being monoclonal antibodies targeting PD-1. They were authorized for clinical use with benefits in various types of cancer such as refractory malignant melanoma[92], Hodgkin lymphoma[93], NSCLC[94], head and neck squamous cell carcinoma[95], urothelial cancer[96], gastric adenocarcinoma[97], colorectal cancer[98] and advanced hepatocellular carcinoma[99] (Table 4). Moreover, pembrolizumab has received general approval for the treatment of all solid tumors with high microsatellite instability and deficiency in the mismatch repair genes. Next, a combination of ipilimumab (anti-CTLA-4) with nivolumab (anti-PD-1) inhibitors was shown to significantly enhance antitumor efficacy and the response rates in patients with colorectal cancer expressing high microsatellite instability phenotype.

In addition to targeting PD-1 and CTLA-4 receptors, PD-L1 has been also confirmed to be useful for immunotherapy. It was demonstrated that increased expression of PD-L1, decreased T cell infiltration and activation, protecting tumor and GCSCs against immune response. In 2016 atezolizumab, an anti-PD-L1 monoclonal antibody received approval for the treatment of several solid cancers, but not yet for gastrointestinal cancers.

The clinical benefits of immunotherapy cannot be questioned. The development of new inhibitors for immune checkpoints or their ligands continues, addressing newly identified regulators Lag-3, Tim-3, TIGIT, V-domain Ig suppressor of T cell activation, *etc*. And this is only the beginning, as many clinical trials are under way to assess the effectiveness of combining these inhibitors either with or without classical chemotherapy in treating gastrointestinal cancers[100].

COMBINATION IMMUNOTHERAPY

To target GCSC and completely eradicate them, it might be necessary to combine these immunotherapy approaches.

Several clinical trials are now proposing interesting strategies for combining immune checkpoints therapy with DC-vaccines or CAR-T technology. Most of these clinical trials are either in the phase of patient recruitment or in phase I. There are several trials that are testing the combination between anti PD-1 compound (nivolumab) and gene-modified T-cells and dendritic cell vaccine targeting cancer-

Table 4 List of approved drugs targeting immune checkpoints for gastrointestinal cancers

Target	Drug	Commercial name	Indication
PD-1	Nivolumab	Opdivo	Hepatocellular carcinoma, colorectal cancer with MSI-H
PD-1	Pembrolizumab	Keytruda	Gastric cancer, hepatocellular carcinoma, colorectal cancer, solid tumors with MSI-H
CTLA-4 and PD-1	Ipilimumab plus nivolumab	Yervoy plus Opdivo	Colorectal cancer with MSI-H

PD-1: Programmed death 1; CTLA-4: Cytotoxic T-lymphocyte-associated protein 4; MSI-H: High microsatellite instability.

testis antigen (CTA). CTAs such as New York esophageal squamous cell carcinoma 1 and melanoma-associated antigen A (MAGEA) are considered excellent candidates for cancer immunotherapy since a large majority of them have their espression limited to the embrionic stem cells, testes, ovaries and endometrium in normal tissue, and are re-expressed in metastatic tumours[101]. MAGEA1-3, MAGEA9, LAGE1, and New York esophageal squamous cell carcinoma 1 were found to be highly expressed in hepatocellular carcinoma, oesophageal, gastric and colorectal carcinoma stem/progenitor cells and associated with poor survival, high risk of tumor recurrence[102,103]. However, there is a small number of CTAs that are expressed on normal tissue as well. Although targeting CTA seems to be a promising strategy, carefull selection of CTA type for immunotherapy is manadatory. Serious neuronal adverse events followed by death were observed during clinical trials using anti-MAGE3 CAR-T cells in patients with solid cancers. Histopathological examination showed that normal neuronal cells also expressed MAGEA proteins that became targets of modified T cell therapy, thus being destroyed[104].

Another approach is targeting of inhibitory immune checkpoints like PD-1 and TIGIT to unblock the NK and T cells activity. Zhang *et al*[105] found that blockade of TIGIT promoted NK cell mediated immunity in a mouse model with colon cancer, and the response was enhanced by additional anti-PD-L1 immunotherapy. Moreover, it seems that the animal model developed a persistent memory immunity that was functional after tumor cell reinfusion.

Combined immune therapies may be very effective having the advantage of addressing both GCSC and TME simultaneously. Thus, they can target, for example, GCSC surface markers with monoclonal antibodies, DC-vaccines or CAR-T therapy, and at the same time, they can reactivate the immune system by blocking the negative signals induced by immune checkpoints in effector immune cells. However, there are some limitations since most of the known solid tumor-associated antigens are expressed also in normal tissues, resulting in damaging off-target toxicity. Therefore, there is a continue effort to identify tumor-specific antigens that can be addressed using immune therapies. Another limiting factor that can influence the clinical response is the level of inflammatory infiltration and the expression of immune checkpoints. Unlike liquid cancers, where immunotherapy has been a real success, in solid tumors, their efficiency has been diminished by the consistency, content and dynamics of TME that modulates the anti-tumor response through access and phenotype of immune cells.

To improve the efficiency and ensure the safety of the treatment it is imperative to carefully select the target antigens, assuring that they are highly immunogenic and expressed only in targeted the cell population.

CONCLUSION

Tumor-immune profiling has highlighted the mechanisms of immune evasion of cancer based not only on CSC properties but also on the interaction of these cells with TME. These include features such as antigen presentation and regulation of immune cells activation and functioning through immunosuppressive elements like immune checkpoints. Novel immunotherapeutic approaches addressed to all these features. There are several approaches that involve expanding of NK and T cells for CSC antigen-specific targeting or dendritic cell-based vaccines against CSCs. However, the most exciting approach is related to immune checkpoints discovery. Targeting PD-1, CTLA-4, Lag-3, Tim-3, and TIGIT, or their respective ligands on CSC allows activation of the immune cells like T-lymphocytes, NK, neutrophils, dendritic cells and destruction of CSC. The main purpose of these approaches is to modify the TME so that tumor cells and CSC become more responsive to chemotherapy.

An important limitation may come from gaining resistance to immunotherapy. It may be caused by the absence of tumor antigens, loss or decrease of MHC expression, alteration of signaling pathways affecting immune cell infiltration, or presence of regulatory T cells or myeloid derived suppressor cells in the tumor microenvironment. In order to prevent resistance and extend the clinical benefits of immunotherapy, it is necessary to better understand the anti-tumor response mechanisms of the strategies discussed here in order to combine them, as combinatorial therapy might be the answer for acquiring long-term remission in cancer therapy.

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MINIREVIEWS

Is the measurement of drain amylase content useful for predicting pancreas-related complications after gastrectomy with systematic lymphadenectomy?

Koki Nakanishi, Mitsuro Kanda, Junichi Sakamoto, Yasuhiro Kodera

ORCID number: Koki Nakanishi (0000-0002-0629-798X); Mitsuro Kanda (0000-0001-5464-3819); Junichi Sakamoto (0000-0002-9675-512X); Yasuhiro Kodera (0000-0002-6173-7474).

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Koki Nakanishi, Mitsuro Kanda, Yasuhiro Kodera, Department of Gastroenterological Surgery (Surgery II), Nagoya University Graduate School of Medicine, Nagoya 466-8550, Japan

Junichi Sakamoto, Tokai Central Hospital, Kakamigahara 504-8601, Japan

Corresponding author: Mitsuro Kanda, FACS, MD, PhD, Department of Gastroenterological Surgery (Surgery II), Nagoya University Graduate School of Medicine, 65 Tsurumai-cho, Showa-ku, Nagoya 466-8550, Japan. m-kanda@med.nagoya-u.ac.jp

Abstract

Many studies investigating postoperative pancreatic fistula (POPF) after gastrectomy, including studies measuring drain amylase content (D-AMY) as a predictive factor have been reported. This article reviews previous studies and looks to the future of measuring D-AMY in patients after gastrectomy. The causes of pancreatic fluid leakage are; the parenchymal and/or thermal injury to the pancreas, and blunt injury to the pancreas by compression and retraction. Measurement of D-AMY to predict POPF has become common in clinical practice after pancreatic surgery and was later extended to the gastric surgery. Several studies have reported associations between D-AMY and POPF after gastrectomy, and the high value of D-AMY on postoperative day (POD) 1 was an independent risk factor. To improve both sensitivity and specificity, attempts have been made to enhance the predictive accuracy of factors on POD 1 as well as on POD 3 as combined markers. Although several studies have shown a high predictive ability of POPF, it has not necessarily been exploited in clinical practice. Many problems remain unresolved; ideal timing for measurement, optimal cut-off value, and means of intervention after prediction. Prospective clinical trial could be imperative in order to develop D-AMY measurement in common clinical practice for gastric surgery.

Key words: Gastric cancer; Drain amylase; Postoperative pancreatic fistula; Pancreas-related complications; Gastrectomy; Early prediction

Core tip: Many studies investigating postoperative pancreatic fistula after gastrectomy, including measurement of drain amylase content (D-AMY) as a predictive factor. This article reviews previous studies and looks to the future of measuring D-AMY in patients

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after gastrectomy. Several studies have reported that the high D-AMY on postoperative day 1 or day 3 was an independent risk factor for postoperative pancreatic fistula. However, issues for clinical use remain unresolved, including the ideal timing of measurement, optimal cut-off value and intervention after prediction. Prospective clinical trials might be indispensable for D-AMY to become a common marker in clinical practice.

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INTRODUCTION

Gastrectomy with radical lymph node dissection, especially peri-pancreatic lymph node dissection, is the mainstay for resectable gastric cancers. Pancreas-related complications, especially postoperative pancreatic fistula (POPF), is one of the most common postoperative complications, and can sometimes lead to serious results, including intra-abdominal abscess, subsequent sepsis, and intraperitoneal bleeding, which usually require prolongation of hospitalization[1,2].

Many studies predicting POPF after gastrectomy have been investigated and age, operation time, body mass index, total gastrectomy, splenectomy, anatomical position of the pancreas, and high value of drain amylase content (D-AMY) have been reported as substantial predictive factors[2-13]. Among those, D-AMY, which is the measurement of the amylase content in drained abdominal fluids taken through an indwelling intra-abdominal drain, is promising because it can be measured objectively regardless of the patient's preoperative condition, type of surgical procedure, and surgeon's skill[14]. However, several problems remain unsolved precluding the implication in common clinical practice.

This article reviews previous reports and looks to the future of measuring D-AMY to predict POPF in gastric cancer surgery.

MECHANISM OF PANCREATIC FLUID LEAKAGE IN GASTRIC CANCER SURGERY

In gastric cancer surgery, the pancreatic duct is not usually transected, and the mechanism of pancreatic fluid leakage after surgery is different from that of pancreatectomy. Mainly, three mechanisms of pancreatic fluid leakage in gastric cancer surgery are reported, presumably caused by the operator and assistant surgeons. The operator can injure the surface of the pancreas by parenchymal and/or thermal injury during the dissection of the suprapancreatic lymph nodes[15]. Second, the assistant could compress and retract the pancreas to achieve a good view of the suprapancreatic area during suprapancreatic lymph node dissection, so called "blunt injury"[16]. Ida *et al*[16] conducted animal experiments using pigs and reported that blunt injury causes pancreatic necrosis and inflammatory cell infiltration, and the value of amylase content around the pancreas increases 2-4 h after the procedure.

Another mechanism is that pancreatic tail mobilization during combined splenectomy or splenectomy with distal pancreatectomy can damage the pancreatic parenchyma, resulting in pancreatic fluid leakage[3]. However, indications for such extended lymphadenectomy for gastric cancer has recently become limited[17].

In this regard, pancreatic fluid leakage involving gastric cancer surgery could mostly be minor leakage, that tends to be subsided spontaneously without clinically relevant pancreas-related postoperative complications.

THE BEGINNING OF STUDIES PREDICTING POPF USING DRAIN AMYLASE CONTENT IN PANCREATIC SURGERY

In pancreatic surgery, the measurement of D-AMY is also used in the diagnostic criteria of POPF[18]. In general, pancreatectomy is recognized as a highly invasive surgery and is associated with a mortality of approximately 5% and a morbidity of 30%-60%[19]. Approximately 16% of patients develop POPF, making it one of the common complications of pancreatectomy[20]. The clinical stratification of POPFs was established by the International Study Group on Pancreatic Fistula (ISGPF) definition in 2005[18]. The presence of POPFs can be determined on postoperative day (POD) 3 by the amylase content in the drained fluid; therefore, the measurement of D-AMY has become common in clinical practice in the field of pancreatic surgery. After pancreatectomy, pancreatic fluid leakage is caused by a disruption of the main pancreatic duct, and D-AMY directly reflects pancreatic fluid leakage[21,22]; therefore, the measurement of D-AMY is a reasonable prediction tool for pancreas-related complications. This concept was later extended to gastric cancer surgery; however, the mechanisms responsible for pancreatic fluid leakage are supposed to differ between pancreatectomy in which the main pancreatic duct is transected, and gastrectomy, which causes some problems. The source of pancreatic fluid leakage after gastrectomy is the seepage of pancreatic juice from the parenchymal damage of the pancreas and blunt damage by compression or retraction[15,16]. Even if the value of D-AMY is high on POD 3, this minor pancreatic leakage seems to subside spontaneously without proceeding to clinical fistula formation[3]. Thus, the establishment of a gastric cancer surgery-specific definition and prediction tool for POPF is desirable.

DEFINITION OF POPF IN GASTRIC CANCER SURGERY

Despite clinical importance, POPF had not been uniformly defined until 2005 when the ISGPF established the definition based on the clinical impact of POPF-related complications[18], and it has been well accepted in the pancreatic surgery community. The stratifications are as follows: Grade A, pancreatic fistulas with no clinical impact, although D-AMY on or after POD 3 is three times more than the upper normal serum amylase level; grade B requires a change in management or adjustment in the clinical pathway; and grade C requires a major change in clinical management and aggressive clinical intervention. This ISGPF classification is sometimes applied in the gastric cancer surgery community. However, validation of applying this definition to POPF following gastrectomy still remains unclear. As described in the previous section, the mechanisms of pancreatic fluid leakage in gastric cancer surgery are different from those in pancreatic surgery, and are shown in Figure 1; POPF of ISGPF grade A is not necessarily clinically significant; in other words, POPF with no clinical impact need not be defined in gastric cancer surgery. In addition, almost all POPFs in gastric cancer surgery are classified as ISGPF grade B, and ISGPF grade C is very rare.

As another definition of POPF, the Clavien-Dindo classification has been adopted, which is a comprehensive evaluation of postoperative complications and has gained widespread acceptance[23,24]. This classification system regards grade II or higher as clinically relevant and grade III or higher as severe complications. However, this definition is sometimes inconsistent with clinical severity, although objective and simple. For example, replacement of drainage tubes under fluoroscopy is classified as Clavien-Dindo grade IIIa, despite minor changes in clinical management and regarded severe complications.

An establishment of a new grading system of POPF after gastrectomy may be necessary. However, it is less frequently used and may not be familiar because the prevalence of POPF after gastrectomy is not as high compared with that after pancreatomy; it occurs in approximately 1.6% of patients who underwent distal gastrectomy[25] and in 2.6% of patients who underwent total gastrectomy[26]. At present, the ISGPF and Clavien-Dindo classification systems have each of their advantages and disadvantages, and it is desirable to use both appropriately.

LITERATURE SEARCH OF STUDIES REPORTING PREDICTIVE VALUE FOR POPF USING DRAIN AMYLASE CONTENT IN GASTRIC CANCER SURGERY

Several studies have reported associations between D-AMY and POPF after gastrectomy, and Table 1 shows these studies in the gastric cancer surgical field. In

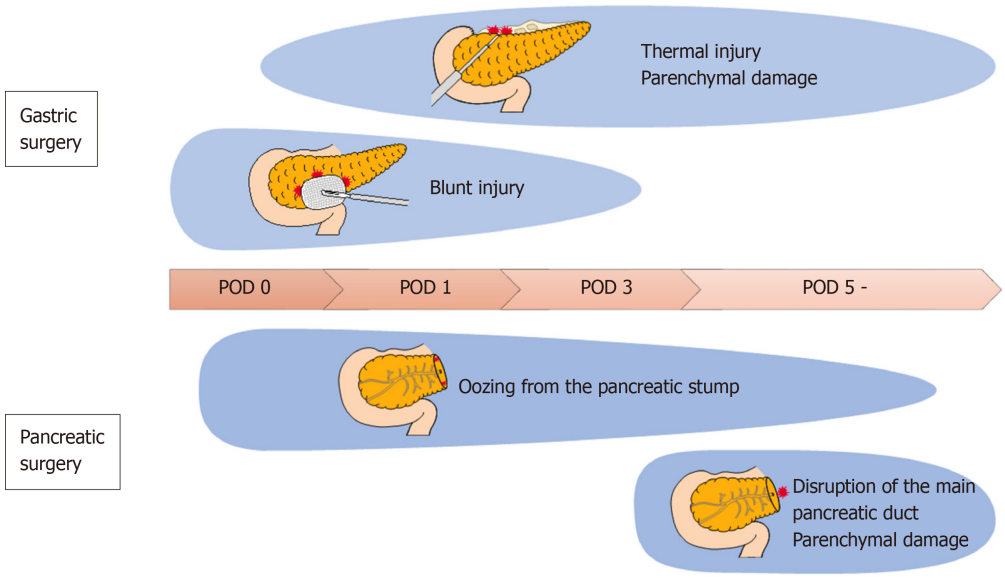

Figure 1 Mechanisms and timings of pancreatic fluid leakage in gastric and pancreatic surgery. POD: Postoperative day.

1997, Sano *et al*[3] reported for the first time that the measurement of D-AMY was useful for POPF after gastrectomy. However, a definition of POPF had not been established at that time, and their study defined it as a condition in which the D-AMY level was more than three times the upper normal serum amylase level for more than 7 d after operation. In their study, the prevalence of POPF in patients with D-AMY ≥ 4000 IU/L on POD 1 was significantly higher compared with that in patients with D-AMY < 4000 IU/L on POD 1, indicating the high level of D-AMY on POD 1 was retained to POD 7, regardless of clinically relevant complications.

The ISGPF established the definition of POPF in 2005, and Iwata *et al*[2] adapted this definition and reported associations between D-AMY and POPF after gastrectomy in 2010. They reported that the prevalence of ISGPF grade A or higher was 16.3% and that D-AMY ≥ 1000 U/L on POD 1 along with body mass index were independent risk factors for POPF. However, the study suffered from the inclusion of a broad spectrum of surgical procedures ranging from the laparoscopic approach for early-stage cancer to extended lymphadenectomy accompanied by splenectomy for advanced cancer. In 2011, there were two studies that defined ISGPF grades B or C as POPF. Miki *et al*[6] reported that the prevalence of POPF after total gastrectomy with D2 lymphadenectomy was 22.1% and that D-AMY ≥ 3398 IU/L on POD 1 was an independent predictor of POPF. Tomimaru *et al*[4] reported that the prevalence of POPF after total gastrectomy with D1 plus or D2 lymphadenectomy was 9.2% and that D-AMY ≥ 5000 IU/L on POD 1 was a predictor of POPF. The above three studies were validated by applying the ISGPF classification and the predictive ability of D-AMY on POD 1, but the surgical procedures were different among studies, which caused the prevalence of POPF and the cut-off values to be inconsistent. In 2012, Kobayashi *et al*[7] adapted the Clavien-Dindo classification and reported that D-AMY ≥ 2000 IU/L on POD 1 and C-reactive protein ≥ 20 mg/dL on POD 3 were predictive of Clavien-Dindo classification grade III or higher POPF. The Clavien-Dindo classification grade III or higher POPF is the same as the ISGPF grade B or higher (grade C) excluding antibiotic treatment. So far, we summarized the studies to evaluate the predictive value of D-AMY on POD 1.

Two time point measurements of D-AMY have been developed to enhance the predictive value of POPF. In 2016, Kanda *et al*[8] reported that D-AMY on POD 1 served as a predictive factor for POPF. In addition, patients whose D-AMY level on POD 3 was retained at ≥ 31.2% of that on POD 1 were more likely to develop POPF after laparoscopic distal gastrectomy[8]. After that, two studies reported that the combined use of D-AMY on POD 1 and POD 3 had a higher predictive performance for POPF compared with each alone[9,10]. The combined use had high sensitivity; however, it did not serve as an early prediction.

A few studies have considered drainage volume[5,27] and concluded that drainage volume was not significant in gastric cancer surgery.

Table 1 Studies measuring drain amylase content for predicting postoperative pancreatic fistula after gastrectomy in patients with gastric cancer

Ref.	Sample size	Surgical procedure	POD 1		POD 3		Definition of POPF
			Data available	Cut-off	Data available	Cut-off	
Sano *et al*[3], 1997	102	OTG, D1 - ≥ D2	Yes	4000 IU/L	No	NG	D-AMY > 3 times more than S-AMY for ≥ 7 days
Iwata *et al*[2], 2010	372	Gastrectomy, D1 - ≥ D2	Yes	1000 IU/L	No	NG	ISGPF definition (grade A/B/C)
Tomimaru *et al*[4], 2011	172	TG, D1 plus - D2	Yes	5000 IU/L	No	NG	ISGPF definition (grade B/C)
Miki *et al*[6], 2011	104	TG, D2	Yes	3398 IU/L	No	NG	ISGPF definition (grade B/C)
Kobayashi *et al*[7], 2015	448	Gastrectomy, D1 - ≥ D2	Yes	1949 IU/L	No	NG	C-D classification (grade III or higher)
De Sol *et al*[5], 2015	53	Gastrectomy,D2	N0	NG	Yes	D-AMY > 3 times more than S-AMY	ISGPF definition (grade B/C)
Kanda *et al*[8], 2016	265	LDG, D1 plus - D2	Yes	904 IU/L	Yes	Retained at ≥ 31.2% of D-AMY on POD 1	C-D classification (grade II or higher)
Taniguchi *et al*[9], 2017	591	Gastrectomy D1- ≥ D2	Yes	2900 IU/L	Yes	2100 IU/L	ISGPF definition (grade B/C)
Kamiya *et al*[10], 2018	801	Gastrectomy D1 plus - ≥ D2	Yes	2218 IU/L	Yes	555 IU/L	C-D classification (grade III or higher)
Wakahara *et al*[12], 2019	327	Gastrectomy D0- D2	No	NG	Yes	761 IU/L	C-D classification (grade II or higher)

POPF: Postoperative pancreatic fistula; POD: Postoperative day; OTG: Open total gastrectomy; D-AMY: Amylase content of drainage tube; S-AMY: Serum amylase content; ISGPF: International Study Group on Pancreatic Fistula; NG: Not given or reported in the study; TG: Total gastrectomy; C-D classification: Clavien-Dindo classification; LDG: Laparoscopic distal gastrectomy.

REMAINING PROBLEMS FOR FUTURE STUDIES

As described in the previous section, the definition of POPF has not been established; in addition, several problems have remained for clinical use, such as timing of measuring D-AMY, optimal cut-off value, and means of intervention after early prediction.

The timing of measuring D-AMY has not been determined. There is a dilemma between early prediction and diagnostic accuracy. With a single predictive marker, there is a limitation to increasing both sensitivity and specificity, and attempts have been made to enhance the predictive accuracy of factors on POD 1 as well as on POD 3 as combined markers[8-10]. However, it has limited clinical use for early prediction and early intervention. In other words, the timing of measurement that has both high diagnostic accuracy and early detection has not been determined.

Second, the cut-off values were different among studies. Differences in the definition of POPF, differences in the surgical procedure, and small-scale retrospective studies prevent the establishment of the optimal cut-off value. From the viewpoint of the mechanism of POPF, the differences in surgical procedures do not affect fistula formation. A large-scale prospective trial is warranted to establish an optimal cut-off value that applies to any surgical procedure.

Third, it is unclear whether early intervention will improve outcomes even if early prediction is successful. Prophylactic antibiotics in gastric cancer surgery are usually administered until the next morning after surgery. Additional prophylactic antibiotic administration may be beneficial for preventing deterioration in particular POPF high-risk patients who underwent pancreaticoduodenectomy[28]; although so far, the benefit is unclear in gastric cancer surgery[29]. Currently, a prospective exploratory randomized trial to evaluate prolonged prophylactic antibacterial drug treatment for patients with high levels of D-AMY on POD 1 after gastrectomy is in progress (UMIN000012152). In addition, the benefit of the measurement of D-AMY content as an indicator of early drainage tube removal is unknown. The prophylactic drain is helpful for the detection of not only POPF but also other serious complications,

including anastomotic leakage, intraoperative bleeding, and injury of the intestine. It is possible that the drainage of intra-abdominal fluids, including pancreatic juice, may prevent subsequent POPF[2]. From the viewpoint of the enhanced recovery after surgery program, the drainage tube should be removed as soon as it is deemed unnecessary in order to reduce drain-related complications and shorten the hospital stay after gastrectomy[30-32]. Additionally, unnecessary drain placement is harmful in terms of intra-abdominal fluid loss. At present, the measurement of D-AMY is not used as an indicator of early drainage tube removal, but if the level of D-AMY is low, the drainage tube can be removed with little concern for later pancreas-related complications.

CONCLUSION

The measurement of D-AMY is promising because of its high predictive ability of POPF, even in the gastric cancer surgical field. However, many problems remain unresolved, *i.e.*, definition of POPF, ideal timing for measurement, optimal cut-off value, and means of intervention after prediction. Prospective clinical trial could be imperative in order to develop D-AMY measurement in common clinical practice for gastric surgery.

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ORIGINAL ARTICLE

Basic Study

Silymarin, boswellic acid and curcumin enriched dietetic formulation reduces the growth of inherited intestinal polyps in an animal model

Bruna Girardi, Maria Pricci, Floriana Giorgio, Mariano Piazzolla, Andrea Iannone, Giuseppe Losurdo, Mariabeatrice Principi, Michele Barone, Enzo Ierardi, Alfredo Di Leo

ORCID number: Bruna Girardi (0000-0002-5593-2046); Maria Pricci (0000-0002-2158-6392); Floriana Giorgio (0000-0001-5209-1256); Mariano Piazzolla (0000-0003-4886-015X); Andrea Iannone (0000-0002-5468-9515); Giuseppe Losurdo (0000-0001-7038-3287); Mariabeatrice Principi (0000-0003-0545-5656); Michele Barone (0000-0001-8284-5127); Enzo Ierardi (0000-0001-7275-5080); Alfredo Di Leo (0000-0003-2026-1200).

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Bruna Girardi, Maria Pricci, Floriana Giorgio, THD SpA, Correggio 42015, Italy

Mariano Piazzolla, Andrea Iannone, Giuseppe Losurdo, Mariabeatrice Principi, Michele Barone, Enzo Ierardi, Alfredo Di Leo, Gastroenterology Section, Department of Emergency and Organ Transplantation, University of Bari, Bari 70124, Italy

Corresponding author: Alfredo Di Leo, MD, PhD, Full Professor, Section of Gastroenterology, Department of Emergency and Organ Transplantation, University of Bari, Piazza Giulio Cesare 11, Bari 70124, Italy. alfredo.dileo@uniba.it

Abstract

BACKGROUND

Some substances of plant origin have been reported to exert an effect in reducing intestinal neoplasm development, especially in animal models. Adenomatous polyposis coli multiple intestinal neoplasia - $Apc^{Min/+}$ is the most studied murine model of genetic intestinal carcinogenesis.

AIM

To assess whether an enriched nutritional formulation (silymarin, boswellic acid and curcumin) with proven "*in vitro*" and "*in vivo*" anti-carcinogenetic properties may prevent inherited intestinal cancer in animal model.

METHODS

Forty adenomatous polyposis coli multiple intestinal neoplasia - $Apc^{Min/+}$ mice were used for the study of cancer prevention. They were divided into two groups: 20 assumed standard and 20 enriched diet. At the 110th d animals were sacrificed. In each group, four subgroups received intraperitoneal bromodeoxyuridine injection at different times (24, 48, 72 and 96 h before the sacrifice) in order to assess epithelial turnover. Moreover, we evaluated the following parameters: Intestinal polypoid lesion number and size on autoptic tissue, dysplasia and neoplasia areas by histological examination of the whole small intestine, inflammation by histology and cytokine mRNA expression by real-time polymerase chain reaction, bromodeoxyuridine and TUNEL immuno-fluorescence for epithelial turnover and apoptosis, respectively. Additionally, we performed western blotting analysis for the expression of estrogen alpha and beta receptors, cyclin D1 and cleaved caspase 3 in normal and polypoid tissues.

RESULTS

Compared to standard, enriched diet reduced the total number (203 *vs* 416) and

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the mean ± SD/animal (12.6 ± 5.0 *vs* 26.0 ± 8.8; $P < 0.001$) of polypoid lesions. In enriched diet group a reduction in polyp size was observed ($P < 0.001$). Histological inflammation and pro-inflammatory cytokine expression were similar in both groups. Areas of low-grade dysplasia ($P < 0.001$) and intestinal carcinoma (IC; $P < 0.001$) were significantly decreased in enriched diet group. IC was observed in 100% in standard and 85% in enriched formulation assuming animals. Enriched diet showed a faster epithelial migration and an increased apoptosis in normal mucosa and low-grade dysplasia areas ($P < 0.001$). At western blotting, estrogen receptor beta protein was well expressed in normal mucosa of enriched and standard groups, with a more marked trend associated to the first one. Estrogen receptor alpha was similarly expressed in normal and polypoid mucosa of standard and enriched diet group. Cleaved caspase 3 showed in normal mucosa a stronger signal in enriched than in standard diet. Cyclin D1 was more expressed in standard than enriched diet group of both normal and polypoid tissue.

CONCLUSION
Our results are suggestive of a chemo-preventive synergic effect of the components (silymarin, boswellic acid and curcumin) of an enriched formulation in inherited IC. This effect may be mediated by the reduction of epithelial proliferation, the increase of apoptosis and the acceleration of villous cell renewal due to dietary formulation intake.

Key words: Intestinal cancer; Familial adenomatous polyposis; Chemopreventive diet; $Apc^{Min/+}$ mice; Boswellia; Curcumin

Core tip: A dietetic formulation containing silymarin, boswellic acid and curcumin has shown *"in vitro"* and *"in vivo"* anti-carcinogenetic properties in animal model of inflammation-related intestinal carcinoma. Herein, we assessed whether it may prevent inherited intestinal cancer in animal model (adenomatous polyposis coli multiple intestinal neoplasia - $Apc^{Min/+}$). Our results showed that the dietetic formulation reduced polypoid lesion number and size on autoptic tissue, histological dysplasia and neoplasia areas. This effect is related to increased epithelial renewal and apoptosis and decreased proliferation. Our data are suggestive of a chemo-preventive synergic effect of the components of the dietetic formulation in inherited intestinal carcinoma.

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INTRODUCTION

Colorectal cancer (CRC) is the conclusive result of a progressive phenomenon that, in most cases, implies a succession of events (normal, pre-cancerous and neoplastic conditions)[1-3]. The progression to cancer may be due to genetic mutations leading to dysplasia and, then, to carcinoma, as in familial adenomatous polyposis and Lynch syndrome (inherited models of CRC). In humans, *APC* gene mutation is the genetic basis to carcinogenesis, making intestinal cells predisposed to cancer promotion and evolution with additional mutations by epigenetic changes, mostly affected by environmental stimuli[4,5].

Some substances of plant origin have been reported to exert an effect in reducing intestinal neoplasm development, especially in animal models. In detail, silymarin, a phytoestrogen compound derived from milk thistle (*Silybum marianum*) may decrease intestinal carcinogenesis through both anti-oxidant and estrogen receptor (ER)-beta agonist properties[6,7]. Previous studies have shown that silymarin is able to hamper

intestinal carcinoma (IC) development in $Apc^{Min/+}$ mice and familial adenomatous polyposis patients with ileal pouch-anal anastomosis[8,9]. Moreover, a case report suggests a similar effect even in Lynch syndrome[10]. Boswellia serrata is a plant with anti-inflammatory properties. Interestingly, boswellic acids, especially Acetyl-11-Keto-beta-Boswellic Acid (AKBA), a component of the gum resin of Boswellia serrata, has been recognized as a promising agent for the prevention of intestinal tumorigenesis in a mouse model of inherited carcinogenesis, *i.e.*, APC multiple intestinal neoplasia (min) animals. The $Apc^{Min/+}$ experimental model mimics familial adenomatous polyposis, even if the disease, differently from humans, is mainly confined to the small bowel and only minimally involves colonic district[11-13]. Curcuma is a phytochemical derived from turmeric (Curcuma longa), a plant similar to ginger. It has been demonstrated to exert anti-inflammatory and anti-neoplastic properties by interacting with several molecular targets, *i.e.*, transcription factors, enzymes, cell cycle proteins, cytokines, receptors and adhesion molecules[14-16].

The background of this study was based on the possibility that a mixture of phytochemicals may result in health benefits more than what supplied by single components[17,18]. Therefore, in a previous experiment we tested "*in vitro*" the effect of the elements of a nutritional combination as well as the complete mixture on the proliferation of cultured colo-rectal neoplastic cells. Every molecule (silymarin, boswellic acids and curcumin) showed a relevant anti-proliferative action in comparison with control samples. In addition, the mixture of the three molecules significantly inhibited cellular growth more than single or double combination[19]. Moreover, in the same study, a nutritional formulation based on the combination of silymarin, boswellic acids and curcumin clearly demonstrated an anti-inflammatory and chemopreventive effect "*in vivo*" in an animal model of colorectal carcinoma arising from inflamed tissue[19].

On these bases, the present study had the primary aim of assessing whether the effect of the nutritional formulation (enriched dietary supplement) could exert an inhibitory activity on intestinal carcinogenesis in $Apc^{Min/+}$ animal model. For this purpose, the dose of every substance was given "*in vivo*" under the maximal effective amount of single components. Furthermore, the doses were in agreement with their bioavailability based on daily intake amount able to achieve an appropriate plasma concentration by a complete intestinal absorption[20]. Additionally, some mechanistic features were investigated.

MATERIALS AND METHODS

Animals

Forty $Apc^{Min/+}$ animals were used for the experimental design. They were kept in controlled conditions of temperature, air and light (from 7 a.m. to 7 p.m.) and received food and water ad libitum. Animals did not receive any surgical or hormonal manipulation, but they were kept anatomically and physiologically intact. All animals received care in agreement with the "Guide for the Care and Use of Laboratory Animals"[21]. The study protocol was approved by the University of Bari Committee for Animal Experimentation (protocol number 6/12).

Dietary features

Dietary procedures were started at the 10^{th} wk of age after a two-week period of settling. Forty wild type animals (20 receiving standard diet and 20 modified diet - enriched formulation, THD SpA, Correggio, Italy) were used for the preliminary assessment of the dietary supplement safety. Forty $Apc^{Min/+}$ animals (20 receiving standard diet and 20 modified diet - enriched formulation) were used for the evaluation of dietary mixture chemopreventive effect. The feeding procedures, used in both phases of the study, were: (1) Standard diet (Harlan Teklad Rodent diet): 18.5% proteins, 3% oils and fats, 6% fibers, 7% crude ash and 65.5% of non-nitrogenous compounds - wheat, maize, toasted soybean meal, corn gluten feed, wheat straw, fish meal, Lucerne meal, mineral bicalcium phosphate, calcium carbonate, sodium chloride, whey powder, soybean oil, yeast and hazelnut skins, poly-vitamin complex (Mucedola Srl, Settimo Milanese, Italy); and (2) Modified Diet (enriched formulation): The standard diet was enriched by a formulation containing silymarin (4 g%), AKBA (3 g%), curcumin (2 g%), maltodextrins (69.553 g%) and excipients (soluble fibers 16.667 g%, citric acid 1 g%, silicon dioxide 1 g%, lignans 0.5 g%, sucralose 0.280 g%, orange flavor 2 g%) and was administered at the cumulative dose of 22.4 mg/100 g of body weight.

The enrichment dietetic formulation was established taking into account that: (1) Mice with a body weight of 20-40 g usually eat about 5 g of daily food amount (16.7

g/100 g of body weight according to Italian Association for Laboratory Animal Science, November 2012)[22]; (2) Daily intake was 0.892 mg of silymarin, 0.672 mg of AKBA and 0.448 mg of curcumin per 100 g of body weight for animal. In detail, these amounts were administered below the maximum order of magnitude of the effective concentrations of the single substances against cancer in animals[20]; and (3) Our supposition was that these substances, similarly to what observed *"in vitro"*, could exert beneficial effects beyond what provided by the single phytochemical.

Animal sacrifice and autoptic tissue collection

$Apc^{Min/+}$ mice were sacrificed 110 d after starting dietetic formulation assumption. All animals received intraperitoneal administration of bromodeoxyuridine (BrdU, 50 mg/kg) for epithelia turnover assessment. In both groups, mice were divided into four subgroups and sacrificed under metaphane anesthesia at 24^{th}, 48^{th}, 72^{th} or 96^{th} h after the injection, respectively. The small bowel from each animal was collected, washed and dissected along the longitudinal axis in order to evaluate both macroscopic and microscopic neoplastic lesions along the whole organ. After macroscopic evaluation, small and large bowel were fixed in 10% buffered formalin and embedded in paraffin. Samples from each animal were frozen at -80 °C for molecular biology and western blotting analyses. Macroscopic evaluation of polypoid lesions was based on their division into three groups according to the size: Small (< 3 mm), intermediate (3-7 mm) and large (> 7 mm).

Histological evaluation

Sections (4 micron thick) were sequentially stained with haematoxylin and eosin for histological examination. Inflammation score (range 0-5) was evaluated in normal tissue according to Yu *et al*[23]. For each animal, the number of low-grade dysplasia (LGD) and high-grade dysplasia (HGD) and IC areas were recorded along the whole small bowel and expressed as mean ± SD/animal. All tissues were examined by two expert observers in blind.

Real time polymerase chain reaction assay of cytokine mucosal expression

Pro-inflammatory cytokine [interferon gamma, interleukin 6 and tumor necrosis factor (TNF) alpha] gene expression was assessed by real time polymerase chain reaction to assess the amount of specific mRNA in frozen samples without polypoid lesions. Results were expressed as fold change compared to control. RNA was extracted from five sections of 10 microns, using QIAgen RNA mini kit (QIAGEN GmbH, Germany). Two-step reverse transcription polymerase chain reaction was performed using first-strand cDNA with a final concentration of 1 × TaqMan gene expression assay, *i.e.*, TNF alpha and glyceraldehyde-3-phosphate-dehydrogenase (Applied Biosystems, Foster City, CA). The final reaction volume was 25 microliters and analyzed in triplicate (all experiments were repeated twice). A non-template control (RNAse-free water) was included on every plate. A further validation of our method was performed by enclosing in each assay fresh samples from at least three healthy mouse colonic mucosa. Specific thermal cycler conditions were employed by real time polymerase chain reaction System (Applied Biosystems, Foster City, CA, United States). A standard curve plus validation experiment were performed for each primer/probe set. A series of 6 serial dilutions (20 to 0.1 ng/microl) of tissue cDNA were used as a template.

Epithelial turnover evaluation by BrdU immuno-fluorescence

BrdU expression in epithelial cells was investigated by a monoclonal mouse antibody cell signaling (Novus Biologicals, Milan, Italy). Sections were rinsed in PBS buffer with TWEEN 0.025% for 10 min and incubated in microwave oven (citric buffer pH 6.0, 10 min, 750 W) for antigen unmasking. Then, they were treated (2 h; room temperature) in 10% goat serum and 1% bovine serum albumin blocking solution. Successively, they were incubated with anti-BrdU antibody diluted 1:50 at 4 °C overnight. Alexa 488 fluorescent-conjugated goat anti-mouse (Invitrogen, Life Technologies, Monza, Italy) at a dilution of 1:200 represented the secondary antibody. All sections were observed with confocal microscopy at 630 × magnification. Ten well-oriented crypt/villous areas of histologically normal small bowel were selected for the analysis. Cell proliferation in the crypts and migration towards mucosal free surface (epithelial turnover) were evaluated, using the highest labeled cell along the villus as marker of the percentage of covered axis[24]. The analysis was performed in four subgroups in both enriched and standard diet in relation to the time of sacrifice after BrdU injection (*i.e.*, 24, 48, 72 and 96 h).

Apoptosis evaluation by TUNEL immune fluorescence

Apoptosis was evaluated by TUNEL (Cell Death Detection kit, Roche, Mannheim,

Germany). Briefly, sections were de-waxed and incubated in 0.1 mol citrate buffer (pH = 6.0) in microwave oven at 350 W for 10 min. Then, sections were incubated with TUNEL probe at 37 °C for one hour and counterstained with TOPRO-3 at a dilution of 1:3000 (Invitrogen Molecular Probes). Slides were analyzed with confocal microscopy at 630 × magnification. The count of marked cells was performed on 10 well-oriented crypt/villous configurations. Labeling index, *i.e.*, percentage of positive cells, was used to quantify apoptosis. Cell count was executed in IC, HGD, LGD and normal tissue. Due to the large specimen size, all histological pictures were found in every section. Two expert observers analyzed the slides in blind.

Western blotting analysis

Frozen samples were homogenized in lysis buffer (25 mmol Tris-HCl pH 7.6, 150 mmol NaCl, 1% NP-40, 1% sodium deoxycholate, 0.1% sodium dodecyl sulphate, protease inhibitor cocktail; Roche Diagnostics, Germany). Lysates underwent centrifugation at 14000 rpm for 30 min a 4 °C. Protein levels were assayed with Bradford method (Bradford Reagent - Bio Rad, Milano, Italy). Aliquots containing 40 µg of total proteins were separated in 4%-12% precast polyacrylamide gels (Invitrogen, Thermo Fisher Scientific, Monza, Italy) and transferred to nitrocellulose membranes (Bio-Rad) for 1 h at 300 mA. Ponceau S stain (Sigma, Milano, Italy) was used to verify the even and complete transfer of proteins onto the membrane. The membranes were blocked with Tris-buffered saline (TBS) containing 5% dry milk powder and 0.1% Tween-20 (Sigma) for 2 h at room temperature. This step was followed by an overnight incubation at 4 °C with primary rabbit antibodies, diluted in TBS 0.1% Tween (T-TBS) with 5% dry milk powder, of ER alpha (Invitrogen cat. no. PA5-34577 - 1 µg/mL), ER beta (Invitrogen cat.no. PA1-310B – 1 µg/mL), Cyclin D1 (Invitrogen cat.no. MA5-14512 - 1:100), Caspase-3 cleaved forms (Invitrogen cat.no. 700182- 0.2 µg/mL) and Beta Actin, as housekeeping protein (Invitrogen cat.no. PA1-183 - 1:1000). After repeated washing with T-TBS, the membranes were incubated for 1 h at room temperature with a horseradish peroxidase conjugated secondary anti-rabbit antibody (Invitrogen cat.no. A27036 - 1:20000). The proteins were detected using an enhanced chemiluminescent substrate (ECL - Super Signal West Pico - Thermo Scientific) for the detection of horseradish peroxidase and the signal density of every protein obtained by the Molecular Imager ChemidocTM (Bio-Rad Laboratories).

Statistical analysis

The comparison of continuous variables in the two groups was performed by Student's *t* test for unpaired data. One-way analysis of variance corrected by Bonferroni's test was used to compare TUNEL expression in normal mucosa, LGD, HGD and IC. Categorical data were evaluated by Fisher's exact test or chi squared test for trend. Values of $P < 0.05$ (two tails) were considered significant. The statistical software GraphPad Prism 5 (San Diego, California, United States) was used.

RESULTS

Enriched diet safety test had been performed in a previous experience[19] and did not show any adverse event.

Macroscopic findings

In the enriched diet group, the total length of the small bowel was similar to that of standard diet group (26.4 cm ± 1.2 cm *vs* 25.7 cm ± 1.3 cm; $P = 0.17$).

A very small amount of polypoid lesions was observed in the colon at sacrifice (0.6 ± 0.8 *vs* 0.8 ± 0.9 in the enriched and standard diet group, respectively; $P = 0.54$). The total number of solid lesions observed in the small bowel of sacrificed animals was 416 in the standard diet and 203 in the enriched diet group. In detail, the number of polypoid lesions per animal in the small bowel was reduced in the enriched compared to the standard diet group (12.6 ± 5.0 *vs* 26.0 ± 8.8; $P < 0.001$) (Figure 1).

The evaluation of polypoid lesion size showed that: (1) In the standard diet group, lesion size was > 7 mm (large) in 9/416 (2.2%), 4-7 mm (intermediate) in 205/416 (49.2%) and < 3 mm (small) in 202/416 (48.6%); (2) In the enriched diet group, the lesion size was > 7 mm (large) in 3/203 (1.4%), 4-7 mm (intermediate) in 57/203 (28.1%) and < 3 mm (small) in 143/203 (70.5%). The test for trend demonstrated that solid lesion size was significantly reduced ($P < 0.001$) in the enriched diet group.

Microscopic findings

There was no significant difference in the histological score of inflammation between the two groups (1.2 ± 0.4 in the standard and 1.5 ± 0.5 in the enriched diet group, P =

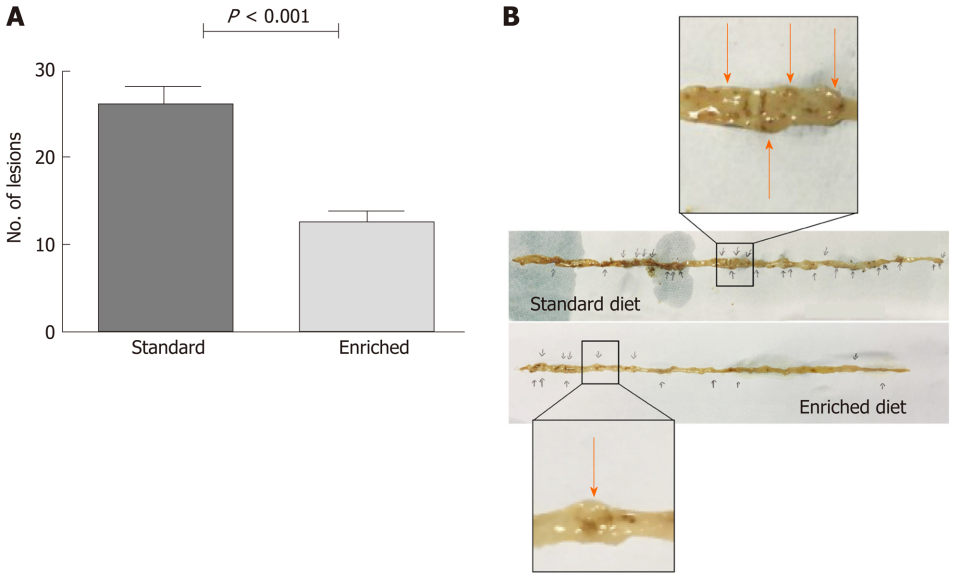

Figure 1 The number of polypoid lesions per animal in the small bowel was reduced in the enriched compared to the standard diet group. A: Number of small bowel solid lesions (mean ± SD/animal) in $Apc^{Min/+}$ mice assuming standard or enriched diet; B: Autopsy macroscopic picture of murine intestine in standard diet (above) and enriched diet (below). Intestinal polypoid lesions are magnified in boxes and highlighted by arrows.

0.09) in normal tissue. Both scores indicate that inflammation was almost absent in $Apc^{Min/+}$ mice in non-dysplastic/neoplastic areas.

The mean number of LGD areas per mouse, recorded along the whole small bowel and expressed as mean ± SD/animal, was 5.4 ± 1.2 in the standard and 3.3 ± 1.2 in the enriched diet group ($P < 0.001$). The number of HGD areas was 4.5 ± 1.0 in the standard and 4.1 ± 1.3 in the enriched diet group ($P = 0.10$). The number of IC areas was 4.5 ± 0.7 in the standard and 2.6 ± 0.7 in the enriched diet group ($P < 0.001$). Results are summarized in Figure 2A, while in Figure 2B and an explanatory example of IC histological picture is shown.

IC was observed in 20/20 (100%) of the standard and 17/20 (85%) of the enriched diet group ($P = 0.22$).

Pro-inflammatory cytokine mRNA expression

Pro-inflammatory cytokine mRNA expression did not statistically differ in normal small bowel of the mice assuming the standard or the enriched diet. In detail, interferon-gamma showed a value of 0.5 ± 0.3 (standard) *vs* 0.5 ± 0.4 (enriched diet) fold-change; interleukin 6 showed a value of 0.4 ± 0.3 (standard) *vs* 0.4 ± 0.3 (enriched diet) fold-change; TNF alpha showed a value of 0.5 ± 0.4 (standard) *vs* 0.8 ± 0.4 (enriched diet) fold-change.

Immunoflorescence findings

Epithelial turnover: In both standard and enriched diet groups, we observed in normal mucosa that positive cells were predominantly located in the crypts after 24 h, while a progression towards the villous district was observed after 48 h. In addition, we found positive cells only near the villous free surface at 72nd and 96th h with a decreasing number through the time progression.

Figure 3 illustrates the percentage of villous axis covered by the highest labeled cell at the 48th h after injection in normal mucosa. This parameter was significantly higher in the enriched than in the standard diet group (82.2 ± 6.4 *vs* 65.3 ± 5.6 percent; $P < 0.001$). In LGD areas of the two groups, we found positive cells in both crypt and villous areas even at 24th h. Positive cells were found only at villous level after 48, 72 and 96 h with a decreasing LI. In HGD and IC the complete alteration of the crypt/villous structure did not allow evaluating the position of BrdU positive cells.

Epithelial apoptosis: Immuno-fluorescent TUNEL expression is shown in Figure 4. Statistical analysis displayed: N < LGD > HGD = IC both in the standard and enriched diet group ($P < 0.001$ for both groups). However, a significant higher level of epithelial apoptosis was observed in the enriched compared to the standard diet group in normal ($P < 0.01$) and LGD ($P < 0.001$) areas.

Western blotting findings

Western blotting findings are summarized in Figure 5. In detail we did not find any

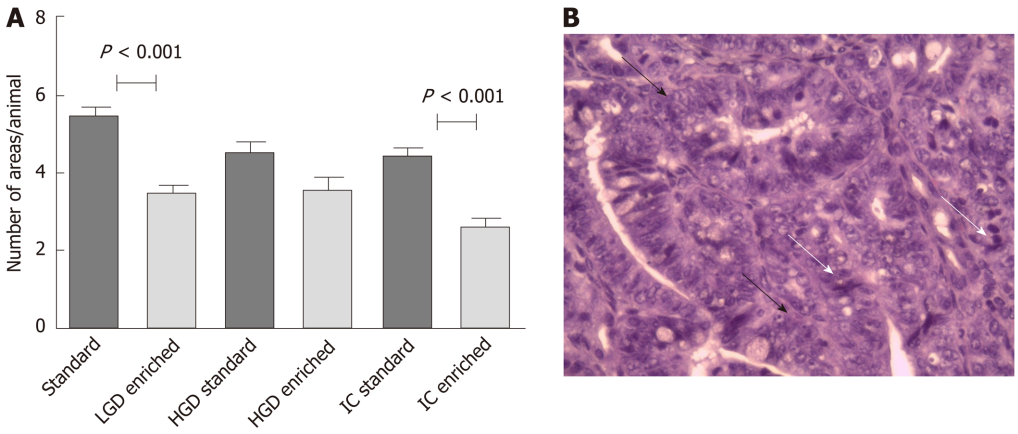

Figure 2 Results summarize and an explanatory example of intestinal carcinoma histological picture. A: Mean number ± SD/mouse of microscopic areas of low-grade dysplasia, high grade dysplasia and intestinal carcinoma. B: A histological picture of intestinal carcinoma (hematoxylin-eosin stain) showing cell crowding and pleomorphism (white arrow), architectural loss and nuclear hyperchromatism (black arrow). LGD: Low-grade dysplasia; HGD: High grade dysplasia; IC: Intestinal carcinoma.

difference in ER alpha expression between normal and polypoid tissue, despite an increased protein level was observed in enriched compared to standard group. Conversely, ER beta protein was poorly expressed in polyps of both groups, while it was well expressed in normal mucosa of enriched and standard diet with a more marked signal in the first group. Cyclin D1 was more expressed showed lower levels in polyps of enriched compared to standard diet animals. Moreover, its expression was less evident in normal tissue of enriched compared to standard diet. The protein of cleaved form of caspase 3 showed a poor expression in polyps of both groups, while evident levels were found in normal mucosa of both groups with a stronger signal in enriched than in standard diet.

DISCUSSION

This study was planned to assess whether an enriched dietary supplement containing silymarin, boswellic acid and curcumin, could protect against polypoid lesion onset in a murine model of inherited intestinal cancer ($Apc^{Min/+}$). The hypothesis of this study was that the combinations of phytochemicals under the maximal effective amount of single components may result in health benefits more than what supplied by single components. In order to support this hypothesis, in a previous experiment we tested *"in vitro"* the effect the elements of a nutritional combination as well as the complete mixture on the proliferation of cultured colo-rectal neoplastic cells. Every molecule (silymarin, boswellic acids and curcumin) showed a relevant anti-proliferative action in comparison with control samples. In addition, the mixture of the three molecules significantly inhibited cellular growth more than single or double combination[19]. Moreover, in the same study, the nutritional formulation based on the combination of silymarin, boswellic acids and curcumin clearly demonstrated an anti-inflammatory and chemopreventive effect in an animal model of colorectal carcinoma arising from inflamed tissue[19].

$Apc^{Min/+}$ model mimics familial adenomatous polyposis even if, unlike humans, almost all of the polyps are located in the small bowel more than in the colon and duodenum[25]. However, the adenoma-carcinoma sequence is well represented[26].

The main result of this study concerns the effect of the dietetic formulation on carcinogenesis. Indeed, we found small bowel carcinomas in the 85% of the animals receiving enriched diet, while cancer was seen in the 100% of the standard diet group. However, enriched diet was associated with a significant decrease in the number and size of polypoid lesions. Finally, in mice assuming enriched diet a significant decrease of areas of dysplasia and carcinoma was detected along the whole small bowel when compared to animals fed with the standard diet.

Despite dietetic formulation has a well-demonstrated anti-inflammatory effect[19], we could not demonstrate it in $Apc^{Min/+}$ animals for the almost absence of mucosal inflammation in normal mucosa. Inflammation evaluation was performed in non-dysplastic/neoplastic tissue, since our aim was to assess its role in carcinogenetic process. Therefore, we did not detect this parameter in pre-cancerous or cancerous lesions, where it is commonly found as a consequence of necrosis and not as a pro-

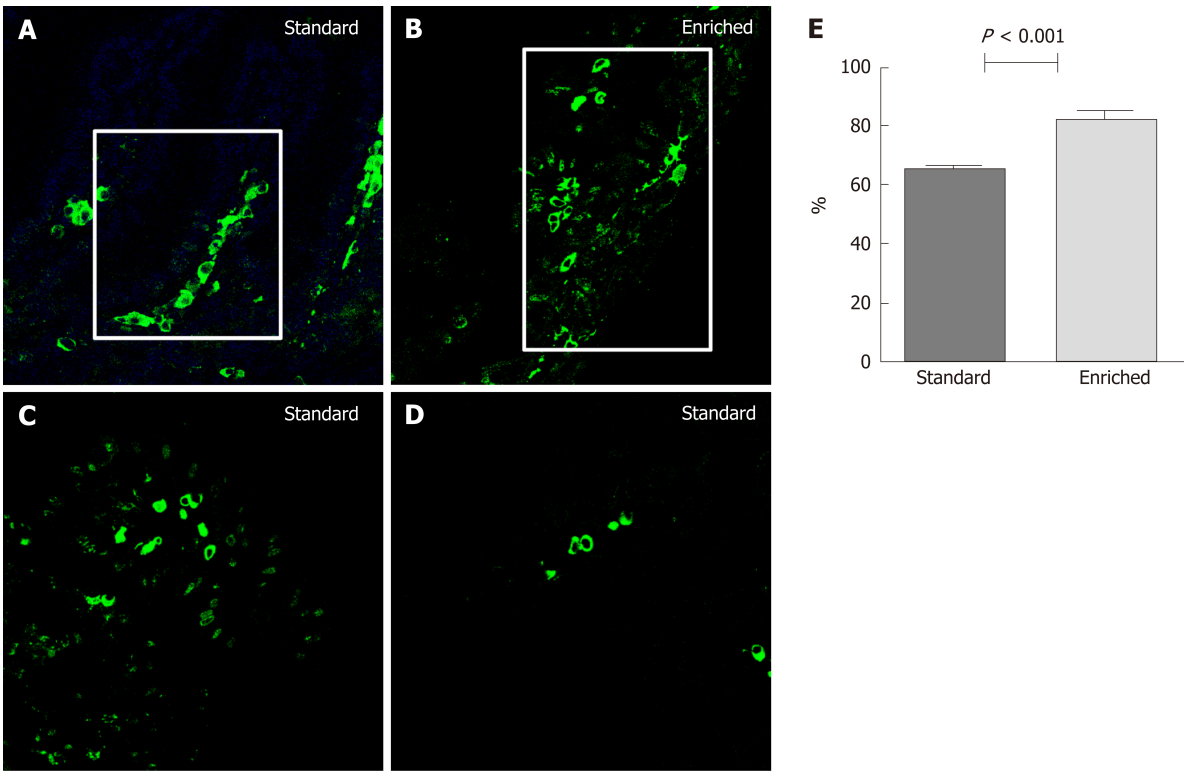

Figure 3 The percentage of villous axis covered by the highest labeled cell at the 48th h after injection in normal mucosa. A, B: Percentage of villous area covered by the highest bromodeoxyuridine labeled cell at the 48th h after injection in normal colonic mucosa in standard (A) and enriched diet group (B); C, D: The pictures at 72th (C) and 96th (D) h in standard group is represented; the findings at these times were the same in the enriched diet group and limited to the tip of villi. Positive cells are stained green (confocal microscopy; magnification: 630 ×); E: Quantitative analysis.

carcinogenetic factor. As expected, we found a very low inflammatory histological score as well as a poor mucosal pro-inflammatory cytokine mRNA expression. Presumably, this finding suggests that anti-neoplastic effect of enriched dietetic formulation may not be mediated by dietetic formulation anti-inflammatory activity. Therefore, other mechanistic potential anti-neoplastic effects may be invoked.

A possible explanation for the anti-neoplastic effect of dietary treatment could be related to the well-demonstrated anti-oxidant and ER beta agonist effects of silymarin[7,27,28]. Indeed, Calabrese *et al*[29] demonstrated that a dietetic supplementation with silymarin induces a significant reduction of number and size of duodenal polyps in patients with familial adenomatous polyposis in the course of the follow up after ileal pouch-anal anastomosis. Moreover, Barone *et al*[8] showed that silymarin is able to lower IC development in $Apc^{Min/+}$ mice and this process is associated with an increased crypt-villous epithelial cell migration and apoptosis. In this study, western blotting analysis demonstrated that silymarin is able to increase beta-ER/alpha ER ratio. Moreover, we have previously observed that beta ERs showed a progressive decline in the progressive steps of intestinal carcinogenesis, *i.e*., normal tissue, LGD, HGD and IC in subjects affected by familial adenomatous polyposis[30]. Interestingly, in the same study we demonstrated that beta-ERs and caspase 3, an early marker of apoptosis, were co-expressed in the same cell and this co-expression declined progressively from normal to neoplastic tissue. In the present study, we found a poor expression of ER beta in polypoid tissue and the presence of high grade dysplasia and carcinoma at this sites may explain this result, since ER beta is poorly expressed in high grade dysplasia and carcinoma areas[30]. However, in macroscopically normal mucosa, ER beta protein was well expressed in normal mucosa of enriched and standard diet with a more marked signal in the first group. Therefore, a stimulation of ER beta by silymarin could have occurred at this level and played a role in anti-neoplastic effect of dietary treatment.

Analogously, boswellic acid has shown interesting properties for the prevention of intestinal tumorigenesis in $Apc^{Min/+}$ mice[11-13]. Its chemopreventive effect was attributed to a collection of activities including antiproliferation and apoptosis induction[12]. In detail, a decrease of proliferative (cyclin D1) and an increase of apoptotic markers (survivin and Bcl-xL) have been described as evidence that anticancer effects of boswellic acid[31].

Curcumin is able to modulate several molecular targets (transcription factors,

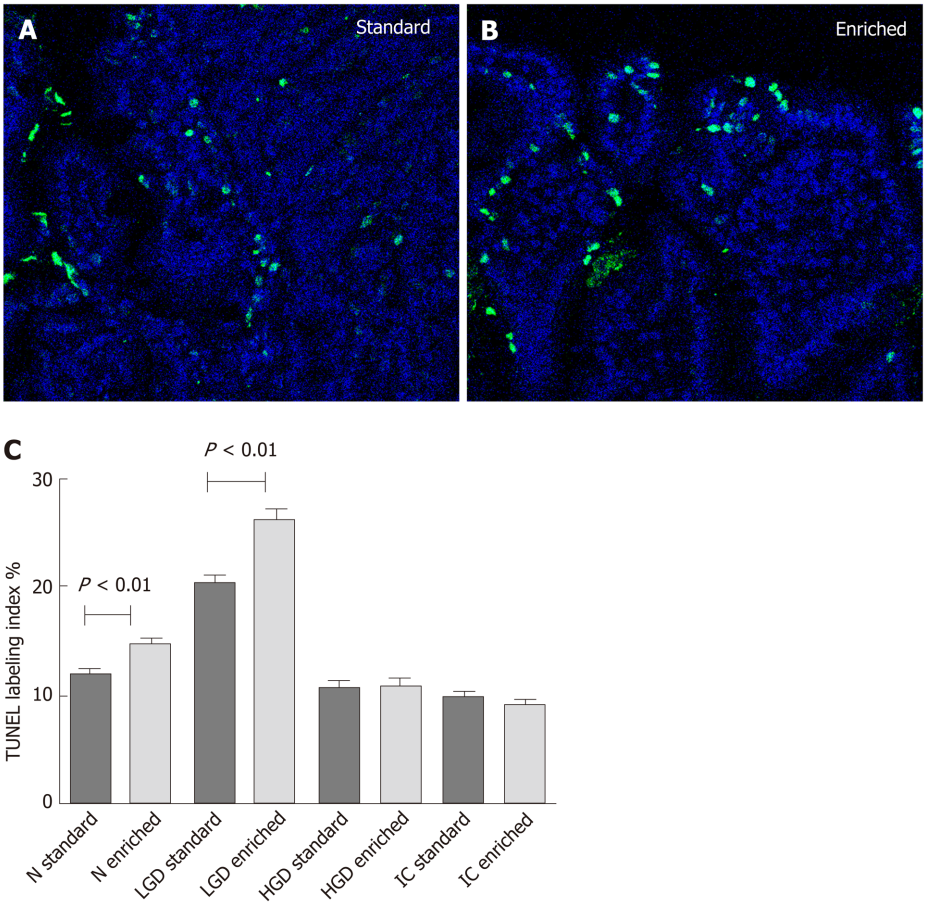

Figure 4 Immuno-fluorescent TUNEL expression. A, B: Immuno-fluorescent TUNEL expression in low grade dysplasia (LGD) areas of both the standard (A) and enriched diet group (B). Positive cells are stained green and negative ones blue (magnification: 630 ×). C: The statistical comparison of labeling index. LGD: Low-grade dysplasia; HGD: High grade dysplasia; IC: Intestinal carcinoma.

enzymes, cell cycle proteins, cytokines, receptors and adhesion molecules) which are involved in carcinogenetic progression[14-16]. A recent meta-analysis by Tabrizi *et al*[32] suggests that taking curcumin-containing supplements may exert antioxidant properties. In detail, Shang *et al*[33] demonstrated that curcumin inhibited CRC cell proliferation and promoted apoptosis by down-regulating DJ-1 to regulate the activity of PTEN/PI3K/AKT pathway.

In the present study we found by western blotting that the dietetic formulation containg silymarin, boswellic acid and curcumin showed some mechanistic effects, which could explain their chemopreventive effect. Thoroughly, ER beta protein was well expressed in normal mucosa of enriched and standard diet, but this trend was more marked in animals assuming enriched diet. On the other hand, ER alpha was similarly expressed in normal and polypoid mucosa of standard and enriched diet group. The protein of cleaved form of caspase 3, moreover, showed in normal mucosa a stronger signal in enriched than in standard diet. Additionally, cyclin D1 was more expressed in standard than enriched diet group of polypoid tissue. In summary, we demonstrated that the dietetic formulation exerts a simultaneous anti-proliferative and pro-apoptotic effect. Moreover, the induction of ER beta increase may explain the silymarin main contribution to the process.

Another result of the present study is related to the effect of enriched diet on epithelial turnover. As expected, in both groups assuming control and supplemented diet, we found positive cells mainly located in the crypts of normal mucosa 24 h after BrdU injection. The step forward of positive cell migration to the villi was seen after 48 h. At this point, the highest labeled cell along the villus as marker of the percentage of covered axis was located much higher in the enriched than in the standard diet group. The mean timing of epithelial cell renewal in the small bowel has been assessed to be about 3 d[34-36]. This information clearly explains why the analysis at 48th h after BrdU injection was the most representative of cellular turnover state in our study. In detail, small bowel epitelial renewal process includes stem cell proliferation in the crypts and following migration towards the tip of the villi with a simultaneous differentiation and the final step of programmed death[37]. Therefore, our results

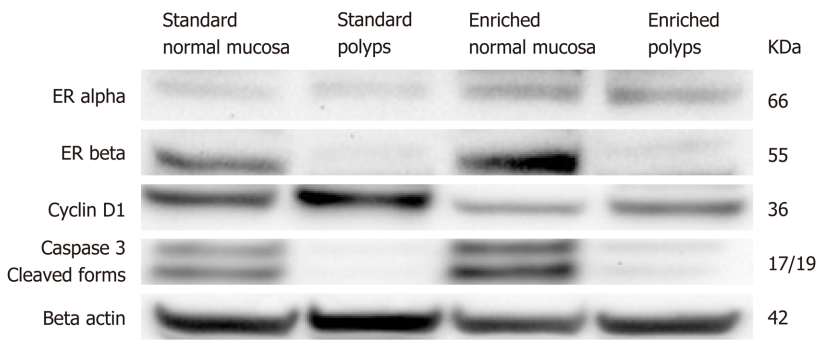

Figure 5 Representative western blotting showing estrogen receptor alpha, estrogen receptor beta, cyclin D1, caspase 3 (cleaved form) in Apc$^{Min/+}$ mice assuming standard or enriched diet evaluated in normal and polypoid tissues; beta actin was used as loading control (panel shows representative bolts of three different experiments performed with similar results).

suggest that epithelial turnover is faster in the enriched compared to the standard diet group. Consequently, enriched diet-related reduction of cellular half-life may decrease the time of exposure of proliferating cells to DNA mutations due to both intrinsic and extrinsic factors[38]. Finally, the enhancement of epithelial renewal induced by dietary supplementation agrees with the increase of apoptosis found in the same group of animals.

The results of the present study suggest an efficient synergic effect of combined diet based on substances of plant origin for the chemoprevention of intestinal genetic carcinogenesis. The synergic effect of our dietetic formulation is suggested even by the dosage used for the individual nutritional components, which turns out to be lower than that used for the single substances for "*in vivo*" experiments on the same animal model[13,39,40].

ARTICLE HIGHLIGHTS

Research background

Some natural products derived from plants may have anti-carcinogenetic effect.

Research motivation

We looked for possible candidate molecules to arrest the development of intestinal cancer in an animal model.

Research objectives

To test a combination of phytochemicals in a mouse model of genetic intestinal carcinogenesis.

Research methods

A combination of silymarin, boswellic acid and curcumin was given to forty adenomatous polyposis coli multiple intestinal neoplasia. Markers of proliferation/apoptosis were examined.

Research results

Compared to standard, enriched diet reduced the total number of polypoid lesions. In enriched diet group a reduction in polyp size was observed. Areas of low-grade dysplasia and intestinal carcinoma were significantly decreased in enriched diet group. Enriched diet showed a faster epithelial migration and an increased apoptosis in normal mucosa and low-grade dysplasia areas. Estrogen receptor beta protein was well expressed in normal mucosa of enriched and standard groups, with a more marked trend associated to the first one. Estrogen receptor alpha was similarly expressed in normal and polypoid mucosa of standard and enriched diet group. Cleaved caspase 3 showed in normal mucosa a stronger signal in enriched than in standard diet. Cyclin D1 was more expressed in standard than enriched diet group of both normal and polypoid tissue.

Research conclusions

Our results are suggestive of a chemo-preventive synergic effect of silymarin, boswellic acid and curcumin in inherited intestinal cancer.

Research perspectives

The dietetic formulation may be promising for patients with a genetic predisposition to develop intestinal polyps and carcinomas.

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ORIGINAL ARTICLE

Retrospective Cohort Study

Lifestyle factors and long-term survival of gastric cancer patients: A large bidirectional cohort study from China

Lu-Lu Zhao, Huang Huang, Yang Wang, Tong-Bo Wang, Hong Zhou, Fu-Hai Ma, Hu Ren, Peng-Hui Niu, Dong-Bing Zhao, Ying-Tai Chen

ORCID number: Lu-Lu Zhao (0000-0001-8344-0498); Huang Huang (0000-0003-0147-9650); Yang Wang (0000-0003-3836-7177); Tong-Bo Wang (0000-0003-2113-3681); Hong Zhou (0000-0002-4172-3411); Fu-Hai Ma (0000-0003-2437-6881); Hu Ren (0000-0001-7274-287X); Peng-Hui Niu (0000-0003-0114-1625); Dong-Bing Zhao (0000-0002-3011-5277); Ying-Tai Chen (0000-0003-4980-6315).

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Lu-Lu Zhao, Yang Wang, Tong-Bo Wang, Hong Zhou, Fu-Hai Ma, Hu Ren, Peng-Hui Niu, Dong-Bing Zhao, Ying-Tai Chen, National Cancer Center/National Clinical Research Center for Cancer/Cancer Hospital, Chinese Academy of Medical Sciences and Peking Union Medical College, Beijing 100021, China

Huang Huang, Department of Environmental Health Sciences, Yale University School of Public Health, New Haven, CT 06520, United States

Yang Wang, Department of General Surgery, Beijing Di Tan Hospital, Capital Medical University, Beijing 100015, China

Corresponding author: Ying-Tai Chen, MD, Professor, National Cancer Center/National Clinical Research Center for Cancer/Cancer Hospital, Chinese Academy of Medical Sciences and Peking Union Medical College, 17 Panjiayuan Nanli, Beijing 100021, China. yingtaichen@126.com

Abstract

BACKGROUND

Lifestyle factors such as body mass index (BMI), alcohol drinking, and cigarette smoking, are likely to impact the prognosis of gastric cancer, but the evidence has been inconsistent.

AIM

To investigate the association of lifestyle factors and long-term prognosis of gastric cancer patients in the China National Cancer Center.

METHODS

Patients with gastric cancer were identified from the China National Cancer Center Gastric Cancer Database 1998-2018. Survival analysis was performed *via* Kaplan-Meier estimates and Cox proportional hazards models.

RESULTS

In this study, we reviewed 18441 cases of gastric cancer. Individuals who were overweight or obese were associated with a positive smoking and drinking history ($P = 0.002$ and $P < 0.001$, respectively). Current smokers were more likely to be current alcohol drinkers (61.3% *vs* 10.1% *vs* 43.2% for current, never, and former smokers, respectively, $P < 0.001$). Multivariable results indicated that BMI at diagnosis had no significant effect on prognosis. In gastrectomy patients, factors independently associated with poor survival included older age (HR =

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1.20, 95%CI: 1.05-1.38, $P = 0.001$), any weight loss ($P < 0.001$), smoking history of more than 30 years (HR = 1.14, 95%CI: 1.04-1.24, $P = 0.004$), and increasing pTNM stage ($P < 0.001$).

CONCLUSION

In conclusion, our results contribute to a better understanding of lifestyle factors on the overall burden of gastric cancer and long-term prognosis. In these patients, weight loss (both in the 0 to 10% and > 10% groups) but not BMI at diagnosis was related to survival outcomes. With regard to other factors, smoking history of more than 30 years conferred a worse prognosis only in patients who underwent gastrectomy. Extensive efforts are needed to elucidate mechanisms targeting the complex effects of lifestyle factors.

Key words: Gastric cancer; Lifestyle factors; Prognosis; Cohort study; Body mass index; Cigarette smoking

Core tip: Lifestyle factors are likely to impact the prognosis of gastric cancer, but the evidence has been inconsistent. We conducted a single-center, large-scale bidirectional cohort study to investigate the association of lifestyle factors with long-term prognosis in patients with gastric cancer in China. Among these patients, weight loss but not body mass index at diagnosis, was related to survival outcomes. With regard to other factors, smoking history of more than 30 years conferred a worse prognosis only in patients who underwent gastrectomy.

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INTRODUCTION

Gastric cancer is the third leading cause of cancer-related mortality and the sixth most common cancer globally[1]. More than 70% of new cases occur in developing countries, and half of the world's total cases occur in Eastern Asia, mainly in China[2]. As the patient population grows, factors contributing to improved or adverse survival are becoming a focus of increasing interest.

Obesity defined as high body mass index (BMI) results from the expansion of white adipose tissue, commonly referred to as fat. To date, evidence for the association between BMI and prognosis in gastric cancer patients has been inconsistent. Some studies[3-8] have reported that being overweight was associated with improved long-term survival for gastric cancer patients who underwent gastrectomy, whereas other results[9-16] showed that BMI was not a prognostic factor. Three studies[17-19] even demonstrated poor survival of gastric cancer patients with higher BMI. However, some previous studies have used coarse categories, such as BMI < 25.0 and ≥ 25[8,15,20,21]. Furthermore, these results may not apply to the Chinese population due to the different BMI categorization criteria for Asians; therefore, the association between BMI and prognosis is unclear in China.

Similarly, the prognostic effects of alcohol drinking and smoking status at diagnosis of gastric cancer are also contradictory, although cigarette smoking is known to be associated with stomach cancer risk[22,23]. Alcohol drinking at diagnosis was reported to decrease survival in patients with gastric cancer in some studies[24,25], while several other studies[26,27] did not confirm this finding. Some studies[28-31] have shown a positive association between smoking and overall survival (OS) in gastric cancer, while other studies[24,26,32] have found that smoking status was not statistically related to prognosis. There is a deficiency in most published studies, especially prospective studies, which have not adjusted for potentially significant covariates such as gastrectomy.

Thus, we conducted a single-center, large-scale bidirectional cohort study in the China National Cancer Center to investigate the three major lifestyle factors

mentioned above - BMI, alcohol drinking, and smoking - and attempted to clarify the association of these factors with the OS of patients with gastric cancer.

MATERIALS AND METHODS

Population

All patient records were abstracted from the China National Cancer Center Gastric Cancer Database. The China National Cancer Center Gastric Cancer Database is a clinical gastric cancer database based on a huge bidirectional cohort, which was sourced from the China National Cancer Center, a single but large-volume institution with patients from all over China from 1998 to 2018. After the diagnosis of gastric cancer was confirmed by pathology, 18441 patients were included in this study. The AJCC 8th edition was used for TNM staging. The median follow-up of gastric cancer patients was 62.7 ± 3.5 mo until December 2018. 1818 patients were lost during the follow-up period with a loss rate of 9.86%. The geographical locations of these gastric cancer patients are shown in Figure 1.

After the analyses of all included gastric cancer patients irrespective of surgery, we further analyzed three detailed subgroups: Consisting of gastrectomy patients, no surgery patients, and only gastric cancer patients with curative gastrectomy. Gastrectomy was defined as surgery with or without D2 lymphadenectomy, while curative gastrectomy was defined as patients who underwent surgery with D2 lymphadenectomy and had negative margins.

Statistical analysis

BMI at diagnosis was calculated as weight at diagnosis (kg) of gastric cancer divided by the square of height (m^2). For the analysis of BMI according to the Asian criteria, patients were stratified according to the following BMI categories: Underweight (< 18.5 kg/m^2), healthy weight (≥ 18.5 to < 23 kg/m^2), overweight (≥ 23 to < 27.5.0 kg/m^2) and obese (≥ 27.5 kg/m^2).

Other lifestyle variables related to cigarette smoking included smoking status (never, current, and former smokers), time since quitting smoking (1-9 and ≥ 10 years), number of cigarettes per day (≤ 20, 21-39, and ≥ 40), and duration of smoking (1-29 and ≥ 30 years). For alcohol drinking, drinking status (never, current, and former drinkers) and amount of alcohol consumed per day (light drinkers, < 15.0 g; moderate drinkers, ≥ 15.0 g to < 53.5 g; and heavy drinkers, ≥ 53.5 g) was included. Subjects who quit smoking or drinking within one year before the present admission were regarded as current smokers or drinkers, respectively.

Categorical variables were compared using the χ^2 test, and continuous variables were analyzed by the Student's *t*-test. Survival curves were plotted for total patients, no-surgery, gastrectomy and only curative gastrectomy groups, respectively, using the Kaplan-Meier method and compared using the log-rank test. Hazard ratios (HRs) and 95% confidence intervals (CIs) were used to estimate the risk of death by employing the multivariate Cox proportional hazards models with adjustment for gender, age, pTNM stage, adjuvant therapies, and gastrectomy. The group with healthy weight (≥ 18.5 to < 23 kg/m^2) was the reference group. A two-sided *P* value less than 0.05 was considered statistically significant. All the statistical analyses were performed using SAS software v9.4 (SAS Institute, Inc., Cary, NC, USA).

RESULTS

Clinical characteristics

In this study, we included 18441 gastric cancer patients diagnosed between 1998 and 2018 (Table 1). Of these subjects, more than half were males (13533, 73.4%) with a median age of 58.5 years. Of the total subjects, 84.5% experienced weight loss at diagnosis when compared with their usual weight, 40.5% had a smoking history, and 33.2% had a drinking history. During the follow-up period, 4717 (25.6%) deaths were recorded in our database.

Compared to those with a healthy BMI at diagnosis, overweight and obese patients had more weight loss (0% to 10%) than patients with healthy weight (81.6% *vs* 81.9% *vs* 75.9%, $P < 0.001$). Underweight patients were more likely to be diagnosed at a later stage than other groups (pTNM IV, 17.0% *vs* 13.7% *vs* 10.6% *vs* 9.6%, $P < 0.001$).

Current drinkers tended to be at a later pTNM stage (stage IV, 44.2% *vs* 12.6% *vs* 11.9%, $P < 0.001$) with a proximal location in the stomach (47.0% *vs* 33.4% *vs* 34.5%) than never or former drinkers. In terms of cigarette smoking, former smokers were older (aged ≥ 66 years, 37.9% *vs* 30.0 *vs* 23.0%, $P < 0.001$) than never or current

Table 1 Comparison of 18441 gastric cancer patients' characteristics

Characteristics, *n* (%)	No. of Patients	BMI at diagnosis				*P* value	Drinkers			*P* value	Smokers			*P* value
		< 18.5	18.5-22.9	23-27.4	⩾ 27.5		Never	Yes			Never	Yes		
								Ex-drinkers	Current-drinkers			Ex-smokers	Current-smokers	
No. of patients	18441 (100)	1193 (6.5)	6872 (37.3)	7310 (39.6)	2196 (11.9)	-	11578 (62.8)	922 (5.0)	5202 (28.2)	-	10244 (55.6)	2315 (12.6)	5156 (28.0)	-
Gender														
Male	13533 (73.4)	758 (63.5)	4893 (71.2)	5682 (77.7)	1667 (75.9)		6992 (62.0)	874 (95.8)	4948 (96.6)		5903 (57.6)	2220 (95.5)	4974 (96.5)	
Female	4819 (26.1)	435 (36.5)	1979 (28.8)	1628 (22.3)	529 (24.1)	< 0.001	4286 (28.0)	38 (4.2)	174 (3.4)	< 0.001	4341 (42.4)	95 (4.1)	182 (3.5)	< 0.001
Age at diagnosis (yr)														
Mean (SD)	58.5	56.7	58.2	59.0	58.5	< 0.001	58.6	60.1	58.2	< 0.001	58.0	62.1	58.1	< 0.001
Younger (≤ 35)	653 (3.5)	127 (10.7)	289 (4.2)	152 (2.1)	61 (2.8)		530 (4.6)	13 (1.4)	91 (1.8)		520 (5.1)	22 (1.0)	93 (1.8)	
Middle-aged (36-65)	12119 (65.7)	682 (57.2)	4604 (67.0)	5063 (69.3)	1524 (69.4)		7373 (63.7)	657 (71.3)	3887 (74.7)		6640 (64.8)	1415 (61.1)	3872 (75.1)	
Older (≥ 66)	5235 (28.4)	383 (32.1)	1976 (28.8)	2090 (28.6)	609 (27.7)	< 0.001	3667 (31.7)	252 (27.3)	1220 (23.5)	< 0.001	3077 (30.0)	877 (37.9)	1188 (23.0)	< 0.001
Weight loss as % of usual weight														
None	2858 (15.5)	164 (13.8)	821 (12.0)	813 (11.1)	276 (12.6)		1629 (14.1)	53 (5.8)	642 (12.3)		1437 (14.0)	261 (11.3)	625 (12.1)	
0-10	13814 (74.9)	766 (64.2)	5217 (75.9)	5964 (81.6)	1798 (81.9)		8830 (76.3)	766 (83.1)	4030 (77.5)		7825 (76.4)	1835 (79.3)	3980 (77.2)	
≥ 10	1769 (9.6)	263 (22.1)	834 (12.1)	533 (7.3)	122 (5.6)	< 0.001	1119 (9.7)	103 (11.2)	530 (10.2)	< 0.001	982 (9.6)	219 (9.5)	551 (10.7)	0.0001
Smoking status														
Never smokers	10244 (55.6)	737 (61.8)	3895 (56.7)	4084 (55.9)	1252 (57.0)		9.081 (78.4)	124 (13.5)	1037 (19.9)		-	-	-	
Smokers	7471 (40.5)	435 (36.5)	2875 (41.8)	3128 (42.8)	922 (42.0)	0.002	2488 (21.5)	795 (86.2)	4159 (80.0)	< 0.001	-	-	-	-
Current smokers	5156 (28.0)	339 (28.4)	2076 (30.2)	2059 (28.2)	604 (27.5)		1733 (15.0)	250 (27.1)	3158 (60.7)		-	-	-	
Ex-smokers	2315 (12.6)	96 (8.1)	799 (11.6)	1069 (14.6)	318 (14.5)	< 0.001	755 (6.5)	545 (59.1)	1001 (19.2)	< 0.001	-	-	-	-
Time since quitting smoking														
Short-term (1-9 yr)	1505 (8.2)	75 (6.3)	535 (7.8)	678 (9.3)	197 (9.0)		474 (4.1)	422 (45.8)	597 (11.5)		0 (0.0)	1505 (65.0)	0 (0.0)	
Long-term (≥ 10 yr)	810 (4.4)	21 (1.8)	264 (3.8)	391 (5.4)	121 (5.5)	< 0.001	281 (2.4)	123 (13.3)	404 (7.8)	< 0.001	0 (0.0)	810 (35.0)	0 (0.0)	< 0.001
No. of cigarettes (per day)														
≤ 20 (1 pack)	5568 (30.2)	338 (28.3)	2190 (31.9)	2318 (31.7)	642 (29.2)		1919 (16.6)	602 (65.3)	3030 (58.3)		0 (0.0)	1770 (76.5)	3798 (73.7)	
21-39	457 (2.5)	22 (1.8)	164 (2.4)	198 (2.7)	62 (2.8)		144 (1.2)	36 (3.9)	276 (5.3)		0 (0.0)	115 (5.0)	342 (6.6)	
≥ 40 (≥ 2 packs)	708 (3.8)	32 (2.7)	250 (3.6)	302 (4.1)	113 (5.2)	< 0.001	228 (2.0)	76 (8.2)	400 (7.7)	< 0.001	0 (0.0)	208 (9.0)	500 (9.7)	< 0.001
Duration of smoking (yr)														
1-29	2805 (15.2)	131 (11.0)	999 (14.5)	1258 (17.2)	383 (17.4)		927 (8.0)	372 (40.4)	1497 (28.8)		0 (0.0)	1230 (53.1)	1575 (30.6)	
≥ 30	4037 (21.9)	267 (22.4)	1652 (24.0)	1604 (21.9)	449 (20.5)	< 0.001	1411 (12.2)	359 (38.9)	2252 (43.3)	< 0.001	0 (0.0)	940 (40.6)	3097 (60.1)	< 0.001
Alcohol consumption														

Never drinkers	11578 (62.8)	862 (72.3)	4520 (65.8)	4549 (62.2)	1347 (61.3)		-	-	-		9081 (88.7)	755 (32.6)	1733 (33.6)	
Drinkers	6124 (33.2)	311 (26.1)	2247 (32.7)	2655 (36.3)	821 (37.4)	< 0.001	-	-	-	-	1161 (11.3)	1546 (66.8)	3408 (66.1)	< 0.001
Current drinkers	5202 (28.2)	272 (22.8)	1909 (27.8)	2249 (30.8)	692 (31.5)		-	-	-		1037 (10.1)	1001 (43.2)	3158 (61.3)	
Ex-drinkers	922 (5.0)	39 (3.3)	338 (4.9)	406 (5.6)	129 (5.9)	< 0.001	-	-	-	-	124 (1.2)	545 (23.5)	250 (4.9)	< 0.001
Amount of alcohol consumption														
Light drinkers (< 15.6 g/d)	102 (0.6)	3 (0.3)	40 (0.6)	48 (0.7)	11 (0.5)		0 (0.0)	15 (1.6)	87 (1.7)		15 (0.2)	42 (1.8)	45 (0.9)	
Moderate drinkers (≥ 15.6 g/d to < 53.5 g/d)	660 (3.6)	30 (2.5)	246 (3.6)	311 (4.3)	62 (2.8)		0 (0.0)	77 (8.4)	583 (11.2)		127 (1.2)	201 (8.7)	329 (6.4)	
Heavy drinkers (≥ 53.5 g/d)	3480 (18.9)	167 (14.0)	1277 (18.6)	1488 (20.4)	501 (22.8)	< 0.001	0 (0.0)	593 (64.3)	2887 (55.5)	< 0.001	620 (6.1)	894 (38.6)	1962 (38.1)	< 0.001
H. pylori														
No	1506 (8.2)	67 (5.6)	561 (8.2)	668 (9.1)	193 (8.8)		837 (7.2)	152 (16.5)	489 (9.4)		734 (7.2)	272 (11.8)	474 (9.2)	
Yes	1167 (6.3)	56 (4.7)	457 (6.7)	503 (6.9)	134 (6.1)	< 0.001	692 (6.0)	74 (8.0)	386 (7.4)	< 0.001	590 (5.8)	169 (7.3)	393 (7.6)	< 0.001
Histologic type														
Well	351 (1.9)	27 (2.3)	107 (1.6)	165 (2.3)	48 (2.2)		214 (1.9)	24 (2.6)	107 (2.1)		185 (1.8)	45 (1.9)	115 (2.2)	
Moderately	5514 (29.9)	257 (21.5)	2004 (29.2)	2390 (32.7)	758 (34.5)		2300 (28.5)	288 (31.2)	1829 (36.2)		2789 (27.2)	844 (36.5)	1787 (34.7)	
Poorly	5824 (31.6)	374 (31.4)	2236 (32.5)	2398 (32.8)	730 (33.2)		3922 (33.9)	233 (25.3)	1567 (30.1)		3643 (35.6)	612 (26.4)	1475 (28.6)	
Undifferentiated, anaplastic	3 (0.0)	0 (0.0)	1 (0.0)	2 (0.0)	0 (0.0)	< 0.001	1 (0.0)	0 (0.0)	2 (0.0)	< 0.001	3 (0.0)	0 (0.0)	0 (0.0)	< 0.001
pathologicT														
p0	61 (0.3)	2 (0.2)	22 (0.3)	27 (0.4)	10 (0.5)		41 (0.4)	3 (0.3)	17 (0.3)		35 (0.3)	7 (0.3)	19 (0.4)	
p1	2778 (15.1)	131 (11.0)	966 (14.1)	1242 (17.0)	412 (18.8)		1810 (15.6)	206 (22.3)	721 (13.9)		1663 (16.2)	347 (15.0)	729 (14.1)	
p2	1524 (8.3)	83 (7.0)	528 (7.7)	647 (8.9)	243 (11.1)		955 (8.3)	75 (8.1)	462 (8.9)		834 (8.1)	192 (8.3)	467 (9.1)	
p3	3340 (18.1)	169 (14.2)	1233 (17.9)	1459 (20.0)	443 (20.2)		2051 (17.7)	176 (19.1)	1093 (21.0)		1702 (16.6)	497 (21.5)	1120 (21.7)	
p4	6545 (35.5)	465 (39.0)	2605 (37.9)	2640 (36.1)	708 (32.2)	< 0.001	4308 (37.2)	266 (28.9)	1882 (36.2)	< 0.001	3888 (38.0)	812 (35.1)	1765 (34.2)	< 0.001
pathologicN														
p0	4964 (26.9)	274 (23.0)	1758 (25.6)	2184 (29.9)	685 (31.2)		3149 (27.2)	304 (33.0)	1436 (27.6)		2785 (27.2)	670 (28.9)	1434 (27.8)	
p1	2426 (13.2)	161 (13.5)	905 (13.2)	1008 (13.8)	313 (14.3)		1577 (13.6)	116 (12.6)	709 (13.6)		1357 (13.3)	301 (13.0)	744 (14.4)	
p2	2549 (13.8)	170 (14.3)	1018 (14.8)	997 (13.6)	327 (14.9)		1639 (14.2)	116 (12.6)	758 (14.6)		1448 (14.1)	316 (13.7)	753 (14.6)	
p3	4045 (21.9)	226 (18.9)	1570 (22.9)	1717 (23.5)	464 (21.1)	< 0.001	2633 (22.7)	175 (19.0)	1193 (22.9)	0.007	2384 (23.3)	545 (23.5)	1079 (20.9)	0.018
pathologicM														
p0	14785 (80.2)	899 (75.4)	5563 (81.0)	6176 (84.5)	1889 (86.0)		9438 (81.5)	770 (83.5)	4373 (84.1)		8348 (81.5)	1971 (85.1)	4273 (82.9)	
p1	2233 (12.1)	203 (17.0)	943 (13.7)	776 (10.6)	211 (9.6)	< 0.001	1460 (12.6)	110 (11.9)	620 (11.9)	< 0.001	1332 (13.0)	231 (10.0)	628 (12.2)	< 0.001
pTNM														
I	3340 (18.1)	157 (13.2)	1155 (16.8)	1490 (20.4)	500 (22.8)		2139 (18.5)	221 (24.0)	16 (0.3)		1928 (18.8)	435 (18.8)	922 (17.9)	
II	2334 (12.7)	137 (11.5)	843 (12.3)	1012 (13.8)	320 (14.6)		1443 (12.5)	147 (15.9)	922 (17.7)		1210 (11.8)	317 (13.7)	782 (15.2)	

Ⅲ	7712 (41.8)	481 (40.3)	3005 (43.7)	3180 (43.5)	917 (41.8)		5009 (43.3)	312 (33.8)	724 (13.9)		4456 (43.5)	1012 (43.7)	2165 (42.0)	
Ⅳ	2233 (12.1)	203 (17.0)	943 (13.7)	776 (10.6)	211 (9.6)	< 0.001	1460 (12.6)	110 (11.9)	2301 (44.2)	< 0.001	1332 (13.0)	231 (10.0)	628 (12.2)	< 0.001
Lauren classification														
Intestinal	2838 (15.4)	126 (10.6)	988 (14.4)	1304 (17.8)	388 (17.7)		1549 (13.4)	250 (27.1)	995 (19.1)		1279 (12.5)	554 (23.9)	962 (18.7)	
Diffuse	2610 (14.2)	154 (12.9)	1017 (14.8)	1095 (15.0)	318 (14.5)		1660 (14.3)	181 (19.6)	726 (14.0)		1547 (15.1)	342 (14.8)	680 (13.2)	
Mixed	1799 (9.8)	68 (5.7)	646 (9.4)	795 (10.9)	271 (12.3)	< 0.001	1010 (8.7)	146 (15.8)	621 (11.9)	< 0.001	897 (8.8)	295 (12.7)	583 (11.3)	< 0.001
Linitis plastica														
No	17321 (93.9)	1132 (94.9)	6611 (96.2)	7059 (96.6)	2115 (96.3)		11149 (96.3)	892 (96.8)	5011 (96.3)		9844 (96.1)	2244 (96.9)	4978 (96.6)	
Yes	181 (1.0)	23 (1.9)	80 (1.2)	59 (0.8)	16 (0.7)	0.005	125 (1.1)	9 (1.0)	44 (0.9)	0.55	117 (1.1)	18 (0.8)	43 (0.8)	0.17
Location														
Proximal GC	6704 (36.4)	386 (32.4)	2409 (35.1)	2856 (39.1)	913 (41.6)		3870 (33.4)	318 (34.5)	2447 (47.0)		3147 (30.7)	1193 (51.5)	2298 (44.6)	
Distal GC	9.992 (54.2)	704 (59.0)	3966 (57.7)	3948 (54.0)	1141 (52.0)		6884 (59.5)	517 (56.1)	2417 (46.5)		6354 (62.0)	933 (40.3)	2545 (49.4)	
Total GC	894 (4.9)	56 (4.7)	353 (5.1)	355 (4.9)	104 (4.7)	< 0.001	558 (4.8)	55 (6.0)	263 (5.1)	< 0.001	507 (5.0)	127 (5.5)	240 (4.7)	< 0.001
Type of gastrectomy														
Gastrectomy	15204 (82.5)	930 (78.0)	5623 (81.8)	6248 (85.5)	1885 (85.8)		9662 (83.5)	773 (83.8)	4278 (82.2)		8533 (83.3)	1961 (84.7)	4230 (82.0)	
No surgery	3146 (17.1)	263 (22.1)	1246 (18.1)	1061 (14.5)	311 (14.2)	< 0.001	1916 (16.6)	148 (16.1)	924 (17.8)	0.019	1711 (16.7)	353 (15.3)	926 (18.0)	0.005
Surgical margin														
Negative	13370 (72.5)	791 (66.3)	5012 (72.9)	5650 (77.3)	1730 (78.8)		8623 (74.5)	684 (74.2)	3899 (75.0)		7622 (74.4)	1755 (75.8)	3841 (74.5)	
Positive	443 (2.4)	36 (3.0)	163 (2.4)	195 (2.7)	41 (1.9)	< 0.001	297 (2.6)	21 (2.3)	120 (2.3)	0.83	263 (2.6)	67 (2.9)	108 (2.1)	0.067
Additional therapy														
No neo/adjuvant	1564 (8.5)	82 (6.9)	520 (7.6)	701 (9.6)	241 (11.0)		925 (8.0)	128 (13.9)	484 (9.3)		840 (8.2)	242 (10.5)	456 (8.8)	
Neoadjuvant	354 (1.9)	25 (2.1)	158 (2.3)	134 (1.8)	33 (1.5)		192 (1.7)	43 (4.7)	113 (2.2)		171 (1.7)	81 (3.5)	97 (1.9)	
Adjuvant	4713 (25.6)	244 (20.5)	1743 (25.4)	2044 (28.0)	621 (28.3)		3057 (26.4)	198 (21.5)	1383 (26.6)		2782 (27.2)	503 (21.7)	1354 (26.3)	
Neo + adjuvant	613 (3.3)	37 (3.1)	222 (3.2)	272 (3.7)	77 (3.5)	< 0.001	353 (3.1)	63 (6.8)	190 (3.7)	< 0.001	319 (3.1)	136 (5.9)	154 (3.0)	< 0.001

H. pylori: *Helicobacter pylori*; GC: Gastric cancer.

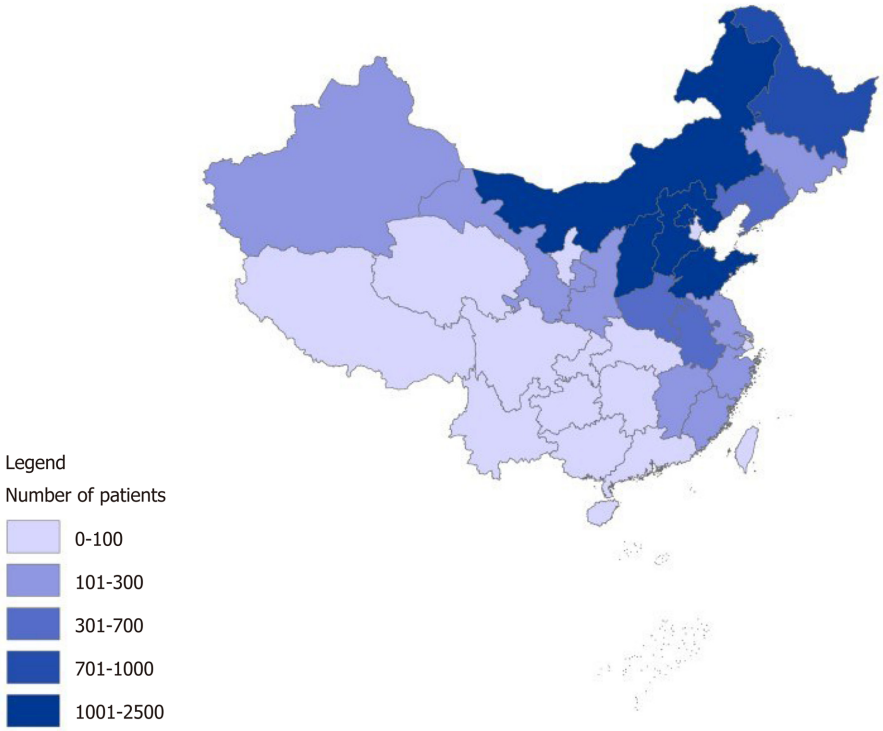

Figure 1 The geographical locations of gastric cancer patients in the China National Cancer Center Gastric Cancer Database, 1998–2018.

smokers. Current smokers were also more likely to be current alcohol drinkers (61.3% *vs* 10.1% *vs* 43.2%, $P < 0.001$) as compared to never or former smokers.

Survival outcomes in univariate analysis

Figure 2 shows the Kaplan-Meier curves for OS of different BMIs at diagnosis in total gastric cancer patients, no surgery, gastrectomy, and only curative gastrectomy patients. The median OS of total patients for each BMI category was as follows: underweight, 12.5 years; healthy weight, 13.0 years; overweight, 13.6 years; and obese, 13.6 years ($P < 0.001$). For no surgery patients, the results were as follows: Underweight, 5.7 years; healthy weight, 5.0 years; overweight, 5.9 years; and obese, 6.1 years ($P = 0.26$). For the gastrectomy group, survival status was as follows: Underweight, 14.0 years; healthy weight, 14.0 years; overweight, 14.2 years; and obese, 14.4 years ($P < 0.001$). For the only curative gastrectomy group, survival status was as follows: Underweight, 14.1 years; healthy weight, 14.3 years; overweight, 14.3 years; and obese, 14.5 years ($P = 0.002$). The 3- and 5-year OS for different gastrectomy groups are presented in Supplementary Table 1.

Kaplan-Meier survival comparisons (Supplementary Figure 1 and Supplementary Table 2) showed no association between smoking status and the OS of gastric cancer patients, even stratified in the only curative gastrectomy group. With regard to alcohol drinking, there was a significant difference between drinking status among no surgery patients ($P = 0.009$), but not in the other groups (Supplementary Figure 2 and Supplementary Table 3). Survival of those with both smoking and alcohol drinking status was also analyzed (Figure 3 and Supplementary Table 4), and significant differences were observed in the total and gastrectomy groups.

Univariate analysis (Supplementary Table 5) showed that overweight and obese patients had better survival than those with normal weight (HR = 0.92, 95%CI: 0.88-0.96, $P < 0.001$ and HR = 0.89, 95%CI: 0.83-0.96, $P = 0.001$, respectively), while the discrepancy in survival rate was attenuated in the gastrectomy group (HR = 0.95, 95%CI: 0.90-1.00, $P = 0.04$ and HR = 0.91, 95%CI: 0.84-0.99, $P = 0.03$, respectively). However, for curative gastrectomy patients, only the overweight group showed an improved survival (HR = 0.94, 95%CI: 0.89-1.00, $P = 0.049$). Weight loss of > 10% of the usual weight was a significant risk factor for mortality in the four groups ($P < 0.001$). As shown in Table 2, smoking history of more than 30 years was a prognostic factor of poor survival for total patients and gastrectomy patients ($P < 0.001$). However, drinking status, even heavy drinking, was not a negative prognostic indicator.

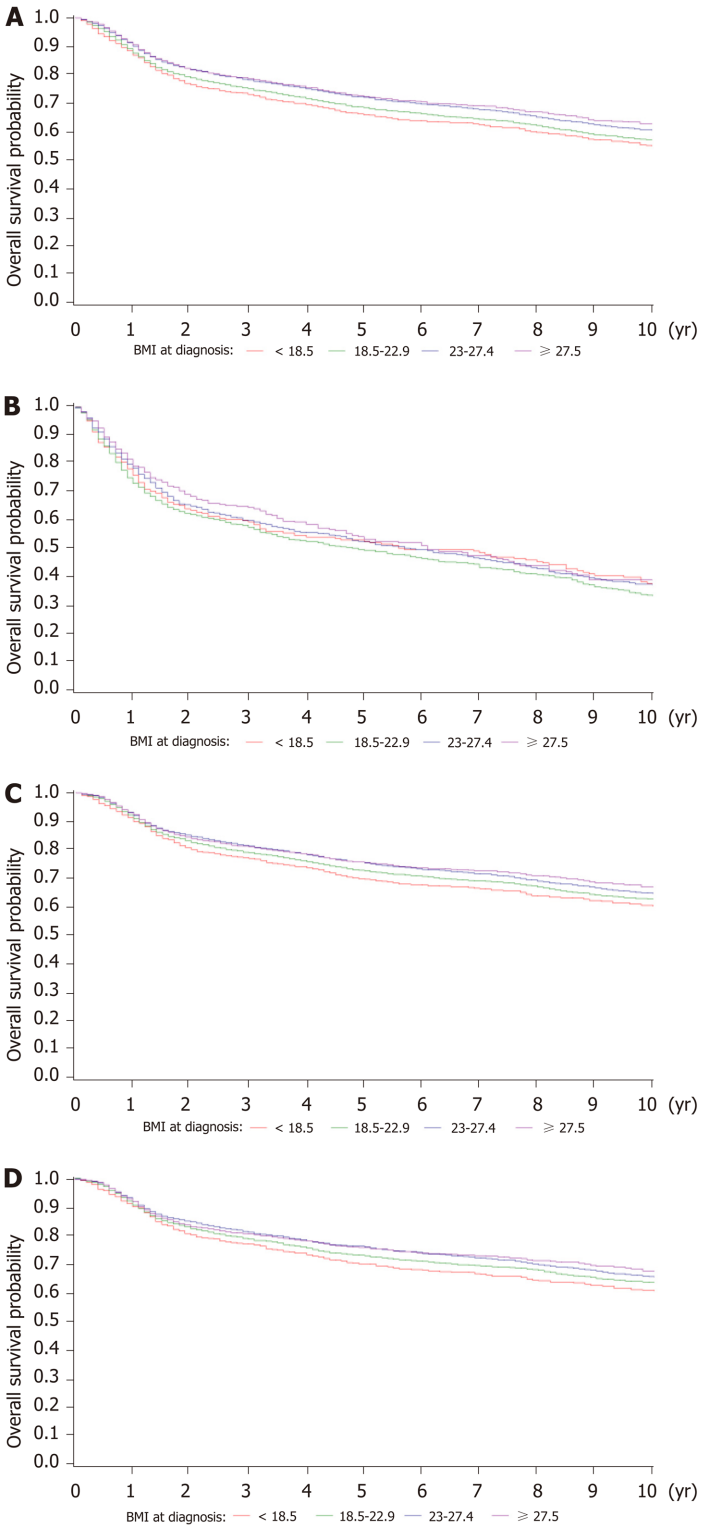

Figure 2 Kaplan-Meier survival curves for overall survival of patients with a different body mass index at diagnosis. A: Total gastric cancer patients; B: No surgery patients; C: Gastrectomy patients; D: Only curative gastrectomy patients. BMI: Body mass index.

Survival outcomes in multivariable analysis

The J-shaped relationship between BMI at diagnosis and survival in patients after multivariate-adjusted analysis of different gastrectomy groups is shown in Figure 4. The HR for total gastric cancer patients was the lowest (HR = 0.90; 95%CI: 0.86-0.94) at a BMI of 25.96, followed by a BMI of 28.20 for no surgery patients, 25.47 for gastrectomy patients, and 25.50 for only curative gastrectomy patients. However, the multivariable results (Table 2) indicated that BMI at diagnosis did not affect prognosis.

Table 2 Multivariate survival analysis

Prognostic factors	Total (*n* = 18322)[1]				No surgery (*n* = 3142)[2]				Gastrectomy (*n* = 15180)[3]				Only curative gastrectomy (*n* = 13695)[4]			
	HR	95%CI		*P* value	HR	95%CI		*P* value	HR	95%CI		*P* value	HR	95%CI		*P* value
		Lower	Upper			Lower	Upper			Lower	Upper			Lower	Upper	
Gender																
Male	1.00				1.00				1.00				1.00			
Female	1.05	1.00	1.11	0.06	1.07	0.97	1.19	0.19	1.05	0.99	1.11	0.13	1.04	0.97	1.11	0.25
Age at diagnosis (yr)																
Younger (≤ 35)	1.00				1.00				1.00				1.00			
Middle-aged (36-65)	1.07	0.95	1.19	0.26	0.94	0.76	1.15	0.54	1.14	0.99	1.30	0.06	1.15	1.00	1.33	0.05
Older (≥ 66)	1.10	0.98	1.23	0.12	0.89	0.72	1.11	0.30	1.20	1.05	1.38	0.01	1.21	1.05	1.40	0.01
BMI (kg/m^2) at diagnosis																
< 18.5	1.00	0.92	1.09	0.99	1.01	0.87	1.18	0.90	1.00	0.90	1.10	0.93	1.02	0.92	1.14	0.72
18.5-22.9	1.00				1.00				1.00				1.00			
23-27.4	0.97	0.93	1.02	0.22	0.92	0.84	1.02	0.11	0.99	0.94	1.05	0.73	0.99	0.94	1.05	0.74
≥ 27.5	0.99	0.92	1.06	0.72	0.95	0.82	1.10	0.48	1.01	0.93	1.10	0.85	1.02	0.93	1.11	0.74
Weight loss as % of usual weight																
None	1.00				1.00				1.00				1.00			
0-10	1.14	1.07	1.20	< 0.001	1.16	1.04	1.30	0.01	1.13	1.05	1.20	0	1.11	1.04	1.19	0
≥ 10	1.24	1.15	1.34	< 0.001	1.22	1.06	1.40	0.01	1.25	1.14	1.38	< 0.001	1.22	1.10	1.34	0
Smoking status																
Never smokers	1.00				1.00				1.00				1.00			
Current smokers	1.02	0.94	1.10	0.66	1.05	0.89	1.23	0.57	1.01	0.92	1.11	0.78	1.03	0.93	1.14	0.58
Ex-smokers	0.93	0.84	1.03	0.17	0.95	0.76	1.19	0.66	0.93	0.82	1.04	0.20	0.91	0.81	1.03	0.15
Time since quitting smoking																
Short-term (1–9 yr)	1.00				1.00				1.00				1.00			
Long-term (≥ 10 yr)	1.04	0.91	1.19	0.55	1.02	0.78	1.34	0.87	1.05	0.90	1.22	0.51	1.07	0.91	1.25	0.42
No. of cigarettes (per day)																
≤ 20 (1 pack)	1.00				1.00				1.00				1.00			
21-39	1.07	0.93	1.22	0.36	1.29	1.00	1.66	0.05	0.98	0.84	1.16	0.84	0.99	0.83	1.17	0.87
≥ 40 (≥ 2 packs)	1.03	0.92	1.16	0.59	0.91	0.72	1.15	0.43	1.09	0.95	1.24	0.22	1.08	0.94	1.24	0.30
Duration of smoking (yr)																
1-29	1.00				1.00				1.00				1.00			
≥ 30	1.08	1.00	1.16	0.05	0.94	0.81	1.09	0.40	1.14	1.04	1.24	0	1.13	1.04	1.24	0.01
Alcohol consumption																
Never drinkers	1.00				1.00				1.00				1.00			
Current drinkers	0.82	0.58	1.18	0.29	1.54	0.71	3.37	0.28	0.75	0.50	1.12	0.16	0.88	0.59	1.32	0.54
Ex-drinkers	0.86	0.59	1.24	0.42	1.33	0.63	2.80	0.46	0.75	0.49	1.16	0.19	0.86	0.56	1.32	0.48
Amount of alcohol consumption																
Light drinkers (< 15.6 g/d)	1.00				1.00				1.00				1.00			
Moderate drinkers (≥ 15.6 g/d to < 53.5 g/d)	1.25	0.86	1.81	0.25	0.97	0.45	2.13	0.95	1.27	0.83	1.94	0.28	1.07	0.70	1.64	0.75
Heavy drinkers (≥ 53.5 g/d)	1.25	0.88	1.79	0.21	0.78	0.37	1.66	0.52	1.37	0.91	2.06	0.13	1.15	0.77	1.73	0.50
pTNM																
I	1.00				1.00				1.00				1.00			
II	1.48	1.33	1.66	< 0.001	3.65	0.45	29.50	0.23	1.48	1.32	1.65	< 0.001	1.38	1.23	1.56	< 0.001
III	2.31	2.12	2.52	< 0.001	4.07	0.56	29.79	0.17	2.29	2.10	2.49	< 0.001	2.19	2.00	2.39	< 0.001
IV	3.19	2.86	3.55	< 0.001	4.41	0.62	31.18	0.14	3.42	3.04	3.86	< 0.001	3.16	2.79	3.58	< 0.001
Type of gastrectomy																
Gastrectomy	1.00				-				-				-			
No surgery	1.51	1.40	1.63	< 0.001	-	-	-	-	-	-	-	-	-	-	-	-

[1]Adjust for gender, age, pTNM stage, adjuvant therapies, gastrectomy.
[2]Adjust for gender, age, pTNM stage.
[3]Adjust for gender, age, pTNM stage, adjuvant therapies.
[4]Adjust for gender, age, pTNM stage, adjuvant therapies.

For total gastric cancer patients, any weight loss ($P < 0.001$), advanced pTNM stage ($P < 0.001$), and gastrectomy status (HR = 1.51, 95%CI: 1.40-1.63, $P < 0.001$) were independently associated with mortality. In the surgery group, factors independently associated with poor survival included older age (≥ 66 years) (HR = 1.20, 95%CI: 1.05-1.38, $P = 0.001$), any weight loss (0 to 10%, HR = 1.13, 95%CI: 1.05-1.20, $P < 0.001$ and > 10%, HR = 1.25, 95%CI: 1.14-1.38, $P < 0.001$), smoking history more than 30 years (HR = 1.14, 95%CI: 1.04-1.24, $P = 0.004$), and advanced pTNM stage ($P < 0.001$). An additional mortality-related factor for the only curative gastrectomy group was middle age (36-65 years) (HR = 1.15, 95%CI: 1.00-1.33, $P = 0.047$).

DISCUSSION

This retrospective study investigated how lifestyle factors in gastric cancer patients were associated with prognosis, including BMI at diagnosis, smoking, and drinking status. A primary finding of this study was that BMI at diagnosis was not independently associated with long-term survival after adjusting for risk factors, although weight loss of both 0 to 10% and > 10% of their usual weight were adverse prognostic factors in gastric cancer patients.

We included 18441 patients in this study, a larger cohort than most previous studies, and followed them for more than ten years. Four subgroups, total patients, no surgery, gastrectomy, and only curative gastrectomy, were analyzed to eliminate the effect of gastrectomy which may affect the survival outcomes. After controlling confounding variables, it was found that percentage weight loss of the usual weight was associated with an increased risk of mortality in gastric cancer patients compared to those without weight loss. It is possible that human adipose tissue may have the function of preserving nutrients and increase the chance of survival when the human body suffers stress, such as anti-cancer treatment[6,33,34]. Weight loss may be due to gastric cancer-induced dysphagia, odynophagia, anorexia or cancer cachexia, thus it has a negative effect on survival[35].

Weight management strategies for gastric cancer patients have attracted a lot of attention; however, the relationship between obesity and cancer prognosis is complex. Many previous retrospective studies have also evaluated the association between BMI and the prognosis of gastric cancer in the general population; however, most of these studies only analyzed patients with gastrectomy. A recent study[6] from Korea, which included a cohort of 7765 patients in a single institution, noted that patients who were overweight or mild-to-moderately obese (BMI 23 to < 30 kg/m^2) preoperatively had better OS than those with healthy weights. This result was similar to that in another study carried out in Japan, which included 7925 patients[8]. The reasons for the above outcomes may be due to the following: Primarily, it is more likely that obese patients who have suffered gastric cancer have less aggressive tumors[36,37], which is consistent with the features in our study, *i.e.* the occurrence of pTNM IV tumors was more common in underweight patients. Furthermore, patients who were overweight or obese could achieve an ideal BMI after gastrectomy, thus acquiring better long-term prognosis[37]. Also, a prospective study[38] involving 1033 patients showed that among patients of 60 and older that lower BMI was associated with all-cause death, displaying a J-shaped pattern (HR= 2.28 for BMI < 18.5; HR = 1.61 for 25 *vs* 23.0 to < 25.0 kg/m^2). Conversely, higher BMI was also reported to promote the peritoneal dissemination of gastric cancer and had a worse survival rate[17]. In summary, the clinical analysis mainly concluded that obesity was associated with improved survival of patients with gastric cancer. McQuade *et al*[39] indicated that high BMI cancer patients had improved response and survival following treatment with targeted therapy and checkpoint blockade immunotherapy, although a mechanistic link was not elucidated[39,40].

Research targeting the tumor microenvironment also investigated the impact of obesity on immune responses during cancer progression and therapy[41-43]. Trevellin *et al*[44] reported that esophageal peritumoral adipose tissue and its secretion of tumor-promoting factors are directly correlated with increased tumor growth. One study cultured periprostatic and subcutaneous adipose tissue with prostate cancer cells and concluded that periprostatic adipose tissue in obese individuals provided a favorable environment for prostate cancer progression[45]. Adipocytes undergoing lipolysis were

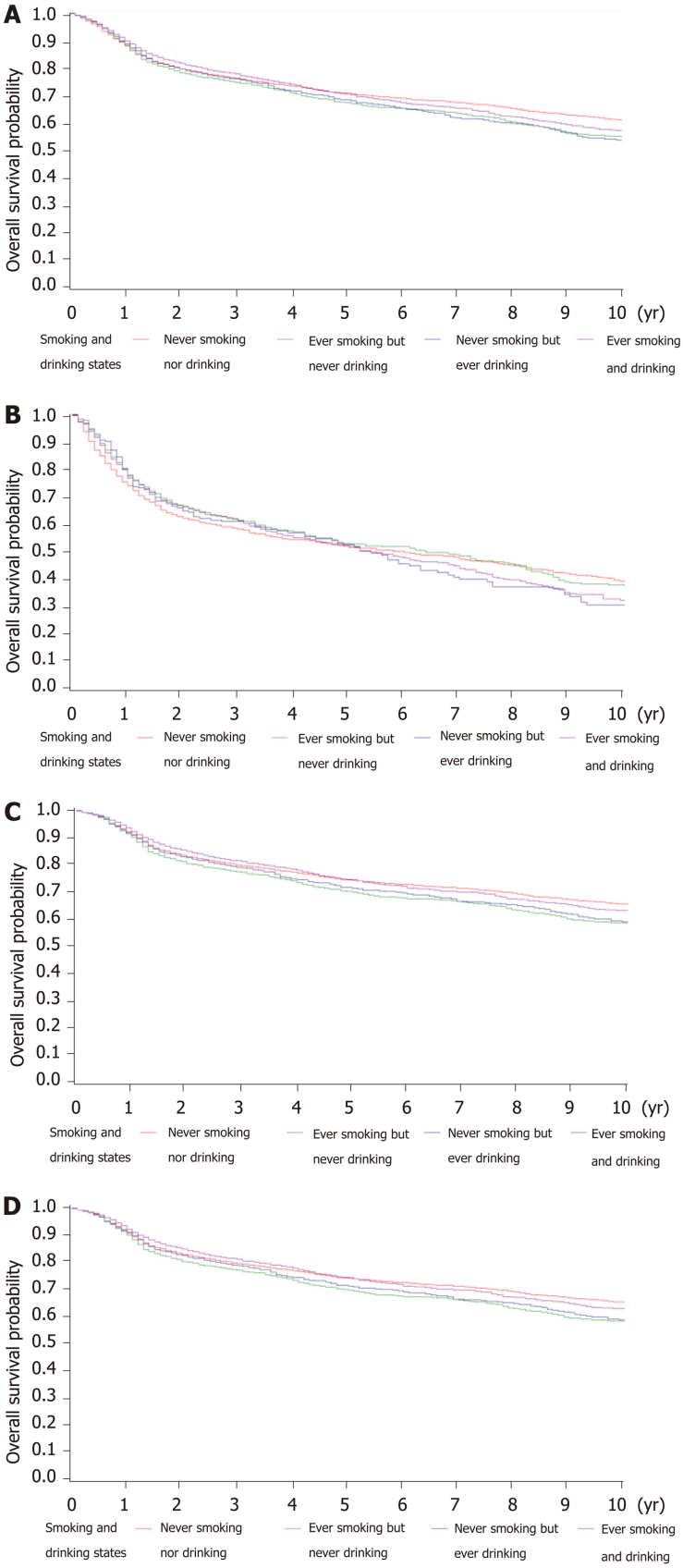

Figure 3 Kaplan-Meier survival curves for overall survival of patients with a history of both smoking and drinking. A: Total gastric cancer patients; B: No surgery patients; C: Gastrectomy patients; D: Only curative gastrectomy patients.

recognized as a source of lipids for cancer cells[46]. Furthermore, a recent study published in Nature Medicine demonstrated that obesity (BMI ≥ 30 kg/m^2) resulted in

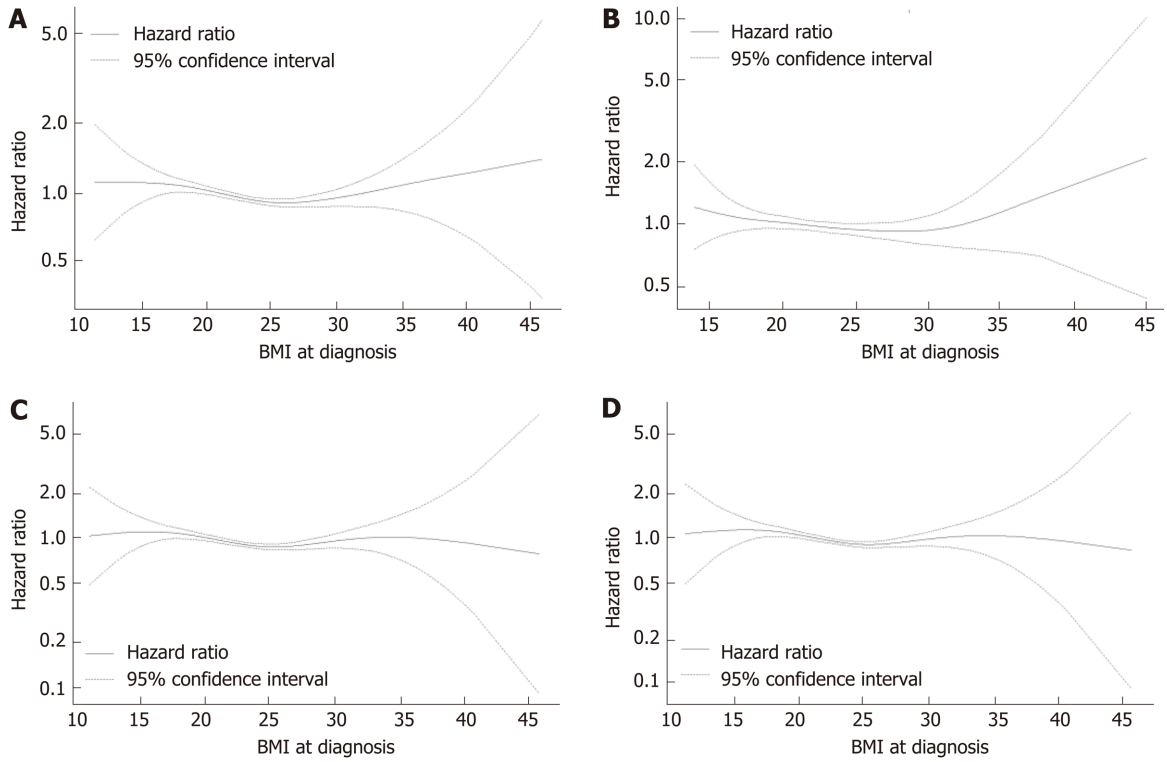

Figure 4 Body mass index at diagnosis and overall survival in multivariate-adjusted analysis. A: Total gastric cancer patients; B: No surgery patients; C: Gastrectomy patients; D: Only curative gastrectomy patients. BMI: Body mass index.

immune aging, tumor progression and PD-1-mediated T cell dysfunction across multiple species and tumor models[43]. However, in our clinical study, BMI at diagnosis was not independently associated with long-term survival in multivariable analyses, even in the stratified gastrectomy group. Further investigations are needed to clarify the paradoxical effects of obesity between clinical and basic research in future studies.

Evidence on the prognostic effect of drinking has been inconsistent. A meta-analysis of 6856 cases from 7 countries showed that drinkers had a lower survival rate, although there was significant heterogeneity among the seven studies included[47]. Our study revealed no significant association between drinking status and long-term prognosis, even in the subgroup of heavy drinkers (≥ 53.5 g/d). These differences may be partly attributed to the genetic distinction of populations with different race and from different regions.

It was found that cigarette smoking was related to gastric cancer risk[22,23,48], and may also have effects on prognosis. It has been demonstrated in most studies that smoking has a null or inverse influence on OS[25-27,49]. Minami *et al*[24] reported a clear association between starting smoking at an earlier age and prognosis. A possible explanation for this is that smoking increases serum estrogen metabolites, which have been postulated to induce a more aggressive tumor at a younger age. Moreover, it has been reported in two published studies[24,31] that the risk of death caused by cancer increases if the patients undergoing curative gastrectomy have a smoking history. This was comparable with our research. In our multivariable analysis, we found that smoking history of more than 30 years conferred a worse prognosis in both the gastrectomy and curative gastrectomy groups. This may indicate that long-term cigarette smoking has a significant effect on the risk of mortality in patients who underwent gastrectomy. Although the cause of this association is unclear, it is possible that smoking has an adverse effect on the pulmonary, circulatory, and immunologic systems, and on wound healing[32,50]. The cumulative chronic toxic effects of long-term smoking may delay the recovery of gastrectomy patients with reduced body condition and cause poor survival outcomes.

Several limitations need to be considered in this study. Firstly, we do not have data on changes in lifestyle factors during treatment, or in the post-treatment phase. These measures collected systematically would allow for a better understanding of whether change following diagnosis is associated with cancer prognosis. Secondly, BMI has been used as the most common measure of indicating obesity due to its simplicity of

measurement and availability. However, waist circumference and actual body composition, particularly fat and muscle percentages, may be more reflective of the degree of obesity. Thirdly, the study was conducted in a single institution; therefore, the results might not be used as a reference for the whole Chinese population. However, the results may provide a reference value as the number of gastric cancer patients was large, and the patients were from Northern and Eastern areas in China. The strength of this study is that the groups analyzed included total patients, no surgery, gastrectomy and curative gastrectomy groups.

In conclusion, our results contribute to a better understanding of lifestyle factors on the overall burden of gastric cancer with regard to long-term prognosis. Among the total patients, weight loss (both the 0 to 10% and > 10% groups) but not BMI at diagnosis was related to survival outcomes. Other factors, such as smoking history of more than 30 years conferred a worse prognosis only in patients who underwent gastrectomy. Extensive efforts are needed to elucidate mechanisms targeting the complex effects of lifestyle factors.

ARTICLE HIGHLIGHTS

Research background

As the gastric cancer patient population grows, lifestyle factors contributing to improved or adverse survival are becoming a focus of increasing interest.

Research motivation

Lifestyle factors such as body mass index (BMI), alcohol drinking, and cigarette smoking, are likely to impact the prognosis of gastric cancer.

Research objectives

To investigate the three major lifestyle factors mentioned above - BMI, alcohol drinking, and smoking - and to clarify the association between these factors and the overall survival of patients with gastric cancer.

Research methods

Patients with gastric cancer were identified from the China National Cancer Center Gastric Cancer Database 1998-2018. Survival analysis was performed *via* Kaplan-Meier estimates and Cox proportional hazards models.

Research results

Patients who were overweight or obese were associated with a positive smoking and drinking history (P = 0.002 and P < 0.001, respectively). Current smokers were more likely to be current alcohol drinkers (61.3% *vs* 10.1% *vs* 43.2% for current, never, and former smokers, respectively, P < 0.001). Multivariable analysis indicated that BMI at diagnosis had no significant effect on prognosis. In gastrectomy patients, factors independently associated with poor survival included older age (HR = 1.20, 95%CI: 1.05-1.38, P = 0.001), any weight loss (P < 0.001), smoking history of more than 30 years (HR = 1.14, 95%CI: 1.04-1.24, P = 0.004), and increasing pTNM stage (P < 0.001).

Research conclusions

Among the total patients, weight loss (both in the 0 to 10% and > 10% groups) but not BMI at diagnosis was related to survival. With regard to other factors, smoking history of more than 30 years conferred a worse prognosis only in gastrectomy patients.

Research perspectives

Factors independently associated with poor survival included older age, any weight loss, smoking history of more than 30 years, and increasing pTNM stage. Extensive efforts are needed to elucidate mechanisms targeting the complex effects of lifestyle factors.

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ORIGINAL ARTICLE

Retrospective Study

Optimal treatment strategies for hepatic portal venous gas: A retrospective assessment

Masanori Gonda, Tatsuya Osuga, Yoshihiro Ikura, Kazunori Hasegawa, Kentaro Kawasaki, Takatoshi Nakashima

ORCID number: Masanori Gonda (0000-0003-2692-5004); Tatsuya Osuga (0000-0001-7316-9833); Yoshihiro Ikura (0000-0001-8599-2245); Kazunori Hasegawa (0000-0003-3270-4689); Kentaro Kawasaki (0000-0002-1891-8886); Takatoshi Nakashima (0000-0003-2051-2057).

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Masanori Gonda, Tatsuya Osuga, Kazunori Hasegawa, Takatoshi Nakashima, Department of Gastroenterology, Takatsuki General Hospital, Takatsuki 5691192, Japan

Yoshihiro Ikura, Department of Pathology, Takatsuki General Hospital, Takatsuki 5691192, Japan

Kentaro Kawasaki, Department of Surgery, Takatsuki General Hospital, Takatsuki 5691192, Japan

Corresponding author: Tatsuya Osuga, MD, PhD, Chief Physician, Doctor, Postdoc, Department of Gastroenterology, Takatsuki General Hospital, 1-3-13 Kosobe-cho, Takatsuki 5691192, Japan. tatsuya.osuga@ajk.takatsuki-hp.or.jp

Abstract

BACKGROUND

Hepatic portal venous gas (HPVG) generally indicates poor prognoses in patients with serious intestinal damage. Although surgical removal of the damaged portion is effective, some patients can recover with conservative treatments.

AIM

To establish an optimal treatment strategy for HPVG, we attempted to generate computed tomography (CT)-based criteria for determining surgical indication, and explored reliable prognostic factors in non-surgical cases.

METHODS

Thirty-four cases of HPVG (patients aged 34-99 years) were included. Necessity for surgery had been determined mainly by CT findings (*i.e.* free-air, embolism, lack of contrast enhancement of the intestinal wall, and intestinal pneumatosis). The clinical data, including treatment outcomes, were analyzed separately for the surgical cases and non-surgical cases.

RESULTS

Laparotomy was performed in eight cases (surgical cases). Seven patients (87.5%) survived but one (12.5%) died. In each case, severe intestinal damage was confirmed during surgery, and the necrotic portion, if present, was removed. Non-occlusive mesenteric ischemia was the most common cause (n = 4). Twenty-six cases were treated conservatively (non-surgical cases). Surgical treatments had been required for twelve but were abandoned because of the patients' poor general conditions. Surprisingly, however, three (25%) of the twelve inoperable

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patients survived. The remaining 14 of the 26 cases were diagnosed originally as being sufficiently cured by conservative treatments, and only one patient (7%) died. Comparative analyses of the fatal (n = 10) and recovery (n = 16) cases revealed that ascites, peritoneal irritation signs, and shock were significantly more frequent in the fatal cases. The mortality was 90% if two or all of these three clinical findings were detected.

CONCLUSION
HPVG related to intestinal necrosis requires surgery, and our CT-based criteria are probably useful to determine the surgical indication. In non-surgical cases, ascites, peritoneal irritation signs and shock were closely associated with poor prognoses, and are applicable as predictors of patients' prognoses.

Key words: Hepatic portal venous gas; Surgical treatment; Conservative treatment; Computed tomography; Intestinal necrosis; Prognostic factor

Core tip: Hepatic portal venous gas caused by intestinal necrosis is a life-threatening condition and requires surgery. Computed tomography findings of free-air, embolism, lack of contrast enhancement of the intestinal wall, and intestinal pneumatosis are useful criteria to determine the surgical indication. In non-surgical cases, ascites, peritoneal irritation signs and shock were closely associated with poor prognoses, and are valuable as predictors of patients' prognoses.

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INTRODUCTION

Hepatic portal venous gas (HPVG) was first presented by Wolfe and Evans[1] in 1955 as a pediatric case, followed in 1960 by the report of Susman and Senturia[2] of an adult case, and was recognized as a fatal condition in patients with serious intestinal damage, including severe intestinal ischemia, enterocolitis, *etc*.[3,4]. Although surgical removal of the damaged portion has generally been considered the sole effective therapy, it has been shown by advanced imaging modalities, including computed tomography (CT), that some of the patients can recover with non-surgical, conservative treatments[3,5-9]. HPVG in the patients who recovered was mostly not associated with intestinal necrosis, suggesting that not all HPVG patients require surgery[9,10]. Conversely, unnecessary laparotomy might have been performed in such mild cases. A robust criterion of surgical indication is necessary to prevent unpredictable under- and/or over-treatments.

The challenging surgery conducted in emergency settings is not applicable to every patient with acute intestinal damage. Some pre-existing conditions, such as poor performance status (PS), severe frailty and extreme exhaustion, may rule out surgery as a therapeutic option. In those cases, to make the best management plan for each non-surgical patient and to explain anticipated outcomes clearly to their families, physicians require reliable prediction indices for estimating the curative potential of non-surgical, conservative treatments.

In this retrospective study, we attempt to determine novel CT-based criteria for deciding surgical indication, and to define prognostic factors in non-surgical conservative treatments of HPVG. A goal of the study is the establishment of optimal treatment strategies against HPVG especially in non-surgical cases.

MATERIALS AND METHODS

Patients

From April 2012 to February 2019, 34 patients (35 cases; one patient was treated twice conservatively) were diagnosed as HPVG and treated at Takatsuki General Hospital. One patient was excluded due to insufficient clinical data, and the remaining 33 patients (19 women and 14 men; aged 34-99 years) were included in this retrospective study. Their chief complaints were abdominal pain (n = 28), nausea/vomiting (n = 12), melena (n = 9) and abdominal fullness (n = 28). Their comorbidities were diabetes (n = 8), cerebral infarction (n = 6), ischemic heart disease (n = 3), pancreatic cancer (n = 1), cerebral palsy (n = 1) and chronic subdural hematoma (n = 1). All the patients presented with acute and serious illnesses, and ten of them were in shock (systolic blood pressure ≤ 90 mmHg) at the initial consultations. This study was reviewed and approved by the ethical committee of Takatsuki General Hospital (Approval No: 2018-1).

The necessity for surgical treatments of these patients was determined mainly based on presence of one or more of the following CT findings: (1) Abdominal free-air; (2) Mesenteric artery embolism; (3) Lack of contrast enhancement of the intestinal wall; and (4) Intestinal pneumatosis. However, for some of the patients who required surgery, laparotomy was abandoned because of poor physical status and socio-medical conditions (inoperable patients). Consequently, the patients were divided into a surgical treatment group and a non-surgical conservative treatment group (including inoperable patients and patients who did not require surgery). Three typical cases are presented below.

Representative cases

Case 1 (surgical case): A 72-year-old female patient, hospitalized for treatment of ischemic heart disease and cerebral infarction complained suddenly of abdominal pain, vomiting and melena. She fell into shock and an abdominal CT was performed immediately, providing a diagnosis of HPVG with intestinal ischemia (Figure 1A). Partial intestinal resection was carried out to save the patient and the pathologic specimen obtained revealed hemorrhagic necrosis associated with pneumatosis (Figure 1B and C), which was concordant with a clinical diagnosis of non-occlusive mesenteric ischemia (NOMI).

Case 2 (inoperable case with recovery): An 86-year-old female patient with dementia and a history of aortic dissection complained of vomiting and melena after her evening meal. She was transferred to our hospital by ambulance. An emergency abdominal CT revealed HPVG and intestinal pneumatosis (Figure 2A). Laparotomy was considered but not performed because of her poor general condition. However, HPVG was alleviated (Figure 2B) by conservative treatment (rehydration and antibiotics), and she survived and recovered.

Case 3 (inoperable fatal case): A 91-year-old female patient with dementia and extremely poor PS [Eastern Cooperative Oncology Group (ECOG) PS 4] complained of vomiting and melena. On admission, abdominal CT showed HPVG and intestinal pneumatosis (Figure 3). Surgical treatment was abandoned because of the expected poor postoperative prognosis. She died the day after admission.

Assessments

The patients' data including clinical backgrounds, physical examination findings, laboratory test results, CT images, and treatment outcomes were analyzed separately for the surgical patients and non-surgical patients. The primary purposes of the analyses were validation of the appropriateness of CT findings-based decision criteria for surgery and development of a prediction index to estimate the mortality of non-surgical patients. Fisher's exact test and Mann-Whitney U-test were used for statistical analysis, and $P < 0.05$ was considered to be significant.

RESULTS

As shown in Figure 4, 20 patients were considered to be suitable for surgical treatments, eight were treated with operations (surgical cases) but 12 were determined to be inoperable cases because of their poor general conditions, *e.g*. ECOG PS 4 (n = 9). Of the eight surgical cases, five patients were originally in shock status, which was resolved preoperatively by rapid rehydration. In 14 cases, CT findings on admission suggested that surgery was not necessary. Consequently, a total of 26 cases (25 patients) were managed conservatively as non-surgical cases, of which 16 cases (15 patients) survived/recovered and 10 died. The overall mortality was 32% (11 of 34

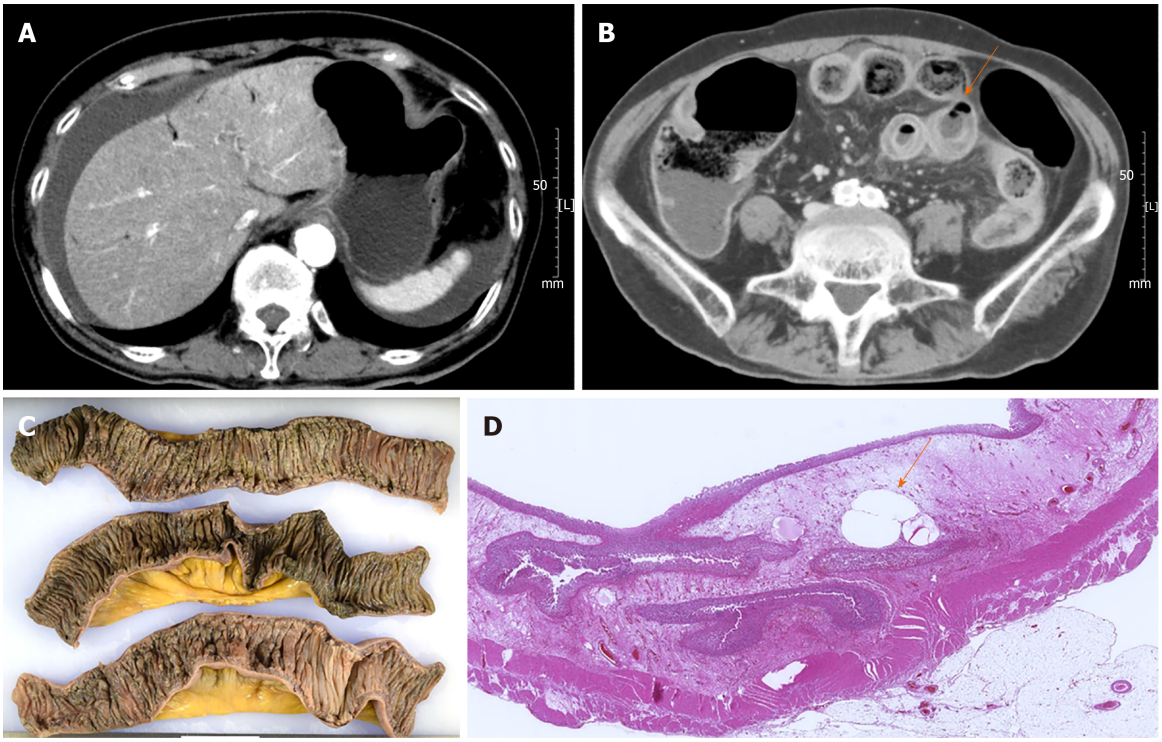

Figure 1 Computed tomography and pathologic findings of case 1. A: Abdominal computed tomography images on admission demonstrate hepatic portal venous gas, ascites; B: Intestinal pneumatosis (arrow); C: The resected small intestine shows hemorrhagic necrosis; D: Air-bubbles in the damaged intestinal wall (arrow).

cases).

Surgical cases

Clinical and pathological details of the eight surgically-treated patients are shown in Table 1. Necrotic portions of the intestine were resected in seven patients, and one patient could be rescued only by separation of intestinal adhesions. Final diagnoses were NOMI (n = 4), clostridium difficile enteritis (n = 1), strangulation ileus (n = 1), superior mesenteric artery thrombosis (n = 1) and gastric perforation (n = 1, a fatal case).

Seven (87.5%) of the eight surgically treated patients survived, and one (12.5%) died (three days post-operation due to sepsis). In contrast, only three (25%) of 12 inoperable patients survived, and nine (75%) died. The difference in the survival rate was statistically significant (P = 0.02), indicating that the decision for operation/laparotomy was very appropriate. This was confirmed by the very low mortality rate (n = 1, 7%) of 14 cases not requiring surgery. In the fatal case, the HPVG had disappeared, and the general condition had also improved, but the patient died of recurrent illness.

Non-surgical cases

Of the 26 non-surgical cases, 16 (61.5%) were cured by conservative treatments (rehydration, antibiotics, ileus tube insertion, *etc*.), but 10 (38.5%) died. Nine of the 10 fatal cases had been defined as inoperable. Detailed clinical and laboratory data of the patients who died or recovered are shown in Table 2.

To determine the critical prognostic factors in the non-surgical cases, comparative analyses were performed between the fatal and recovery cases (Table 2). Rates of ascites (80% *vs* 31%), peritoneal irritation sign (80% *vs* 12.5%) and shock (60% *vs* 0%) were significantly higher in the fatal cases. Of the laboratory test results, leukocyte counts were significantly higher in the fatal cases than the recovery cases (median 13400 *vs* 9050 /μL; P = 0.025). Base excess (median -6.2 *vs* 1.8 mEq/L) tended to be lower, and plasma levels of CRP (median 12.84 *vs* 2.39 mg/dL) and lactic acid (median 36 *vs* 26 mg/dL) tended to be higher in the fatal cases, but the differences were not significant.

A predictive index was developed using the three statistically significant clinical/non-laboratory factors, *i.e*. ascites, peritoneal irritation sign and shock. Most of the fatal cases (90%) presented two or three of the factors, while none of the recovery cases presented two or three of the factors (Table 3). This indicates that prediction of mortality with detection of two or all of the three factors is a superior

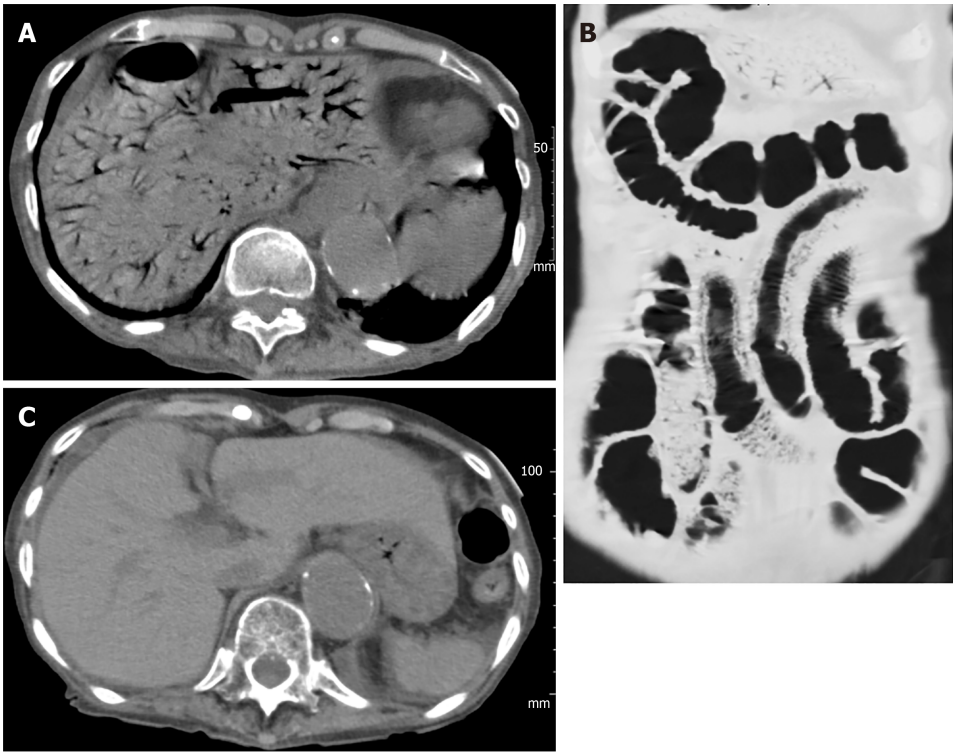

Figure 2 Abdominal computed tomography images of case 2. A: Extensive hepatic portal venous gas; B: Intestinal pneumatosis were found on the first hospital day; C: However, hepatic portal venous gas disappeared on the seventh hospital day.

index of sensitivity 90% and specificity 100%. Notably, the three inoperable patients who recovered, without exception, had none or only one of the three factors. The predictive accuracy was not improved by adding the leukocyte counts as a fourth factor (data not shown).

DISCUSSION

HPVG has been recognized as a serious condition that is associated with poor prognosis and requires urgent surgical treatment. Formerly its mortality was reported to be 75%-90%[3,11] but this has improved recently to 29%-56%[6,10,12] with an increase in the detection rate and advances in therapy. At the same time, non-surgical cases have become more common[6-9] but reliable guidelines to select the optimal treatment for each patient have not yet been established.

The pathologic mechanisms of HPVG are summarized as (1) intramural gas-producing bacterial proliferation; (2) elevated intraluminal pressure because of bowel obstruction, endoscopic procedures, *etc*.; and (3) air-translocation through damaged/necrotic mucosa[3,7]. Kinoshita *et al*[10] reported that the etiologies /underlying conditions of HPVG were mesenteric ischemia (43%), digestive tract dilation (12%), intraperitoneal abscess (11%), ulcerative colitis (4%), gastric ulcer (4%), complications from endoscopic procedures (4%), intraperitoneal tumors (3%), and others (15%). HPVG of our patients were related to NOMI (n = 12, including eight suspicious cases), ischemic enterocolitis (n = 5), superior mesenteric arterial thrombosis (n = 3) , ileus [strangulation (n = 1) or non-strangulation (n = 4)], constipation (n = 4), postoperative intestinal necrosis (n = 1), clostridium difficile enteritis (n = 1), acute pancreatitis (n = 1), gastric perforation (n = 1), and bladder cancer invading the rectum (n = 1). The proportion of these background conditions and the overall mortality in our cases (32%) were almost identical with those of other recent reports, indicating that our HPVG group was standard and not at all unusual.

Through the retrospective observation of this standard HPVG group, we validated the appropriateness of our original CT findings-based determination to select subjects for surgical treatment. The four CT findings, *i.e*. abdominal free-air, mesenteric artery embolism, lack of contrast enhancement of the intestinal wall, and intestinal pneumatosis, which are hallmarks of intestinal perforation and/or severe ischemia/necrosis[13], seem to be appropriate as convenient decision criteria for laparotomy. A similar preoperative assessment was previously reported from a group

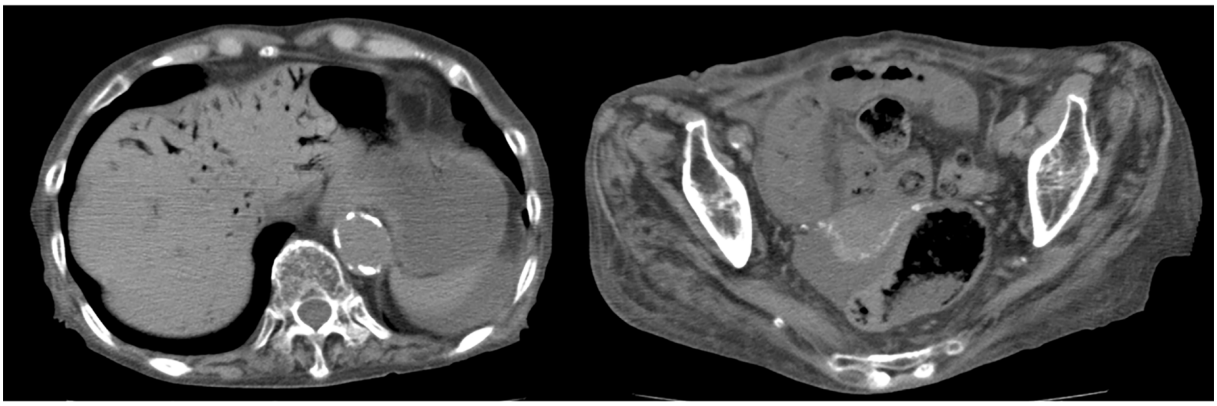

Figure 3 Abdominal computed tomography images of case 3 on admission. Left lobe hepatic portal venous gas and the moderate amount of ascites are seen.

of Japanese surgeons[14]. Our CT-based simple method provides not only comparable accuracy but also superior convenience.

In addition, we found reliable clinical indices for predicting the mortality of non-surgical patients with HPVG, including inoperable cases. Non-surgical patients who have more than two of three clinically obtainable factors, ascites (by CT), peritoneal irritation (by physical examination), and shock (by checking vital signs), are thought to be in life-threatening conditions. Critical intestinal ischemia is associated frequently with perforation, sepsis and peritonitis, and hence, ascites, peritoneal irritation and shock are thought to be its typical manifestations. An analogues prediction system using Acute Physiology and Chronic Health Evaluation (APACHE) II was proposed by Yoo *et al*[15]. However, because APACHE II requires several items of laboratory data[16,17], this predictive algorithm may be difficult to disseminate as a general procedure. Very recently, similar emergency medicine scorings also have been suggested for use for the identical purpose[18,19]. Same as APACHE II, they are not specific to abdominal illnesses, and seem not to be perfect as predictors of HPVG.

The importance of laboratory test results for determination of surgical indication and for prediction of non-surgical patients' prognoses was also examined, as had been done in previous analogous studies. Although some of these tended to show greater degrees of abnormal values in patients who died than in those who survived, the differences were not statistically significant. Only leukocyte counts were significantly higher in the fatal cases than in the recovery cases, but were not a contributory factor to the mortality prediction. As a result, we were able to develop a quite simple diagnostic algorithm composed of characteristic CT findings and physical examination findings, to provide the optimal treatment for each HPVG patient. With the progression of an aging society, the incidence of HPVG, especially of inoperable cases, is inevitably increasing. Our two-step decision and prediction process may be useful not only for selection of surgical cases but also for considering non-surgical but intensive treatments for such inoperable patients. In other words, a strategic non-surgical management may be recommended in the future to HPVG patients who have 0-1 of the high-mortality factors (ascites, peritoneal irritation sign and shock). Alternatively, a challenging surgery may be considered in patients who have 2-3 of the factors, regardless of their background conditions. A further validation study, such as a prospective study, may be required to generalize our novel treatment strategy for HPVG.

Table 1 Preoperative findings, outcomes and final diagnosis of the surgical cases (*n* = 8)

Case	Age	Gender	Final diagnosis	Outcome	Chief complaints	Shock[1]	Peritoneal irritation signs	Ascites[2]	Intestinal pneumatosis	LOCE in intestinal wall	Free air	WBC (μL)	CRP (mg/dL)	BE (mmol/L)	Lactate (mg/dL)
1[3]	72	F	NOMI	Recovery	Abdominal pain, nausea	+	+	2+	-	+	-	26000	1.57	-4.9	18
2	74	M	Clostridium enteritis	Recovery	Nausea, vomiting	-	+	2+	-	+	-	15400	16	5.3	7
3	65	F	NOMI	Recovery	Abdominal pain	-	-	-	-	+	+	31500	7.39	-7	13
4	86	M	Gastric perforation	Death	Abdominal pain, vomiting	+	+	3+	+	+	+	7800	0.17	-11.3	73
5	69	M	NOMI	Recovery	Abdominal pain	+	+	2+	+	-	-	13200	10.8	2.3	14
6	71	M	Mesenteric artery thrombosis	Recovery	Abdominal pain, vomiting	+	+	1+	+	+	+	22500	1.81	-6.3	46
7	84	M	NOMI	Recovery	Abdominal fullness	+	+	2+	+	NE	-	36700	15.54	-5.5	47
8	34	M	Strangulation ileus	Recovery	Abdominal fullness	-	NE	-	+	+	-	10000	4.67	-3.1	42

[1]≤ Systolic blood pressure 90 mmHg.
[2]Semiquantitative evaluation as – (none).
[3]Shown as case 1 in the case presentation.
1+: Small amount; 2+: Moderate amount; 3+: Large amount. LOCE: Lack of contrast enhancement; BE: Base excess; NE: Not examined; NOMI: Non-occlusive mesenteric ischemia.

Table 2 Clinical data comparison between the non-surgical recovery and the non-surgical death cases

	Recovery (*n* = 16)	Death (*n* = 10)	*P* value
Age [median (range)]	86 (56-92)	84 (72-99)	$P > 0.999$[1]
Gender (M:F)	4:12	4:6	$P = 0.665$[2]
Shock (≤ systolic BP 90 mmHg) (%)	0 (0%)	6 (60%)	$P = 0.001$[2]
Peritoneal irritation (%)	2 (13%)	8 (80%)	$P = 0.001$[2]
Ascites (%)	5 (31%)	8 (80%)	$P = 0.041$[2]
Intestinal pneumatosis (%)	8 (50%)	7 (70%)	$P = 0.428$[2]
WBC (/μL) [median (range)]	9050 (4200-31800)	13400 (9900-19000)	$P = 0.025$[1]
CRP (mg/dL) [median (range)]	2.39 (0.11-28.41)	12.84 (0.1-33.26)	$P = 0.355$[1]
BE (mmol/L) [median (range)]	1.8 (-8.4 – 14.6)	-6.2 (-18.2 – 6.8)	$P = 0.071$[1]
Lactate (mg/dL) [median (range)]	26 (9-63)	36 (11-120)	$P = 0.231$[1]

[1]Mann-Whitney-*U* test.
[2]Fisher's exact test. A significant *P* value is shown on underline. BE: Base excess.

Table 3 Mortality prediction in the 26 non-surgical cases by the three factors [Ascites, peritoneal irritation sign (muscular defense and/or rebound tenderness), and shock]

	Recovery	Death
0-1 Factor	16	1
2-3 Factors	0	9

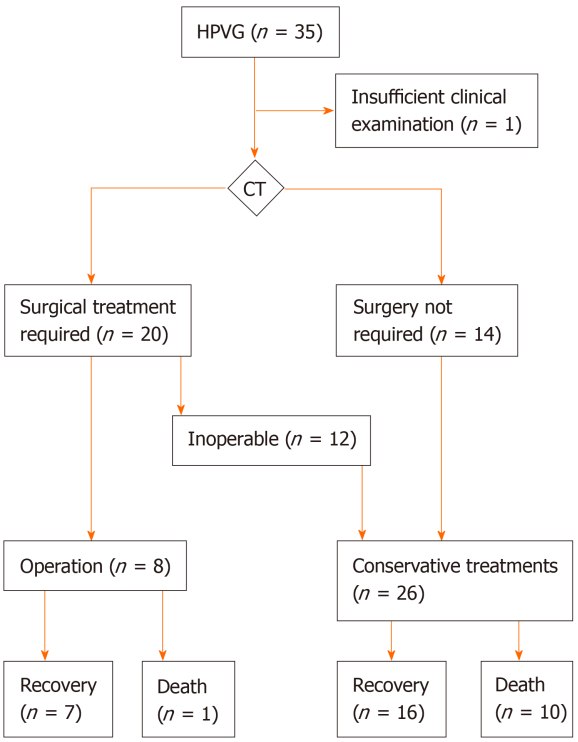

Figure 4 A treatment decision flowchart and outcomes. CT: Computed tomography; HPVG: Hepatic portal venous gas.

ARTICLE HIGHLIGHTS

Research background

Hepatic portal venous gas (HPVG) is generally recognized as a life-threatening sign in patients with serious intestinal damage. While most of such patients require surgical treatments, some

patients can recover without surgery.

Research motivation
We aimed to establish an optimal treatment strategy for HPVG, *i.e.*, how to select surgical or conservative treatments.

Research objectives
We tested accuracy of our original computed tomography (CT)-based selection criteria. Additionally, we found if there were reliable prognostic factors in non-surgical cases.

Research methods
Thirty-four cases of HPVG were included. Surgical indication had been decided by CT findings, including free-air, embolism, lack of contrast enhancement of the intestinal wall, and intestinal pneumatosis. Their clinical findings and treatment outcomes were analyzed separately in the surgical cases and non-surgical cases.

Research results
Of eight surgical cases, seven patients (87.5%) survived but one (12.5%) died. All the surgical patients had severe intestinal damage and the necrotic portions were resected. In addition to 14 cases without surgical indication, 12 inoperable cases were defined as non-surgical cases (total 26 cases). Three (25%) of the 12 inoperable patients survived. Only one patient (7%) died among the 14 patients diagnosed as being surgery unnecessary. Comparative analyses of the fatal (n = 10) and recovery (n = 16) cases revealed that ascites, peritoneal irritation signs, and shock were significantly more frequent in the fatal cases. The mortality was 90% if two or all of these three clinical findings were detected.

Research conclusions
Our CT-based criteria were useful to determine the surgical indication for HPVG patients. In non-surgical cases, ascites, peritoneal irritation signs and shock were closely associated with poor prognoses, and are applicable as predictors of patients' prognoses.

Research perspectives
Our two-step decision and prediction process may be applicable not only for selection of surgical cases but also for considering non-surgical but intensive treatments for such inoperable patients.

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ORIGINAL ARTICLE

Retrospective Study

Clinical and microbiological characteristics of patients with biliary disease

Xue-Xiang Gu, Meng-Pei Zhang, Yan-Feng Zhao, Guang-Ming Huang

ORCID number: Xue-Xiang Gu (0000-0001-7574-6606); Meng-Pei Zhang (0000-0001-6475-6171); Yan-Feng Zhao (0000-0001-6475-6172); Guang-Ming Huang (0000-0003-1127-5803).

Author contributions: Zhao YF and Huang GM designed the research and Gu XX wrote the paper; Gu XX and Zhang MP analyzed the data and performed the research; Zhao YF contributed new reagents and analyzed data.

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Informed consent statement: All study participants, or their legal guardian, provided informed written consent.

Conflict-of-interest statement: The authors declare no conflicts of interest.

Data sharing statement: Data sets are available from the corresponding author. The presented data are anonymized, and the risk of identification is low.

Xue-Xiang Gu, Department of Gastroenterology, The Affiliated Huaian No.1 People's Hospital of Nanjing Medical University, Huaian 223001, Jiangsu Province, China

Meng-Pei Zhang, Guang-Ming Huang, Department of Gastroenterology, The Second Affiliated Hospital of Nanjing Medical University, Nanjing 210011, Jiangsu Province, China

Yan-Feng Zhao, Center of Laboratory Medicine, The Second Affiliated Hospital of Nanjing Medical University, Nanjing 210011, Jiangsu Province, China

Corresponding author: Yan-Feng Zhao, MD, PhD, Assistant Professor, Teacher, Center of Laboratory Medicine, The Second Affiliated Hospital of Nanjing Medical University, 121 Jiangjiayuan, Nanjing 210011, Jiangsu Province, China. zhaoyanfeng@njmu.edu.cn

Abstract

BACKGROUND

Biliary diseases are common digestive system disorders which may combine with biliary tract infection such as cholecystitis or cholangitis. Thus, rapid identification of the bacteria and their antibiotic susceptibility profiles are crucial for reducing the mortality of patients with biliary tract infection.

AIM

To identify bacterial species and antibiotic susceptibility for antibacterial therapy and analyze bile cultivation risk factors for increasing detection rates.

METHODS

This retrospective study was conducted from July 2008 to July 2017. In total, 1339 bile samples which were collected during therapeutic endoscopic retrograde cholangiopan-creatography or percutaneous transhepatic cholangiodrainage or other biliary surgeries or biliary drainage were obtained to characterize pathogen spectra, antibiotic susceptibility, and clinical features. Clinical data including age, sex, comorbidities, clinical symptoms, protopathies, and history of biliary tract diseases and surgeries were collated from hospital medical records. Species identification and initial drug susceptibility were further identified by biochemical characterization using the VITEK 2 Compact test.

RESULTS

Positive microbiological findings were observed in 738 samples. The most frequently encountered strains were gram-negative bacteria (74.94%), including *Escherichia coli* (37.78%), *Pseudomonas aeruginosa* (8.96%), and *Klebsiella pneumoniae* (10.29%). Bile bacteria were largely sensitive to carbapenems, piperacillin/tazobactam, and gentamicin. Gram-negative strains had low

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susceptibility to ceftriaxone, quinolones and ampicillin. Almost the same micro-organisms were present in patients with malignant and benign diseases. The number of samples with *Klebsiella pneumoniae* in the bile culture were significantly different between patients with malignant and benign diseases (55 *vs* 30; P = 0.019). Age (P < 0.001), fever (P < 0.001), history of biliary tract diseases and surgeries (both P < 0.001), benign disease (P = 0.002), and the comorbidity chronic renal insufficiency (P = 0.007) affected the positive rates of the bile samples.

CONCLUSION
Gram-negative bacteria were the most commonly isolated biliary bacteria. We determined the major factors associated with positive detection rates. Microbiological analysis of bile samples allowed accurate antibiotic treatments.

Key words: Microorganism; Antibiotic susceptibility; Bile culture; Biliary diseases; Retrospective study; Detection rate

Core tip: In this large ten year retrospective study, we analyzed bacterial species in bile and their antibiotic susceptibility for antibacterial therapy, and analyzed bile cultivation risk factors to increase detection rates. The most frequently encountered strains were gram-negative bacteria, which were largely sensitive to carbapenems, piperacillin/tazobactam, and gentamicin. Almost the same micro-organisms were present in patients with malignant and benign diseases. Major risk factors for positive detection rates were age, fever, history of biliary tract diseases and surgeries, benign diseases, and the comorbidity chronic renal insufficiency.

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INTRODUCTION

Biliary diseases are common digestive system disorders and include gallstones, gallbladder polyps, gallbladder carcinoma, and cholangiocarcinoma. These diseases may be combined with biliary tract infection such as cholecystitis or cholangitis. Normally, bile is a lipid-rich sterile solution produced in the liver[1,2]. However, bacterial colonization of bile has been found in some healthy people, and due to the normal bile flow, such individuals have no clinical symptoms. Bacteria may proliferate through the retrograde intrusion path owing to obstruction of the normal excretion of bile by tumors, stones, or worms that increase the pressure within the biliary ducts. These bacteria may also invade through the blood and lymphatic system. Furthermore, certain interventions such as surgery or endoscopic manipulations may negatively influence human defense mechanisms. Thus, bacteria translocate into the circulation, causing infection, possibly leading to severe sepsis and septic shock or even multiple organ dysfunction syndrome and eventually death[3-5]. Thus, rapid identification of the bacteria and their antibiotic susceptibility profiles is crucial for reducing the mortality of patients with biliary tract infection[6]. However, bile culture requires time and has lower positive rates. Moreover, insufficient data are available regarding the microbiological flora of the biliary tract, and most studies were conducted decades ago[7,8]. In addition, microbes show both regional and temporal variations[9].

Furthermore, there is no agreement concerning an optimum initial antibiotic therapy[5,10], and few data are available regarding the antibiotic susceptibility profiles of bacteria isolated from bile[11]. In addition, the rapid development of multidrug-resistant bacteria complicates the choice of an appropriate empiric antimicrobial therapy even more.

Thus, the aim of this study was to identify bacterial species and their antibiotic susceptibility for early empiric antibacterial therapy and to analyze risk factors in

order to increase the detection rates of bile cultivation in patients with biliary diseases.

MATERIALS AND METHODS

Patient characteristics

In total, 1339 bile samples were collected between July 2008 and July 2017 from 1339 patients who underwent therapeutic endoscopic retrograde cholangiopancreatography or percutaneous transhepatic cholangiodrainage or other biliary surgeries or biliary drainage at the Second Affiliated Hospital of Nanjing Medical University. For this retrospective study, clinical data were collected from medical records. The following variables from medical records were included in our research: Age, sex, comorbidities, clinical symptoms, protopathies, and history of biliary tract diseases and surgeries (Table 1). The study was conducted in accordance with the principles of the Declaration of Helsinki, and the study protocol was approved by the ethics committee of the Second Affiliated Hospital of Nanjing Medical University.

Reagents and microbiological sampling

Bacterial isolates were obtained from the Second Affiliated Hospital of Nanjing Medical University, a large hospital in Jiangsu Province, China, between July 2008 and July 2017. They were confirmed by classic microbiological methods including Gram stain and catalase. Species identification and initial drug susceptibility were further identified by biochemical characterization using the VITEK 2 Compact test (bioMérieux, Lyon, France). *Escherichia coli* (*E. coli*) ATCC25922, *Pseudomonas aeruginosa* (*P. aeruginosa*) ATCC 27853, and *Staphylococcus aureus* ATCC 29213 were used as the quality control strains. Imipenem, meropenem, ceftriaxone, ampicillin, piperacillin/tazobactam, quinolones, gentamicin, vancomycin, and linezolid were purchased from Oxoid Ltd (Basingstoke, United Kingdom). Minimum inhibitory concentrations of the antibiotics were determined using the broth dilution method, E test (bioMérieux), or disk diffusion methods according to Clinical Laboratory Standards Institute guidelines.

Statistical analysis

All statistical analyses were performed using the SPSS software (Chicago, IL, United States). Between-group analyses were conducted using *t*-test or χ^2 test. A P value of < 0.05 was considered to indicate statistical significance.

RESULTS

Microbiological characteristics

As shown in Figure 1, 738 of 1339 bile samples showed positive culture results (55.12%); 826 bacterial isolates were identified from 738 bile samples. Of these 738 samples, 652 contained single bacterial species, 84 had simultaneous growth of two different bacterial species, and two had simultaneous growth of three diverse bacterial species. In total, 619 strains were gram-negative bacteria (74.94%), 189 strains were gram-positive bacteria (22.88%), and 18 strains were fungi (2.18%). At the family level, the most frequently isolated pathogens were *Enterobacteriaceae* (472; 57.14%) and *Enterococcaceae* (109; 13.20%). The most common gram-negative bacterial strains at the species level were *E. coli* (312; 37.78%), *Klebsiella pneumoniae* (*K. pneumoniae*) (85; 10.29%), and *P. aeruginosa* (74; 8.96%). The most frequently detected gram-positive pathogenic bacteria were *Enterococcus faecium* (51; 6.17%), *E. avium* (32; 3.87%), and *E. faecalis* (21; 2.54%). Of the 18 fungal strains identified, 14 (77.78%) belonged to *Candida albicans*, and the remaining four represented *C. tropicalis* (16.67%) and *C. parapsilosis* (5.55%).

Antibiotic susceptibility and bacterial resistance profiles

We analyzed the most common isolates in our study for susceptibility to antibiotics. Overall, for isolated *E. coli*, ceftriaxone and ampicillin resistance was observed in 251/312 cases (80.45%) and 277/312 isolates (88.78%), respectively. Ampicillin showed activity against 27.45% of the *E. faecium* isolates, and quinolones were active against 33.33% of the isolates. Furthermore, 78.38% of *P. aeruginosa* strains were resistant to ceftriaxone, and 100% of the isolates were resistant to ampicillin. Ceftriaxone and ampicillin resistance were observed in 71.76% and 95.29% of the *K. pneumoniae* isolates, respectively (Table 2).

Table 1 Clinical characteristics and factors related to bile culture positive rates

	Group 1 (*n* = 601) (%)	Group 2 (*n* = 738) (%)	Total (*n* = 1339) (%)	*P* value
Age (mean in years)	60.93 ± 14.99	63.62 ± 14.72	62.42 ± 14.90	0.000[a]
Sex (male)	293 (48.75)	375 (50.81)	668 (49.89)	0.453
Clinical manifestations				
Fever	62 (10.32)	206 (27.91)	268 (20.01)	0.000[a]
Abdominal pain	459 (76.37)	606 (82.11)	1065 (79.54)	0.906
Jaundice	252 (41.93)	327 (44.31)	579 (43.24)	0.382
Benign diseases	405 (67.39)	554 (75.07)	959 (71.62)	0.002[a]
Malignant diseases	196 (32.61)	184 (24.93)	380 (28.38)	
History of biliary tract diseases	191 (31.78)	369 (50.00)	560 (41.82)	0.000[a]
History of biliary tract surgery	128 (21.30)	329 (44.58)	457 (34.13)	0.000[a]
Comorbidities				
Diabetes	77 (12.81)	86 (11.65)	163 (12.17)	0.519
Hypertension	170 (28.29)	182 (24.66)	352 (26.29)	0.134
Brain infarction	52 (8.65)	56 (7.59)	108 (8.07)	0.477
Coronary heart disease	30 (4.99)	33 (4.47)	63 (4.71)	0.655
Chronic bronchitis	14 (2.33)	19 (2.57)	33 (2.46)	0.774
Chronic renal insufficiency	4 (0.67)	19 (2.57)	23 (1.72)	0.007[a]

Group 1: 601 cases with negative bile culture; Group 2: 738 cases with positive bile culture. Significance differences were calculated using the χ^2 or *t*-test.
[a]$P < 0.05$.

Clinical characteristics

The patients admitted to our hospital had a mean age of 62.42 years (SD, 14.90; range, 1-97 years) and were mostly men (n = 668) and aged > 65 years (n = 619). Of 1339 selected bile samples, 959 were collected from patients with benign diseases such as gallstones, cholecystitis, and gallbladder polyps. In addition, 380 bile samples were collected from patients with malignant diseases such as adenocarcinoma of the duodenal papilla, pancreatic cancer, gallbladder carcinoma, and cholangiocarcinoma. The most common clinical symptoms were abdominal pain (82.23%), fever (20.01%), and jaundice (43.24%). Major comorbidities were diabetes (163 cases), hypertension (352 cases), brain infarction (108 cases), coronary heart disease (63 cases), chronic bronchitis (33 cases), and chronic renal insufficiency (23 cases). Altogether, 560 patients had a history of biliary tract diseases, and 457 patients underwent biliary tract surgeries (Table 1).

Factors for bile culture-positive rates

We compared the main clinical differences between 738 cases with positive bile culture results (group 2) and 601 cases with negative bile culture results (group 1) (Table 1). We found that older patients (≤ 60 *vs* > 60) had high positive rates ($P < 0.05$). There was a statistically significant difference between the two groups with and without clinical manifestations such as fever (62 of 601, 10.32% *vs* 206 of 738, 27.91%; $P < 0.001$). The patients with benign diseases had higher positive rates that those with malignant diseases (405 of 601, 67.39% *vs* 554 of 738, 75.07%; $P = 0.002$). We also found statistically significant differences in patients with a history of biliary tract diseases and surgeries (191 of 601, 31.78% *vs* 369 of 738, 50% and 128 of 601, 21.30% *vs* 329 of 738, 44.58%; both $P < 0.001$). Four patients in group 1 and 19 patients in group 2 had chronic renal insufficiency ($P = 0.007$).

Distribution of bile bacteria and cause of diseases

We found that the most common strains in patients (n = 554) with benign diseases were *E. coli* (231; 41.7%), *P. aeruginosa* (55; 9.93%), *K. pneumoniae* (55; 9.93%), and *E. faecium* (39; 7.04%). The predominant strains identified in patients (n = 184) with malignant diseases were *E. coli* (81; 44.02%), *K. pneumoniae* (30; 16.30%), *P. aeruginosa* (19; 10.33%), and *E. faecium* (12; 6.52%). Both for benign and malignant disease, the prevalence was almost the same. A significant difference was observed between patients with malignant and those with benign diseases with regard to *K. pneumoniae* bile cultures (55 *vs* 30; $P = 0.019$; Table 3).

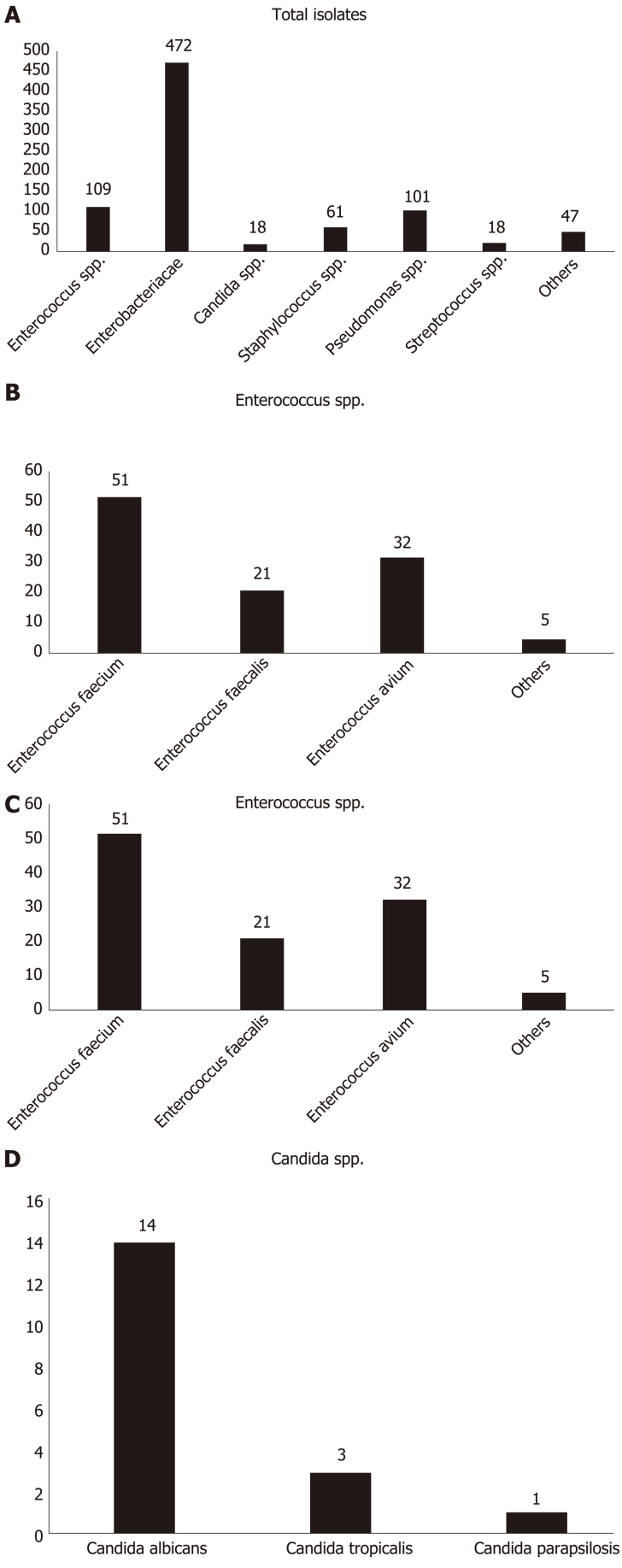

Figure 1 Microbiological distribution. Numbers of bacteria at the family and species level isolated from bile samples. A: Total; B: *Enterococcus* spp.; C: *Enterobacteriaceae*; D: *Candida* spp.

Table 2 Antibiotic susceptibility and resistance profile of bacteria

Antibiotic	*Escherichia coli* (*n* = 312) (%)	*Pseudomonas aeruginosa* (*n* = 74) (%)	*Klebsiella pneumoniae* (*n* = 85) (%)	*Enterococcus faecium* (*n* = 51) (%)
Ceftriaxone	251 (80.45%)	58 (78.38%)	61 (71.76%)	NA
Ampicillin	277 (88.78%)	74 (100%)	81 (95.29%)	37 (72.55%)
Piperacillin/tazobactam	37 (11.86%)	9 (12.16%)	18 (21.18%)	28 (54.90%)
Quinolones	204 (65.38%)	11 (14.86%)	34 (40.00%)	34 (66.67%)
Carbapenems	0 (0%)	17 (22.97%)	2 (2.35%)	NA
Vancomycin	NA	NA	NA	0 (0%)
Gentamicin	144 (46.15%)	9 (12.16%)	43 (50.59%)	18 (35.29%)
Linezolid	NA	NA	NA	0 (0%)

NA: Not applicable.

DISCUSSION

It has been reported that biliary pathogenic bacteria may be associated with intestinal flora distribution, such as *E. coli*, *K. pneumoniae*, and *Enterococcus*[12-14]. In the present study, we analyzed 1339 bile samples over ten years and established that biliary bacteria were mainly gram-negative bacteria, accounting for 74.94%, the rest included 22.88% of gram-positive bacteria and 2.18% of fungus. *E. coli* (37.78%) and *K. pneumoniae* (10.29%) were the most common gram-negative bacteria, and *Enterococcus* (13.20%) and *Staphylococcus* (7.38%) were the main gram-positive bacteria. Therefore, at the species level, our results, are consistent with previous results[12-14]. Normally, due to the protective effects of bile salts, flushing of bile, and phagocytosis by Kupffer cells[1,15], the numbers of bacteria in the biliary tract are low. In a previous study, gut microbes were established to shift to the bile ducts and liver *via* the duodenal papilla and portal system following obstruction of the biliary tract, which caused infection[16]. In our study, the mixed infection rate was lower than the individual bacterial infection rate (11.65% *vs* 88.35%), which was different to previous reports[17,18]. This may have been because we did not perform anaerobic bacteria cultivation and the wide application of antimicrobial agents.

In the past, the combination of ampicillin and an aminoglycoside was considered to be the first choice for treatment of biliary tract infection. However, due to increasing resistance to penicillin and kidney toxicity of aminoglycoside, empiric therapy was changed. Current guidelines now recommend treatment with third-generation cephalosporins or a penicillin/beta-lactamase inhibitor-based agent for empiric therapy of biliary bacteria by intravenous infusion[19]. In conclusion, bacterial resistance has changed. In our study, gram-negative strains had low susceptibility to ceftriaxone, quinolones and ampicillin, which is inconsistent with the guidelines. This high resistance may be related to common inappropriate use of these antibiotics, the selection of third-generation cephalosporins and no classification of quinolones. Thus, ceftriaxone and ampicillin were not recommended. On the other hand, they were reasonably susceptible to piperacillin/tazobactam and carbapenems. However, not all patients with biliary infections are treated with carbapenems and piperacillin/tazobactam due to cost issues and emerging resistance. It was reported that univariate risk factors for biliary multidrug resistant bacteria were male sex, nosocomial acute cholangitis, prior antibiotic exposure and prior biliary stenting[13]. We analyzed the risk factors for this high ceftriaxone resistance rate with regard to *E. coli* (Supplementary Table 1). Unfortunately, we did not find any relevant risk factors. Further in-depth analysis is required in the future. In our study, the resistance rates of *E. faecium* were exceedingly high. In our series, gentamicin and piperacillin/tazobactam led to insignificant susceptibility rates, and only narrow-spectrum antibiotics such as vancomycin were effective. These findings must be considered during future empiric antibiotic treatments.

We suspected that microbiological profiles may be related to different diseases. Therefore, we analyzed the differences in microbiological profiles of patients with benign and malignant diseases. However, we found almost the same micro-organisms were positively cultured, and *E. coli*, *Enterococcus*, *P. aeruginosa*, and *K. pneumoniae* were the most common bacteria present in patients with malignant and benign diseases. We also attempted to demonstrate a possible association between bactibilia and the emergence of malignant diseases, such as *Helicobacter pylori*, which is associated with pancreatic cancer[20] and biliary tract cancer[21]. However, only a

Table 3 Distribution of bacteria identified in 738 bile samples with positive bile culture based on the different diseases caused by them

Bacteria	Benign diseases (*n* = 554) (%)	Malignant diseases (*n* = 184) (%)	Total (*n* = 738) (%)	*P* value
Escherichia coli	231 (41.7)	81 (44.02)	312 (42.28)	0.580
Enterococcus faecium	39 (7.04)	12 (6.52)	51 (6.91)	0.810
Klebsiella pneumoniae	55 (9.93)	30 (16.30)	85 (11.52)	**0.019**[a]
Pseudomonas aeruginosa	55 (9.93)	19 (10.33)	74 (10.03)	0.876
Proteus mirabilis	14 (2.53)	7 (3.8)	21 (2.85)	0.367
Staphylococcus	40 (7.22)	21 (11.41)	61 (8.27)	0.074

Main bacteria were identified in 738 bile samples from 738 patients; 554 patients with benign diseases and 184 with malignant diseases. Significant differences were calculated using the χ^2 test.
[a]$P < 0.05$.

significant difference was observed between patients with benign diseases and those with malignant diseases with regard to *K. pneumoniae* ($P = 0.019$).

When the bile duct is obstructed, bacteria in the bile proliferate and inflammation occurs. Thus, choosing suitable antibiotics according to the profiles of the different bacteria identified is essential. However, in clinical practice, there is a certain time delay in obtaining bacterial culture and drug susceptibility results. At the same time, positive cultivation rates are low. Therefore, analyzing the factors that affect the positive cultivation rates of patients to increase detection rates is important. We found that older patients presented with high positive rates, possibly because they had comorbid diseases and had low immunity to resist bacteria. Moreover, weakened gastrointestinal motility, decreased secretion of gastric acid and bile, and lower gastric acid concentration and intestinal disorders in older people promoted the growth of bacteria. Abdominal pain, fever, and jaundice were the most frequently observed clinical manifestations in patients with biliary diseases[10]. However, in our study, we found that only patients with fever tended to present with high positive rates, possibly because fever had higher specificity than other symptoms.

Salvador *et al*[22] revealed that patients with benign diseases had higher positive rates than those with malignant diseases ($P = 0.002$), which is consistent with our findings, but is contrary to those of another study[14]. This may be due to other reasons. According to some investigators, sphincter of Oddi function in patients is normal before the onset of malignant disease. Normal function can adjust the flow of bile and pancreatic juices to maintain normal bile duct pressure. In addition, it can prevent reflux of duodenal contents. When sphincter of Oddi dysfunction occurs, it will lead to obstruction of the biliary tract and growth of bacteria. Furthermore, patients with benign diseases had higher rates of bile duct stones than those with malignant diseases in our study, which would also lead to obstruction of the biliary tract and the growth of biliary pathogenic bacteria.

A history of biliary tract diseases and surgeries was also another risk factor ($P < 0.001$). Previous research has shown that biliary tract diseases such as gallstones provide beneficial conditions for adhesion, growth, and propagation of pathogenic bacteria. In addition, the symptoms of these diseases cause disorders of bodily function and change the tissue structure, which decreases the ability to remove the bacteria. There is evidence of damage to the normal structure of the sphincter of Oddi[23] and influence on the function of bile ducts[24] due to previous surgeries such as endoscopic retrograde cholangiopancreatography. Similar surgical interventions can easily lead to duodenal–biliary reflux, mucosal hyperemia edema, and anastomosis of the biliary stricture, which creates beneficial conditions for rapid multiplication of bacteria.

We analyzed the different comorbidities of patients with high positive rates. In contrast to the findings of other studies, few of the observed comorbidities had a significant risk for positive rates[25]. We only found chronic renal insufficiency ($P = 0.007$) to be significant. The kidneys of patients with chronic renal insufficiency may have long-term serious injuries. As a result, their lymphocyte levels decrease, neutrophil function is damaged, and immune function is weakened.

In conclusion, Gram-negative bacteria were the most commonly isolated biliary bacteria. Risk factors such as age, fever, history of biliary tract diseases and surgeries, benign diseases, and comorbidities such as chronic renal insufficiency positively influenced the detection rates. Bile samples for microbiological analysis may enable a more accurate selection of antibiotic treatments.

ARTICLE HIGHLIGHTS

Research background

Biliary diseases may combine with biliary tract infection such as cholecystitis or cholangitis which possibly lead to severe sepsis and septic shock or even multiple organ dysfunction syndrome and eventually death. However, bile culture requires more time and has lower positive rates. Most related studies were conducted decades ago and lack a large sample size.

Research motivation

Using a large sample size and ten years of study, we fully understand the bacterial species and antibiotic susceptibility for antibacterial therapy in patients with biliary diseases.

Research objectives

The identification of bacterial species and their antibiotic susceptibility for early empiric antibacterial therapy are crucial for reducing the mortality of patients with biliary tract infection.

Research methods

Clinical data were collected from hospital medical records. Species identification and initial drug susceptibility were further identified by biochemical characterization using the VITEK 2 Compact test. All statistical analyses were performed using the SPSS software. Between-group analyses were conducted using the *t*-test or χ^2 test.

Research results

The most frequently encountered strains were gram-negative bacteria (74.94%), including *Escherichia coli* (37.78%), *Pseudomonas aeruginosa* (8.96%), and *Klebsiella pneumoniae* (10.29%). Bile bacteria were largely sensitive to carbapenems, piperacillin/tazobactam, and gentamicin. We found almost the same micro-organisms present in patients with malignant and benign diseases. Age ($P < 0.001$), fever ($P < 0.001$), history of biliary tract diseases and surgeries (both $P < 0.001$), benign disease ($P = 0.002$), and the comorbidity chronic renal insufficiency ($P = 0.007$) affected the positive rates of the bile samples.

Research conclusions

We found that gram-negative strains had low susceptibility to ceftriaxone, quinolones and ampicillin. In addition, some risk factors such as age, fever, history of biliary tract diseases and surgeries, benign diseases, and the comorbidity chronic renal insufficiency positively influenced the detection rates. Bile samples for microbiological analysis may enable a more accurate selection of antibiotic treatments.

Research perspectives

The risk factors for antibiotic resistance rate and bacterial resistance genes should be analyzed.

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ORIGINAL ARTICLE

Retrospective Study

Development and validation of a prediction model for microvascular invasion in hepatocellular carcinoma

Lin Wang, Yue-Xinzi Jin, Ya-Zhou Ji, Yuan Mu, Shi-Chang Zhang, Shi-Yang Pan

ORCID number: Lin Wang (0000-0003-0136-6306); Yue-Xinzi Jin (0000-0001-7817-0366); Ya-Zhou Ji (0000-0002-8291-7343); Yuan Mu (0000-0002-2759-6858); Shi-Chang Zhang (0000-0002-6587-2518); Shi-Yang Pan (0000-0001-9320-6988).

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Lin Wang, Yue-Xinzi Jin, Ya-Zhou Ji, Yuan Mu, Shi-Chang Zhang, Shi-Yang Pan, Department of Laboratory Medicine, The First Affiliated Hospital of Nanjing Medical University, Nanjing 210029, Jiangsu Province, China

Lin Wang, Yue-Xinzi Jin, Ya-Zhou Ji, Yuan Mu, Shi-Chang Zhang, Shi-Yang Pan, National Key Clinical Department of Laboratory Medicine, Jiangsu Province Hospital, Nanjing Medical University, Nanjing 210029, Jiangsu Province, China

Corresponding author: Shi-Yang Pan, MD, PhD, Professor, Department of Laboratory Medicine, The First Affiliated Hospital of Nanjing Medical University, No. 300, Guangzhou Road, Nanjing 210029, Jiangsu Province, China. sypan@njmu.edu.cn

Abstract

BACKGROUND

Microvascular invasion (MVI) is an important prognostic factor affecting early recurrence and overall survival in hepatocellular carcinoma (HCC) patients after hepatectomy and liver transplantation, but it can be determined only in surgical specimens. Accurate preoperative prediction of MVI is conducive to clinical decisions.

AIM

To develop and validate a preoperative prediction model for MVI in patients with HCC.

METHODS

Data from 454 patients with HCC who underwent hepatectomy at the First Affiliated Hospital of Nanjing Medical University between May 2016 and October 2019 were retrospectively collected. Then, the patients were nonrandomly split into a training cohort and a validation cohort. Logistic regression analysis was used to identify variables significantly associated with MVI that were then included in the nomogram. We evaluated the discrimination and calibration ability of the nomogram by using R software.

RESULTS

MVI was confirmed in 209 (46.0%) patients by a pathological examination. Multivariate logistic regression analysis identified four risk factors independently associated with MVI: Tumor size [odds ratio (OR) = 1.195; 95% confidence interval (CI): 1.107–1.290; $P < 0.001$], number of tumors (OR = 4.441; 95%CI: 2.112–9.341; $P < 0.001$), neutrophils (OR = 1.714; 95%CI: 1.036–2.836; $P = 0.036$), and serum α-fetoprotein (20–400 ng/mL, OR = 1.955; 95%CI: 1.055–3.624; $P =$

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0.033; >400 ng/mL, OR = 3.476; 95%CI: 1.950–6.195; $P < 0.001$). The concordance index was 0.79 (95%CI: 0.74–0.84) and 0.81 (95%CI: 0.74–0.89) in the training and validation cohorts, respectively. The calibration curves showed good agreement between the predicted risk by the nomogram and real outcomes.

CONCLUSION
We have developed and validated a preoperative prediction model for MVI in patients with HCC. The model could aid physicians in clinical treatment decision making.

Key words: Microvascular invasion; Nomogram; Hepatocellular carcinoma; Discrimination and calibration; Neutrophils; Early recurrence

Core tip: Microvascular invasion (MVI) is an established risk factor for early recurrence and a poor prognosis in patients with hepatocellular carcinoma, but it can be confirmed only by postoperative pathology. Our study identified four predictors independently related to MVI based mainly on laboratory parameters and established a nomogram to predict the presence of MVI preoperatively. The model showed good performance in the evaluation of discrimination and calibration ability and could help optimize treatment options in the clinic.

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INTRODUCTION

Hepatocellular carcinoma (HCC) represents one of the most common malignancies worldwide. It is the third leading cause of cancer-related deaths[1]. Hepatectomy and liver transplantation are considered the most effective treatments and provide a curative opportunity for selected patients. However, the prognosis of HCC is still poor, with a recurrence rate of more than 50% at 5 years after resection[2,3] due to frequent blood vessel invasion resulting in intrahepatic and extrahepatic metastases.

Vascular invasion is usually related to tumor metastasis, recurrence, and poor outcomes and is divided into macrovascular invasion and microvascular invasion (MVI) in HCC. Macrovascular invasion can be diagnosed by an imaging examination. Generally, patients with macrovascular invasion have no chance of radical resection or liver transplantation. In contrast, as a pathological concept, MVI can be confirmed only in surgical specimens. MVI is defined as a microscopic cancer cell nest in vessels lined with endothelial cells that is commonly observed in the small branches of the portal vein in adjacent liver tissues and occasionally in the hepatic artery, bile duct, and lymphatic vessels[4]. In the presence of MVI, tumor cells can spread and metastasize in the liver to form a portal vein tumor thrombus or multiple lesions or distant metastasis. It has been reported that the incidence of MVI in HCC patients ranges from 15% to 57%[5]. MVI is a definite factor leading to the early recurrence and poor long-term survival outcomes of HCC after resection and liver transplantation. The preoperative identification of MVI is beneficial to therapeutic decisions. Unfortunately, there is no effective and accurate prediction method before surgery.

Currently, a number of studies on the preoperative prediction of the risk of MVI in HCC and risk factors related to MVI have been carried out: The risk factors include tumor characteristics, serum tumor markers, imaging features, and gene tags. As a new strategy of combining multiple factors to predict MVI, a clinical prediction model has become a research focus. In particular, many radiomics models have been developed for diagnosing MVI preoperatively and noninvasively[6,7]. However, due to the lack of standardization in radiomics and overreliance on the subjective judgment of diagnostic radiologists, the accuracy and practicality of the radiomics model are still controversial[8]. Moreover, some radiological parameters are too specialized and

thus cannot be understood and applied by clinicians. By contrast, routine laboratory tests are more common and easy to control and standardize, and data from different sources are accurate and comparable.

The purpose of this study was to identify clinical variables significantly associated with the risk of MVI and develop and validate a new clinical prediction model for the presence of MVI in patients with HCC before hepatectomy based on laboratory parameters.

MATERIALS AND METHODS

Study design and participants

We retrospectively searched the hepatosurgical database of the First Affiliated Hospital of Nanjing Medical University from May 2016 to October 2019 to identify patients who were diagnosed with HCC histologically and underwent hepatic resection. The diagnosis of HCC followed the Guidelines for Diagnosis and Treatment of Primary Liver Cancer in China (2017 Edition)[4]. The inclusion criteria were as follows: (1) Age 18 years or older; (2) Underwent hepatectomy; and (3) Diagnosed with HCC confirmed by histology. The exclusion criteria were as follows: (1) History of HCC treatment; (2) Received antiviral treatment within 3 mo preoperatively; (3) Preoperative overt bacterial infection or trauma within 2 weeks; (4) History of other cancers; (5) Unclear pathologic diagnosis of MVI; and (6) Incomplete laboratory data. Finally, eligible patients were included in the study. Data on HCC patients collected from May 2016 to March 2019 were used as the training dataset, and data on HCC patients collected from April 2019 to October 2019 were used as the validation dataset. The current study was approved by the Institutional Ethics Committee of the First Affiliated Hospital of Nanjing Medical University.

Clinicopathological variables

All patients received a routine preoperative examination within 2 wk before hepatectomy that included whole blood count, blood biochemistry, coagulation function, hepatitis B immunology, and serum α-fetoprotein (AFP) tests and an imaging examination [abdominal ultrasonography, computed tomographic scan of the abdomen, and contrast-enhanced magnetic resonance imaging (MRI)]. Anatomic or nonanatomic resection was performed after the clinical evaluation, and all the obtained surgical specimens were histologically assessed to determine the presence of MVI and the Edmondson-Steiner grade by two pathologists. As previously described, MVI refers to the presence of tumor cell clusters in the blood vessels lined with endothelial cells only under microscopic observation. Imaging parameters mainly included the number of tumors and tumor size. For the derivative indicators involved, the neutrophil–lymphocyte ratio (NLR), platelet–lymphocyte ratio (PLR), and systemic immune-inflammation index (SII) were calculated as follows: NLR = neutrophil count/lymphocyte count, PLR = platelet count/lymphocyte count, and SII = platelet count × neutrophil count/lymphocyte count. The albumin-bilirubin (ALBI) grade was computed by the following formula: $0.66 \times \log_{10}$ (bilirubin μmol/L) − 0.085 × (albumin g/L). According to the previously described cut-off points, the patients were divided into three grades: ALBI grade 1 (≤ -2.60), ALBI grade 2 (> -2.60 to -1.39) and ALBI grade 3 (> -1.39)[9]. Serum AFP and hepatitis B immunology were measured by electrochemiluminescence immunoassays using a Cobas e602 automated analyzer (Roche, Germany). A Sysmex XN series automated hematology analyzer (Sysmex, Japan) and a Sysmex CS5100 automated blood coagulation analyzer (Sysmex, Japan) were used to determine the complete blood count and coagulation function, respectively. A Beckman Coulter AU5800 analyzer (Beckman Coulter, United States) was used to determine blood biochemistry.

Statistical analysis

Categorical variables are displayed as the number and percentage, and continuous variables are presented as the median [interquartile range (IQR)]. Categorical variables were compared using the chi-square test or Fisher's exact test. Comparisons of continuous variables between two different groups were conducted using the Mann-Whitney test. A univariate logistic regression analysis was used to assess the significance of each variable in the training cohort for the prediction of MVI. All variables with $P < 0.05$ in the univariate logistic regression analysis were incorporated into a multivariate logistic regression analysis. The nomogram for the prediction of MVI was established based on the results of the multivariate logistic regression analysis by using the rms package of R, version 3.6.1 (http://www.r-project.org/). To evaluate the prediction performance of the nomogram, we calculated the concordance

index (C-index) with 1000 bootstrap samples to measure discrimination (the model's ability to distinguish between HCC patients with and without MVI) and generated calibration plots to measure calibration (consistency between the predicted probability and observed frequency of patients with MVI). The optimal cut-off value of the nomogram was determined by maximizing the Youden index. Additionally, we performed decision curve analysis (DCA) to evaluate the clinical usefulness and net benefits of the developed model. A *P* value less than 0.05 was considered statistically significant. All statistical analyses were performed using SPSS software, version 22 (SPSS, Inc., Chicago, IL) and R, version 3.6.1 (http://www.r-project.org/). This report followed the Transparent Reporting of a Multivariable Prediction Model for Individual Prognosis Or Diagnosis (TRIPOD) guidelines[10].

RESULTS

Patient characteristics

During our study period, a total of 522 patients were diagnosed with HCC histologically and underwent hepatectomy. Ultimately, 454 patients met the inclusion criteria. Among them, 339 patients whose data were collected between May 2016 and March 2019 formed the training cohort, and 115 patients whose data were collected between April and October 2019 formed the validation cohort (Figure 1). The clinicopathologic characteristics of the patients are summarized in Table 1. The median ages of patients in the training and validation cohorts were 57 and 59 years, respectively. The number of male patients was significantly higher than that of female patients. Approximately 80% of all patients had hepatitis B virus (HBV) infection. The histological examination confirmed MVI in 157 (46.3%) patients in the training cohort and 52 (45.2%) patients in the validation cohort. There was no significant difference in the distribution of variables between the training and validation cohorts except for the red blood cell distribution width (RDW), albumin (ALB), and ALBI grade.

Preoperative predictors of MVI

The results of the univariate logistic regression analysis of the clinical features in the training cohort are shown in Table 2. Tumor size [odds ratio (OR) = 1.214; 95% confidence interval (CI): 1.155–1.332; $P < 0.001$], number of tumors (OR = 5.174; 95%CI: 2.611–10.252; $P < 0.001$), serum AFP (for 20–400 *vs* ≤ 20 ng/mL, OR = 1.936; 95%CI: 1.100–3.407; $P = 0.022$; for ≥400 *vs* ≤ 20 ng/mL, OR = 4.546; 95%CI: 2.687–7.691; $P < 0.001$), neutrophils (OR = 1.989; 95%CI: 1.289–3.069; $P = 0.002$), NLR (OR = 1.927; 95%CI: 1.244–2.983; $P = 0.003$), PLR (OR = 1.945; 95%CI: 1.261–3.000; $P = 0.003$), SII (OR = 2.170; 95%CI: 1.404–3.352; $P < 0.001$), and ALP (OR = 1.677; 95%CI: 1.078–2.610; $P = 0.022$) were significant preoperative risk factors associated with MVI in the univariate analysis, and all these predictors with a *P* value less than 0.05 were selected for the multivariate analysis. In the multivariate analysis, multiple tumors (OR = 4.441; 95%CI: 2.112–9.341; $P < 0.001$), large tumor size (OR = 1.195; 95%CI: 1.107–1.290; $P < 0.001$), high neutrophil level (OR = 1.714; 95%CI: 1.036–2.836; $P = 0.036$), and high serum AFP level (for 20–400 *vs* ≤ 20 ng/mL, OR = 1.955; 95%CI: 1.055–3.624; $P = 0.033$; for ≥400 *vs* ≤ 20 ng/mL, OR = 3.476; 95%CI: 1.950–6.195; $P < 0.001$) were independently associated with the presence of MVI (Table 3).

Development and validation of a nomogram for preoperative MVI prediction

Based on the results of the multivariate analysis, we chose tumor size, number of tumors, neutrophils, and serum AFP for model development. The nomogram for predicting the presence of MVI in patients with HCC preoperatively is presented in Figure 2. The probability of MVI can be estimated by using this nomogram to calculate the total points for each patient. Further analysis indicated that the nomogram has excellent performance in distinguishing the absence or presence of MVI. In the training cohort, the C-index was 0.79 (95%CI: 0.74–0.84), and in the validation cohort, the C-index was 0.81 (95%CI: 0.74–0.89). According to the maximum Youden index, the optimal cut-off value for the prediction probability of the nomogram was 0.40. The sensitivity, specificity, negative predictive value, and positive predictive value when the model was used to differentiate between the presence and absence of MVI were 77.7%, 70.9%, 78.7%, and 69.7%, respectively, in the training cohort and 69.2%, 68.3%, 72.9%, and 64.3%, respectively, in the validation cohort (Table 4).

In addition, we generated calibration curves to evaluate the calibration of the prediction model. Calibration curves demonstrated acceptable model calibration, with good agreement between the observed frequency and predicted probability of patients with MVI in both datasets (Figure 3). Figure 4 illustrates the decision curves

Table 1 Comparison of participant characteristics in the training and validation cohorts

Characteristic	Training cohort (*n* = 339)	Validation cohort (*n* = 115)	*P* value
Median age (IQR), yr	57 (49, 65)	59 (51, 67)	0.141
Gender			
Male	284 (83.8)	104 (90.4)	0.080
Female	55 (16.2)	11 (9.6)	
Tumor size, cm	4.5 (3.0, 8.0)	4.0 (2.5, 7.0)	0.095
Number of tumors			
Single	285 (84.1)	89 (77.4)	0.104
Multiple	54 (15.9)	26 (22.6)	
Child-Pugh grade			
A	315 (92.9)	104 (90.4)	0.388
B	24 (7.1)	11 (9.6)	
Clinical stage			
I	241 (71.1)	80 (69.6)	0.727
II	86 (25.4)	29 (25.2)	
III	12 (3.5)	6 (5.2)	
Etiology			
Hepatitis B	253 (74.6)	93 (80.9)	0.175
Non-hepatitis B	86 (25.4)	22 (19.1)	
AFP, ng/mL			
≤ 20	142 (41.9)	46 (40.0)	0.122
20–40	81 (23.9)	38 (33.0)	
≥ 400 L	116 (34.2)	31 (27.0)	
WBC, 10^9/L			
≤ 4.0	83 (24.5)	33 (28.7)	0.371
> 4.0	256 (75.5)	82 (71.3)	
Neutrophils, 10^9/L			
≤ 3.0	167 (49.3)	65 (56.5)	0.178
> 3.0	172 (50.7)	50 (43.5)	
PLT, 10^9/L			
≤ 125	128 (37.8)	44 (38.3)	0.923
> 125	211 (62.2)	71 (61.7)	
RDW			
≤ 13.0	119 (35.1)	54 (47.0)	0.024
> 13.0	220 (64.9)	61 (53.0)	
NLR			
≤ 2.0	150 (44.2)	60 (52.2)	0.141
> 2.0	189 (55.8)	55 (47.8)	
PLR			
≤ 100	166 (49.0)	65 (56.5)	0.161
> 100	173 (51.0)	50 (43.5)	
SII			
≤ 300	173 (51.0)	66 (57.4)	0.238
> 300	166 (49.0)	49 (42.6)	
PT, sec			
≤ 13.0	250 (73.7)	92 (80.0)	0.179
> 13.0	89 (26.3)	23 (20.0)	
FIB, g/L			
≤ 2.0	90 (26.5)	24 (20.9)	0.225
> 2.0	249 (73.5)	91 (79.1)	
ALB, g/L			
≤ 40	192 (56.6)	50 (43.5)	0.015
> 40	147 (43.4)	65 (56.5)	

ALT, U/L			
≤ 40	204 (60.2)	77 (67.0)	0.196
> 40	135 (39.8)	38 (33.0)	
AST, U/L			
≤ 35	160 (47.2)	62 (53.9)	0.213
> 35	179 (52.8)	53 (46.1)	
GGT, U/L			
≤ 45	120 (35.4)	38 (33.0)	0.647
> 45	219 (64.6)	77 (67.0)	
TB, μmol/L			
≤ 19	257 (75.8)	85 (73.9)	0.683
> 19	82 (24.2)	30 (26.1)	
ALP, g/L			
≤ 120	210 (61.9)	71 (61.7)	0.968
> 120	129 (38.1)	44 (38.3)	
GLU, mmol/L			
≤ 6.1	278 (82.0)	97 (84.3)	0.567
> 6.1	61 (18.0)	18 (15.7)	
ALBI grade			
1	164 (48.4)	71 (61.7)	0.017
2	171 (50.4)	41 (35.7)	
3	4 (1.2)	3 (2.6)	
MVI			
Absent	182 (53.7)	63 (54.8)	0.839
Present	157 (46.3)	52 (45.2)	
Edmondson-Steiner classification			
I–II	142 (41.9)	43 (37.4)	0.396
III–IV	197 (58.1)	72 (62.6)	

IQR: Interquartile range; AFP: α-fetoprotein; WBC: White blood cells; PLT: Platelets; RDW: Red blood cell distribution width; NLR: Neutrophil–lymphocyte ratio; PLR: Platelet–lymphocyte ratio; SII: Systemic immune-inflammation index; PT: Prothrombin time; FIB: Fibrinogen; ALB: Albumin; ALT: Alanine aminotransferase; AST: Aspartate transaminase; GGT: γ-glutamyltransferase; TB: Total bilirubin; ALP: Alkaline phosphatase; GLU: Glucose; ALBI: Albumin-bilirubin; MVI: Microvascular invasion.

for the clinical model to predict the correct diagnosis of MVI in patients with HCC in both cohorts. DCA was used to evaluate the net benefit under different clinical decisions at a certain threshold probability. The model was useful between threshold probabilities of 48%–89%.

DISCUSSION

According to the statistical analysis of nonrandom split-data from a large retrospective cohort, we developed and validated a new preoperative prediction model for MVI in patients with HCC. The obtained nomogram could effectively distinguish between patients with and without MVI preoperatively and showed good agreement between the predicted probability and actual frequency of MVI.

MVI is common in HCC and reflects the high invasion and metastasis capacities of the tumor early. Even in small HCCs (< 3 cm), the incidence of MVI is still above 20%[11,12], and MVI is an important hidden danger of postoperative recurrence and poor outcomes. The Guidelines for Diagnosis and Treatment of Primary Liver Cancer in China (2017 Edition) emphasize that MVI is an important basis for assessing the risk of recurrence of HCC and the choice of treatment options and should be used as a routine pathological examination index[4]. In our study, the incidence of MVI was close to 46% in a total of 454 patients, and the incidence in small HCCs was 21.4%, consistent with the literature.

Additionally, the presence of MVI often affects the choice of clinical treatment options and postoperative efficacy. Cucchetti *et al*[13] reported that, compared with nonanatomical resection, anatomical resection can reduce the early recurrence rate after hepatic resection for early-stage HCC patients with poor differentiation or with MVI. Mazzaferro *et al*[14] demonstrated that preoperative assessment of the absence of

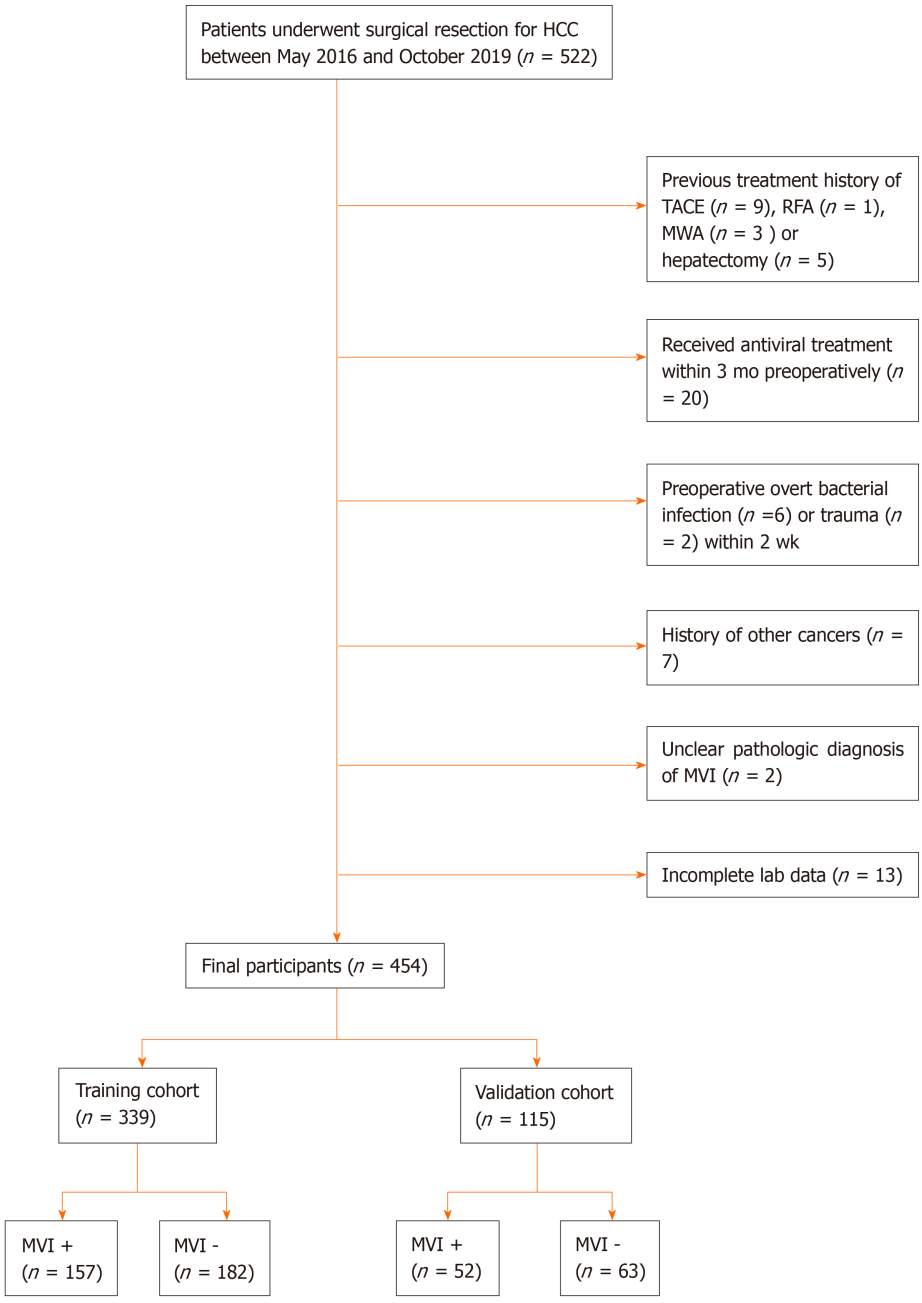

Figure 1 Flow chart of the study population. HCC: Hepatocellular carcinoma; TACE: Transcatheter arterial chemoembolization; RFA: Radiofrequency ablation; MWA: Microwave ablation; MVI: Microvascular invasion.

MVI is crucial for selecting candidates for transplantation. For patients without MVI, the Milan criteria can be expanded to achieve the same expected survival outcomes as patients within the Milan criteria, whereas the presence of MVI doubles the hazard of recurrence and death. Vitale *et al*[15] reported that MVI has a strong negative impact on the benefit of liver transplantation and that hepatic resection should be preferred to liver transplantation in HCC patients within the Milan criteria who are predicted to be at high risk for MVI before surgery. Therefore, how to accurately predict MVI to optimize the treatment plan is the main problem faced by surgeons. However, there is no uniform scheme or standard for the preoperative prediction of MVI both in China and other countries.

Previous studies have confirmed that tumor diameter, number of tumors, AFP, protein induced by vitamin K absence or antagonist-II (PIVKA-II), inflammation-related indicators, *etc*. are independent risk factors for MVI, but the univariate analyses lack sensitivity and specificity for MVI prediction, resulting in limited clinical applications. Therefore, some clinical prediction models that combine clinical features, laboratory parameters, and imaging characteristics have been established to

Table 2 Univariate logistic regression analysis of preoperative data for microvascular invasion presence in the training cohort

Variable	OR (95%CI)	*P* value
Age, yr	0.981 (0.962–1.001)	0.062
Gender, male *vs* female	1.488 (0.823–2.689)	0.188
Number of tumors, multiple *vs* single	5.174 (2.611–10.252)	< 0.001
Tumor size, cm	1.214 (1.155–1.332)	< 0.001
Etiology, non-hepatitis B *vs* hepatitis	0.837 (0.511–1.370)	0.479
AFP, ng/mL		
20–40 *vs* ≤ 20	1.936 (1.100–3.407)	0.022
≥ 400 *vs* ≤ 20	4.546 (2.687–7.691)	< 0.001
WBC, 10^9/L, > 4.0 *vs* ≤ 4.0	1.117 (0.711–1.927)	0.537
Neutrophils, 10^9/L, >3.0 *vs* ≤ 3.0	1.989 (1.289–3.069)	0.002
PLT, 10^9/L, > 125 *vs* ≤ 125	1.375 (0.883–2.143)	0.159
RDW, > 13.0 *vs* ≤ 13.0	1.116 (0.713–1.748)	0.630
NLR, > 2.0 *vs* ≤ 2.0	1.927 (1.244–2.983)	0.003
PLR, > 100 *vs* ≤ 100	1.945 (1.261–3.000)	0.003
SII, > 300 *vs* ≤ 300	2.170 (1.404–3.352)	< 0.001
PT, sec, > 13 *vs* ≤ 13	1.514 (0.931–2.462)	0.094
ALB, g/L, > 40 *vs* ≤ 40	0.949 (0.617–1.460)	0.812
ALT, U/L, > 40 *vs* ≤ 40	0.882 (0.570–1.366)	0.575
AST, U/L, > 35 *vs* ≤ 35	1.275 (0.831–1.958)	0.266
GGT, U/L, > 45 *vs* ≤ 45	1.486 (0.947–2.334)	0.085
TB, μmol/L, > 19 *vs* ≤ 19	1.297 (0.788–2.133)	0.307
ALP, U/L, > 120 *vs* ≤ 120	1.677 (1.078–2.610)	0.022
FIB, g/L, > 2.0 *vs* ≤ 2.0	1.397 (0.852–2.290)	0.185
GLU, mmol/L, > 6.1 *vs* ≤ 6.1	0.904 (0.518–1.579)	0.723
ALBI grade, 1 *vs* 2 and 3	1.266 (0.825–1.942)	0.281

AFP: α-fetoprotein; WBC: White blood cells; PLT: Platelets; RDW: Red blood cell distribution width; NLR: Neutrophil–lymphocyte ratio; PLR: Platelet–lymphocyte ratio; SII: Systemic immune-inflammation index; PT: Prothrombin time; FIB: Fibrinogen; ALB: Albumin; ALT: Alanine aminotransferase; AST: Aspartate transaminase; GGT: γ-glutamyltransferase; TB: Total bilirubin; ALP: Alkaline phosphatase; GLU: Glucose; ALBI: Albumin-bilirubin; OR: Odds ratio; CI: Confidence interval.

make accurate MVI predictions more likely. Lei *et al*[16] developed a nomogram that combines seven factors, namely, nodule number, tumor diameter, capsule, serum AFP, platelet count, hepatitis B virus DNA load, and typical dynamic pattern of tumors on contrast-enhanced MRI, for the preoperative prediction of MVI in HBV-related HCC within the Milan criteria. Xu *et al*[17] created a new algorithm based on large-scale clinico-radiologic and radiomic features, including AST, AFP, tumor margin, growth pattern, capsule, peritumoral enhance, radio-genomic venous invasion and, radiomic score, that showed good performance in predicting MVI for patients with HCC.

As opposed to radiomic characteristics, our model was built from routine laboratory parameters and has potential advantages in standardization and popularization. In our report, tumor size, number of tumors, neutrophil count, and serum AFP were identified as independent risk factors significantly associated with MVI. Although PIVKA-II has been reported to be a predictor of MVI[18], it was eliminated as an initial variable in the data analysis, because PIVKA-II is not a routine laboratory test for HCC in our institution. HBV is the most important leading cause of HCC in China, whereas our results indicate that 20% of HCC is unrelated to HBV. The univariate analysis showed no significant difference between non-HBV- and HBV-related HCC. Compared to previous studies that limited the predicted population to patients with HBV-related HCC, our nomogram has a greater application scope.

Another notable predictor included was neutrophils. A number of circulating inflammatory markers from routine laboratory parameters, such as neutrophil, lymphocyte, and platelet counts and combined inflammatory scores, have been reported to have prognostic or clinically predictive value in patients with HCC[19]. As the most reported inflammatory score, NLR has been included in some nomograms to

Table 3 Multivariate logistic regression analysis of preoperative data for microvascular invasion presence in the training cohort

Variable	β	OR (95%CI)	*P* value
Number of tumors, multiple *vs* single	1.491	4.441 (2.112–9.341)	< 0.001
Tumor size, cm	0.178	1.195 (1.107–1.290)	< 0.001
Neutrophils, 10^9/L, > 3.0 *vs* ≤ 3.0	0.539	1.714 (1.036–2.836)	0.036
AFP, ng/mL			
20–400 *vs* ≤ 20	0.670	1.955 (1.055–3.624)	0.033
≥ 400 *vs* ≤ 20	1.246	3.476 (1.950–6.195)	< 0.001

AFP: α-fetoprotein; OR: Odds ratio; CI: Confidence interval.

calculate the prediction probability of MVI in patients with HCC before surgery[20,21]. In our report, the neutrophil count was independently associated with MVI, and this is currently rarely reported. Hepatocarcinogenesis has been proven to be inextricably linked to inflammation. Most HCCs are accompanied by a background of chronic liver disease. Although the etiology and mechanisms vary, inflammation in HCC is uniform. The intricate interaction between the tumor itself and its microenvironment and the host immune response forms the basis for the progression of inflammation-driven HCC. A large multicenter cohort study demonstrated that a high level of neutrophils is the only significant and independent risk factor for driving progression and a poor prognosis in HCC compared to lymphocytes and platelets[22]. In recent years, the mechanism by which neutrophils exert protumoral activity has gradually been revealed. Neutrophils may be classified into several subtypes due to phenotypic switching mediated by the tumor microenvironment and show polarization, plasticity, and protumor/antitumor functions[23]. It has been proven that tumor-associated neutrophils can promote the development of HCC and therapeutic resistance by recruiting macrophages and Treg cells. Furthermore, neutrophils can form neutrophil extracellular traps (NETs) to capture circulating tumor cells and promote tumor metastasis[24-26]. The above evidence indicates the important role of neutrophils in the development of HCC, which requires attention and further research.

From a clinical point of view, surgeons often have to consider both risks and benefits when making treatment decisions. As mentioned above, MVI-positive patients who undergo anatomical resection to reduce the recurrence rate also face the risks of bleeding and liver failure due to the large resection range. Another issue to address is the allocation between the selection of suitable transplant candidates and the scarce liver resources in reality. Therefore, through clinical decision analysis, we provide the threshold probability range of the model with clinical net benefit that could help clinicians balance the risks and benefits under different conditions.

Undeniably, our study still had some limitations. First, all the data analyzed in this study were obtained from a single institution, and data from other centers are needed to further verify the reliability of the model. Second, the neutrophil count was considered an important predictor in our study. As a common inflammatory marker in peripheral blood, a rise in neutrophil levels usually occurs due to infections or injuries. However, "antiviral treatment", "infection", and "trauma" were set as the only three control conditions in our model. In fact, the neutrophil count can also fluctuate under the influence of various factors, such as time, eating, exercise, pain, and emotion. How to avoid or correct the effects of these factors on neutrophils is a challenge.

In conclusion, we have developed and validated a preoperative prediction model for MVI in patients with HCC. With the inclusion of two tumor features (number of tumors and tumor size) and two laboratory parameters (serum AFP and neutrophil count), our prediction model could effectively differentiate between HCC patients with and without MVI and provide a reliable basis for clinicians to optimize preoperative decisions.

Table 4 Accuracy of the nomogram in predicting the risk of microvascular invasion at the optimal threshold value

Variable	Value (95%CI)	
	Training cohort	Validation cohort
Sensitivity, %	77.7 (71.1–84.3)	69.2 (56.3–82.2)
Specificity, %	70.9 (64.2–77.5)	68.3 (56.4–80.1)
Positive predictive value, %	69.7 (62.8–76.6)	64.3 (51.3–77.2)
Negative predictive value, %	78.7 (72.3–85.0)	72.9 (61.2–84.6)
Positive likelihood ratio	2.67 (2.10–3.40)	2.18 (1.45–3.27)
Negative likelihood ratio	0.31 (0.23–0.42)	0.45 (0.30–0.69)
Concordance index	0.79 (0.74–0.84)	0.81 (0.74–0.89)
Predicted probability[1]	0.40	0.40

[1]Predicted probability refers to the optimal cut-off value for microvascular invasion prediction based on the maximum Youden index. CI: Confidence interval.

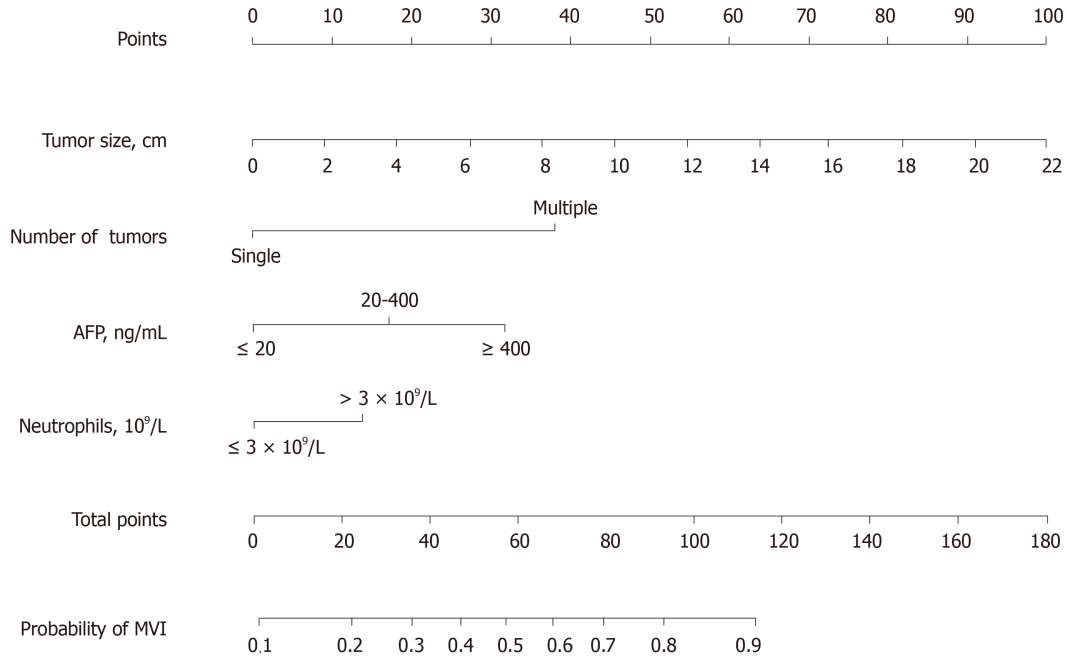

Figure 2 Nomogram for predicting the presence of microvascular invasion preoperatively in patients with hepatocellular carcinoma. When using the nomogram, find the position of each variable on the axis and the corresponding point vertically. Then, add the points of all variables, and determine the prediction probability of microvascular invasion on the bottom axis. AFP: α-fetoprotein; MVI: Microvascular invasion.

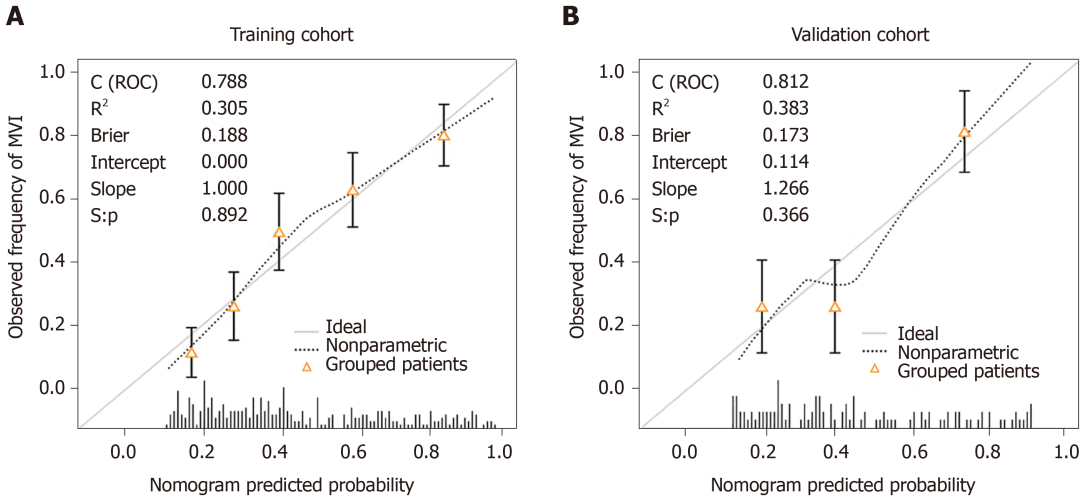

Figure 3 Calibration curves of the clinical prediction model. A: Calibration plot for predicting microvascular invasion in the training cohort; B: Calibration plot for predicting microvascular invasion in the validation cohort.

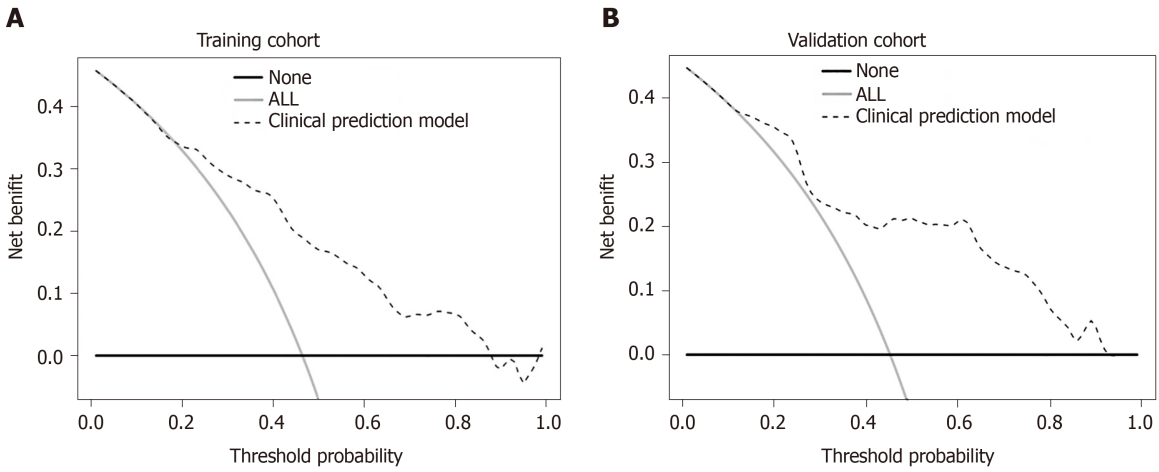

Figure 4 Decision curve analysis for the prediction model. The gray and black lines indicate patients that were microvascular invasion (MVI) positive or negative, respectively. The dashed line represents the net benefit of the nomogram at different threshold probabilities. The net clinical benefit was calculated as the true-positive rate minus the weighted false-positive rate. A: Decision curve analysis for MVI in the training cohort; B: Decision curve analysis for MVI in the validation cohort.

ARTICLE HIGHLIGHTS

Research background

Microvascular invasion (MVI) is a definite risk factor of early recurrence and poor surgical outcomes of hepatocellular carcinoma (HCC). Accurate preoperative prediction of MVI is helpful for the choice of clinical treatment options and evaluation of postoperative efficacy.

Research motivation

Histologic examination of the surgical specimens is the only reliable method to diagnose MVI. There is an urgent need for an effective tool to predict MVI preoperatively.

Research objectives

This study aimed to construct a new prediction model, mainly based on routine laboratory parameters, to achieve more accurate prediction for MVI in patients with HCC before surgery.

Research methods

In this retrospective study, data from 454 patients with HCC who underwent hepatectomy were collected and nonrandomly split into a training cohort and a validation cohort. Univariate and multivariable logistic regression analyses were performed to identify variables significantly associated with MVI, and a new preoperative prediction model for MVI was established and further validated.

Research results

The incidence of MVI was 46.0% in patients with hepatectomy. Tumor size, number of tumors, neutrophils, and serum α-fetoprotein were identified as independent significant factors associated with MVI. A nomogram was established and showed good performance in the evaluation of discrimination and calibration.

Research conclusions

This prediction model could effectively predict MVI with good discrimination and calibration ability.

Research perspectives

Data from other centers are needed to further validate the clinical usability of this novel model.

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ORIGINAL ARTICLE

Prospective Study

Macrophage inhibitory cytokine-1/growth differentiation factor-15 in premalignant and neoplastic tumours in a high-risk pancreatic cancer cohort

Robert Sean O'Neill, Sam Emmanuel, David Williams, Alina Stoita

ORCID number: Robert Sean O'Neill (0000-0002-9576-2248); Sam Emmanuel (0000-0003-1022-1539); David Williams (0000-0002-0808-0311); Alina Stoita (0000-0001-9460-2149).

Author contributions: O'Neill RS contributed to the writing of the manuscript; Emmanuel S provided the statistical analysis; Stoita A and Williams D as senior authors designed the prospective study, performed screening and revised the manuscript.

Institutional review board statement: The study protocol was reviewed and approved by the Ethics Committees of St Vincent's Hospital, Sydney, NSW, Australia.

Clinical trial registration statement: This is not a clinical trial and therefore does not need to be registered under the clinical trials. Registration applies only to randomised control trials. There is no control arm, nor health intervention, the study looks at a new biomarker in an established pancreatic screening program. Results are not given to the patients and there is no intervention.

Informed consent statement: All study participants provided written informed consent prior to study enrolment.

Conflict-of-interest statement: The authors declare no competing interests.

Robert Sean O'Neill, David Williams, Alina Stoita, Department of Gastroenterology, St Vincent's Hospital, Sydney 2010, Australia

Sam Emmanuel, Alina Stoita, St Vincent's Clinical School, Faculty of Medicine, University of New South Wales, Sydney 2010, Australia

Corresponding author: Alina Stoita, FRACP, MBBS, Assistant Lecturer, Doctor, Department of Gastroenterology, St Vincent's Hospital Sydney, 390 Victoria Street, Sydney 2010, Australia. alina.stoita@svha.org.au

Abstract

BACKGROUND

Pancreatic cancer (PC) is a leading cause of cancer related mortality worldwide, with poor survival due to late diagnosis. Currently, biomarkers have limited use in early diagnosis of PC. Macrophage inhibitory cytokine-1 or growth differentiation factor-15 (MIC-1/GDF15) has been implicated as a potential serum biomarker in PC and other malignancies.

AIM

To determine the role of MIC-1/GDF15 in detecting pre-malignant pancreatic lesions and neoplastic tumours in an asymptomatic high-risk cohort part of Australian Pancreatic Cancer Screening Program.

METHODS

A feasibility prospective single centre cohort study was performed. Participants recruited for yearly surveillance with endoscopic ultrasound (EUS) had serial fasting blood samples collected before EUS for MIC-1/GDF15, C-reactive protein and carbohydrate antigen 19-9. Patients were stratified into five groups based on EUS findings: Normal; pancreatic cysts, branch-duct intraductal papillary mucinous neoplasm; diffuse non-specific abnormalities; and neoplastic tumours. MIC-1/GDF15 serum levels were quantified using ELISA. Participants in whom EUS demonstrated abnormalities but not malignancy were closely followed up with magnetic resonance imaging (MRI) or computed tomography.

RESULTS

One hundred twenty participants were prospectively recruited from 2011-2018. Forty-seven participants (39.2%) had an abnormal EUS and five participants (4.2%) were diagnosed with neoplastic tumours, three by EUS (two pancreatic

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and one liver) and two by MRI/computed tomography (breast cancer, bladder cancer), which were performed for follow up of abnormal EUS. Baseline serum MIC-1/GDF15 was a significant predictor of neoplastic tumours on receiver operator characteristic curve analysis [area under curve (AUC) = 0.814, P = 0.023]. Baseline serum MIC-1/GDF15 had moderate predictive capacity for branch-duct intraductal papillary mucinous neoplasm (AUC = 0.644) and neoplastic tumours noted on EUS (AUC = 0.793), however this was not significant (P = 0.188 and 0.081 respectively). Serial serum MIC-1/GDF15 did not demonstrate a significant percentage change between a normal and abnormal EUS (P = 0.213). Median baseline MIC-1/GDF15 was greater in those with neoplastic tumours (Median = 1039.6, interquartile range = 727.0-1977.7) compared to those diagnosed with a benign lesion (Median = 570.1, interquartile range = 460.7-865.2) on EUS and MRI (P = 0.012).

CONCLUSION
In this pilot study MIC-1/GDF15 has predictive capacity for neoplastic tumours in asymptomatic individuals with a genetic predisposition for PC. Further imagining may be warranted in patients with abnormal EUS and raised serum MIC-1/GDF15. Larger multicentric prospective studies are required to further define the role of MIC-1/GDF15 as a serological biomarker in pre-malignant pancreatic lesions and neoplastic tumours.

Key words: Growth differentiation factor 15; Cytokines; Pancreatic neoplasms; Digestive system neoplasms; Pancreatic diseases; Biomarkers; Diagnostic screening programs

Core tip: In this prospective cohort study in an asymptomatic population at high risk of developing pancreatic cancer due to a genetic predisposition serum baseline macrophage inhibitory cytokine-1 or growth differentiation factor-15 was shown to be a significant predictor of neoplastic tumours (both pancreatic and extra-pancreatic).

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INTRODUCTION

Macrophage inhibitory cytokine-1 (MIC-1), also known as growth differentiation factor-15 (GDF-15) is a distant member of the transforming growth factor (TGF-b) superfamily of cytokines, with its original role being identified as a gene expressed in the context of macrophage activation[1,2]. MIC-1/GDF15 is present in the serum of all individuals with a wide normal range 150-1150 pg/mL[3]. MIC-1/GDF15 has been implicated in regulation of inflammation, metabolism and carcinogenesis, with previous literature demonstrating serum elevation in acute inflammatory conditions, congestive heart failure, renal failure and anti-inflammatory use[4-7]. More recent studies have focused on its role in malignancy, being one of the few secreted proteins induced by p53 activation and its expression was initially postulated to stimulate apoptosis in cancer cells[8-10]. More recently it was suggested that MIC-1/GDF15 directly modulates the biology of tumour progression from initial tumorigenesis to metastasis[11]. In addition to this, MIC-1/GDF15 protein and mRNA was noted to be elevated both in cancer tissue specimens along with peripheral serum samples. MIC-1/GDF15 has been implicated in colorectal cancer, with serum levels being elevated in patients with premalignant colonic polyps, and subsequently increasing with disease progression, including metastasis, along with predicting disease outcome[12-15]. In addition to this, other studies have identified a potential role of MIC-1/GDF15 in prostate[16], breast[17], pancreatic[18-20], ovarian[21], endometrial[22] and lung cancer[23]. Although the role of MIC-1/GDF15 as a biomarker in malignancy has been explored, there is still ongoing discussion regarding its precise function in malignancy, with

researchers hypothesising that MIC-1/GDF15 enhances anti-tumour immunity in the early stages of malignancy, along with stimulating tumour cell spread through promoting tumour angiogenesis as demonstrated in oesophageal squamous cell carcinoma[24].

When analysing the role of MIC-1/GDF15 in pancreatic cancer (PC), at a molecular level it has been demonstrated to promote pancreatic cell invasion through its interaction with the transcription factor Twist1[25]. In the clinical domain, MIC-1/GDF15 has been demonstrated to be elevated in the serum of PC patients compared to both healthy controls and those with benign pancreatic tumours, as well as being reported to be beneficial in the diagnosis of pancreatic adenocarcinoma[18,26]. While few individual studies show that MIC-1/GDF15 is more sensitive than carbohydrate antigen 19-9 (CA19-9) in the diagnosis of PC, a meta-analysis[27] published in 2018 shows that MIC-1/GDF15 has a comparable diagnostic accuracy to CA19-9 in diagnosis of PC. Further preliminary studies have demonstrated that MIC-1/GDF15 is superior to CA19-9 in differentiating PC from chronic pancreatitis and when used in combination with CA19-9 it improves further the diagnostic accuracy of differentiating PC form chronic pancreatitis and healthy controls[27-29]. A recent meta-analysis published the diagnostic sensitivity and specificity for MIC-1/GDF15 in diagnosing PC as 80% and 85% respectively, with an area under curve (AUC) of 0.894[27]. In addition to this, MIC-1/GDF15 was found to have a positive predictive value of 78.3%, and a negative predictive value of 78.6%[30,31].

In light of the current emerging evidence that advocates for MIC-1/GDF15 as a potential serological marker of malignancy, the aim of this study was to determine the value of MIC-1/GDF15 as a serological marker of pancreatic pre-malignant lesions and neoplastic tumours in an asymptomatic high-risk population being screened for pancreatic malignancy in an established PC screening program.

MATERIALS AND METHODS

Eligible participants were enrolled in the Australian Pancreatic Cancer Screening study for high-risk individuals performed at St Vincent's Hospital in Sydney, Australia which had started in 2011. The study was approved by St Vincent's Hospital Ethics Committee (HREC/10/SVH/33) and uses annual endoscopic ultrasound (EUS) as a screening modality. Asymptomatic individuals with a hereditary predisposition to PC were recruited between May 2011-May 2018 (Inclusion criteria Supplementary file 1). Participants were referred by Australian Family Cancer Clinics, the Australian Familial Pancreatic Cancer Registry, medical practitioners or participants had self-referred. At enrolment participants completed a questionnaire detailing past medical history, smoking and alcohol intake, and basic parameters such as height and weight. Participants were excluded from the study if they had a concurrent diagnosis of active malignancy or were not medically suitable for EUS (renal failure, congestive heart failure, human immunodeficiency virus) thus controlling for conditions that could have influenced MIC-1/GDF15 level.

MIC-1/GDF15, CA19-9 and C-reactive protein (CRP) levels were determined on a fasting 10 mL blood sample collected from the participants at the time of EUS. CRP levels was used to control for inflammatory conditions that could have increased MIC-1/GDF15 level. When malignancy was detected, EUS fine need aspiration was performed. Participants in whom EUS demonstrated abnormalities but not malignancy were closely followed up with magnetic resonance imaging (MRI) or computed tomography (CT) (if claustrophobic) and repeat EUS in 3-6 mo as per study protocol. MIC-1/GDF15, CRP and CA19-9 were repeated when a follow up EUS become abnormal.

Statistical analyses were performed using IBM SPSS statistics for Windows (Version 25.0. Armonk, NY). The baseline characteristics of the study population were stratified according to EUS findings: Normal EUS, pancreatic cyst, branch-duct intraductal papillary mucinous neoplasm (BD-IPMN), diffuse non-specific abnormalities (*e.g.*, hyperechoic foci, strands, lobularity) and solid neoplastic tumours. Further analysis was then performed on those diagnosed with neoplastic tumours on EUS and subsequent MRI/CT.

Fisher's exact test (2-tailed) was used to compare categorical characteristics between respective groups. Continuous baseline characteristics including age, body mass index (BMI), number of cigarettes smoked daily, weekly alcohol intake and age of drinking initiation were evaluated for an association with MIC-1/GDF15 serum levels using Spearman rank correlation. An ANOVA test was used to compare normally distributed continuous variables, whereas a Kruskal-Wallis test was used to compare non-normally distributed continuous variables with two or more samples.

Mann-Whitney U test was used to compare non-normally distributed continuous variables. A receiver operating characteristic curve (ROC) of MIC-1/GDF15 was generated for its ability to determine the presence or absence of pancreatic cyst, BD-IPMN, diffuse non-specific abnormality or neoplastic tumours on EUS using serum levels adjusted for variables shown to either be significantly related to MIC-1/GDF15 concentrations in this study, or have shown to correlate with MIC-1/GDF15 in previous studies. This included: Age, gender, BMI, history of colonic polyps, smoking status, alcohol use, metformin use, past history of cancer, nonsteroidal anti-inflammatory drug (NSAID), and aspirin use. All analyses performed were 2-sided and statistical significance was defined as $P < 0.05$.

RESULTS

A total of 120 asymptomatic participants based on the EUS results were stratified as follows; (1) Normal EUS (n = 74, 61.7%) as the control group; (2) Pancreatic cyst (n = 25, 20.8%); (3) BD-IPMN (n = 9, 7.5%); (4) Diffuse non-specific abnormalities (n = 9, 7.5%); and (5) Solid neoplastic tumours (n = 3, 2.5% which included pancreatic adenocarcinoma, pancreatic neuroendocrine tumour and liver cancer), outlined in Table 1. Two further neoplastic tumours: One breast cancer and a bladder cancer were identified on further imaging (MRI pancreas and CT abdomen) performed for close monitoring of a diffusely abnormal pancreas.

Study population characteristics

Table 1 outlines the baseline characteristics of the 120 subjects. The median age of participants diagnosed with BD-IPMN on EUS was higher compared to their counterparts, however this was not statistically significant (P = 0.388). There was no significant difference in the number of first-degree relatives (FDR) (P = 0.947) or second-degree relatives (SDR) diagnosed with PC (P = 0.432) between groups. The median age of those diagnosed with neoplastic tumours on EUS was higher compared to those with a normal EUS, however this was not statistically significant (P = 0.519). Furthermore, those with neoplastic tumours identified on EUS had a higher median number of cigarettes smoked per week (Median = 20) compared to the other groups, however this was not significant (P = 0.929). Participants diagnosed with neoplasia on EUS had a higher serum MIC-1/GDF15 [Median = 849.1, interquartile range (IQR) = 604.9-849.1] compared to the other groups however this was not significant (P = 0.178) but approached significance when compared to participants with a normal EUS (P = 0.061) (Figure 1). Percentage change between serial MIC-1/GDF15 was not significant in those participants who had a normal EUS and subsequent abnormal EUS (tumour, BD-IPMN, cyst, diffuse abnormality) (P = 0.213). Median serum CA19-9 was greatest in patients with an EUS indicative of malignancy, this approached significance (P = 0.058) when compared to the other groups included in the analysis.

Correlation of MIC-1/GDF15 with population variables

Baseline MIC-1/GDF15 was significantly correlated with advancing age for the entire cohort (correlation coefficient = 0.602, $P < 0.01$) and age of youngest PC diagnosis (correlation coefficient = 0.223, P = 0.015). Increasing BMI did not correlate with increasing serum MIC-1/GDF15 (P = 0.548). The number of cigarettes smoked per day, and number of drinks per week did not correlate with increased baseline serum MIC-1/GDF15 values in this population (P = 0.138 and P = 0.451 respectively).

The total number of both FDR and SDR diagnosed with PC had a significant negative correlation with baseline serum MIC-1/GDF15 (correlation coefficient = -0.190, P = 0.038). The number of FDR diagnosed with PC did not correlate with baseline serum MIC-1/GDF15 (P = 0.238), however the number of SDR diagnosed with PC had a significant negative correlation with baseline serum MIC-1/GDF15 (correlation coefficient = -0.225, P = 0.014).

Baseline serum MIC-1/GDF15 did not correlate with gender (P = 0.176), BRCA2 status (P = 0.097), ethnicity (P = 0.570) or Jewish background (P = 0.606). Further analysis of dichotomous variables demonstrated that baseline serum MIC-1/GDF15 was significantly greater in those with a history of cancer ($P < 0.001$), history of diabetes (P = 0.001), those taking oral hypoglycaemic medication (P = 0.001) and history of coronary artery disease (P = 0.005), hypercholesterolaemia (P = 0.013) and colon polyps (P = 0.005). Serum MIC-1/GDF15 levels were elevated in those participants taking aspirin regularly (P = 0.019) and metformin (P = 0.001). Baseline serum MIC-1/GDF15 was not elevated in those with regular NSAID, folate or antidepressant use (P = 0.863, 0.928 and 0.172 respectively) in this study population.

ROC curve for capacity of MIC-1/GDF15 to identify premalignant lesions on EUS

Table 1 Characteristics of participants in pancreatic cancer screening program based on endoscopic ultrasound results

Baseline characteristics	Normal EUS (*n* = 74)	Pancreatic Cyst (*n* = 25)	BD-IPMN (*n* = 9)	Diffuse abnormality (*n* = 9)	Neoplastic tumours on EUS (*n* = 3)	*P* value
Age (yr), mean (SD)	55.0 (9.8)	57.3 (7.9)	60.1 (10.0)	59.3 (8.8)	57.7 (4.5)	0.388
Age quartile, *n* (%)						
Quartile 1 (35-50)	23 (31.1)	5 (20.0)	1 (11.1)	2 (22.2)	0 (0.0)	
Quartile 2 (51-56)	17 (23.0)	7 (28.0)	3 (33.3)	1 (11.1)	1 (33.3)	
Quartile 3 (57-63)	20 (27.0)	6 (24.0)	2 (22.2)	3 (33.3)	2 (66.7)	
Quartile 4 (64-78)	14 (18.9)	7 (28.0)	3 (33.3)	3 (33.3)	0 (0.0)	
BMI, mean (SD)	27.3 (5.2)	27.8 (5.4)	26.8 (4.2)	31.6 (3.4)	24.0 (5.2)	0.117
BMI quartile, *n* (%)						0.013[1]
Quartile 1 (19.5-23.8)	18 (24.3)	6 (24.0)	4 (44.4)	0 (0.0)	2 (66.7)	
Quartile 2 (23.9-27.2)	22 (29.7)	8 (32.0)	0 (0.0)	0 (0.0)	0 (0.0)	
Quartile 3 (27.3-30.4)	18 (24.3)	6 (24.0)	2 (22.2)	3 (33.3)	1 (33.3)	
Quartile 4 (30.5-46.7)	16 (21.6)	5 (20.0)	3 (33.3)	6 (66.7)	0 (0.0)	
Gender, *n* (%)						0.362
Female	51 (68.9)	18 (72.0)	5 (55.6)	4 (44.4)	1 (33.3)	
Male	23 (31.1)	7 (28.0)	4 (44.4)	5 (55.6)	2 (66.7)	
BRCA2 positive, *n* (%)	10 (13.5)	7 (28.0)	0 (0.0)	3 (33.3)	2 (66.7)	0.032[1]
First degree relatives with PC, *n* (%)						0.947
0	3 (4.1)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	
1	43 (58.1)	16 (64.0)	4 (44.4)	5 (55.6)	2 (66.7)	
2	21 (28.4)	5 (20.0)	5 (55.6)	4 (44.4)	1 (33.3)	
3	7 (9.5)	4 (16.0)	0 (0.0)	0 (0.0)	0 (0.0)	
Second degree relative with PC, *n* (%)						0.432
0	23 (31.1)	9 (36.0)	5 (55.6)	1 (11.1)	2 (66.7)	
1	17 (23.0)	9 (36.0)	0 (0.0)	7 (77.8)	1 (33.3)	
2	20 (27.0)	3 (12.0)	2 (22.2)	1 (11.1)	0 (0.0)	
3	8 (10.8)	3 (12.0)	2 (22.2)	0 (0.0)	0 (0.0)	
4	6 (8.1)	1 (4.0)	0 (0.0)	0 (0.0)	0 (0.0)	
Youngest PC diagnosis, median (IQR)	50 (44-64.5)	60 (46-66)	65 (45.5-68.5)	53 (38-70)	75 (22-75)	0.519
Ethnicity, *n* (%)						0.848
Asian	1 (1.4)	1 (4.0)	0 (0.0)	0 (0.0)	0 (0.0)	
Caucasian	70 (94.6)	24 (96.0)	9 (100.0)	9 (100.0)	3 (100.0)	
Other	3 (4.1)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	
Jewish origin, *n* (%)	5 (6.8)	7 (28.0)	1 (11.1)	1 (11.1)	0 (0.0)	0.079
Ashkenazi	5 (7.4)	6 (24.0)	0 (0.0)	1 (11.1)	0 (0.0)	0.121
Medical history						
Personal history of cancer, *n* (%)	13 (17.6)	5 (20.0)	3 (33.3)	4 (44.4)	1 (33.3)	0.350
Diabetes, *n* (%)	4 (5.4)	1 (4.0)	1 (11.1)	2 (22.2)	0 (0.0)	0.434
Insulin, *n* (%)	0 (0.0)	0 (0.0)	0 (0.0)	1 (14.3)	0 (0.0)	0.184
Oral hypoglycaemic medication, *n* (%)	4 (7.4)	3 (16.7)	1 (16.7)	1 (14.3)	0 (0.0)	0.840
Smoking status, *n* (%)						0.188

Never smoked	32 (47.8)	17 (68.0)	5 (55.6)	6 (66.7)	2 (66.7)	
Stopped smoking	32 (47.8)	7 (28.0)	4 (44.4)	3 (33.3)	0 (.0)	
Still smoking	3 (4.5)	1 (4.0)	0 (.0)	0 (.0)	1 (33.3)	
Cigarettes per day, Median (IQR)	13.5 (6.0-20.0)	12.5 (6.3-23.8)	12.0 (1.0-12.0)	10.0 (5.0-10.0)	20.0 (20.0-20.0)	0.929
Cigarettes per day quartile, *n* (%)						0.963
Quartile 1 (1-6)	11 (30.6)	2 (25.0)	1 (33.3)	1 (33.3)	0 (0.0)	
Quartile 2 (7-12)	7 (19.4)	2 (25.0)	1 (33.3)	1 (33.3)	0 (0.0)	
Quartile 3 (15-20)	14 (38.9)	2 (25.0)	0 (0.0)	0 (0.0)	1 (100.0)	
Quartile 4 (25-75)	4 (11.1)	2 (25.0)	1 (33.3)	1 (33.3)	0 (0.0)	
Years smoking, *n* (%)						0.629
< 10	12 (33.3)	3 (37.5)	2 (50.0)	1 (33.3)	0 (0.0)	
11-20	11 (30.6)	3 (37.5)	0 (0.0)	1 (33.3)	0 (0.0)	
21-30	8 (22.2)	1 (12.5)	2 (50.0)	1 (33.3)	0 (0.0)	
31-40	4 (11.1)	1 (12.5)	0 (0.0)	0 (0.0)	1 (100.0)	
41-50	1 (2.8)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	
> 50	12 (33.3)	3 (37.5)	2 (50.0)	1 (33.3)	0 (0.0)	
Alcohol consumption, *n* (%)						0.209
Daily	19 (25.7)	7 (28.0)	0 (0.0)	3 (33.3)	2 (66.7)	
Weekly	14 (18.9)	5 (20.0)	1 (11.1)	2 (22.2)	0 (0.0)	
Social	5 (6.8)	5 (20.0)	2 (22.2)	2 (22.2)	1 (33.3)	
No history of chronic consumption	36 (48.6)	8 (32.0)	6 (66.7)	2 (22.2)	0 (0.0)	
Drinks per week, Median (IQR)	6.0 (3.0-15.0)	4 (2.0-10.0)	2.5 (1.0-6.0)	6.0 (1.0-15.0)	21.0 (1.0-21.0)	0.331
Drinks per week quartile, *n* (%)						0.328
Quartile 1 (1 - 3)	16 (25.8)	6 (31.6)	5 (62.5)	2 (28.6)	1 (33.3)	
Quartile 2 (4 - 6)	19 (30.6)	4 (21.1)	2 (25.0)	2 (28.6)	0 (0.0)	
Quartile 3 (7 - 14)	11 (17.7)	7 (36.8)	0 (0.0)	1 (14.3)	0 (0.0)	
Quartile 4 (15 - 35)	16 (25.8)	2 (10.5)	1 (12.5)	2 (28.6)	2 (66.7)	
Age of first drink, Median (IQR)	18.0 (17.0-18.0)	20.0 (18.0-25.0)	19.0 (18.0-21.0)	17.0 (15.0-20.0)	18.0 (15.0-18.0)	0.033[1]
Years drinking, *n* (%)						0.129
< 10	2 (3.4)	2 (11.8)	0 (0.0)	0 (0.0)	0 (0.0)	
11-20	11 (18.6)	3 (17.6)	3 (37.5)	0 (0.0)	0 (0.0)	
21-30	13 (22.0)	6 (35.3)	0 (0.0)	2 (28.6)	1 (33.3)	
31-40	22 (37.3)	5 (29.4)	2 (25.0)	1 (14.3)	2 (66.7)	
41-50	8 (13.6)	1 (5.9)	2 (25.0)	2 (28.6)	0 (0.0)	
> 50	2 (3.4)	0 (0.0)	1 (12.5)	2 (28.6)	0 (0.0)	
Biochemistry						
CRP, Median (IQR)	1.3 (0.6-2.5)	1.7 (0.7-4.2)	1.4 (0.5-1.9)	0.8 (0.6-4.4)	0.8 (0.3-0.8)	0.835
CA19-9, Median (IQR)	9.0 (6.0-16.0)	9.0 (7.0-15.8)	9.0 (5.7-15.0)	16.0 (8.5-19.5)	47.0 (22.0-47.0)	0.058
MIC-1/GDF15, Median (IQR)	558.2 (449.6-715.3)	574.3 (448.5-830.3)	659.3 (484.2-1077.3)	553.2 (512.9-967.0)	849.1 (604.9- 849.1)	0.178

Quartiles were created using the entire cohort, which were split into 4 groups for the appropriate measurements. Percentages for variables such as cigarettes, drinking *etc.* are CUMULATIVE, *i.e.*, ignores variables which did not have a number, presumably because the patient doesn't drink/smoke. Biochemistry of MIC-1 is at baseline. An ANOVA test was used to compare normally distributed continuous variables, whereas a Kruskal-Wallis test was used to comparing ordinal and non-normally distributed continuous variables. A Fisher's exact test (2-tailed) was used to compare dichotomous variables.
[1]Denotes statistical significance. EUS: Endoscopic ultrasound; BD-IPMN: Branch duct intraductal mucinous papillary neoplasia; BMI: Body mass index; PC: Pancreatic cancer; IQR: Interquartile range; CRP: C-reactive protein; CA19-9: Cancer antigen 19-9.

Baseline serum MIC-1/GDF15 was a poor predictor of abnormal EUS in our cohort of asymptomatic high-risk patients as determined using a ROC curve for the capacity for

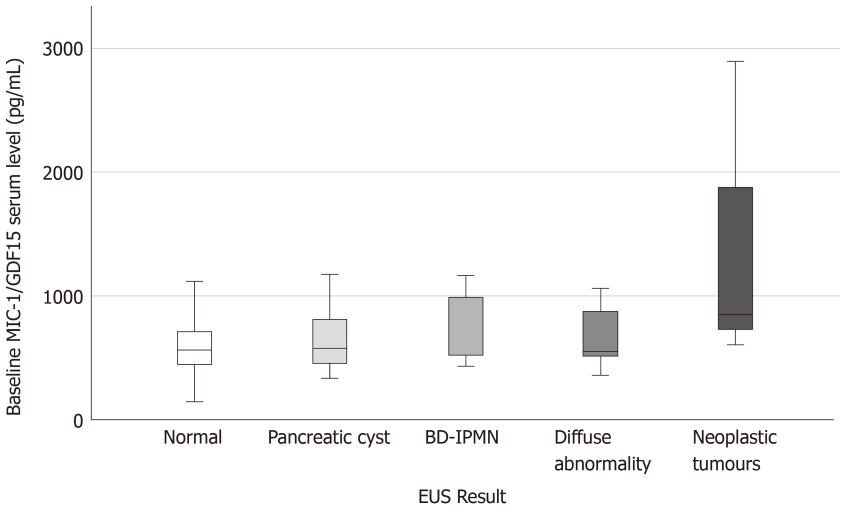

Figure 1 Boxplot of baseline medium serum macrophage inhibitory cytokine-1 or growth differentiation factor-15 levels by group with 95% confidence interval errors bars in participants with a normal endoscopic ultrasound, branched duct intraductal papillary mucinous neoplasm, pancreatic cyst, diffuse abnormality and neoplastic tumours/malignancy detected by endoscopic ultrasound. BD-IPMN: Branched duct intraductal papillary mucinous neoplasm; EUS: Endoscopic ultrasound; MIC-1/GDF15: Macrophage inhibitory cytokine-1 or growth differentiation factor-15.

MIC-1/GDF15 to predict an abnormal EUS. The MIC-1/GDF15 serum level, when adjusted for aspirin use, alcohol intake per week, smoking status, BMI, NSAID use, history of colonic polyps, gender, metformin use and age had an AUC of 0.576 (95%CI: 0.454-0.698) (P = 0.234) (Figure 2A). Similarly, baseline serum MIC-1/GDF15 could not predict BD-IPMN (AUC = 0.644, 95%CI: 0.414-0.875, P = 0.223) (Figure 2B), pancreatic cyst (AUC = 0.347, 95%CI: 0.162-0.532, P = 0.131) (Figure 2C) and diffuse abnormalities (AUC = 0.510, 95%CI: 0.254-0.764, P = 0.935) (Figure 2D). In those with neoplastic tumours diagnosed on EUS and subsequent biopsy (n = 3), the AUC was 0.793, however this was not statistically significant (P = 0.081) (Figure 3).

ROC curve for capacity of MIC-1/GDF15 to identify neoplastic tumours on EUS and subsequent imaging MRI/CT

Baseline MIC-1/GDF15 was a significant predictor of neoplastic tumours diagnosed on EUS and MRI/CT (n = 5) with an AUC=0.814 (95%CI: 0.657-0.970, P = 0.023) (Figure 4). In this asymptomatic cohort three neoplastic tumours were diagnosed on EUS and two other malignancies were diagnosed on further imaging performed to monitor the pancreas (one breast cancer on MRI pancreas and one bladder cancer on CT abdomen). In addition to this, median baseline serum MIC-1/GDF15 in asymptomatic patients found to have neoplastic tumours (Median = 1039.6, IQR = 727.0-1977.7) was significantly greater than benign lesions (Median = 570.1, IQR = 460.7-865.2) (P = 0.012) as demonstrated in Figure 5.

DISCUSSION

PC is a leading cause of cancer mortality worldwide, with a very poor survival rate due to late diagnosis, primarily due to symptoms presenting at advanced stages of the disease. The prognosis correlates strongly with pathological stage at the time of diagnosis, and despite advances in medicine in the last forty years, the 5-year survival has increased only from 4% to 7%[32]. As a result, efforts are made in detecting PC early at asymptomatic stage and multiple PC screening programs in high risk individuals have been established around the world. These screening programs target individuals with a genetic predisposition for developing PC (people with hereditary cancer syndromes due to known mutations and familial PC). Current screening modalities rely on pancreatic imaging (EUS and MRI) and biomarkers are at research level. Ideally, we need an early sensitive and specific serological marker that can be used as a first line screening tool in a high-risk population and help select cases that need further investigations, such as EUS or MRI. CA19-9 is not sensitive enough to be a marker for early detection of PC, having a specificity of 77%, sensitivity 75%, a positive predictive value of 0.5%-0.9%[33,34] and can be increased in other conditions

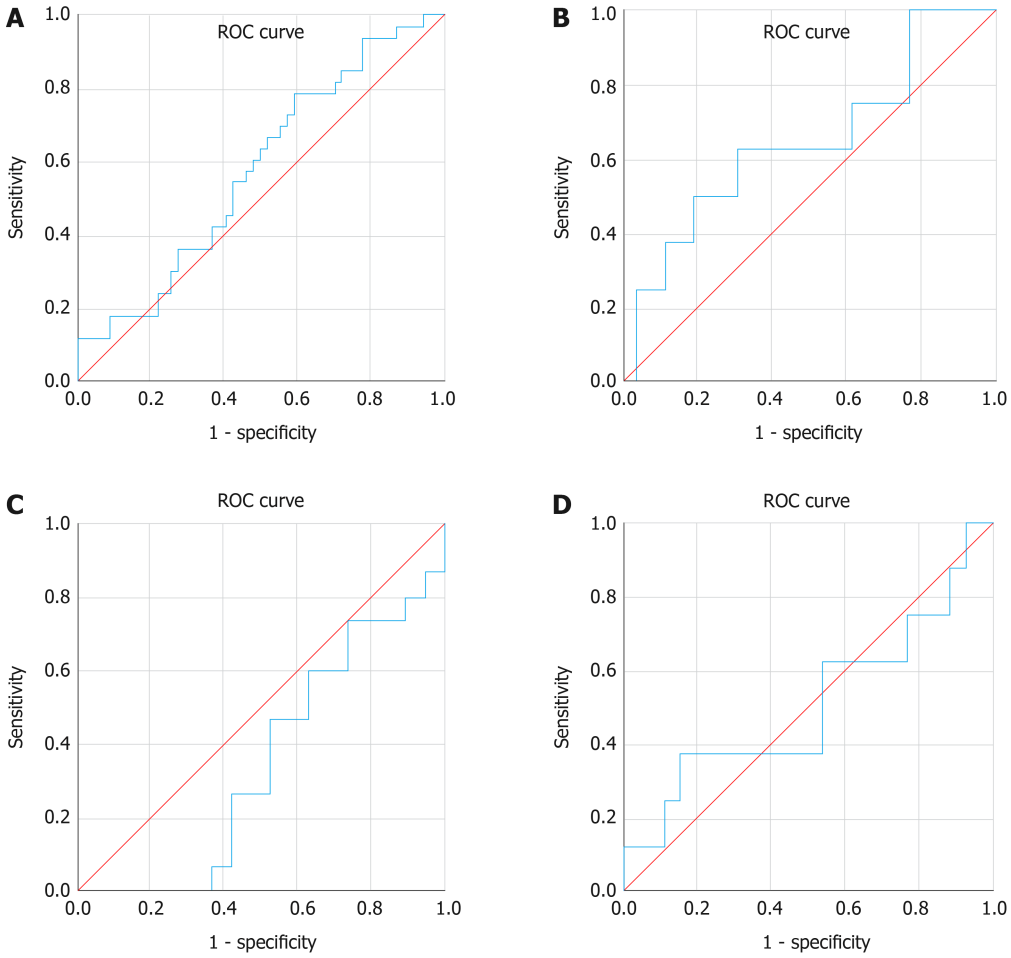

Figure 2 Receiver operating characteristic curve generated for the capacity of macrophage inhibitory cytokine-1 or growth differentiation factor-15 to predict abnormal endoscopic ultrasound results. A: Abnormal endoscopic ultrasound (AUC = 0.576, 95%CI: 0.454-0.698, *P* = 0.234); B: Branched duct intraductal papillary mucinous neoplasm (AUC = 0.664, 95%CI: 0.414-0.875, *P* = 0.223); C: Pancreatic cyst (AUC = 0.347, 95%CI: 0.162-0.532, *P* = 0.131); D: Diffuse abnormality (AUC = 0.510, 95%CI: 0.254-0.764, *P* = 0.935). ROC: Receiver operating characteristic.

such as biliary obstruction. Similarly, carcinoembryonic antigen (CEA) has no utility in early detection of PC with a sensitivity and specificity of 65%[35].

MIC-1/GDF15 has been explored as a novel candidate tumour marker for PC with initial results proving to be elevated in the serum of patients with PC compared to healthy controls and those with benign lesions[18]. As MIC-1/GDF15 can be increased in other malignancies, studies report an increase in its diagnostic specificity if CA19-9 is used in combination with MIC-1/GDF15[28,30]. In addition to this, serum MIC-1/GDF15 has been proven to be more sensitive than CA19-9 in detecting early-stage PC. Importantly, MIC-1/GDF15 had a sensitivity of 63.1% in detecting patients with CA19-9-negative PC[26].

In this feasibility prospective cohort study in an asymptomatic population at high risk of developing PC undertaking yearly screening with EUS, serum baseline MIC-1/GDF15 was shown to be a significant predictor of neoplastic tumours (both pancreatic and extra-pancreatic) after ROC curve analysis, with an AUC of 0.814 (*P* = 0.023). In addition, those diagnosed with neoplastic tumours on EUS or MRI/CT had a higher median baseline MIC-1/GDF15 compared to those diagnosed with benign lesions on EUS. Baseline serum MIC-1/GDF15 had a significant positive correlation with advancing age and age of PC diagnosis in family members. Further analysis of the screening cohort demonstrated that serum MIC-1/GDF15 was elevated in those with a family history of cancer, history of diabetes, current metformin use and those with previous colonic polyps.

When evaluating the utility of serum baseline MIC-1/GDF15 comparing to EUS results only, using ROC curve analysis, we found that it was best utilised when used in participants who were diagnosed with solid neoplastic tumours or BD-IPMN on EUS, with AUCs of 0.793 and 0.644 respectively, with solid tumours diagnosed on EUS approaching significance despite having only 3 cases. These results demonstrated that MIC-1/GDF15 is elevated in participants with pre-malignant and

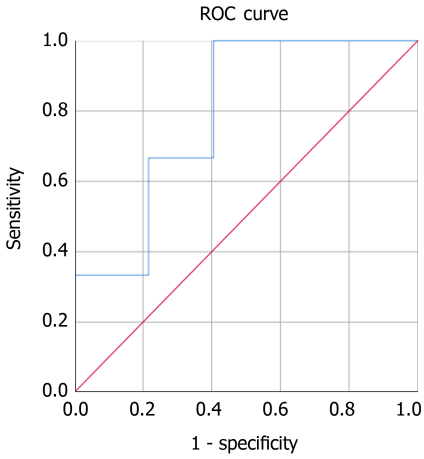

Figure 3 Receiver operating characteristic curve generated for the capacity of macrophage inhibitory cytokine-1 or growth differentiation factor-15 to predict solid neoplastic tumours on endoscopic ultrasound (AUC = 0.793, *P* = 0.081, *n* = 3). ROC: Receiver operating characteristic.

neoplastic tumours, and seems to bear similar predictive value to prostate-specific antigen testing for prostate cancer and the faecal occult blood test for colonic adenoma[36-39]. Previously Koopmann *et al*[18] were able to demonstrate an AUC for MIC-1/GDF15 of 0.81 for the detection of pancreatic adenocarcinoma, and when used in combination with CA19-9, this increased to 0.87.

Compared with previous studies that evaluate the role of MIC- 1/GDF15 in patients with known PC or other malignancies our study design is unique. This is a pilot study, the first to the authors knowledge, to evaluate serum MIC-1/GDF15 in an asymptomatic population at high risk of malignancy in an established PC screening program. Based on the inclusion criteria (patients with a genetic predisposition for PC) these participants are at risk of developing other malignancies not just pancreatic, as shown in our cohort where three non-pancreatic malignancies were found at an asymptomatic stage (liver, breast and bladder cancer). This study shows that baseline MIC-1/GDF15 is elevated in patients with neoplastic tumours and could be potentially used to guide further investigations such as MRI or CT if EUS is negative for PC.

The authors acknowledge that due to the nature of the screening program, the recruitment of asymptomatic high-risk participants is time intensive and the subsequent low incidence of abnormal EUS results and malignant lesions are two limitations of this prospective study. Further larger prospective multi-centre cohort studies are required to further assess the value of MIC-1/GDF15 in screening for malignancy in this type of cohort.

The authors echo the findings of Wang *et al*[40] who stated that serum MIC-1/GDF15 should be interpreted cautiously due to the potential for a broad range of values in the general population and the need to control for multiple confounding factors, particularly inflammation promoting an elevated MIC-1/GDF15 serum level. We controlled for conditions that influence MIC-1/GDF15 levels by using CRP as marker of active inflammation and excluding patients with congestive heart failure, renal failure, human immunodeficiency virus and known malignancy.

Although this study was not able to detect a significant change in serum MIC-1/GDF15 in participants who had a normal then subsequent abnormal EUS , further studies should endeavour to explore whether percentage change in MIC-1/GDF15 is indicative of tumorigenesis in populations at high risk for developing cancer.

A limitation of the use of MIC-1/GDF15 as a biomarker is a wide normal serum range. Serial monitoring of an individual's MIC-1/GDF15 serum level would identify those with increasing levels, even those that were within the normal range. It is the aim of this screening program to implement serial serum MIC-1/GDF15 to assess if with a large enough sample size and long-term follow-up, a statistically significant result can be achieved.

Future studies should aim to further evaluate and analyse MIC-1/GDF15 in both the general population and in patients at risk of malignancy due to a genetic predisposition to determine how this serum biomarker can be better applied in the clinical setting with intention to facilitate its progressive implementation regularly in the clinical domain, along with being further assessed in the academic setting[40].

In conclusion, this pilot study, the first of its kind to implement MIC-1/GDF15 as a screening tool in an asymptomatic population with a genetic predisposition of

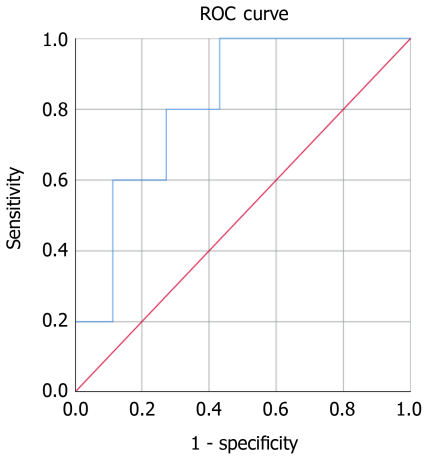

Figure 4 Receiver operating characteristic curve generated for the capacity of macrophage inhibitory cytokine-1 or growth differentiation factor-15 to predict solid neoplastic tumours identified on endoscopic ultrasound and magnetic resonance imaging or computed tomography in an asymptomatic population (AUC = 0.814, 95%CI: 0.657-0.970, *P* = 0.023, *n* = 5). ROC: Receiver operating characteristic.

developing PC, provides moderate support to the previous findings that MIC-1/GDF15 is elevated in patients with neoplastic tumours, however the sample size used to assess this was small. In addition, this study highlights that an elevated MIC-1/GDF15 in the context of a negative pancreatic EUS in a high risk of malignancy cohort may warrant further investigation to determine whether an occult malignancy exists.

While population based screening is difficult to implement due to wide range of normal values and its elevation in select disease processes, MIC-1/GDF15 might be better suited for screening for malignancy in patients with hereditary cancer syndromes where baseline and serial measurement can be used in combination with other validated serological markers to overcome many of these limitations and potentially select patients who require further investigations.

Larger multicentric prospective studies are required to further define the role of MIC-1/GDF15 as a serological biomarker in pre-malignant pancreatic lesions and neoplastic tumours.

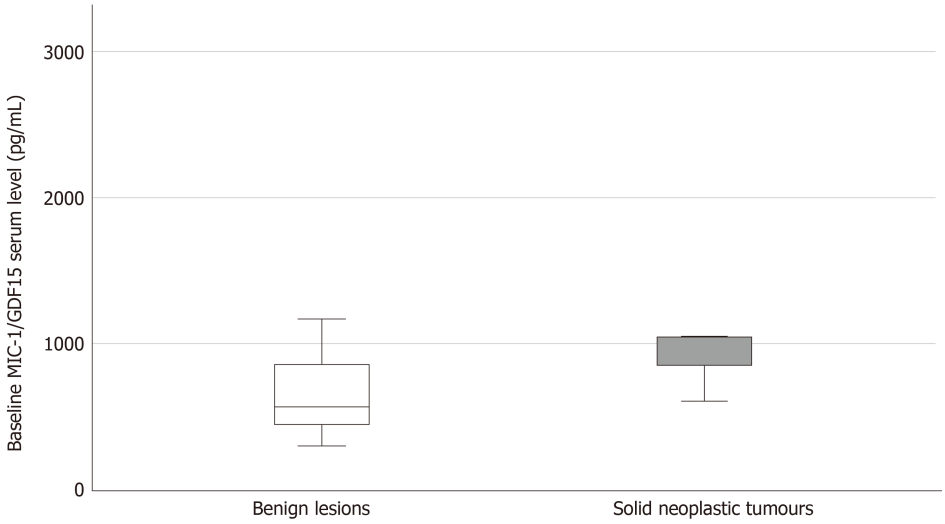

Figure 5 Boxplot of median baseline macrophage inhibitory cytokine-1 or growth differentiation factor-15 in participants diagnosed with benign pancreatic abnormalities (*n* = 42) and solid neoplastic tumours (*n* = 5) on endoscopic ultrasound and magnetic resonance imaging or computed tomography. MIC-1/GDF15: Macrophage inhibitory cytokine-1 or growth differentiation factor-15.

ARTICLE HIGHLIGHTS

Research background

Early detection of pancreatic cancer (PC) is a key priority in order to improve survival. Macrophage inhibitory cytokine-1 or growth differentiation factor-15 (MIC-1/GDF15) is a novel candidate tumour marker for PC with initial results proving to be elevated in the serum of patients with PC compared to healthy controls and those with benign lesions.

Research motivation

We need an early sensitive and specific serological marker that can be used as a first line screening tool in patients at risk of PC and help select cases that need further investigations, such as endoscopic ultrasound (EUS) or magnetic resonance imaging. This study evaluates the role of MIC-1/GDF15 in patients at high risk of developing PC.

Research objectives

This is a pilot study to determine the role of MIC-1/GDF15 in detecting pre-malignant pancreatic lesions and neoplastic tumours in an asymptomatic high-risk cohort part of Australian Pancreatic Cancer Screening Program and correlate with imaging finding.

Research methods

Participants recruited for yearly surveillance with EUS had serial fasting blood samples collected for MIC-1/GDF15, C-reactive protein and carbohydrate antigen 19-9. Patients were stratified into five groups based on EUS findings. MIC-1/GDF15 serum levels were quantified using ELISA and correlations of MIC-1/GDF15 with population variables and imaging findings were performed. A receiver operating characteristic curve of MIC-1/GDF15 was generated for its ability to determine the presence or absence of neoplastic tumours , pancreatic cysts, branch-duct intraductal papillary mucinous neoplasm and diffuse non-specific abnormality using serum levels adjusted for variables shown to either be significantly related to MIC-1/GDF15 concentrations in this study, or have shown to correlate with MIC-1/GDF15 in previous studies.

Research results

One hundred twenty participants were recruited over 8 years. Baseline serum MIC-1/GDF15 was a significant predictor of neoplastic tumours on receiver operating characteristic curve analysis. Baseline serum MIC-1/GDF15 had moderate predictive capacity for branch-duct intraductal papillary mucinous neoplasm (AUC = 0.644) and neoplastic tumours noted on EUS (AUC = 0.793), however this was not significant (P = 0.188 and 0.081 respectively). Serial serum MIC-1/GDF15 did not demonstrate a significant percentage change between a normal and abnormal EUS. Median baseline MIC-1/GDF15 was greater in those with neoplastic tumours compared to those diagnosed with a benign lesion.

Research conclusions

MIC-1/GDF15 has predictive capacity for neoplastic tumours in asymptomatic individuals with a genetic predisposition for PC. Further imagining may be warranted in patients with raised serum MIC-1/GDF15 and abnormal EUS.

Research perspectives

This pilot study is the first of its kind to implement MIC-1/GDF15 as a screening tool in an asymptomatic population with a genetic predisposition of developing PC. Our study is a feasibility study and we hope our results will start a new wave of research (larger, multicentric, prospective trials) into investigating the role of this biomarker in early detection of neoplastic tumours to validate our finding and provide further characterisation of this biomarker.

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CASE REPORT

Small bowel racemose hemangioma complicated with obstruction and chronic anemia: A case report and review of literature

Ji-Xin Fu, Ya-Nan Zou, Zhi-Hao Han, Hao Yu, Xin-Jian Wang

ORCID number: Ji-Xin Fu (0000-0002-9215-9144); Ya-Nan Zou (0000-0001-7273-129X); Zhi-Hao Han (0000-0001-5280-3989); Hao Yu (0000-0002-4119-0432); Xin-Jian Wang (0000-0001-8248-0551).

Author contributions: Fu JX designed the report, reviewed the literature, and wrote the paper; Zou YN, Yu H, and Wang XJ performed the surgery; Han ZH analyzed and interpreted the imaging findings; Fu JX and Yu H collected the patient's clinical data; Fu JX and Wang XJ analyzed the data and revised the paper; all authors have read and approved the final version of this manuscript.

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Ji-Xin Fu, Hao Yu, Xin-Jian Wang, Department of Gastrointestinal Surgery, Weihai Central Hospital, Weihai 264200, Shandong Province, China

Ya-Nan Zou, Department of Anesthesiology, Weihai Central Hospital, Weihai 264200, Shandong Province, China

Zhi-Hao Han, Department of Radiology, Weihai Central Hospital, Weihai 264200, Shandong Province, China

Corresponding author: Xin-Jian Wang, MPhil, Chief Doctor, Director, Professor, Surgeon, Department of Gastrointestinal Surgery, Weihai Central Hospital, No. 3, Mishandong Road Xi, Wendeng District, Weihai 264200, Shandong Province, China. wdwxj@126.com

Abstract

BACKGROUND

Gastrointestinal hemangiomas are rare benign tumors. According to the size of the affected vessels, hemangiomas are histologically classified into cavernous, capillary, or mixed-type tumors, with the cavernous type being the most common and racemose hemangiomas being very rare in the clinic. Melena of uncertain origin and anemia are the main clinical manifestations, and other presentations are rare. Due to the rarity of gastrointestinal hemangiomas and lack of specific manifestations and diagnostic methods, preoperative diagnoses are often delayed or incorrect.

CASE SUMMARY

We report a 5-year-old girl who presented with abdominal pain, nausea, and vomiting for a duration of 10 h. The laboratory studies showed prominent anemia. Computed tomography and contrast-enhanced computed tomography of the abdomen revealed a small bowel obstruction caused by a giant abdominal mass. Segmental resection of the ileal lesions was performed through surgery, and the final pathology results revealed a diagnosis of racemose hemangioma complicated by a small bowel obstruction and simultaneous chronic anemia.

CONCLUSION

The current report will increase the understanding of the diagnosis and treatment of gastrointestinal hemangiomas and provide a review of the related literature.

Key words: Gastrointestinal hemangioma; Racemose hemangioma; Small bowel obstruction; Chronic anemia; Computed tomography; Case report

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Core tip: Gastrointestinal hemangiomas are rare benign tumors, and small bowel racemose hemangiomas complicated by obstructions and chronic anemia are even more rare clinically. Here, we report a 5-year-old girl who presented with abdominal pain, nausea, and vomiting for a duration of 10 h. The preoperative examination revealed an acute obstruction and anemia. A segmental resection of the ileum was performed, and the final pathology revealed a small bowel racemose hemangioma complicated by an obstruction and simultaneous chronic anemia. To improve the diagnosis and treatment of gastrointestinal hemangiomas, we present this unusual report and review some of the related literature.

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INTRODUCTION

Gastrointestinal hemangiomas are rare benign tumors, representing 0.05% of all gastrointestinal tumors[1]. These tumors usually present in young people with no sex predilection. Their main clinical manifestation is gastrointestinal bleeding of uncertain origin, which is defined as chronic or recurrent gastrointestinal bleeding of an unknown cause. Other forms of presentation include obstruction, intussusception, intramural hematoma, perforation, and platelet sequestration[2]. According to the size of the affected vessels, hemangiomas are histologically classified into cavernous, capillary, or mixed-type tumors, with the cavernous type being the most common and racemose hemangioma being very rare in the clinic[3]. In the gastrointestinal tract, these tumors are more frequently found in the jejunum. Computed tomography (CT) and contrast-enhanced computed tomography (CECT) are the main methods for diagnosing such lesions preoperatively, and capsule endoscopy is significantly helpful for diagnosing small bowel lesions[4]. Surgical resection is the ideal treatment. This study presents the unusual case of a 5-year-old girl who underwent segmental resection, and the final pathology results revealed a small bowel racemose hemangioma complicated by an obstruction and simultaneous chronic anemia. A review of the current literature was also provided to contextualize the findings of the present study.

CASE PRESENTATION

Chief complaints

A 5-year-old female child was admitted to the Emergency Department of our hospital complaining of abdominal pain, nausea, and vomiting for a duration of 10 h.

History of present illness

The patient suddenly developed abdominal pain 10 h ago, which was total abdominal pain accompanied by nausea and vomiting. There was no pulsatile vomiting. The vomitus was the previously ingested food and yellow-green bile-like substance, and she vomited three times. There was no hematemesis, no fever, no chest tightness or suffocation, and no diarrhea. The abdominal symptoms gradually became aggravated.

History of past illness and personal and family history

The patient was born after a full-term pregnancy by spontaneous vaginal delivery and had a history of iron deficiency anemia for 1 year. Prior to this admission, the patient had been treated with supplemental iron as recommended by her pediatrician for her symptoms but had shown no improvement. Her parents were healthy, and there were no close relatives. Her mother had a healthy pregnancy.

Physical examination

On the physical examination, her heart rate was 99 beats per minute, and her blood pressure was 12/8 KPa. There were no lesions in the oropharynx, and her neck was supple. The lungs were clear, and her heart rate was regular, without a murmur. Her abdomen was soft, and an abdominal mass could be felt on the left lower abdomen, which was tender. The neurologic examination was unremarkable.

Laboratory examinations

The white cell count was 5.41 × 10^9/L, with 77.8% of neutrophils; hemoglobin was 78 g/L, with a hematocrit level of 27.7%, and the platelet count was 356 × 10^9/L. The serum ferritin level was less than 1.0 μg/L (normal range: 15-200). The electrocardiogram and chest X-ray were normal.

Imaging examinations

An initial imaging evaluation by ultrasound revealed an enormous tumor mass in the middle of the abdomen and pelvis with an inhomogeneous echo pattern that was 10.3 cm × 4.0 cm in size, and several strong echoes and grid-like structures could be seen in the mass with a low blood flow signal on color Doppler flow imaging.

The abdominal lesions were further evaluated by an abdominal CT scan and CECT. The former revealed an ill-circumscribed mass of mixed density in the left lower abdomen that extended to the pelvis. There were multiple high-density nodes in the mass (Figure 1A). The latter revealed that the mass exhibited heterogeneous enhancement following contrast administration. In the venous phase, there were thick and tortuous blood vessels in the mass, which were connected to each other by a honeycomb or racemose appearance (Figure 1B-1D).

FINAL DIAGNOSIS

Considering the large abdominal mass in a young woman with multiple calcifications, the most likely preoperative diagnosis was a teratoma complicated by a small bowel obstruction. However, the final diagnosis by histopathology was small bowel racemose hemangioma complicated by an obstruction and anemia (Figure 2).

TREATMENT

Laparoscopy was performed, and the result revealed a 10 cm × 4 cm lesion on the ileum; a vascular nature was suspected due to the bluish purple coloration, compressibility, and presence of varices on the surface (Figure 3). The mass invaded the intestinal canal and required a dilated proximal intestinal and segmental small bowel resection.

OUTCOME AND FOLLOW-UP

The patient was discharged without immediate complications on the 8th day, and the hemoglobin increased to 123 g/L at the second month after the operation.

DISCUSSION

Hemangiomas are defined as congenital benign vascular lesions that are venous malformations, not true tumors. Hemangiomas are classified into cavernous, capillary, or mixed tumors; the cavernous type is the most common, and racemose hemangioma is very rare[3]. According to the biological characteristics of hemangioma, Fishman *et al*[5] divided them into two categories: Hemangioma and vascular malformations. According to the angiographic findings, vascular malformations can be divided into high-flow and low-flow types, and racemose hemangioma is a complex high-flow type of arteriovenous malformation, which accounts for approximately 1.5% of all hemangiomas and mostly occurs in the head, neck, and limbs[6,7]. Hemangiomas of the gastrointestinal tract are rare, accounting for only 0.05% of all intestinal neoplasms and 7%-10% of all benign tumors of the small bowel[8]. According to the literature, small bowel racemose hemangiomas with obstructions and chronic anemia were rarely reported, which makes our case even more unusual.

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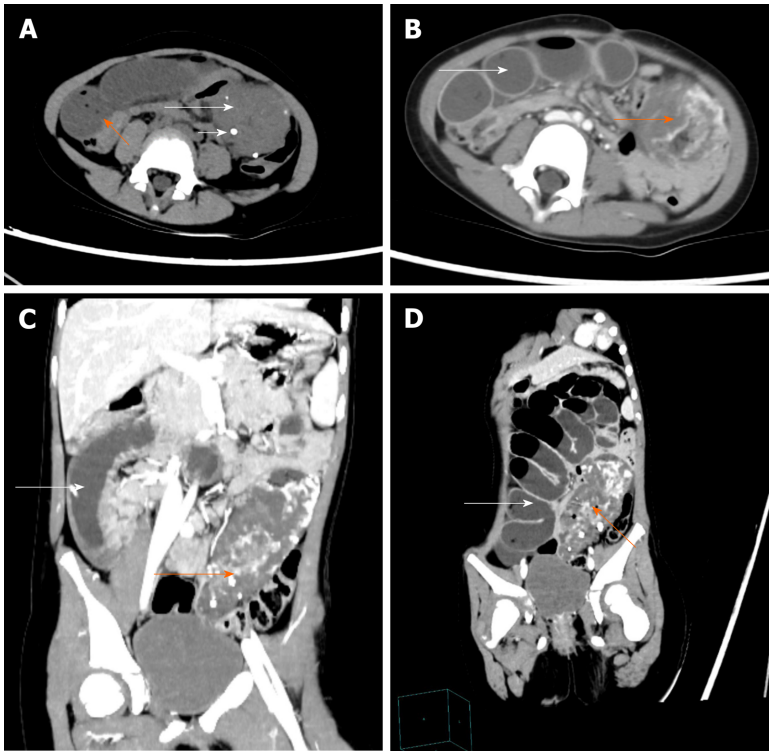

Figure 1 Pre-operative abdominal computed tomography and contrast-enhanced computed tomography images. A: Abdominal computed tomography image showing an ill-circumscribed mass of mixed density in the left lower abdomen (long white arrow) with proximal small bowel dilatation (orange arrow) and multiple nodes with high density in the mass (short white arrow); B-D: Abdominal contrast-enhanced computed tomography images revealing that the mass exhibited heterogeneous enhancement following contrast administration and there were thick and roundabout blood vessels in the mass (orange arrow). There were multiple dilated intestines and air-fluid level within the intestine (white arrow).

(http://www.wanfangdata.com.cn/index.html), and China National Knowledge Infrastructure (CNKI; http://kns.cnki.net/kns/brief/default_result.aspx) databases were investigated between 2009 and 2019 to analyze the clinicopathological features and outcomes of patients with gastrointestinal hemangiomas by searching for MeSH terms and keywords such as "hemangioma", "capsule endoscopy", "double balloon enteroscopy", "anemia", and "gastrointestinal bleeding". The reference lists were screened to identify additional relevant studies, and a standardized form was used for data extraction. Finally, there were approximately 25 cases of gastrointestinal hemangiomas[9-31]. The patient information is summarized in Table 1 to analyze the clinicopathological features (Table 2). The mean age of the patients with gastrointestinal hemangioma was 42.9 years (range: 0-75 years). The sex distribution included 14 males and 11 females (Male:Female = 1.27:1), which is consistent with the results of Durer C *et al*[14]. Gastrointestinal hemangiomas were mainly located in the jejunum and ileum, accounting for 36% and 24% of all gastrointestinal hemangiomas, respectively. The sizes of the gastrointestinal hemangiomas ranged widely from 0.3 cm to 32.5 cm, and the average size was approximately 7.44 cm. In our case, the patient was a 5-year-old girl, and the lesion was confirmed to be located in the ileum with a size of 9 cm x 6 cm.

Clinically, gastrointestinal hemangiomas are symptomatic in 90% of cases, unlike other benign tumors of the gastrointestinal tract that tend to present as an incidental finding[32]. The most frequent sign is chronic gastrointestinal bleeding, which causes anemia of an unknown origin and rarely leads to massive bleeding. Occasionally, these tumors may cause intestinal obstructions, intussusception, intramural hematoma, perforation, and platelet sequestration[2]. Among the 25 patients analyzed in our literature, melena, which was observed in 11 (44%) patients, was the main clinical symptom, followed by anemia in 7 (28%), and dizziness in 5 (20%). However, shock and intestinal obstructions caused by gastrointestinal hemangioma were only observed in 1 (4%) patient. Based on the histological examinations, there have been 15 reported cases of cavernous hemangioma, 3 cases of capillary hemangioma[16,26,28], 2 cases of racemose hemangioma[29,31], 1 case of hemolymphangioma[18], and 1 case of hemangiolymphangioma[19]. Overall, acute intestinal obstruction and chronic anemia

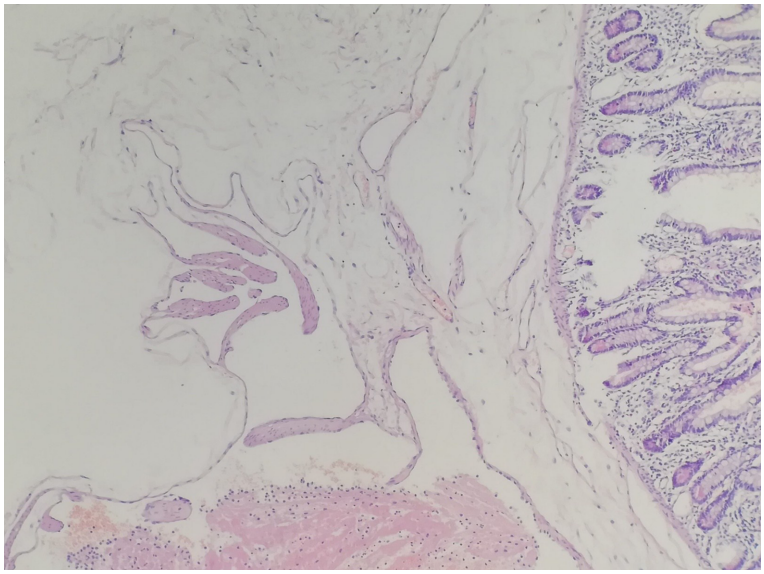

Figure 2 Postoperative histopathological image reveals a small bowel racemose hemangioma (HE, × 100).

caused by a small intestinal racemose hemangioma as in our case are extremely rare.

Gastrointestinal hemangioma is difficult to diagnose preoperatively, especially for small intestinal hemangiomas. Since the most frequent clinical presentation in these patients is gastrointestinal bleeding, the patients frequently undergo gastroscopy and colonoscopy studies with normal results, as in the reported case. However, when the lesions are located in the stomach or colorectal region, gastroscopy and colonoscopy can still have great value in the diagnosis and treatment of this disease. In the literature, among the 25 patients, 10 hemangiomas were located in the stomach or colorectum, 9 of which were diagnosed by endoscopy before the operation. A simple abdominal X-ray may be useful if phleboliths (50% of cases), obstructions, or perforations are present[1,33]. In our literature review, phleboliths were recognized overlying the right sacrum by a preoperative abdominal X-ray in one case[11]. CT and CECT are fundamental tools in the preoperative diagnosis of gastrointestinal hemangiomas, especially in emergency situations, because of their speed, availability, and ability to diagnose extraintestinal lesions. Due to the large degree of vascularity, gastrointestinal hemangiomas are homogenously and significantly enhanced on CECT. Magnetic resonance imaging (MRI), unlike CT, can demonstrate blood flow in the lesion without the administration of contrast medium, and phleboliths are usually void of signal on T1- and T2-weighted images[1]. For colorectal hemangioma, preoperative MRI can define the size of the lesion, which has great significance for treatment. In our retrospective analysis of 25 patients, half of all positive results before surgery were acquired by CT and/or CECT, and 16% were from MRI. Small bowel video capsule endoscopy (VCE) is a noninvasive imaging test and can be recommended when the source of the bleeding remains unidentified after upper and lower endoscopy. On the other hand, double-balloon enteroscopy (DBE) is an invasive and highly sensitive diagnostic tool that provides both therapeutic and diagnostic interventions[2]. There were 15 cases of small intestinal hemangioma in our literature, 9 of which were preoperatively diagnosed by small bowel VCE and 6 by DBE. Undoubtedly, small bowel VCE and DBE are very important for the diagnosis of small intestinal hemangiomas. However, small bowel VCE and DBE are not suitable for critical patients with gastrointestinal hemangiomas, such as those with massive hemorrhage, intestinal obstructions, or intussusception. In addition, 30% of the results were false positives, and 20% of the examinations were incomplete[11].

Based on the literature we reviewed and the CT images of our patient, we summarized the following features of gastrointestinal hemangiomas: (1) CT scan: Tumors tend to appear with mixed density, and there is a blurred boundary between the tumor and the surrounding intestinal tissue; additionally, multiple calcifications representing phleboliths can be recognized inside the tumor on approximately half of all CT studies; (2) CECT: The masses exhibit heterogeneous enhancement following contrast administration; in the venous phase, thick and tortuous blood vessels are present inside the tumor on CT images, and phleboliths can be found in some cases; and (3) For racemose hemangiomas, the characteristic CT manifestations include a dilated feeding artery, malformed vessels, and thick and tortuous draining veins[11,32,34]. Phleboliths are secondary to thrombosis of the intralesional vessels and subsequent

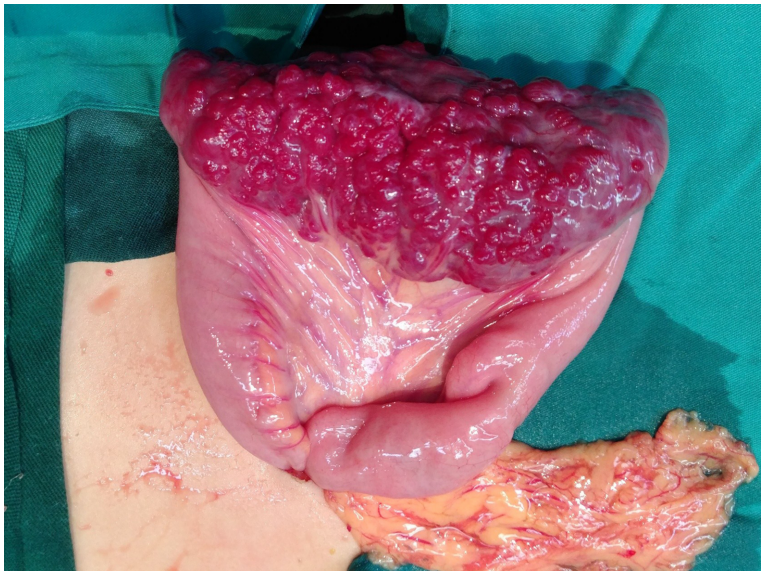

Figure 3 Intraoperative image showing that there was a 10 cm × 4 cm lesion on the ileum with bluish purple coloration and compressible varices on its surface.

partial or total calcifications of the thrombus and are an important diagnostic criterion that can be observed in 26%-50% of adult patients, especially in young patients, and phleboliths are virtually pathognomonic of hemangiomas if they are grouped[33-35]. Phleboliths and malformed vessels were evident in our case.

The main treatment for hemangiomas is surgical resection of the affected segment[1,10,36]. Since hemangiomas never metastasize to the lymph nodes or distant organs, local resection is sufficient. However, in some cases of polypoid lesions accessible by endoscopy, especially those located in the stomach or colorectal region, it may be possible to perform polypectomy and cauterization. However, these are still controversial options because of the risk for uncontrollable bleeding and intestinal perforation. In the literature, surgical resection was still the main treatment for gastrointestinal hemangiomas and was applied in 80% of all cases. However, there was an endoscopic resection performed for a stomach hemangioma with a size of 4 cm × 2 cm, resulting in a good clinical course[18]. In terms of drug therapy, Kaya *et al*[12] reported a case of neonatal gastric hemangioma successfully cured by propranolol. However, in symptomatic hemangiomas, which may be associated with potentially life-threatening massive bleeding, perforations, intestinal obstructions, and intussusception, surgical resection is the preferred treatment option. Gastrointestinal hemangiomas usually have a satisfying prognosis, and there is no evidence in the literature on the recurrence of hemangiomas[10,13]. Our patient underwent partial small bowel resection, and two months after the operation, her hemoglobin increased to 123 g/L, with a hematocrit level of 40.6%.

CONCLUSION

In conclusion, hemangiomas of the small intestine are a rare but significant source of gastrointestinal tract bleeding. Since the main symptoms of hemangiomas are not specific, the clinical diagnosis is often delayed or incorrect, and the preoperative diagnosis was mistaken for a teratoma in our case. However, rare pathologies do occur and most importantly, they can present in an unspecific presentation. Therefore, we can say that although gastrointestinal hemangiomas are rare tumors, they should be considered in the differential diagnoses of patients, especially children, who present with gastrointestinal bleeding of an obscure origin or other abdominal symptoms.

Table 1 Gastrointestinal hemangiomas reported in the literature between 2009 and 2019

Ref.	Age (yr)	Sex	Presentation	Preoperative diagnosis study	Hemangioma size (cm)	Location	Histology	Treatment
Attash *et al*[9]	3	M	Hematemesis, anemia	EGD, CECT	17.6 × 13.2	Stomach	Cavernous	Resembling sleeve gastrectomy
Peng *et al*[10]	47	M	Fatigue, dizziness, melena	VCE, CECT	50 × 15	Ileum	Cavernous	Partial small bowel resection
Ocampo *et al*[11]	29	M	Anemia	Abdominal X-ray, CECT	10	Ileum	ND	Segmental small bowel resection
Kaya *et al*[12]	2 d	F	Melena	EGD	ND	Stomach	ND	Propranolol
Fernandes *et al*[13]	56	F	Hematochezia, dizziness	VCE, VECT	14	Ileum	Cavernous	Laparotomy and vascular tumor resection
Durer *et al*[14]	66	M	Anemia	VCE, DBE	2.5	Jejunum	Cavernous	Surgery
Amati *et al*[15]	20	F	Abdominal distention, pain	CT	28 × 26 × 12	Sigmoid colon	Cavernous	Resection of the sigmoid
Wang *et al*[16]	73	M	Melena, weakness, dizziness	VCE, DBE	2 × 1	Ileum	Capillary	Laparotomy
Andrade *et al*[17]	44	F	Melena	Colonoscopy, MRI	7.5 × 3.5	Rectal	ND	Managed conservatively
Li *et al*[18]	68	M	Epigastric discomfort	EUS, CECT	4 × 2	Stomach	Hemolymphangioma	Endoscopic ultrasonography treatment
Iwaya *et al*[19]	70	M	Anemia, melena	VCE, DBE	2 × 1.7 × 1.2	Jejunum	Hemangiolymphangioma	Laparoscopic small bowel resection
Vitor *et al*[20]	18	M	Melena	Colonoscopy, MRI	ND	Rectum	Cavernous	Iron supplementation
Parker *et al*[21]	32	M	Abdominal pain, anorexia, constipation	CT biopsy, MRI	14 × 7 × 7	Ileum	Cavernous	Laparotomy
Ganesananthan *et al*[22]	65	M	Rectal bleeding	Colonoscopy, CT	ND	Left colon and rectum	Cavernous	Managed Conservatively
Takase *et al*[23]	62	M	Anemia, melena	VCE, DBE	1.5	Jejunum	Cavernous	Laparoscopic enterectomy
	52	M	Anemia	CECT, VCE, DBE	1	Ileum	Cavernous	Laparoscopic enterectomy
Kuo *et al*[24]	20	F	Lower abdominal pain, postprandial bloating	CECT	5.6 × 4.6 × 1.5	Jejunum	Cavernous	Segmental resection
Zhang *et al*[25]	44	M	Melena	VCE	ND	Jejunum	Cavernous	Laparotomy with segmental resection
Moein Jahromi *et al*[26]	75	F	Anemia, melena	VCE, DBE	2.7 × 1.7	Jejunum	Capillary	Laparoscopic partial small bowel resection
Fu *et al*[27]	54	F	Painless rectal bleeding	Colonoscopy, CT, MRI	ND	Rectum	Cavernous	3-D laparoscopically assisted surgery
	22	F	Recurrent intermittent rectal bleeding	Colonoscopy, CT	ND	Rectum	Cavernous	3-D laparoscopically assisted surgery
Liao *et al*[28]	11	F	Hematochezia, palpitation, cold sweat	Angiography	1.2 × 1.0	Jejunum	Capillary	Laparoscopic segmental resection

Hu *et al*[29]	31	F	Melena, dizziness	Angiography	0.3 × 0.3	Jejunum	Racemose hemangioma	Laparoscopic segmental resection
Lian *et al*[30]	57	M	Abdominal pain with anus exhausting and defecating	CT	16 × 12 × 8	Jejunum	Cavernous	Reduction of the volvulus and segmental resection
Li *et al*[31]	54	F	Hematemesis, melena, dizziness	EGD, CECT	2 × 2 × 2	Stomach	Racemose hemangioma	Local excision of lesion

ND: Not described; VCE: Video capsule endoscopy; DBE: Double-balloon enteroscopy; CT: Computed tomography; MRI: Magnetic resonance imaging; EUS: Endoscopic ultrasonography; CECT: Contrast-enhanced computed tomography; EGD: Esophagogastroduodenoscopy.

Table 2 Clinicopathological features of gastrointestinal hemangiomas

No. of cases	25		
Age	42.92 ± 22.75 (0-75)	Preoperative diagnosis study	
Sex		CT or CECT	14 (56%)
Male	14 (54%)	Colonoscopy, EGD, or EUS	9 (36%)
Female	11 (44%)	VCE	9 (36%)
Location		DBE	6 (24%)
Jejunum	9 (36%)	MRI	4 (16%)
Ileum	6 (24%)	Angiography	2 (8%)
Colorectum	6 (24%)	Abdominal X-ray	1 (4%)
Stomach	4 (16%)	Histology	
Size	7.44 ± 8.60[1] (0.3-32.5)	Cavernous hemangioma	15 (68.2%)
Main symptom		Capillary hemangioma	3 (13.7%)
Melena	11 (44%)	Racemose hemangioma	2 (9.1%)
Anemia	7 (28%)	Hemolymphangioma	1 (4.5%)
Dizziness	5 (20%)	Hemangiolymphangioma	1 (4.5%)
Abdominal distention or pain	5 (20%)	Treatment	
Rectal bleeding or Hematochezia	5 (20%)	Operation	20 (80%)
Hematemesis	2 (8%)	Endoscopy	1 (4%)
Fatigue or weakness	2 (8%)	Medication (propranolol)	1 (4%)
Anorexia or postprandial bloating	2 (8%)	Iron supplementation	1 (4%)
Palpitations or cold sweat	1 (4%)	None	2 (8%)
Anus exhausting and defecating	1 (4%)		

[1]The size was the average value of the longitudinal, transverse, and axial lengths of the tumor.

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