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REVIEW

Clinical implications of diabetes in chronic liver disease: Diagnosis, outcomes and management, current and future perspectives

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Abstract

Diabetes mellitus (DM) is common in liver cirrhosis (LC). The pathophysiological association is bidirectional. DM is a risk factor of LC and LC is a diabetogenic

condition. In the recent years, research on different aspects of the association DM and LC has been intensified. Nevertheless, it has been insufficient and still exist many gaps. The aims of this review are: (1) To discuss the latest understandings of the association of DM and LC in order to identify the strategies of early diagnosis; (2) To evaluate the impact of DM on outcomes of LC patients; and (3) To select the most adequate management benefiting the two conditions. Literature searches were conducted using PubMed, Ovid and Scopus engines for DM and LC, diagnosis, outcomes and management. The authors also provided insight from their own published experience. Based on the published studies, two types of DM associated with LC have emerged: Type 2 DM (T2DM) and hepatogenous diabetes (HD). High-quality evidences have determined that T2DM or HD significantly increase complications and death pre and post-liver transplantation. HD has been poorly studied and has not been recognized as a complication of LC. The management of DM in LC patients continues to be difficult and should be based on drug pharmacokinetics and the degree of liver failure. In conclusion, the clinical impact of DM in outcomes of LC patients has been the most studied item recently. Nevertheless many gaps still exist particularly in the management. These most important gaps were highlighted in order to propose future lines for research.

Key Words: Diabetes mellitus; Liver cirrhosis; Hepatogenous diabetes; Clinical implications; Therapy

Core Tip: The prevalence of diabetes mellitus (DM) and impaired glucose tolerance in patients with liver cirrhosis (LC) is around 30% and 40% respectively. DM is a risk factor for LC and LC is a diabetogenic condition. Two types of diabetes associated with LC have emerged: Type 2 DM and hepatogenous diabetes (HD). However HD has not been recognized as a complication of LC. It is widely accepted that DM increases complications and mortality in cirrhotic patients. DM treatment is quite difficult due to liver failure. In the present review we will discuss the most recent information published in this field, pointing out the gaps that still exist in the subject.

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INTRODUCTION

For some time, type 2 diabetes mellitus (T2DM) has been suggested as a risk factor for chronic liver disease (CLD)[1]. Besides, the diabetogenic nature of liver cirrhosis (LC) was described and the term "hepatogenous diabetes" (HD) was coined hepatogenous diabetes (HD) in order to differentiate it from T2DM and attributed it to hepatic dysfunction[2].

In 1994, Bianchi *et al*[3] demonstrated for the first time the negative predictive capability of DM on the outcome of LC patients by observing a significant increase in mortality due to liver complications. Up to date, it is known that DM and impaired glucose tolerance (IGT) are common in LC patients worldwide, particularly in this era of nonalcoholic fatty liver disease (NAFLD) and obesity. From the beginning of this millennium, research on the different aspects of the association between DM and LC has been progressively intensified giving rise to important results. Therefore, we decided to critically review this scientific evidence generated so far highlighting the gaps that still exist in this complex field in order to propose future lines for research.

We also attempt (based on available evidence) to catalyze the recognition of HD as an entity with its own epidemiologic, physiopathological, and clinical implications for patients with LC, creating a template for future refinement of this condition.

EPIDEMIOLOGY

Between 20% and 60% of patients with LC may have overt DM, from 60% to 80% may have IGT and close to 100% insulin resistance (IR)[4,5]. The prevalence of impaired glucose homeostasis seems to be

increased by the severity of CLD. The prevalence of T2DM and HD in compensated LC patients has been determined in 19.2% and 21.5%, respectively[6]. NAFLD, malnutrition, alcohol abuse, hepatitis C virus (HCV) or hepatitis B virus (HBV) infection and primary hemochromatosis are associated to an increased risk of HD[7]. In 2000, the National Health and Nutrition Examination Survey carried out in United States, demonstrated a 3-fold increased prevalence of DM in HCV carriers compared with the average population[8]. In other studies, the extent of fibrosis in patients with hepatitis B and C correlated with an increased prevalence of DM[9,10].

Many clinical studies have shown that DM may increase the severity and accelerate the progression of liver failure leading to a significant increase in liver complications and mortality[4,11]. In the other side, HD has been found to have well defined clinical and pathophysiological characteristics that allow to differentiate it from T2DM[12,13]. HD can be suspected in LC patients without personal or family history of DM and metabolic syndrome (MS)[12].

Notwithstanding, the conceptual term "hepatogenous diabetes" is not included in the currently valid national and international classification systems describing etiologies of DM[14], neither HD is accepted as a complication of LC by the World Health Organization (WHO). Maybe for this reason, the diagnosis and treatment of IGT and DM in LC patients are often overlooked by physicians as was shown by a questionnaire applied to 576 gastroenterologists in Germany. The 90% and 40% of physicians underestimated the prevalence of IGT and DM in cirrhotic patients respectively[15]. In another study, it was found that, in contrast to other complications associated with cirrhosis, HD was underestimated even among medical staff from highly specialized hospital departments[16].

PATHOPHYSIOLOGY

The pathophysiological relationship between DM and LC is bidirectional. In one side, T2DM may lead to liver disease in the context of the MS and NAFLD and, in the other side, LC is a diabetogenic condition[17,18]. The pathophysiology of liver disease due to T2DM (NAFLD) is not discussed in this text as it can be reviewed elsewhere. In contrast, liver failure, portosystemic shunts, hyperinsulinemia, increased glucagon, growth hormone, insulin-like growth factor, free fatty acids and cytokines that induce peripheral IR and β-cells dysfunction play a significant pathogenic role in HD[17,18] (Figure 1).

IR and hyperinsulinemia

The liver plays a key role in glucose metabolism as the major site of glycogen synthesis and gluconeogenesis. Hepatocellular functional impairment results in abnormal glycogen synthesis and decreased hepatic capacity for glycogen deposits[13]. IR in peripheral tissues (adipose and muscular tissues) and liver dysfunction play a central role in the glucose metabolism disturbance[19,20]. Reduced insulin clearance by the damaged liver and portosystemic shunts result in hyperinsulinemia which is potentiated by raised levels of contra-insulin hormones (glucagon, growth hormone, insulin-like growth factor) and free fatty acids and cytokines[20,21].

Hyperinsulinemia can be detected in the early stages of CLD, both in the fasting and postprandial state. A major precipitating factor of hyperinsulinemia is the reactive insulin hypersecretion by the pancreas for compensation of peripheral IR in muscle tissue and impaired hepatic glucose utilization.

Pancreatic beta cells adaptation

Inadequate early increase in insulin secretion and decreased hepatic glucose production are often observed in LC even in the absence of DM. The progressive loss of insulin secretion culminates in a step-wise fashion in DM[22]. The trigger seems to be glucotoxicity from chronic hyperglycemia, which causes secretory impairment of pancreatic β-cells[23].

NAFLD AND DM

NAFLD is the most common CLD in the world and its prevalence in the general population is between 17% and 46%. NAFLD is closely related to MS and DM. For this reason an international consensus panel of experts have recommended the redefinition of this disease with the term of metabolism associated fatty liver disease[24,25]. Notwithstanding, NAFLD may also affect lean or non-obese subjects in the absence of other metabolic risk factors[26]. Lean NAFLD is most commonly seen in the Asian population where the parameters for defining obesity are different than those of western population. Non-alcoholic steatohepatitis (NASH) is the severe manifestation since it causes steatosis, inflammation, ballooning and fibrosis which can progress to cirrhosis and hepatocellular carcinoma (HCC). The prevalence of NASH is estimated at 2%-3%[24].

The pathophysiological relationship between DM and NAFLD is bidirectional and complex. On the one hand, T2DM has been suggested as a strong risk factor for NAFLD, LC and HCC[17,18]. In a recently published study with 561 patients with T2DM attending primary care outpatient clinics and

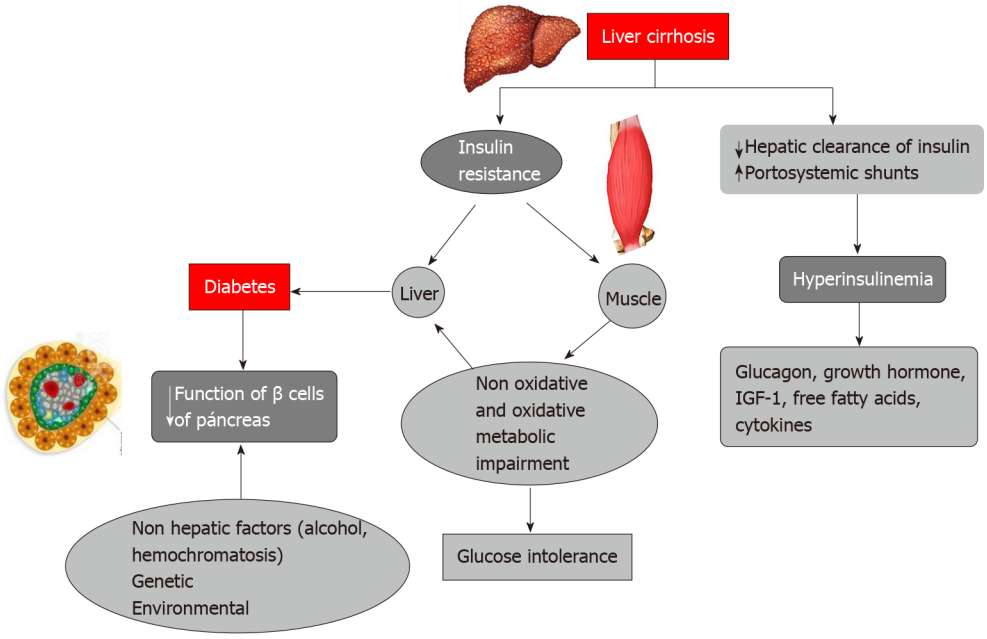

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Figure 1 Pathophysiology of hepatogenous diabetes in cirrhotic liver. Hepatogenous diabetes develops directly from insulin resistance in the liver, and indirectly from impaired glucose metabolism due to insulin resistance in muscle. Hyperinsulinemia can result from reduced insulin clearance by the damaged liver and the presence of portosystemic shunts. With progression of diabetes, there is a reduction in the sensitivity of pancreatic b cells due to glucotoxicity, and reduced production of insulin. IGF: Insulin-like growth factor.

unaware of having NAFLD, 15% showed moderate-to advanced fibrosis by transient elastography and confirmed with liver biopsy. Only a minority of patients showed elevated aspartate aminotransaminase or alanine aminotransaminase[27].

On the other hand, NAFLD in the absence of metabolic disorders may be a risk factor for incidental DM and MS, as it has been demonstrated in lean subjects with NAFLD[28,29]. However, the two conditions have additional common risk factors (Figure 2).

DM in lean patients with NAFLD has clinical characteristics similar to HD. Compared with obese NAFLD patients, leans or non-obese tend to be younger, have lower levels of fasting plasma glucose (FPG), glycated hemoglobin (HbA1c), blood pressure, and homeostasis model assessment IR index (HOMA-IR), and lower prevalence of MS[30]. However, compared with healthy individuals, they tend to have more dyslipidemia and higher FPG, body mass index (BMI), visceral fat, blood pressure and HOMA-IR[31].

HCV AND DM

Numerous prospective studies have shown higher prevalence of DM in patients with chronic hepatitis C virus (CHC) compared to those with HBV liver infection or without liver disease[8,32]. A meta-analysis of 32 studies found that DM was associated with CHC regardless of the presence of fibrosis or LC[33]. The prevalence of DM in patients without LC was 12.6% to 17% and that of IGT was 40%[20,34].

The HCV has diabetogenic properties through several mechanisms. First, autoimmune phenomena, as massive stimulation of the immune system induced by HCV may result in the nonspecific activation of potentially self-reactive lymphocytes that might develop autoimmunity, inducing an immune cascade that could culminate in islet cell dysfunction in susceptible individuals[35]. As a consequence, organ nonspecific antibodies are more frequent in HCV-positive patients with mixed cryoglobulinemia and diabetes than in non-diabetic HCV-negative patients with mixed cryoglobulinemia[36]. Second, direct cytotoxicity to islet β-cells, as rough endoplasmic reticulum morphological changes have been observed in the β-cells of HCV-infected patients, accompanied by reduced glucose-stimulated insulin release[35]. Third, blockade of insulin receptors at the cellular level, as HCV core up-regulates suppressor of cytokine signaling 3 expression that induces proteasomal degradation of insulin receptor substrates 1 and 2 (which are central molecules of the insulin-signaling cascade) and increases gluconeogenesis[37].

The CHC patients with DM have been shown to have an attenuated DM phenotype: They are thinner and have lower levels of low-density lipoprotein (LDL) cholesterol, which could be due to hypobetalipoproteinemia as a result of binding competition between HCV and hepatic LDL receptor, giving rise to

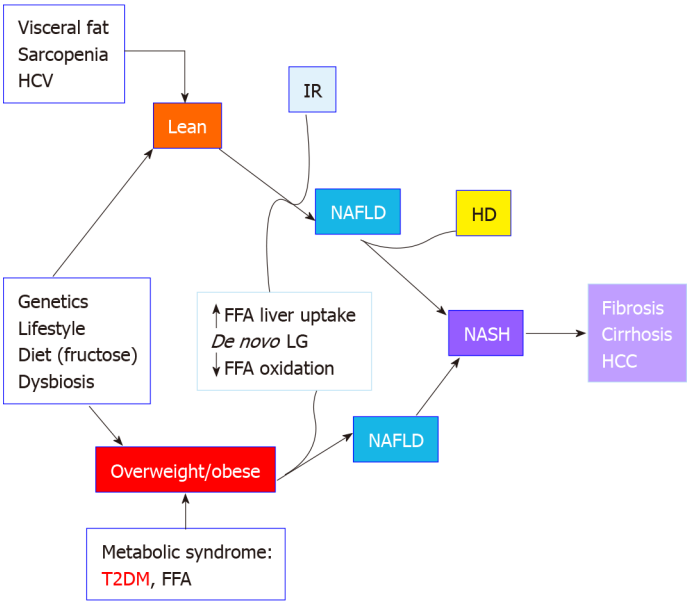

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Figure 2 The pathophysiological relationship between diabetes mellitus and nonalcoholic fatty liver disease. This is bidirectional: On the one hand, type 2 diabetes mellitus (T2DM) is a strong risk factor (alone or as part of metabolic syndrome) for nonalcoholic fatty liver disease (NAFLD), liver cirrhosis and hepatocellular carcinoma. On the other hand, NAFLD in the absence of metabolic disorders is a risk factor for incidental DM as it has been demonstrated in lean subjects with NAFLD. In both cases genetics, [PNPLA3 rs738409 polymorphism (G allele), SREBF-2 rs133291 C/T polymorphism, TM6SF2 rs58542926 C>T and CETP rs12447924 and rs1259700 polymorfisms], as well as sedentary life style, diet and dysbiosis may also play an important role. HCV: Hepatitis C virus; IR: Insulin resistance; NAFLD: Nonalcoholic fatty liver disease; HD: Hepatogenous diabetes; FFA: Free fatty acids; LG: Lactoglobulin; T2DM: Type 2 diabetes mellitus; NASH: Non-alcoholic steatohepatitis; HCC: Hepatocellular carcinoma.

steatosis[38] which is frequently observed in this disease.

Numerous studies have shown that DM has negative clinical implications for the outcomes of HCV liver infection with or without LC[39]. It has been demonstrated that regardless of BMI and age, DM can accelerate the progression of CHC to LC and HCC. In patients with compensated cirrhosis, DM induces an increased risk of decom-pensation with the onset of liver failure and significant increase in mortality [11,40,41]. DM was also associated with an increased risk for HCC development in treatment-naïve CHC patients in Asia. Furthermore, LC and an early DM diagnosis further increased the risk of HCC development in patients diagnosed with both CHC and DM.

With the introduction of direct-acting antiviral (DAA) drugs for the treatment of HCV liver infection, the eradication rate is close to 100% regardless of viral genotype and degree of fibrosis. In multiple studies, the elimination of the virus had a short- and medium-term beneficial effect on DM in CHC patients. In these studies, patients showed improved blood glucose and insulin levels, insulin sensitivity and HbA1c values. These changes were independent of BMI, age and degree of fibrosis[42,43].

In one of these studies with 893 CHC patients, 15.7% with LC, the persistence of normalization of glucose metabolism parameters was demonstrated 44.5 mo after virus eradication[43]. In a recent systematic review and metanalysis HCV eradication with DAAs produced a significant mean reduction in HbA1c levels of 0.45% [95% confidence interval (CI): 0.60-0.30%; $P < 0.001$] and in FPG levels of 22.03 mg/dL (95%CI: 41.61-2.44 mg/dL; $P = 0.03$)[44]. Nevertheless, not all studies have obtained similar results, this may be due to differences in study design, sample size and time of follow up[45]. For this reason, large prospective cohort studies using appropriate stratifications are urgently needed to evaluate the extent of such an amelioration.

CLINICAL MANIFESTATIONS OF DM IN LC, HD

Diagnosis of HD may be difficult since clinical manifestations in the early stages of liver disease are absent. FPG and HbA1c may be normal and in most patients, an oral glucose tolerance test (OGTT) is required for diagnosis[46]. As liver failure progresses, DM becomes clinically manifested[6]. Some clinical parameters may be useful for distinguishing between T2DM and HD. The time of onset is important, as T2DM usually precedes while HD follows the occurrence of LC. Nevertheless, this distinction may be difficult when the two conditions are simultaneously detected. In this case, presence of MS, family history of DM and vascular complications are less frequent, whereas liver-related complications are more frequent in HD compared to T2DM[4,47,48].

Finally, unlike T2DM, orthotopic liver transplantation (LT) may reverse or improve HD confirming its origin from liver dysfunction[49] (Table 1).

Diagnosis

In a study comparing patients with HD to those with T2DM, the ratios of postprandial plasma glucose to FPG, fasting insulin and HOMA-IR index were significantly higher in patients with HD[50]. Consequently, the diagnosis of HD often requires an OGTT. It has been determined that around 50% to 70% of patients with compensated LC will require this test in order to diagnose IGT or DM[4,6,48]. In addition, research is being made to identify genetic and biochemical markers aimed to establish the differentiation among the two types of DM more precisely[50,51].

OGTT

In 1997, the American Diabetes Association determined the cut-off value of FPG in 126 mg/dL for the diagnosis of DM because it corresponded to a 2 h value of 200 mg/dL in the OGTT[52]. A study in 60 LC patients based on the results of OGTT, however, showed that mean values of FPG levels corresponding to a 2 h value of 200 mg/dL were lower (107 mg/dL)[53]. Besides, nine of 42 patients (21%) with FPG levels < 110 mg/dL from this study, were diagnosed with DM using OGTT.

Therefore, lower FPG levels may be required in LC patients for predicting IGT or DM, so the use of lower cut-off values to diagnose HD should be considered in these patients.

HbA1c

HbA1c levels > 6.5%, are used to diagnose DM[54]. However, a previous study showed that LC patients with DM had lower HbA1c levels (mean 5.7%), and 40% of patients with compensated LC had levels below in the non-DM range[55]. The poor diagnostic performance of HbA1c in LC patients is due to the curvilinear relationship between HbA1c and erythrocyte turnover, which can occur in patients with advanced LC as a result of hemorrhage related to portal hypertension and coagulopathy, hemolysis caused by splenomegaly and impaired erythropoiesis due to bone marrow suppression[56]. HbA1c values can also be affected by blood transfusion, which are frequently prescribed to LC patients[57]. A study showed that in non-anemic CLD patients with DM with HbA1c < 7%, the decrease in liver functional reserve is associated with worsening of parameters of glycemic variability determined with continuous glucose monitoring. Mean blood glucose levels and the difference between highest and lowest blood glucose increased significantly with worsening of liver functional reserve[58].

IMPLICATIONS OF DM IN LC

T2DM or HD are associated with numerous complications and high mortality in patients with LC (Tables 2 and 3).

Complications

Hepatic encephalopathy: Several studies have linked DM to an increased incidence of hepatic encephalopathy (HE) in patients with LC. Among patients with HCV-related LC, the severity of HE was higher in DM than in non-DM patients[59]. In a further study in LC patients, the association between DM and HE was independent of the model for end-stage liver disease (MELD) score[39]. In a large prospective study, LC patients with DM had more episodes of first-time overt HE compared to those without DM in one year. In addition, a greater proportion of first-time HE progressed beyond grade 2 in DM patients. Notably, the proportion of Child-Pugh class C LC was lower in the DM group, which suggested that DM conferred an additional risk of HE irrespective of liver disease severity[60].

In a recent study, the risk of both covert and overt HE was more pronounced among patients with poor glycaemic control[61]. Finally, DM increased significantly the risk of HE after a trans-jugular intrahepatic portosystemic shunt (TIPSS)[62].

Variceal haemorrhage: Hyperglycaemia may lead to splanchnic hyperaemia and increased portal pressure which may increase the risks of haemorrhage[59,63]. In a prospective study, DM was associated with increased hepatic venous pressure gradient, variceal haemorrhage (VH), and Child-Pugh's score. Postprandial hyper-glycaemia had a significant association with VH within 6 mo[64]. In another study, DM was a risk factor for rebleeding following endoscopic variceal ligation[65]. In a retrospective study, DM was also an independent predictor of in-hospital death in LC patients with acute gastro-intestinal bleeding[66]. Finally, in another study, LC patients with DM had a higher incidence of re-bleeding and hospitalizations, and a higher mortality rate than those without DM[67].

Infectious complications

An impaired immunological response has been observed in patients with DM and LC[68]. DM was an independent predictor of bacterial infections in hospitalized patients with LC[40]. In hospitalized LC patients, the prevalence of bacterial infections was significantly higher in DM compared to non-DM

Table 1 Clinical differences between hepatogenous diabetes and type 2 diabetes mellitus

Variables	Hepatogenous diabetes	Type 2 diabetes mellitus
Onset	After cirrhosis onset	Before cirrhosis onset
Clinical presentation	Normal FPG and HbA1c; Abnormal OGTT	Increased FPG and HbA1c
Metabolic risk Factors	Less frequent	More frequent
Vascular complications	Less frequent	More frequent
Liver complication	More frequent	Less frequent
Effect of OLT	Reversal or improvement	Non modification
Mortality	More than non-diabetics	More than non-diabetics

FPG: Fasting plasma glucose; OGTT: Oral glucose tolerance test; OLT: Orthotopic liver transplantation; HbA1c: Glycated hemoglobin.

Table 2 Studies depicting implications of diabetes on complications of patients with liver cirrhosis

Ref.	Design	Population, *n*	Outcomes	Limitations
Sigal *et al*[59], United States, 2006	Cross-sectional	65 HCV-LC; 31% diabetics	HE and severe HE was higher in diabetics. DM was independent risk factor for HE	Small sample size. HE was not standardized
Tietge *et al*[81], Germany, 2004	Case-control, prospective	100 LC, 35% diabetics, 62 post-LT	Pre-LT IGT or DM was the major risk factor for post-LT DM	Only 31 patients were prospectively evaluated
Takahashi *et al*[77], Japan, 2011	Prospective	203 CHC	Two hours post-challenge hyperglycaemia associated with HCC	Patients received IFN
Jeon *et al*[64], Republic of Korea, 2013	Prospective	195 LC, 55.4% with HD	HD correlated with HVPG, VH and large varices. Most patients with VH within 6 mo, had post-prandial hyperglycaemia	Risk stratification of varices and prophylaxis for VH were not taken into account
Zheng *et al*[75], China, 2013	Retrospective case-control	1568 CLD, 852 with HCC	DM associated with increased risk of HCC regardless of cirrhosis. Synergistic interaction between DM and HBV for HCC	Hospital based study. Temporal relationship between DM and HCC could not be established
Yang *et al*[63], Taiwan, 2014	Prospective	146 LC, 25% diabetics	DM was predictor of VH. Patients with VH had worse glycaemic control (HBA1c ≥ 7%)	DM associated with decompensated cirrhosis, renal disease and VH
Jepsen *et al*[60], Denmark, 2015	Database from randomized trials	863 LC, 22% diabetics	Diabetics had more episodes of first-time overt HE in one year. First-time HE progression beyond grade 2 higher in diabetics	Diagnosis of DM was not standardized. Vaptan could be a confounder
Yang *et al*[73], United States, 2016	Retrospective	739 LC, 34% diabetics	DM increased the risk of HCC in patients with non-HCV cirrhosis	Single-centre probably with referral bias
Tergast *et al*[69], Germany, 2018	Prospective case-control	475 decompensated LC, 118 diabetics	DM increased risk for SBP and was higher with HbA1c values ≥ 6.4%	Criteria for diagnosis of DM not clearly defined
Wang *et al*[65], China, 2020	Retrospective	207 LC, 137 diabetics; 68 had HD	Rebleeding rate following EST or EVL higher in diabetics, including HD at 1, 3, and 6 mo	Relatively small number of patients with shorter follow-up
Labenz *et al*[61], Germany, 2020	Prospective	240 LC, 27% diabetics	DM associated with covert HE at inclusion and follow-up. The risk of covert HE and overt HE was more pronounced when HbA1c ≥ 6.5%	Spontaneous porto-systemic shunts, GIB, drugs were not taken into account

DM: Diabetes mellitus; EST: Endoscopic sclerotherapy; EVL: Endoscopic variceal ligation; HCC: Hepatocellular carcinoma; HCV: Hepatitis C virus; HD: Hepatogenous diabetes; HE: Hepatic encephalopathy; IGT: Impaired glucose tolerance; OGTT: Oral glucose tolerance test; VH: Variceal hemorrhage; LT: Liver transplantation; HVPG: Hepatic venous pressure gradient; HBV: Hepatitis B virus; IFN: Interferon; GIB: Gastrointestinal bleeding; SBP: Spontaneous bacterial peritonitis; LC: Liver cirrhosis; HbA1c: Glycated hemoglobin.

subjects[68]. In a prospective study in LC patients with ascites, those with DM had an increased risk of developing spontaneous bacterial peritonitis (SBP) and the incidence of SBP was significantly higher when HbA1c values were ≥ 6.4%[69]. In a retrospective analysis of LC patients with DM, uncontrolled DM was associated with an increased overall risk of bacterial infection and a higher hospital mortality rate in the elderly[70].

Table 3 Prospective and retrospective studies depicting implications of diabetes on mortality of patients with liver cirrhosis

Ref.	Design	Population	Outcomes	Limitations
Bianchi *et al*[3], Italy, 1994	Retro-prospective	354 LC, 98 with DM	5-yr survival rate: DM: 41%, non-DM 56%	Diagnosis of DM not standardized
Holstein *et al*[4], Germany, 2002	Prospective	52 LC, 71% with DM	5.6-yr survival rate after diagnosis of LC: 51% of HD patients. 80% of deaths were cirrhosis-related causes	Small sample size. Comparative outcome data of non-DM patients not available
Moreau *et al*[79], France, 2004	Prospective	75 LC and refractory ascites	DM, older age, and HCC were predictors of poor survival. The survival rate of patients without DM was higher	OGTT was not used to diagnose DM
Nishida *et al*[48], Japan, 2006	Prospective	56 LC, 38% diabetics	The 5-yr survival rate was 94%, 68% and 56%, with NGT, IGT and DM, respectively	Small sample size
Quintana *et al*[80], México, 2011	Prospective	110 compensated LC, 45% diabetics	2.5 yr cumulated survival years: DM: 48 *vs* non-DM: 69% ($P < 0.05$). DM was not predictor of death	Maybe DM death- prediction capability was masked by Child-Pugh C score
García-Compeán *et al*[78], México, 2014	Prospective	100 compensated LC and normal FPG	Patients with IGT + DM had lower 5-yr cumulated survival rate. Death causes in 90 % were cirrhosis related	Small sample size
Elkrief *et al*[40], Canada, 2014	Retrospective	348 HCV-LC, 40% diabetics	DM significantly associated with ascites, renal dysfunction, infections, HCC and mortality during the follow-up period	Retrospective. Potential errors in the diagnosis of DM
Khafaga *et al*[67], Egypt, 2015	Case-control	60 LC, 50% diabetics	Diabetics had higher incidence of VH, hospitalizations, HE and mortality rate	Small sample size
Qi *et al*[66], China, 2015	Retrospective	145 LC, 29 diabetics	In-hospital mortality was higher in diabetics	Small number of patients
Hoehn RS *et al*[82], United States, 2015	Retrospective	12442 pos- LT, 24% with DM	Diabetic recipients had longer hospitalization, higher peri-transplant mortality and 30-d readmission rates	More diabetic patients were on haemodialysis and received allografts from older donors
Rosenblatt *et al*[70], United States, 2021	Retrospective	906559 LC with DM, and 109694 uncontrolled DM	Uncontrolled DM associated with increased risk of bacterial infection and increased risk of death in elderly patients	Subject to administrative error. Criteria for DM was not standardized

DM: Diabetes mellitus; FPG: Fasting plasma glucose; HCC: Hepatocellular carcinoma; HCV: Hepatitis C virus; HD: Hepatogenous diabetes; HE: Hepatic encephalopathy; IGT: Impaired glucose tolerance; NGT: Normal glucose tolerance; OGTT: Oral glucose tolerance test; VH: Variceal hemorrhage; LT: Liver transplantation; LC: Liver cirrhosis.

HCC

The increased risk of HCC in diabetic patients was reported in two large meta-analyses[71,72]. DM was also found to increase the risk of mortality in HCC patients[71]. However, the published studies are somewhat inconsistent on this issue. In a retrospective study with LC patients, DM did not increas the risk of HCC in those with HCV infection compared to other causes of liver disease[73]. Another study in patients with chronic hepatitis B, reported a higher prevalence of DM among HCC patients without LC than in those with LC[74]. A large case control study found that DM was associated with an increased risk of HCC regardless of the prevalence of LC[75]. In another study, DM could not be confirmed as a major risk factor for HCC in general; however, DM did become an independent predictor when "traditional" risk factors such as LC, alcohol abuse, hepatitis B or C and smoking were excluded[76]. The inconsistent criteria for diagnosing DM may be an important cause of this discrepancy. Recently, it was observed that while high FPG levels were not associated with HCC, 2 h post-challenge hyperglycaemia remained as significant predictor for HCC development in HCV-RNA-positive patients [77].

Mortality

Many studies have indicated that DM significantly reduced the survival rate in patients with CLD and LC[3,4,40,48,66,67,78,79]. Nevertheless, only some of them have been prospective. Bianchi *et al*[3] reported the adverse impact of DM on the 5-year cumulated survival in a study in patients with LC. Refractory ascites, DM, older age, and HCC, but not Child-Pugh score were independent predictors of poor survival[3]. Even subclinical abnormalities in glucose homeostasis have been found to adversely affect prognosis. In a recent prospective study of 100 compensated LC patients with normal FPG, those with abnormal OGTT had lower 5-year cumulated survival than those with a normal test[78]. In a similar prospective study, the cumulative 5-year survival rates of patients were 94.7%, 68.8% and 56.6%, in those with normal glucose tolerance, IGT and DM, respectively[48]. In another study, DM had an

impact on survival only in patients with a baseline MELD score < 10. This implies that the severity of CLD may mask the deleterious effect of DM[40,80]. The results of these studies suggest that DM can be detected more clearly as reliable predictor of morbidity and mortality in the early stages of LC. In the advanced stages, its effect can be masked by other complications of LC. Therefore, further studies, based on the dynamic assessment of glycaemic parameters using OGTT, are needed to obtain a robust conclusion on this important issue (Table 3).

LT

Pre-transplant DM is the major risk factor for DM after LT (7%-45%)[81-83]. Increased FPG levels were a risk factor for new-onset DM after LT[84]. Pre-operative β-cell function determined by an OGTT may be a useful predictive tool for the recurrence of DM after LT[85]. Post-LT DM is associated with increased risk of graft rejection, severe complications and mortality[81,86,87]. A study on adult LT recipients showed that post-LT DM incidence was 34.7%, 46.9%, and 56.2% at 1, 3, and 5 years, respectively, with overall survival rates of 90%, 80.9%, and 71.7%, respectively. The post-LT DM group had more rejection episodes and worse 5-year survival rates[86]. Persistent or new-onset DM after LT is also associated with cardiovascular disease, biliary complications, renal dysfunction, infections and graft rejection[81, 87]. In patients with HCV-related LC, a pre-existing or new onset DM is associated with increased risk of HCV recurrence and hepatic fibrosis after LT[88]. Some studies have demonstrated improvement in glucose homeostasis after LT[49,89]. In a study where LT failed to cure overt DM in 33% of patients, a persistently reduced β-cell function was found[88]. However, normalized glucose production and insulin sensitivity after LT have the potential to reverse β-cell dysfunction and thus lead to remission in most cases of HD[90].

MANAGEMENT OF DM IN LC

In the absence of specific guidelines, the treatment of DM in patients with LC (T2DM or HD) starts from the general principles of management of T2DM, according to current established guidelines[91].

Lifestyle

Diet and physical activity are a cornerstone of T2DM management. On the one hand, prevalence of obesity and NAFLD is increasing worldwide and > 10% of weight reduction has been shown to significantly reduce inflammation and fibrosis in patients with this disease[92]. On the other hand, malnutrition remains a common feature among LC individuals (20%-50%), mostly in those with decompensated liver disease[93]. Both obesity and malnutrition may be associated with sarcopenia, causing a major risk factor for frailty, conditions associated with a higher rate of severe complications [94].

Diet

A moderate caloric restriction is recommended for overweight/obese LC patients in order to achieve a weight reduction of > 5% to 10%, but paying a special attention to maintain an adequate protein intake to avoid loss of muscle mass (85%). A widely accepted approach is to supply at least 35 kcal/kg body weight/d, using the actual body weight, then subtracting a 5%, 10%, or 15% in case of mild, moderate, or severe ascites, respectively, plus an additional 5% in case of peripheral oedema[93].

Protein intake should be increased up to 1.2-1.5 g/kg body weight/d to avoid sarcopenia, unless moderate-to-severe renal insufficiency is present, but oral protein supplements, especially branched-chain amino acids[95], or short-term enteral or parenteral nutrition[93] may be necessary in some patients. The common deficits of vitamins should be corrected with supplementation[93].

Physical activity

Physical exercise is associated with increments of insulin sensitivity and is highly recommended in patients with NAFLD. However it may be limited in LC patients by the presence of asthenia, sarcopenia, and ascites[96,97]. A combination of aerobic and resistance training of moderate intensity is also recommended[93], as it may result in the concurrent improvement of muscle function and mass.

Pharmacological therapy

Despite the growing problem of management of DM in patients with CLD the existing literature data, especially on newer antidiabetic agents is very limited and furthermore, no guidelines exist. The recommended use of antidiabetic drugs and insulins is based mostly on available data on pharmacokinetics and safety drug studies taking into account the degree of liver dysfunction and the presence of comorbidities[98] (Tables 4 and 5).

Non-insulin agents

The inhibitors of alpha-glucosidase such as the acarbose, inhibit α-glucosidases, which contribute to

Table 4 Kinetics, metabolism and excretion of the currently available anti-hyperglycaemic drugs[102]

Drug	Half life	Metabolism	Excretion
Short-acting insulins			
Human	140 min	Proteolytic degradation	
Lyspro	80 min	Proteolytic degradation	
Aspart	80 min	Proteolytic degradation	
Glulisine	80 min	Proteolytic degradation	
Long-acting insulins			
Human-NPH	6.6 h	Proteolytic degradation	
Glargine	12.1 h	Proteolytic degradation	
Levemir	5-7 h	Proteolytic degradation	
Degludec	25 h	Proteolytic degradation	
Glargine-300	19 h	Proteolytic degradation	
Sulfonylureas			
Glibenclamide	10 h	Liver 100%	Urines 50%; feces 50%[1]
Glimepiride	9 h	Liver 100%	Urines 60%; feces 40%[1]
Gliclazide	10-11 h	Liver 100%	Urines 80%; feces 20%
Glipizide	2-5 h	Liver 90%	Urines mainly
Meglitinides			
Repaglinide	1 h	Liver 100%	Bile 92%; urines 8%
Biguanides			
Metformin	1.5-3 h	Not metabolised	Urines 100%
Thiazolidinediones			
Pioglitazone	3.7 h	Liver 100%	Feces 55%; urines 45%
DPP-4 inhibitors			
Sitagliptin	8–24 h	Limited	Urines
Vildagliptin	1.5–4.5 h	Limited	Urines
Saxagliptin	2–4 h	Moderate	Urines
Linagliptin	10–40 h	Extensive	Feces
Alogliptin	12–21 h	Limited	Urines
GLP-1RAs			
Exenatide	2.4 h	Proteolytic degradation	Renal
Liraglutide	13 h	Proteolytic degradation	No specific organ
Lixisenatide	3 h	Proteolytic degradation	Renal
Exenatide LAR	5-6 d	Proteolytic degradation	Renal
Dulaglutide	5 d	Proteolytic degradation	No specific organ
Semaglutide	7 d	Proteolytic degradation	No specific organ
α-glicosidase inhibitors			
Acarbose	4 h	Intestine	Urines 35%; feces 65%
SGLT2 inhibitors			
Dapaglifozin	10-13 h	Glucuronidation	Urines 33%; feces 42%
Canaglifozin	12.9 h	Glucuronidation	Urines 75%; feces 21%
Empaglifozin	12.4 h	Glucuronidation	Urines 54%; feces 41%

Ertugliflozin	17 h	Glucuronidation	Urines 50%; feces 41%

[1]Excreted as weakly active metabolite.

DPP-4: Dipeptidyl peptidase 4; GLP-1RAs: Glucagon-like peptide 1 receptor agonists; SGLT2: Sodium-glucose cotransporter 2; LAR: Long-acting release; NPH: Neutral protamine Hagedorn.

degradation of disaccharides in the intestine. It results in reduction in the absorption of carbohydrates and in the risk of postprandial hyperglycemia. Its safety has been evaluated in patients with DM and CLD[99,100]. Its use was associated with a significant reduction of fasting and postprandial hyperglycemia, HbA1c and C-peptide as well as improvement of mild HE in compensated cirrhosis [100].

The secretagogues sulfonylureas and glinides are extensively metabolized by the liver in a cytochrome P450-dependent manner and may accumulate in LC patients[99], thus increasing the risk of hypoglycemia[101]. Thus, it is recommended to avoid these agents in patients with moderate-to-severe liver failure.

The insulin-sensitizing agent metformin is not metabolized by the liver[102]. It has been associated with risk of lactic acidosis. However, this complication was reported only in anecdotal cases, particularly with concomitant alcohol intake[103]. Chronic use of metformin has been associated with a reduced risk of HCC[104-107], reduced liver-related complications[105,107], and increase survival in LC patients[105-107].

Probably, glycemic control of the patients contributed for obtaining these effects. Nevertheless, an independent anti oncogenetic mechanism has been recently described experimentally[108]. The other insulin-sensitizing agents, thiazolidinediones, are metabolized entirely by the liver, so they accumulate in patients with hepatic failure[101]. Therefore, their use is restricted to patients with Child-Pugh class A LC, also because of the fluid retention and decrease in bone mineral density caused by these drugs[109]. The significant reduction of liver fibrosis reported in NASH patients with and without T2DM supports the use of these drugs in early-stage LC due to this aetiology.

Despite the fact that all the inhibitors of dipeptidyl peptidase 4 (DPP-4) are metabolized by the liver, their use is generally allowed in patients with Child-Pugh class A or B LC with no dose adjustment[101,110]. A dose reduction of these drugs is however required in case of estimated glomerular filtration rate (eGFR) < 50 mL · min^{-1} · 1.73m^{-2}, except for linagliptin, which is not excreted by the kidney[111]. Notwithstanding, in a recently published population based cohort study with 2828 DPP-4 inhibitor user and nonuser patients with T2DM and LC, the incidence rate of decompensated cirrhosis during follow-up was significantly higher for DPP-4 inhibitor users. The adjusted hazard ratios (aHRs) (95%CI) of variceal bleeding and hepatic failure were 1.67 (1.11-2.52) and 1.35 (1.02-1.79), respectively, for DPP-4 inhibitor users over nonusers. The risk of all-cause mortality, HCC, and major cardiovascular events were not statistically different[112]. In another population-based, retrospective cohort study including patients with DM and LC treated with GLP-1 receptor agonists (GLP-1RAs), DPP-4 inhibitors, sulfonylureas or sodium-glucose co-transporter-2 (SGLT-2) inhibitors, GLP-1RAs use was associated to significantly reduced number of liver related complications compared to DPP-4 inhibitors and sulfonylureas. In contrast, complication rates were similar when GLP-1RAs and SGLT-2 inhibitors were directly compared[113]. The results of these studies suggest that patients using DPP-4 inhibitors should heave a tight monitoring.

The GLP-1RAs, are not metabolized by the liver and, hence, no dose adjustment is required[101,110]. As pharmacokinetic information in patients with end-stage liver disease are available only for liraglutide, dulaglutide, and semaglutide, so the use of these GLP-1RAs is allowed in Child-Pugh class A or B patients, whereas that of exenatide and lixisenatide should be restricted to Child-Pugh class A individuals[101,110]. The use of exenatide and lixisenatide should be avoided also if eGFR is < 30 mL · min^{-1} · 1.73 m^{-2}, as these agents are excreted by the kidney[111]. The reported beneficial impact of this class of drugs on NASH may support their use in patients with NASH-related LC. Conversely, these agents may not be suitable for malnourished sarcopenic individuals, due to their energy wasting effect [101].

The inhibitors of the SGLT-2 are all metabolized in the liver, but have significant accumulation only in severe liver failure[114]. Therefore, the use of SGLT2 inhibitors is allowed in Child-Pugh class A and B LC with no dose adjustment[101], unless impaired renal function is present[111]. Their diuretic properties might be useful for potentiating the effect of therapy with loop diuretics and mineralocorticoid receptor agonists[115], though they may cause dehydration that may further reduce effective plasma volume and precipitate renal dysfunction[101].

In general terms, the use of non-insulin agents (except secretagogues) is generally allowed in mild-to-moderate LC, whereas all of them should be avoided in severe LC, in which insulin represents the sole treatment option[101]. In HD, metformin alone may be sufficient. However, drugs potentially capable of preserving β-cell function (*e.g.*, thiazolidinediones, incretin-based drugs, and SGLT2 inhibitors) may be also suggested[101].

Table 5 Use of anti-hyperglycaemic agents in cirrhotic individuals according to Child-Pugh class[102]

Drug	Child-Pugh class A	Child-Pugh class B	Child-Pugh class C
Short-acting insulins			
Human	Allowed	Allowed	Allowed (dose reduction)
Lyspro	Allowed	Allowed	Allowed
Aspart	Allowed	Allowed	Allowed
Glulisine	Allowed	Allowed	Allowed
Long-acting insulins			
Human-NPH	Allowed	Allowed	Allowed (dose reduction)
Glargine	Allowed	Allowed	Allowed
Levemir	Allowed	Allowed	Allowed
Degludec	Allowed	Allowed	Allowed
Glargine-300	Allowed	Allowed	Allowed
Sulfonylureas			
Glibenclamide	Not recommended	Contraindicated	Contraindicated
Glimepiride	Allowed (caution)	Not recommended	Contraindicated
Gliclazide	Allowed (caution)	Not recommended	Contraindicated
Glipizide	Allowed (caution)	Not recommended	Contraindicated
Meglitinides			
Repaglinide	Allowed (caution)	Not recommended	Contraindicated
Biguanides			
Metformin	Allowed	Allowed (dose reduction)	Contraindicated
Thiazolidinediones			
Pioglitazone	Allowed	Contraindicated	Contraindicated
DPP-4 inhibitors			
Sitagliptin	Allowed	Allowed	Contraindicated
Vildagliptin	Contraindicated	Contraindicated	Contraindicated
Saxagliptin	Allowed	Allowed	Contraindicated
Linagliptin	Allowed	Allowed	Contraindicated
Alogliptin	Allowed	Allowed	Contraindicated
GLP-1RAs			
Exenatide	Allowed	Contraindicated	Contraindicated
Liraglutide	Allowed	Contraindicated	Contraindicated
Lixisenatide	Allowed	Allowed	Contraindicated
Exenatide LAR	Allowed	Allowed	Contraindicated
Dulaglutide	Allowed	Allowed	Contraindicated
Semaglutide	Allowed	Allowed	Contraindicated
α-glicosidase inhibitors			
Acarbose	Allowed	Allowed (caution)	Contraindicated
SGLT2 inhibitors			
Dapaglifozin	Allowed	Allowed	Contraindicated
Canaglifozin	Allowed	Allowed	Contraindicated
Empaglifozin	Allowed	Allowed	Contraindicated

Ertugliflozin	Allowed	Allowed	Contraindicated

DPP-4: Dipeptidyl peptidase 4; GLP-1 RAs: Glucagon-like peptide 1 receptor agonists; SGLT2: Sodium-glucose cotransporter 2; NPH: Neutral protamine Hagedorn; LAR: Long-acting release.

Insulin

As human insulin is metabolized by insulinase in the liver, it may be necessary to reduce dosage[116]. Conversely, as no significant changes in the kinetics of insulin analogues, either both short-acting[117], or long-acting insulins[101,116] have been reported, no dose adjustment is required for these agents.

For these reasons, use of insulin (with preference for insulin analogues) is allowed at all stages of cirrhosis (Table 5) and represents the first-choice treatment in LC patients with DM. Insulin requirements can be high in patients with compensated cirrhosis and low in decompensated patients. Few clinical studies have evaluated its long-term effects and safety. In a recently published retrospective cohort study, insulin use in people with T2DM and compensated LC was associated with higher risks of hypoglycemia, cardiovascular events, liver-related complications, and mortality than insulin nonusers. However, no information regarding important risk factors such as body weight, physical activity, alcohol consumption, and cigarette smoking was given and effective glycemic control or treatment adherence was not evaluated[118]. More studies are needed to confirm these findings. Anyway, insulin treatment should be started with close monitoring to avoid hypoglycemia. The insulin regimen may consist of basal insulin only or a combination of basal and prandial insulin (basal-plus or basal-bolus).

In the Figure 3 an algorithm for the diagnosis and management of DM in CLD and LC is depicted.

Treatment of post-transplantation DM

HD may be improved with LT, nevertheless it may persist in 30% of cases or DM may occur *de novo* after LT due to several factors with diabetogenic potential, such as immunosuppressant treatment, viral infections, and donor- and procedure-related factors[101].

The intra-operative and immediate post-LT periods are often characterized by severe hyperglycemia that may be transient and reverse with appropriate management[101]. Intravenous or subcutaneous intensive insulin therapy using validated algorithms is the standard of care, as a strict intra-operative glycemic control is recommended to reduce the associated increased risk of morbidity and mortality [119]. With reduction of steroid dose, insulin requirement rapidly decreases and insulin treatment may be interrupted in many instances[101].

Specific guidelines for the treatment of post-LT DM were released in 2014[83]. While lifestyle measures are identical to those for T2DM patients[91], there are insufficient data to recommend specific anti-hyperglycemic agents[101]. Indeed, all the available agents can be used to treat post-transplant DM [120], with limitations in case of renal dysfunction[121].

CONCLUSION

DM and IGT are common in LC patients worldwide, particularly in this current era of NAFLD and obesity. The pathophysiological relationship of DM and LC is bidirectional. Over the years, the evidences that LC is a diabetogenic condition have been consolidated and the mechanisms are better understood. High-quality evidences have also determined that DM is associated to increased complications and death in CLD patients. Although the existence of two types of DM in LC patients has been confirmed, the practical usefulness of taxonomic separation of the two types of DM is unknown. In part because they have not been separately studied.

However, the research carried out to date has permitted to clearly understand the pathophysiologic mechanisms of HD and to define its clinical characteristics. Despite this, HD is not currently recognized as secondary diabetes nor as a complication of LC.

Based on the arguments presented in this review, we think that, it is time to classify LC-associated DM into T2DM and HD in order to standardize clinical research studies, which will make it possible to evaluate separately their impact on outcomes of LC patients. It is also urgent to determine standardized therapeutic guidelines for this vulnerable patients based on prospective randomized clinical trials with great number of patients and long term follow up taking into account clinical surrogates such as complication and mortality rates. Because these patients are referred from primary care levels to specialized services, we believe that the hepatologists should have the basic knowledge in the management of uncomplicated DM and equally the diabetologists should have the basic competences in the early detection and management of CLD. Complicated and severe patients should be treated by a multidisciplinary team.

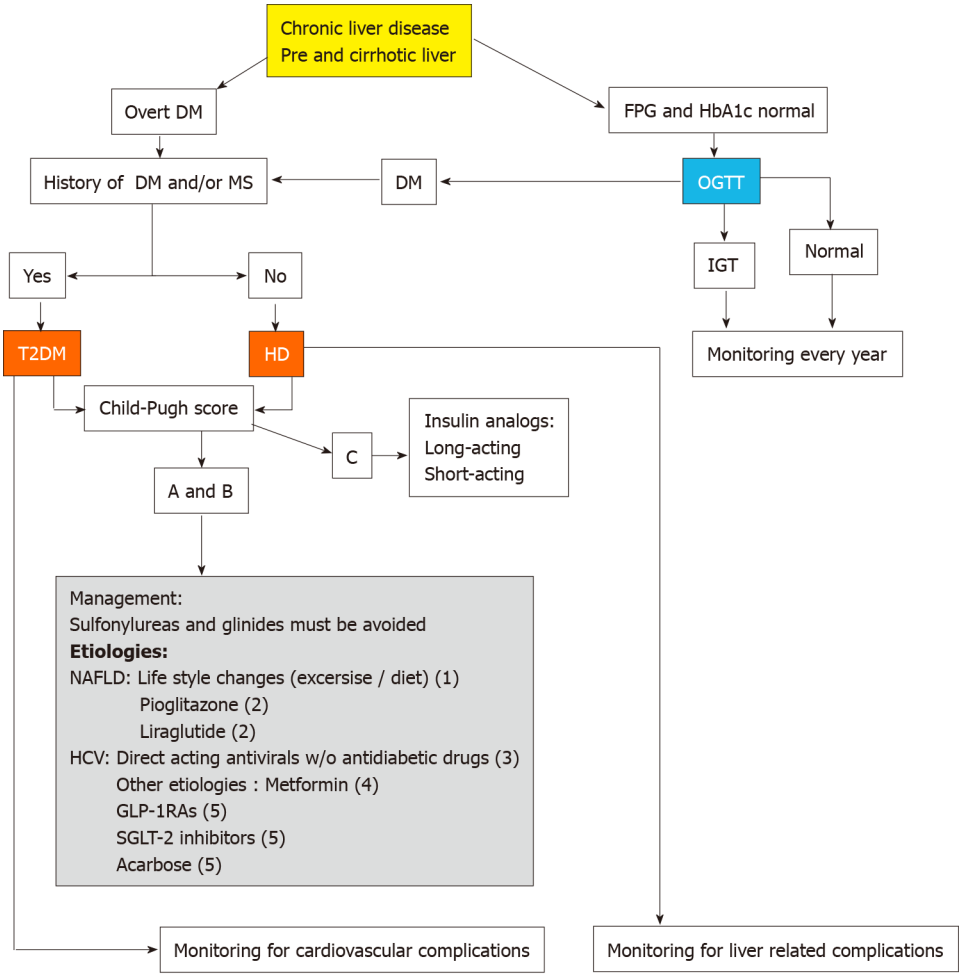

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Figure 3 Algorithm for diagnosis and management of diabetes mellitus and nonalcoholic fatty liver disease based on the published evidences. As follows: (1) This treatment has been evaluated only in nonalcoholic fatty liver disease (NAFLD); (2) These drugs have been evaluated in NAFLD showing improvement of non-alcoholic steatohepatitis and fibrosis; (3) Direct-acting antiviral have demonstrated improvement of short and long term glycemic control after hepatitis C virus eradication; (4) Long term administration of metformin has demonstrated association to significant reduction of liver related complications, hepatocellular carcinoma and mortality; and (5) GLP-1 receptor agonists and sodium-glucose co-transporter-2 inhibitor drugs have demonstrated effectiveness for glycemic control and good tolerance in liver cirrhosis patients. NAFLD: Nonalcoholic fatty liver disease; DM: Diabetes mellitus; GLP-1Ras: GLP-1 receptor agonists; SGLT-2: Sodium-glucose co-transporter-2; HCV: Hepatitis C virus; FPG: Fasting plasma glucose; HbA1c: Glycated hemoglobin; HD: Hepatogenous diabetes; MS: Metabolic syndrome; OGTT: Oral glucose tolerance test; IGT: Impaired glucose tolerance.

FOOTNOTES

Author contributions: García-Compeán D, Orsi E, Kumar R, Gundling F and Nishida T made a bibliographic research and wrote sections of the manuscript, reviewed and corrected the final text; Cueto-Aguilera ÁN made a bibliographic research; Villarreal-Pérez JZ, González-González JA and Pugliese G critically reviewed the manuscript; García-Compeán D conceived and coordinated the whole project.

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MINIREVIEWS

Mixed neuroendocrine–nonneuroendocrine neoplasms of the gastrointestinal system: An update

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Abstract

Mixed neuroendocrine-nonneuroendocrine neoplasms (MiNENs) of the digestive tract are a rare heterogeneous group of tumors that present many challenges in terms of diagnosis and treatment. Over the years, the diagnostic criteria, classification, and clinical behavior of these tumors have been the subjects of ongoing debate, and the various changes in their nomenclature have strengthened the challenges associated with MiNENs. This review is performed to provide an understanding of the key factors involved in the evolution of the designation of these tumors as MiNEN, highlight the current diagnostic criteria, summarize the latest data on pathogenesis and provide information on available treatments. Moreover, this work seeks to increase the awareness about these rare neoplasms by presenting the clinicopathological features and prognostic factors that play important roles in their behavior and discussing their different regions of origin in the gastrointestinal system (GIS). Currently, the MiNEN category also includes tumors in the GIS with a nonneuroendocrine component and epithelial tumors other than adenocarcinoma, depending on the organ of origin. Diagnosis is based on the presence of both morphological components in more than 30% of the tumor. However, this value needs to be reconfirmed with further studies and may be a limiting factor in the diagnosis of MiNEN by biopsy. Furthermore, available clinicopathological data suggest that the inclusion of amphicrine tumors in the definition of MiNEN is not supportive and warrants further investigation. The diagnosis of these tumors is not solely based on immunohistochemical findings. They are not hybrid tumors and both components can act independently; thus, careful grading of each component separately is required. In addition to parameters such as the metastatic state of the tumor at the time of diagnosis and the feasibility of surgical resection, the aggressive potential of both components has paramount importance in the choice of treatment. Regardless of the organ of origin within the GIS, almost MiNENs are tumors with poor prognosis and are frequently encountered in the elderly and men. They are most frequently reported in the colorectum, where data from molecular studies indicate a monoclonal origin; however, further studies are required to provide additional support for this origin.

Key Words: Mixed neuroendocrine–nonneuroendocrine neoplasms; Mixed adeno neuroendocrine carcinoma; Gastrointestinal system; Liver; Pancreas; Gallbladder

Core Tip: Mixed neuroendocrine-nonneuroendocrine neoplasms of the gastrointestinal system are a rare heterogeneous group of tumors that present many challenges in terms of diagnosis and treatment. Current data indicate that they are more frequent in the colon and rectum and that most of them consist of aggressive tumors that have poor prognoses in older men. Their correct diagnosis with the proposed criteria and the separate assessment of the grade of each component are crucial in terms of determining the treatment. Although studies have indicated a monoclonal origin, further studies are needed to determine whether these molecular changes could become treatment targets.

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INTRODUCTION

In epithelial tumors, cells with both neuroendocrine and nonneuroendocrine features coexist in varying amounts, and these tumors occur in almost all organs, including those of the GIS. Although these rarely encountered neoplasms are well known in terms of pathology, debates remain about their diagnosis, classification, pathogenesis, behavior and treatment, and some points are still controversial.

The presence of different proportions of each component in a mixed epithelial tumor (each of which can account for 1% to 99% of the tumor) can result in a wide variety of morphologically heterogeneous tumors as well as different classifications and diagnostic difficulties in pathology[1]. More importantly, these conditions have created problems for oncologists in determining the component that should be targeted primarily in treatment. In addition, the assignment of different definitions to mixed epithelial tumors has led to great inconsistencies in the data obtained from previous studies, especially in determining the prognostic parameters that affect their behavior[2,3]. Therefore, different criteria have recently been introduced for simplifying the diagnosis and classification of these tumors based on the identification of prognostic parameters that may enable effective treatment for mixed epithelial tumors.

The purpose of this review is to highlight the definition of mixed neuroendocrine-nonneuroendocrine neoplasms (MiNENs), summarize the current approaches for their histopathological diagnosis and molecular findings related to their pathogenesis and address current approaches in their treatment. Moreover, this work is performed to raise awareness about these rare tumors of the GIS by presenting localization-specific demographic and clinicopathological findings.

DEFINITION OF MiNEN

Mixed tumors were first described by Cordier[4] at the beginning of the last century as neoplasia in the gastrointestinal tract consisting of adenocarcinoma and neuroendocrine components. However, their classification as a different group and their subcategories under this heading were not recommended until proposed by Levine in 1967[5]. Accordingly, three main subtypes have been proposed based on their nonneuroendocrine and neuroendocrine features: collision tumors, combined tumors, and amphicrine tumors. However, this nomenclature has not been entirely accepted. In addition to the challenges posed by their pathological heterogeneity, many different definitions (some repetitious or overlapping) have been provided for mixed epithelial neoplasms composed of both neuroendocrine and nonneuroendocrine components, which has led to further confusion among clinicians and pathologists by leading to considerable variability in published data[2]. In recent years, many attempts have been made to elucidate the clinical and biological meanings of the different combinations of components and simplify the diagnosis and classification, with the primary goal of developing precise diagnostic criteria that can be used to produce a proper prognostic classification for the management of patients. Standardizing the terminology to provide a prognostic classification of mixed neoplasms of the digestive tract was first suggested by Capella *et al*[6] in 2000. Accordingly, the term "mixed exocrine–endocrine tumor" was used by the World Health Organization (WHO) to define these neoplasms[7]. This category included previous subcategories suggested by Levine[5], whereas adenocarcinomas or

squamous cell carcinomas with scattered neuroendocrine cells were excluded based on previous data about the absence of a relationship between the presence of neuroendocrine cells and prognosis. To emphasize the diagnosis, a subjective cutoff value of 30% was determined for each component to define mixed tumors. Ten years later, the term "mixed exocrine–endocrine tumor" was substituted by "mixed adeno-neuroendocrine tumor" (MANEC)[8]. Since the two components of mixed tumors of the GIS are not always constituted by adenocarcinomas and neuroendocrine adenocarcinoma (NEC), the term MANEC was not sufficient to describe these combinations. This situation encouraged many researchers to find another term that included other associations. In 2016, La Rosa *et al*[2] proposed the umbrella term "mixed nonneuroendocrine and neuroendocrine neoplasm" (MiNEN). Introduced first for the pancreas by the 2017 WHO classification of tumors of endocrine organs, this term is currently used for all mixed neoplasms of the GIS[9]. According to the WHO, mixed neoplasms consisting of an adenoma and a neuroendocrine tumor (NET) should be classified as MANETs and should not be included in the MiNEN group.

DIAGNOSIS OF MiNEN

Pathological evaluation of H&E-stained sections is essential for detecting the neuroendocrine and nonneuroendocrine components for the diagnosis of MiNEN. These findings must be confirmed by immunohistochemical (IHC) evaluation. IHC markers used in detecting and grading the endocrine component should be accompanied by markers appropriate for the type of nonneuroendocrine component (Figure 1). The properties and applications of these markers for neuroendocrine neoplasia (NEN) will be briefly mentioned here. However, comprehensive information on this subject can be reviewed in a previous study[10]. Although several biomarkers, including neuron-specific enolase (NSE), CD57, protein gene product 9.5 (PGP 9.5), insulinoma-associated protein 1 (INSM1) and somatostatin receptor subtype 2A (SSTR2A), have been described to date, the most widely used and reliable neuroendocrine markers are chromogranin A, synaptophysin, and CD56[11,12]. In the nonneuroendocrine component, adenocarcinomas express carcinoembryonic antigen, CA 19-9, cytokeratins 7, 19, and AE 1/3. The immunohistochemical features of other tumors that make up this component are presented below according to their localization in different organs of the GIS[10-12].

Both tumor components must account for at least 30% of the whole neoplasm for the diagnosis of MiNEN[8]. This cutoff is arbitrary and was proposed in 1987. The basis for determining this value is the assumption that the prognosis is influenced by the predominant histological component and prevents the management of these cases without consideration of treatment guidelines[1,5]. However, this value has not been reaffirmed in systematic studies. Moreover, the possibility of the negative influence of a small component of high-grade NEN on tumor behavior should not be overlooked[13,14]. This quantitative threshold also poses problems for tissue biopsies. The discrimination of both components could not be performed accurately according to the likelihood of their presence in random biopsies, thus leading to potential underestimations of the frequency of MiNEN diagnosis[15]. Recently, it has been argued that a cutoff value is not mandatory for diagnosing MiNEN because the latest molecular information in the modern classification of these neoplasms has made it possible to demonstrate that both components are clonally related[16].

Another point to be considered is that the determination of NEN on a purely quantitative basis may cause problems in diagnosis, especially with the use of IHC. Indeed, it has been reported that achieving a diagnosis of MiNEN only by quantification with IHC findings has caused inconsistencies and confusion in terminology[17]. Therefore, the WHO has clearly stated that the findings of IHC alone are not sufficient for the diagnosis of MiNEN, and those histopathological findings should be present in each morphological component[9,18].

To date, the definition of MiNEN also excludes nonneuroendocrine neoplasms in which scattered tumor cells express neuroendocrine markers without the presence of neuroendocrine morphology (Table 1). Since neuroendocrine markers can be positive in many nonneuroendocrine tumors, including poorly differentiated adenocarcinomas, performing IHC alone may lead to an overdiagnosis of MiNEN [19-21]. Therefore, it is highly recommended to avoid the application of neuroendocrine markers to tumors that do not have a morphological neuroendocrine component to overcome this difficulty.

Another issue that should be considered is that the diagnosis of MINEN must be given in cases that have not undergone neoadjuvant treatment. Many studies have observed that the number of neuroendocrine cells may increase after treatment (especially chemotherapy) in GIS adenocarcinomas[22-24]. The effective mechanisms of this phenomenon are not fully known and await clarification, and the latest WHO classification does not include these tumors in the MiNEN group.

As pointed out previously, the behavior of MiNEN does not correspond to the average of the two components,such as hybrid neoplasia, but the sum of the two components because each of component can independently progress and metastasize[17,25]. For this reason, it is crucial to detect and evaluate each component separately during pathological examination to determine the treatment of the tumor. Although the nonneuroendocrine component of many MiNENs is more frequently composed of adenocarcinomas, it may differ according to the location within the GIS (see below), which should be

Table 1 Discrimination among mixed neuroendocrine-nonneuroendocrine neoplasms, neuroendocrine tumors, and carcinomas according to pathological parameters

Morphology	Dual differentiation present			Dual differentiation absent		
Immunohistochemisty						
Neuroendocrine markers	(-)	(+)	(+)	(+)	(-)	Few (+) cells[1]
Nonneuroendocrine markers	(+)	Few (+) cells	(+)	(+)	(+)	(+)
Diagnosis	Carcinoma	NEN	MiNEN	Amphicrine tumor	Carcinoma	Carcinoma

[1]These cells frequently express synaptophysin, the expression of other neuroendocrine markers is rare.
NEN: Neuroendocrine neoplasia.

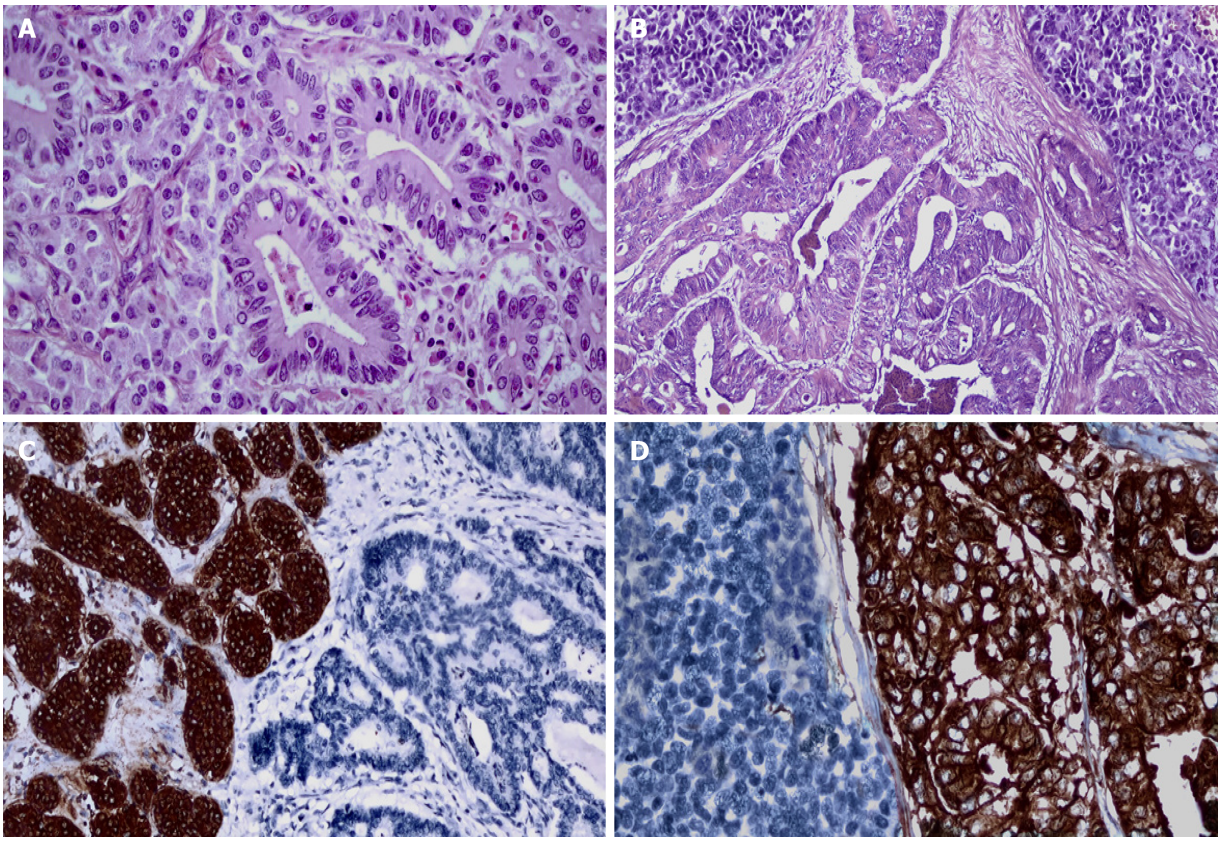

Figure 1 Examples of histopathological and immunohistochemical findings in gastrointestinal mixed neuroendocrine-nonneuroendocrine neoplasms. A: Gastric mixed neuroendocrine-nonneuroendocrine neoplasms (MiNEN) composed of a NET (lower left) intermingled with an adenocarcinoma (x 400); B: Colonic MiNEN constituted from a neuroendocrine carcinoma and an adenocarcinoma (x 200); C. Diffuse immunostaining with synaptophysin in the neuroendocrine component of a colonic MiNEN (x 200); D: The adenocarcinoma component of this MiNEN shows diffuse positivity with CK20 (x 400).

considered during evaluation. This part of the tumor should be graded according to the type of nonneuroendocrine component of MiNEN. Similarly, neuroendocrine components should be evaluated according to the WHO classification and tumors should be graded according to the Ki-67 index percentage and mitotic count.

SUBTYPES OF MiNEN

These tumors are subdivided into three categories. While collision MiNEN is defined as two coexisting cell populations that remain separate without transition, composite MiNEN involves two morphologically distinct components that coexist in an intermingled population[25]. To date, true collision tumors consisting of two independent neoplasms arising in the same organ, even if they abut one another, should not be considered MiNEN unless these components are presumed to be clonally related[18]. The last group includes amphicrine tumors composed of a morphologically one-cell population that displays

the phenotypes of neuroendocrine and adenocarcinoma phenotypes. These cells show coexisting morphological, immunohistochemical, and ultrastructural properties that present both neuroendocrine and exocrine differentiation. Although these tumors have been described for many years in many locations, including the GIS, there is no consensus regarding their relationship with MiNENs. These rare neoplasms have been observed in the stomach, pancreas, appendix, and colon[26-28]. In the appendix, where they are observed relatively more frequently, they are now classified as goblet cell adenocarcinomas[29]. Although this nomenclature does not reflect the amphicrine characteristics of the cells, the term goblet cell carcinoid has been used for many years to avoid misclassification. A recent elegant study by Huang *et al*[30] provided evidence that amphicrine carcinomas arising from the stomach and intestine are distinct tumors with different clinicopathological and pancancer transcriptome features. The latter revealed that although amphicrine neoplasms show similarities to adenocarcinomas, they are not similar to NENs. However, since the nature of NENs was not specified in the study, this finding awaits further investigation. In an elegant study comparing the similarities and differences in genetic alterations between gastric amphicrine carcinomas and MiNENs, Sun *et al*[31] observed that the copy number (CN) characteristics of gastric amphicrine carcinomas were different from those of MiNENs based on a hierarchical clustering analysis, thus supporting that amphicrine carcinoma is a separate entity from MiNENs. In addition, a higher CN level of C5 (complement C5) was observed in amphicrine carcinomas than in MiNENs, suggesting that these tumors might benefit more from C5 inhibitors than MiNENs.

Currently available data also suggest that the inclusion of amphicrine tumors in the definition of MiNEN is not supported based on clinical and pathological features. Moreover, the fact that amphicrine tumors are a subject of debate does not exclude their consideration in the differential diagnosis of MiNEN.

PATHOGENESIS OF MiNEN

Although the carcinogenesis of MiNEN has not been clarified, recent studies suggest that they are derived from a single precursor cell that has the capacity for dual differentiation after the initiation of carcinogenesis. Studies on colorectal MiNEN have demonstrated that both components share common driver genetic aberrations in critical oncogenes and/or their protein products, including tumor protein p53 (TP53), retinoblastoma tumor corepressor 1 (RB1), adenomatous polyposis coli (APC), phosphatase and tensin homolog (PTEN), Kirsten rat sarcoma viral oncogene homolog (KRAS), v-myc avian myelocytomatosis viral oncogene homolog (MYC), phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit alpha (PI3KCA), and v-raf murine sarcoma viral oncogene homolog B (BRAF)[32-36]. Recent data have also suggested that microsatellite instability (MSI) and prostaglandin E2 receptor 4 (PTGER4) activation are also involved during the evolution of MiNEN[35]. Many studies have provided essential data about common driver genetic aberrations[32-34,37,38] . Indeed, both components display loss of heterozygosity (LOH) at multiple loci and mutations in key oncogenes with high allele frequencies compared to exclusive alterations of a single component, thus supporting their common clonal origin hypothesis, at least in the earlier steps of carcinogenesis[32-34,37,38].

In a more recent molecular study in gastric tumors with targeted DNA sequencing, a great majority of mutations were shared by both ADC and NEC components, and among them, TP53 was the most commonly mutated gene (69.2%)[39]. A subset of TP53-wild-type tumors had a microsatellite-unstable phenotype or amplifications in various oncogenes, including ERBB2 and NMYC. While differentially altered genes of ADC components were significantly associated with receptor tyrosine kinase signaling pathways, differentially altered genes of NEC components were significantly associated with the NOTCH signaling pathway, thus providing evidence for a possible clonal origin of ADC and NEC components of MiNENs[39].

Due to additional genetic alterations in poorly differentiated NEC (PDNEC), it is suggested that two different components activate separate genetic pathways at some step of carcinogenesis[32,36,38,40]. Another finding supporting a common carcinogenic pathway is that a proportion of either PDNEC or MiNEN of the stomach and colorectum has increased methylation with a mismatch repair-deficient phenotype[41-44]. Since these tumors had less aggressive behavior, similar to sporadic colon adenocarcinoma in the elderly, it is proposed that mismatch repair deficiency could be a pathway between PDNEC/MiNEN and adenocarcinoma[21,25]. In addition, the neuroendocrine component of PDNEC carries mutations specific to the organ from which it originates and is similar to adenocarcinoma in localization; meanwhile, these alterations are different from a common neuroendocrine alteration. When CDK2A and APC mutations were compared in the PDNEC of the colon and pancreas, it was observed that the CDK2A mutation was higher in pancreatic PDNEC compared with APC mutations, which were higher in the colon; this finding was consistent with adenocarcinomas originating from these organs [45]. The comparison of MiNEN with their pure neuroendocrine counterparts in the colon showed that while the former shares a similar copy number aberration profile with adenocarcinoma, the latter displays different structural aberrations; thus, the developmental pathway suggests that MiNEN is related to the nonneuroendocrine component but not to neuroendocrine carcinomas[34,35]. As noted

above, exclusive alterations in the neuroendocrine component carry a higher number of aberrations and an imbalance of alleles with a more aggressive phenotype, thus leading some authors to suggest that nonneuroendocrine components give rise to the neuroendocrine component through transdifferentiation, where c-myc and SMARC4 are potentially involved[32,46]. A significant increase in the number of neuroendocrine marker-expressing cells following neoadjuvant therapy in PDNEC supports the development of a neuroendocrine component through the adenoma-adenocarcinoma sequence[22,24]. The presence of cases of MiNEN that combine PDNEC and adenoma without adenocarcinoma is also postulated as evidence of a common carcinogenic pathway[47]. Evidence has also been obtained that the two components demonstrate distinct genetic patterns, suggesting that some MiNENs have polyclonal origins[35,37,38]. Interestingly, well-differentiated NET components of MiNEN do not share similar genetic alterations observed in their adenoma/adenocarcinoma counterparts, such as LOH of APC, KRAS, and TP53, although they do display specific alterations that are usually found in NETs (but not PDNECs), such as LOH of VHL, which may also represent true collision MiNEN with an independent carcinogenic pathway[42,47].

MANAGEMENT OF MiNEN

Although there is no complete consensus on the treatment of MiNEN, the presence of tumor metastasis at the time of diagnosis and histopathological MiNEN grading of the tumor play a vital role in the choice of therapeutic options[20]. The classification of MiNEN according to the grade malignancy is presented in Table 2.

Localized MiNEN

In all localized MiNENs, curative-intent surgery is recommended as the first treatment of choice, if available. Even in high-grade MiNENs, because of their less aggressive behavior than pure NECs, tumors with an acinar component of the pancreas belonging to this category have benefited from such treatment[48-50]. In the same group, although combinations of etoposide (VP16) and platinum salt or a combination of 5-fluorouracil (5FU) with irinotecan (IRI)- or oxaliplatin (OX)-based preoperative and postoperative chemotherapy have been used in tumor management similar to PDNEC therapy, the role of adjuvant therapy is still not completely defined[44,51-53]. Moreover, in the intermediate group, recent studies suggest replacing this therapy with a combination of 5FU and IRI and/or OX or gemcitabine (GEM) and/or OX for treatment parallel to the chemotherapy applied to adenocarcinomas, and in some cases, radiotherapy has been used as a treatment option in addition to chemotherapy[3,54-57].

Metastatic MiNEN

Unfortunately, extensive surgery does not seem to be an option in this group because the risk outweighs the benefits, especially in high-grade MiNENs. Therefore, systemic chemotherapy is generally performed according to the type present at metastatic sites. In patients with both components diagnosed either at the metastatic site or the primary tumor, therapy is based on the most aggressive component[3,54-57]. Since some intermediate-grade MiNENs with predominant NET components frequently express type 2 somatostatin receptors, such cases may also benefit from long-acting somatostatin analogs and peptide irradiation nucleotide therapy[58-60]. Although MANET is not categorized as MiNEN, its neuroendocrine component can metastasize. Therefore, pure NET-based chemotherapy and peptide irradiation nucleotide therapy are recommended for their treatment. At present, Akt/mTOR mutations have not been investigated in MiNENs. However, some patients who benefit from everolimus have been described[61].

Despite all these findings, the fact that further studies in large series are needed to define the treatment of patients more precisely with MiNEN should not be overlooked. Future studies aiming to determine the molecular vulnerability of both components in MiNEN cases in the GIS diagnosed based on criteria recommended for the diagnosis of these tumors may allow for the development of targeted therapies against both components and improve their treatment.

The treatment approach in MiNENs in both groups is briefly presented in Tables 3 and 4.

ORGAN-SPECIFIC CLINICOPATHOLOGICAL FINDINGS

Esophagus and gastroesophageal junction

Recently, a comprehensive systematic review and a multicenter study performed by Frizziero *et al*[3,15] showed that esophageal MiNENs accounted for between 5.9% and 15.9% of all GIS MiNENs. These tumors, which account for approximately one-quarter of the NENs observed in this localization, show an apparent male predominance and are observed at advanced ages (6th decade) in the distal third of the esophagus[2,19,62]. Although the tumor consists of NEC and squamous cell carcinoma (SCC) in

Table 2 Classification of mixed neuroendocrine-nonneuroendocrine neoplasms depending on the grade of malignancy[15]

Grade of MiNEN	Nonneuroendocrine component	Endocrine component
High-grade	Adenocarcinoma	PDNEC, NET G3[1]
	Squamous cell carcinoma	
	Cholangiocarcinoma	
	Ductal and acinar cell carcinoma	
	Ductal adenocarcinoma	
High-grade	Acinar cell carcinoma	NET, G1 or G2[1]
	Ductal adenocarcinoma	
	Acinar cell carcinoma	
Intermediate grade	Adenocarcinoma	NET, G1 or G2[1]
Low-grade[2]	Adenoma	NET, G1 or G2

[1]Grading should be performed according to the latest WHO classification.
[2]Those tumors are not included in the mixed neuroendocrine-nonneuroendocrine neoplasms category.
MiNEN: Mixed neuroendocrine-nonneuroendocrine neoplasms; PDNEC: Poorly differentiated neuroendocrine carcinoma; G: Grade; NET: Neuroendocrine tumor.

Table 3 Summary of the therapy protocols applied to localized mixed neuroendocrine-nonneuroendocrine neoplasms

Surgery	Complete resection is possible			Complete resection is not possible		
MiNEN grade	High	Intermediate	Low	High	Intermediate	Low
Chemotherapy	PDNEC-like	ADC-like	NR	PDNEC-like	ADC- like	NET-like

MiNEN: Mixed neuroendocrine-nonneuroendocrine neoplasms; ADC: Adenocarcinoma; PDNEC: Poorly differentiated neuroendocrine carcinoma; NET: Neuroendocrine tumor.

Table 4 Summary of the therapy protocols applied to metastatic mixed neuroendocrine-nonneuroendocrine neoplasms

Metastatic component	Defined		Not defined
	One component	Two components	
Chemotherapy target	The metastatic component	The most aggressive component	The most aggressive component in the primary tumor

most cases, there are rare adenocarcinoma cases, particularly adenocarcinoma, in the background of Barrett's esophagus[40,63,64]. Molecular data indicate their monoclonal origin, as demonstrated by LOH, RB1, TP53, and alterations in TP63, SOX2, DVL3, PTEN, PIK3A, and KRAS[40]. However, the monoclonal origin of esophageal MiNEN with a nonendocrine component composed of adenocarcinoma on the background of Barrett's metaplasia remains to be elucidated, and some authors postulate that this group reflects a true collision tumor rather than MiNEN[64].

In the esophagus, because the discrimination of basaloid SCC and small-cell NEC (SNEC) has paramount importance and may pose a diagnostic pitfall in routine microscopic evaluation, immunohistochemical staining for high molecular weight cytokeratins, p63, and p40, is highly recommended to discriminate basaloid SCC from SCNEC[17].

Unfortunately, their lower metastatic capacity (25% *vs* 54%) and longer survival time (28 *vs* 15 mo) relative to pure PDNEC do not change their poor prognosis[65]. Another important finding is the predictive role of the Ki-67 proliferation index of NEC for prognosis[15,66]. More recently, any statistically significant difference in OS between gastroesophageal GEP MiNEN *vs* colorectal MiNEN was detected[66].

Stomach

These tumors constitute 6%–20% of MiNENs located in the GIS, and 7% of NENs are located in the stomach[14,15,67]. Similar to those in the esophagus, stomach tumors are observed in elderly patients (5^{th}-6^{th} decades) and show a male predominance. Based on the macroscopic appearance of these tumors,

which are observed equally in the corpus and antrum of the stomach, they are not different from adenocarcinomas, and specific diagnostic findings on endoscopy have not been obtained[19]. Most of these aggressive tumors consist of well-differentiated adenocarcinomas, and the PDNEC component is mainly located deeper in the organ[68,69]. Although cases composed of adenocarcinoma and NETs have been recorded, it is suggested that the term MANEC can be retained for MiNEN at this location[19]. MiNENs composed of gastric NEN and adenocarcinoma have been described in the setting of chronic atrophic gastritis, etiological factors have not yet been entirely identified[70]. Recent molecular studies indicated a monoclonal origin[37,44,71]. Ishida *et al*[72] compared the molecular pathology of poorly differentiated NEC and MiNEN of the stomach by whole-exome sequencing. The analysis revealed recurrent mutations in 62% of TP53 cases, and they were more frequent in MiNENs than in NECs. Frameshift mutations of APC were observed in two MiNEN cases. In cases of MiNEN, two histological components shared mutations in TP53, APC, and ZNF521, whereas alterations in CTNNB1, KMT2C, PTEN, and SPEN were observed in neuroendocrine components only. They concluded that TP53 is a single, frequently mutated gene in gastric NEC and MiNEN, and alterations in other genes are less common, thus resembling the mutation profiles of gastric adenocarcinomas. Another interesting previous finding is the presence of ATRX gene mutations (primary partial loss) in 37% of cases involving a substantial proportion of gastric MiNEN[73]. However, these findings should be investigated in further studies. Gastric MiNENs are tumors with a poor prognosis that show lymph node and distant metastases at the time of initial diagnosis, and the prognosis is slightly better than that of pure PDNEC[15,74,75]. Similar to these findings, in a recent study including 401 patients, the 5-year disease-free survival was 51.1%, which was significantly better than that of NEC (47,6%) and worse than that of adenocarcinoma (57,8%). Furthermore, in the same series, advanced stages and lymph node metastasis were independent risk factors related to distant recurrence[76].

Small intestines

MiNENs of the small intestines frequently in the duodenum, where they are mainly located in the ampulla[77-81]. MiNENs of the jejunum and ileum are exceedingly rare, similar to PDNECs encountered in these regions[19]. They are equally observed in both sexes and older patients. While adenocarcinoma constitutes the nonneuroendocrine component of tumors in many cases, rare cases of SCC have also been recorded[77,78]. Since the neuroendocrine component is frequently located in the deeper part of the intestinal wall, they are frequently diagnosed as adenocarcinoma from biopsies[81]. The histological subtype of adenocarcinoma forming these tumors was also found to be associated with tumor behavior. MiNENs with intestinal-type adenocarcinoma have a better prognosis than those with the pancreaticobiliary subtype[20,25]. However, ampullary MiNENs are aggressive tumors with a poor prognosis and generally present at advanced stages[15,17]. This finding contrasts with MiNEN in the other part of the duodenum, which combine intestinal-phenotype adenocarcinoma and a well-differentiated somatostatin-secreting NET[20]. They are mostly superficial and not highly aggressive, and distant metastasis is rare.

Appendix

The last WHO classification of MiNEN indicated that these tumors were composed of two morphologically recognizable components generally represented by adenocarcinoma and NEC, and this classification has provided knowledge about mixed tumors in the appendix[18]. The exclusion of goblet cell carcinoids, which are currently determined to be amphicrine tumors, from the MiNEN group has led to the limited applicability of past clinicopathological data on these tumors located in the appendix, thus necessitating further evaluation of the findings related to this group[29]. A few studies conducted in the recent past indicate that MiNENs constitute 10% of malignancies at this location. On the other hand, a systematic review showed that among the lower gastrointestinal tract organs, these tumors were most frequently (60.3%) localized in the appendix[82]. An interesting finding is that the age-adjusted incidence for MiNENs increased from 0.01/100000 person-years to 0.07/100000 person-years (range 2004-2016), with an annual percentage change (APC) of 13.8%[83]. This finding can be attributed to the increase in clinical recognition and better diagnostic technologies over the years. They are observed in advanced age (between 58-60 years) mostly encountered incidentally and discovered at advanced stages [56,83,84]. Although recent studies indicate that these tumors do not show sex predilection, new findings that APC shows significant differences according to sex (13.81% in females *vs* 12.24% for males) need to be clarified[83].

Their overall survival rate is 6.5 years, which is better than that of signet ring cell carcinomas (2.1 years) and worse than that of goblet cell adenocarcinomas (13.8 years) and pure NETs (39.4 years)[56, 84]. In a more recent study, the prognosis of 315 patients with MiNEN was compared with that of other histological subtypes in the appendix, including NETs, NECs, goblet cell carcinoma, signet ring cell carcinoma, mucinous adenocarcinoma and nonmucinous adenocarcinoma, based on the surveillance, epidemiology and end results program 18 registries. The overall 5-year survival rate was 57.4%, and the level of invasion was the only independent factor influencing tumor behavior. In addition, multivariate analysis demonstrated that the prognosis of MiNENs was worse than that of NETs, NECs, goblet cell carcinoma, and mucinous adenocarcinoma but better than that of nonmucinous adenocarcinoma and signet ring cell carcinoma[83].

Their pathological differential diagnosis should include goblet cell carcinomas, tubular-type carcinoids and pure NETs with glandular configurations.

Colon and rectum

Tumors in this region constitute more than half of all MiNENs of the GIS[67,85]. In particular, evidence has shown that they are observed more frequently than pure NETs in resections performed in this region[85]. Their distribution among NENs is 14-20% and 1%-3% in the colon and rectum, respectively. They are more common in men than women and observed at an advanced age (6th decade)[25,67,85]. Although they do not have specific clinical findings, they have been defined in the background of inflammatory bowel diseases[86-89]. As a result of their more frequent detection in these regions, the number of molecular studies performed in MiNEN exceeds that of other localizations, and they have provided important data regarding their pathogenesis. The same genetic alterations in both components strongly support their monoclonal origin from a common precursor progenitor cell[33,35,37,38,42]. Parallel to these findings, a recent case showed that in addition to microsatellite instability due to MLH1 promoter methylation, the same mutations affecting the *ARID1A*, *ASXL1*, *BLM*, and *RNF43* genes occur in both components, as determined by a multigene next-generation sequencing panel. On the other hand, BRCA2 has been explicitly altered in the neuroendocrine area. Although the latter observation suggested that BRCA2 could be a potential new target for MiNEN, the lack of this alteration in the nonneuroendocrine part of the tumor requires further consideration concerning intratumor heterogeneity[90].

Macroscopically, these tumors form masses (average 5 cm) without distinguishing features from adenocarcinomas. Histologically, most cases are composed of adenocarcinoma and NEC. Rare cases in which the nonneuroendocrine component consisted of squamous cell carcinoma have also been noted [43,91]. If the neuroendocrine component consists of PDNEC, it is consistently observed in metastases. In comparison, adenocarcinomas are observed in one-third of cases[47]. Such tumors show aggressive behavior and have poor prognosis (overall survival: 12.2 mo) according to the Ki67 proliferative index of the NEC component as well as the MSI status and stage[42,43,67]. Recently, a systematic review demonstrated that if the neuroendocrine component consists of NETs, it is unknown which component will be encountered in metastatic sites because of a higher grade, and the predominance of one component does not warrant their presence in metastatic sites[47,92,93]. This finding emphasizes the complexity of MiNENs and the need for an accurate morphological description of all components. A recent systematic review also demonstrated that in MiNENs of the lower gastrointestinal tract, the site of origin in those with metastatic disease at diagnosis appeared to influence prognosis. The median survival time was 12.3 mo for those with primary colonic tumors *vs* 11.7 mo for those with primary anorectal tumors, with hazard ratios of 1.13 *vs* 0.80, respectively[82].

Pancreas

Pancreatic MiNENs are rare tumors in which the nonneuroendocrine component can be formed by ductal or acinar carcinoma[18]. In addition, tumors with a nonneuroendocrine part consisting of ductal and acinar carcinomas are extremely rare and defined as mixed ductal-acinar-neuroendocrine carcinomas[94]. Tumors with mixed ductal–neuroendocrine carcinoma are rare and account for approximately 0.5%–2% of all ductal adenocarcinomas and 5% of all NENs arising from this organ[27]. There is no sex predilection. Although the average age of onset is 68, they are observed in a wide age range, from 21 to 68 years old. They usually consist of NEC accompanying ductal adenocarcinoma[95-97]. They can be located anywhere in the organ and produce clinical symptoms similar to ductal adenocarcinoma without specific clinical findings. Because of the rare nature of these tumors, molecular data are scarce and limited[46,67,98]. Therefore, the diagnosis should be performed using adenocarcinoma and neuroendocrine tumor-specific markers separately and morphologically because they should be clearly distinguished from ductal adenocarcinomas with entrapped islets and NETs with entrapped ductules in the differential diagnosis[17,27]. In the former, the islands present an ovoid shape and have regular contours constituted by endocrine cells without atypia, and express all hormones, which is inconsistent with a predominant cell line of tumor cells; in the latter, however, the absence of atypia of ductal cells , the lack of aberrant P53 staining and the low proliferative index are essential clues in the differential diagnosis (Figure 2). An increase in the number and size of Langerhans islands that accompany chronic obstructive pancreatitis is another diagnostic pitfall in the differential diagnosis. Previous reports indicated that mixed ductal–neuroendocrine carcinomas are aggressive tumors with poor prognoses (5-year survival is 0%)[18,98]. More recently, lymph node metastasis was indicated as an adverse prognostic factor of disease-specific survival in 7 patients with mixed ductal–neuroendocrine carcinomas[99]. Similar findings were also observed by Zhang *et al*[92] in a larger number of patients. Although data for surgically resected cases are very limited in the literature, a cohort study reported that the median survival was 15.3 mo and all cases died due to disease[100]. However, in a recent study evaluating 8 cases with a median follow-up of 21 mo, the overall survival was 88 mo and the 5-year OS was 58%. In addition, the survival of these tumors was better than that of pancreatic ductal adenocarcinomas; thus, further investigation is warranted[101].

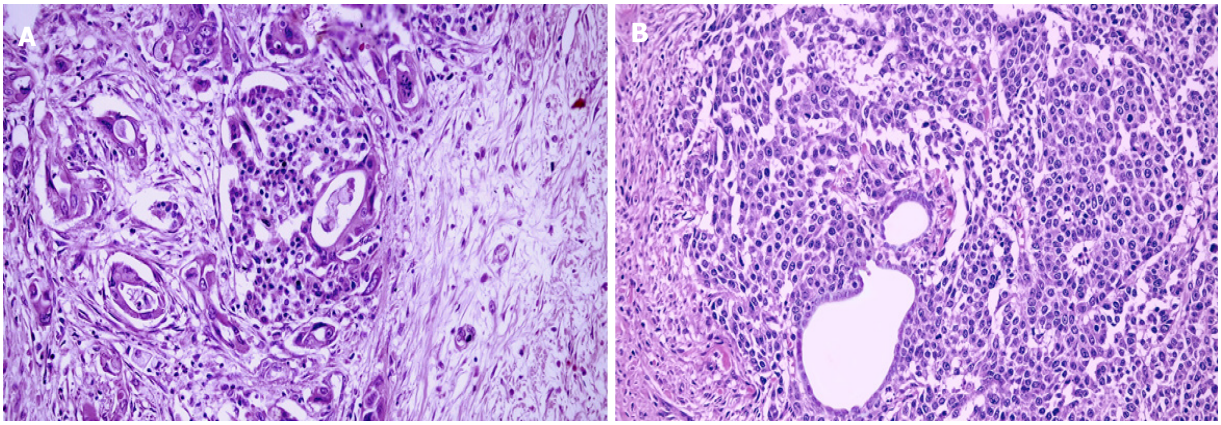

Figure 2 Histopathological pitfalls in the diagnosis of mixed neuroendocrine-nonneuroendocrine neoplasms of the pancreas. A: A ductal adenocarcinoma of the pancreas surrounding and invading an islet in the background of chronic pancreatitis (x 200). The islet has regular contours despite an invasion; B: A neuroendocrine tumor of the pancreas with entrapped two ductulus without atypia. Such areas should be evaluated carefully to avoid a misdiagnosis of mixed neuroendocrine-nonneuroendocrine neoplasms (x 200).

Cases in which the neuroendocrine component consists of NETs have been reported, although their morphology resembles true collision tumors and their monoclonal origin remains to be proven[95,96].

Mixed acinar–neuroendocrine carcinomas are rare and account for nearly one-fifth of all pancreatic ACCs[49,94]. The features of these tumors do not differ from the macroscopic features of ACC, and the tumors are quite large. In the differential diagnosis, morphological and IHC findings should be evaluated together, such as in ductal carcinoma. It is worth noting that 20%-30% of ACCs show a small neuroendocrine cell population, and this morphologically undetected component should not lead to the diagnosis of MiNEN[49]. Immunohistochemical staining with trypsin and bcl-10 [monoclonal antibody directed against the C-terminal portion of bcl-10 (clone 331.3)] is very useful for identifying the acinar component[102] (Figure 3). However, it should be kept in mind that a significant portion of the ACC is stained with synaptophysin.

Moreover, since pure NETs of the pancreas have a better prognosis than these tumors, advanced immunohistochemical evaluations with bcl-10 and trypsin are recommended for all tumors with neuroendocrine-appearing pancreatic neoplasms that show a high mitotic index, abundant necrosis, and evident nucleoli[17]. Although these tumors seem to share the genetic changes observed in pure ACCs, they do not show characteristic mutations that can be found in pancreatic NETs[103,104].

A recent study suggested that c-MYC alterations are involved in mechanisms leading to the neuroendocrine differentiation of ACCs[46]. Surgical resection and tumor stage are the most important prognostic factors, and the reported 5-year survival rate is 30%–50% for patients who undergo surgery [49,105].

Liver

The nonneuroendocrine component of MiNENs is mostly hepatocellular carcinoma (HCC) and less frequently cholangiocellular carcinoma, and they are rare liver tumors encountered at advanced ages (43-84 years) predominantly in men[2,106,107]. The neuroendocrine components of these tumors are predominantly NECs, and they have a dismal prognosis, with many cases presenting distant metastasis at the time of diagnosis[2,108,109]. More recently, the 1-year cumulative survival rate of patients was reported to be 53%[107].Although the pathogenesis has not been fully elucidated; neuroendocrine differentiation from existing HCC has been suggested[108,109].

Gallbladder and biliary tract

MiNENs of the gallbladder and biliary tract account for 10% of all biliary carcinomas and 2% of all hepatobiliary carcinomas[110,111]. Although considered rare, adenocarcinoma is detected in approximately 30% of NENs, particularly in the gallbladder, and these tumors constitute 35% of NENs in this region, thus indicating that they are more frequent than previously described[79,110]. While the age range is relatively wider than that of many MiNENs, these tumors are observed at an advanced age (mean: 65 years), which is similar to those in other regions of the GIS. However, compared with the male dominance observed in other MiNENs, these tumors are more common in females[18]. The close relationships between MiNENs and inflammatory diseases in these locations suggest that inflammation plays a role in pathogenesis. Recent findings indicate that the NEC component of the tumor is composed of large cell NECs in a great majority of cases (59%), and NECs are incidentally discovered during imaging studies without any specific clinical findings[112]. In parallel, specific findings that differ from the findings for adenocarcinomas on macroscopic examination have not been observed. Although a considerable portion of the tumors are confined to the gallbladder wall at the time of

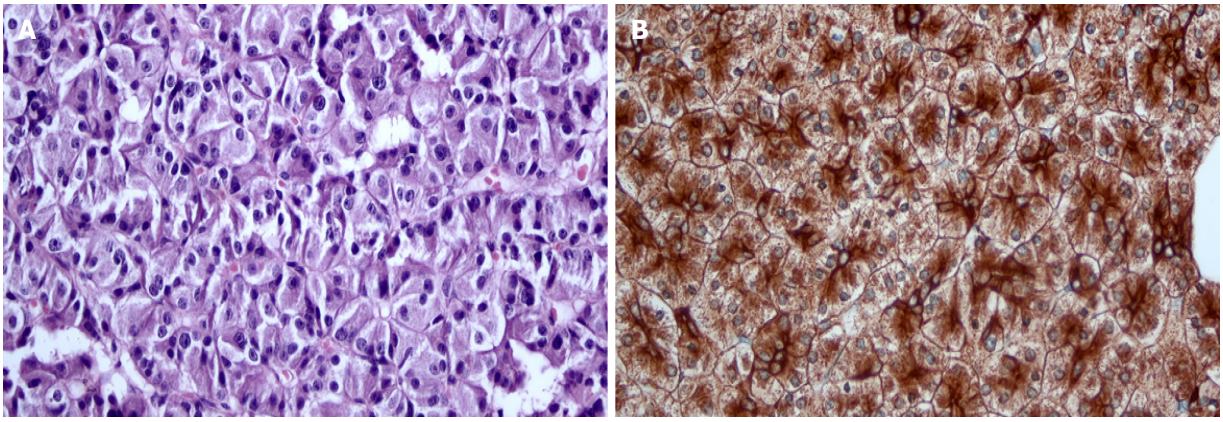

Figure 3 Acinar carcinoma of the pancreas. A: The tumor is composed of cells that demonstrate the presence of monomorphic nuclei, sometimes forming minute lumens. Tumor cells are in a monolayer with basally located nuclei and have a granular eosinophilic cytoplasm (x 400); B: Bcl-10 expression with higher staining in the apical portion of tumor cells (x 400).

diagnosis, one-fifth of the cases have serosa and one-third have adjacent organ invasion[16]. Therefore, the rarity of distant metastases in these tumors does not exclude the possibility that half of them metastasize to neighboring organs (liver, peritoneum, lymph nodes) at the time of diagnosis. On histopathologic evaluation, the nonneuroendocrine component often consists of adenocarcinoma and rarely consists of squamous cell carcinoma or carcinoma with sarcomatous or osteosarcomatous differentiation[112,113]. Few cases with intracystic papillary neoplasms (IPNs) have also been reported[16]. The NEN component, which is usually more deeply located, often consists of NEC. Molecular studies support that MiNENs in this region also consist of a common precursor[16,114]. Although studies on IPN have indicated that the endocrine component originates from these areas, further studies are needed to support this finding. The one-year survival was four times higher in patients with organ-confined tumors than in those with distant metastases, revealing that distant metastasis is the most effective predictive parameter for the course of the disease, thus emphasizing the importance of staging [79,110]. A recent systemic review based on 53 studies to predict the clinicopathological features and prognosis of biliary MiNENs, including gallbladder MiNENs, showed a median overall survival time of 21 mo. In addition, radical resection and small morphological subtype were independent prognostic factors associated with higher overall survival, and radical resection (R0) and younger age (< 65 years) were associated with higher recurrence-free survival time[115].

CONCLUSION

In conclusion, MiNENs of the GIS are a rare group of heterogeneous and aggressive tumors that should be diagnosed in patients without neoadjuvant therapy. Morphological findings are indispensable for their histopathological diagnosis. Evaluations based solely on the percentage of cells stained with IHC may lead to overdiagnosis. Since the current therapeutic approach depends on the grade of MiNEN, each component should be evaluated and graded separately. Although many studies support that these tumors are monoclonal, at least in the early stages of carcinogenesis, these data require additional research support.

Similarly, the 30% cutoff value should be reaffirmed by systematic studies because the possibility of the negative influence of a small component of high-grade NEN on tumor behavior should not be ignored. The deep localization of the NEN component in many organs is another potential limitation leading to their underestimation in biopsies. As recent findings suggest that amphicrine tumors may belong to a different tumor category, more studies are needed to reach a complete conclusion regarding these tumors.

In summary, new cases diagnosed as MiNENs in the GIS according to the currently proposed categories will increase awareness of these tumors, provide new data and eliminate diagnostic controversies of the past.

FOOTNOTES

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ORIGINAL ARTICLE

Basic Study

Mucosal bacterial dysbiosis in patients with nodular lymphoid hyperplasia in the terminal ileum

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Abstract

BACKGROUND

Nodular lymphoid hyperplasia (NLH) in the small intestine is a rare benign lesion characterized by multiple small nodules on the intestinal surface. Patients with terminal ileal NLH may experience long-term abdominal pain, diarrhea, and abdominal distension, among other symptoms. Supplementation with probiotics could mitigate these symptoms. NLH is linked to the immune system, and it may result from accumulation of plasma-cell precursors due to a maturational defect during the development of B lymphocytes. The intestinal microbiome plays an essential role in the immune system. Thus, we speculate that the gut flora plays a key role in terminal ileal NLH.

AIM

To explore the correlation between intestinal flora and terminal ileal NLH.

METHODS

We collected mucosal biopsy samples that were obtained *via* colonoscopy from 15 patients with terminal ileal NLH (the test group) and 15 normal subjects (the control group). We subsequently performed 16S-rRNA gene amplicon sequencing of these samples, and the results were evaluated using alpha diversity, beta diversity and microbial composition analyses. The Phylogenetic Investigation of Communities by Reconstruction of Unobserved States was used to predict the

metabolic pathways and orthologous groups according to the Kyoto Encyclopedia of Genes and Genomes database.

RESULTS

Compared with the control group, the terminal ileal NLH group showed an increased alpha diversity ($P < 0.05$). The overall intestinal microbiota in the NLH group was significantly different from that of the control group ($P < 0.05$), implying that there was the dysbiosis in the terminal ileal NLH patients. The relative abundance of phylum Bacteroidetes was significantly lower in the NLH group, while that of Patescibacteria and Campilobacterota was significantly higher. The genus *Bacteroides* was the dominant gut microbiota in both groups, but its abundance was significantly lower in the test group than it was in the control group. Conversely, the relative abundances of *Haemophilus, Streptococcus, Pseudomonas, Actinomyces, TM7X, Fusobacterium nucleatum, Parvimonas, Granulicatella, Helicobacter*, and the [*Eubacterium*] *nodatum group* were significantly higher in the test group than they were in the control group. In addition, several altered metabolic pathways, orthologous groups, and modules were found. For example, the Peptidoglycan biosynthesis and Aminoacyl tRNA biosynthesis were both increased in the test group.

CONCLUSION

Maintaining the microbial balance and supplementing targeted protective bacteria could improve symptoms and potentially reduce the risk of lymphoma transformation in patients with terminal ileal NLH.

Key Words: Hyperplasia; Bacteroides; Small intestine; Microbiome; Helicobacter pylori; Colonoscopy

Core Tip: Nodular lymphoid hyperplasia (NLH) in the small intestine is a rare benign lesion characterized by multiple small nodules on the surface of the intestine. To explore the correlation between the intestinal flora and terminal ileal NLH, we performed bacterial 16S rRNA gene sequencing of mucosal samples from patients with terminal ileal NLH. Our results reveal that specific microflora may act on the mucosa of the small intestine and cause terminal ileal NLH. Therefore, maintaining the balance of intestinal flora and supplementing targeted protective bacteria may improve terminal ileal NLH symptoms and potentially reduce the risk of lymphoma transformation.

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INTRODUCTION

Nodular lymphoid hyperplasia (NLH) in the small intestine is a rare benign lesion, and its incidence has not yet been determined. It is characterized by multiple small nodules on the surface of the intestine, which are found to be present in the lamina propria and superficial submucosa of the intestine[1]. The diagnosis of NLH is mainly based on endoscopic and histological examinations, markedly including the presence of hyperplastic lymphoid follicles and mitotically active germinal centers with well-defined lymphocytic mantles[2]. Terminal ileal NLH is a type of NLH, and patients with terminal ileal NLH demonstrate multiple symptoms that seriously reduce the individual's quality of life, such as chronic diarrhea, abdominal pain, hematochezia, anemia, hypoproteinemia, among other symptoms[3]. Patients with irritable bowel syndrome are more likely to accompany with NLH[4]. During the clinical diagnosis and treatment of patients with terminal ileal NLH, we found that probiotics could improve gastrointestinal symptoms.

NLH may be associated with a risk factor for intestinal lymphoma, as the resolution of gastrointestinal tract nodular lymphoid hyperplasia has been shown following chemotherapy for extraintestinal lymphoma[5]. However, the pathogenesis of NLH has not been fully elucidated yet. A frequently proposed hypothesis implicates an intestinal antigenic trigger, possibly infectious, that leads to the repetitive stimulation and eventual hyperplasia of the lymphoid follicles, which may originate

from proliferative plasma cell precursors related to a maturational defect during the development of B lymphocytes[6]. NLH has been reported in patients with human immunodeficiency virus, common variable immunodeficiency, Giardia lamblia infection, helicobacter pylori (*H.pylori*) infection, familial adenomatous polyposis, and Gardner's syndrome[7]. The intestinal microbiome plays an essential role in the immune system; gut microbiota that colonize the human intestinal tract form a mutual symbiotic relationship with the host and play a key role in regulating the host immune system and metabolism[8, 9]. Conventionally, alterations in the gut microbiota are closely related to inflammatory bowel disease, obesity, colonic adenoma, and colorecatal cancer[10-13], but whether the gut flora plays a role in NLH is unclear.

In this study, we performed bacterial 16S rRNA gene amplicon sequencing of mucosal tissue samples to study the gut microflora dysbiosis that is associated with terminal ileal NLH to determine what alterations occur in microflora and explore the correlation between the intestinal microflora and terminal ileal NLH. Moreover, we used molecular bioinformatic technology to predict the metabolic pathways that are involved in terminal ileal NLH, which will provide the possibility for further targeted intervention therapy.

MATERIALS AND METHODS

Clinical trial design and sampling

A total of 30 patients who underwent a colonoscopy in the Digestive Endoscopy Center at Jiading Branch of Shanghai General Hospital (Shanghai, China) from January 2021 to April 2021 were recruited for this study. A total of 15 Patients with terminal ileal NLH (11 males and 4 females aged 24-44 years)were assigned to the test group, while 15 healthy volunteers (7 males and 8 females aged 30-44 years)were assigned to the control group after undergoing a routine physical examination. There were no statistically significant differences in the general data between the groups ($P > 0.05$). Among the 15 patients with terminal ileal NLH, the most common symptom was diarrhea, followed by abdominal pain and abdominal distension.

Endoscopic images were reviewed and confirmed by the endoscopic team. Terminal ileal NLH was diagnosed using endoscopy and histopathology (Figure 1). We confirmed that there was no history of antibiotic or probiotic administration within the previous two months for all the subjects. Patients with diabetes mellitus, inflammatory bowel disease, previous colon resection, colorectal cancer, or a body mass index ≥30 kg/m^{2}were excluded. This research was approved by the research ethics boards of Shanghai General Hospital (2021KY085), and written informed consent was obtained from all the patients before sample collection. We collected mucosal biopsy samples that were obtained *via* colonoscopy from both groups. All samples were frozen immediately after sampling and stored at -80 °C.

DNA extraction/isolation

Microbial genomic DNA was extracted from each sample using the E.Z.N.A.® Stool DNA Kit (Omega Bio-tek, Inc., GA) according to the manufacturer's instructions. The samples were suspended in 790 μl of sterile lysis buffer (4M guanidine thiocyanate; 10% N-lauroyl sarcosine; and 5% N-lauroyl sarcosine-0.1 M phosphate buffer [pH, 8.0]) in a 2-ml screw-cap tube containing 1 gof glass beads (0.1mm BioSpec Products, Inc., United States). This mixture was vortexed vigorously and subsequently incubated at 70°C for 1 h. After incubation by bead beating for 10 min at maximum speed, the extracted DNA was stored at -20 °C for further analysis.

PCR amplification

The V3-V4 region of the bacterial 16S ribosomal RNA gene from each sample was amplified using the universal bacterial primers F1 and R2 (5′-CCTACGGGNGGCWGCAG-3′and 5′-GACTACHVGGGTATCTAATCC-3′); these primers correspond to positions 341 to 805 in the Escherichia coli 16S rRNA gene. The PCR reactions were run in a T100™ Thermal Cycler PCR system (Bio-Rad Laboratories, Inc., United States) using the following protocol: 3 min of denaturation at 95 °C, followed by 21 0.5-min denaturation cycles at 94 °C, 0.5 min of annealing at 54 °C, and 0.5 min of elongation at 72 °C, with a final 5 min extension at 72 °C.

Sequencing

The amplicons from different samples were purified using Hieff NGS® DNA Selection Beads (Yeasen-Biotech Co., Ltd., Shanghai, China). The products were indexed and mixed at equal ratios for sequencing by Shanghai Mobio Biomedical Technology Co., Ltd. using the Miseq platform (Illumina Inc., United States) according to the manufacturer's instructions.

Data availability

The raw sequencing data from the 16S rRNA gene V3-V4 regions and the accompanying information

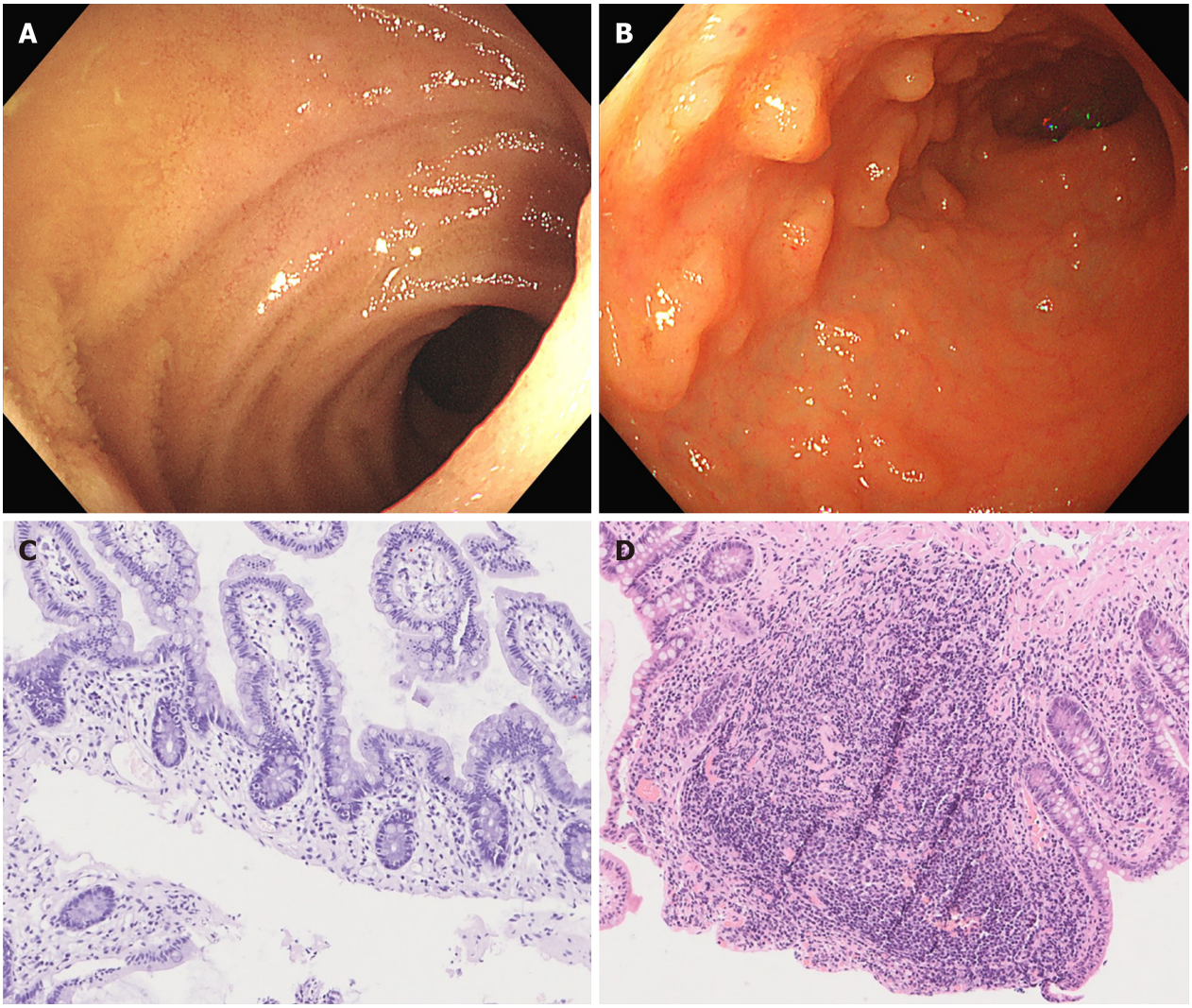

Figure 1 Endoscopic images and pathological characteristics of nodular lymphoid hyperplasia and normal mucosa in the terminal ileum.
A: Endoscopic image of a normal terminal ileum from a healthy volunteer; B: Endoscopic image of nodular lymphoid hyperplasia (NLH) in terminal ileum of a patients; C: Pathological characteristics of normal terminal ileal mucosa (×100; scale bar, 200 μm); D: Pathological characteristics of terminal ileal NLH: hyperplasic lymphoid follicles germinal centers (×100; scale bar, 200 μm).

are available in the Sequence Read Archive database under accession number PRJNA759383.

Bioinformatics analysis

Clean data was extracted from the raw data using USEARCH version 11.0.667 (http://www.drive5.com/usearch/). Quality-filtered sequences were clustered into unique sequences and sorted in order of decreasing abundance to identify representative sequences using UPARSE according to the UPARSE Operational Taxonomic Units (OTUs) analysis pipeline, with singletons being omitted. OTUs were classified based on a 97% similarity after the chimeric sequences were removed using UPARSE version 7.1 (http://drive5.com/uparse/), after which they were annotated using the SILVA reference database (SSU138). The number of common OTUs in both groups was calculated and the results were shown using a Venn diagram.

Alpha diversity, which reflects the diversity of microbiome community, was obtained by analyzing the ACE estimator, Chao 1 estimator, Shannon-Wiener diversity index, and Simpson diversity index using Mothur version 1.42.1. The larger the Chao 1 or ACE index, the higher the gut flora abundance, whereas the higher the Shannon or Simpson index, the higher the community diversity.

To visualize the structural diversity of the gut microbiome in the discovery group, we used a principal coordinates analysis (PCoA) and nonmetric multidimensional scaling (NMDS) plots based on the Bray-Curtis distances. The corresponding statistical significance of the beta diversity was measured separately using an Adonis analysis.

To compare the microbial communities at each taxonomic level between the groups, significant between-group differences in the microbial composition were analyzed using a Wilcoxon rank-sum test. A linear discriminant analysis effect size (LEfSe) was used to show the maximum difference in the microbial structures between the groups (LEfSe version 1.1, https://github.com/SegataLab/Lefse) to

determine the specific bacterial taxa and predominant bacteria that are related to terminal ileal NLH. The results of the microbiome heatmap analysis, as provided by a random forests model, revealed a discriminatory intestinal microbiome between the two groups.

The Phylogenetic Investigation of Communities by Reconstruction of Unobserved States (PICRUSt) 2 version 2.4.1 (https://github.com/picrust/picrust2/wiki) was used to predict different metabolic pathways and orthologous groups between the groups according to the yoto Encyclopedia of Genes and Genomes (KEGG) database.

Statistical analysis

Non-parametric Mann-Whitney U tests were used to determine if there were significant differences between the groups. A Student's t-test was performed using SPSS for Windows, version 20.

RESULTS

OTU clustering and alpha diversity analysis of the mucosal intestinal microflora

In total, 1530914 usable sequences were obtained from 30 samples using Illumina high-throughput sequencing technology. From these, 1263179 high-quality sequences were selected, with an average of 42106 sequences per sample. Using 97% as the similarity cutoff, we generated 554 OTUs. The Venn diagram showed that 500 of the 554 OTUs were shared by both groups, whereas 33 OTUs were unique to the test group, and 21 were specific to the control group (Figure 2A).

The alpha diversity of the intestinal mucosal microflora was higher for the test group than it was for the control group. As estimated by the observed OTUs in each sample and the ACE and Chao indexes, which reflect the richness of the species diversity, the microbial diversity was significantly higher in the test group than it was in the control group ($P < 0.05$).The index values for the alpha diversity analysis are shown in Figures 2B-F.

The resulting rarefaction curves indicate that the microbial richness of the sampled guts was near saturation at the applied sequencing depth, which was sufficient to identify most of the bacterial community members in each individual. The Shannon-Wiener curve based on the OTUs was already flat, indicating that our sequencing depth was already adequate. The specaccum species accumulation curves revealed that the OTU richness approached saturation in all the samples (Supplementary Figure 1).

Analysis of the beta diversity based on OTU levels

To evaluate the similarities between all samples, the ecologic distances, which were calculated based on the Bray-Curtis distances, were visualized using a PCoA plot. A certain tendency of separation was found between both groups, indicating that the bacterial flora differences in the overall structure of gut microbiota existed between the groups (Figure 3A). Moreover, a non-metric multidimensional scaling analysis based on the Bray-Curtis distances showed a significant difference in gut microbiomes between both groups (Figure 3B).

Additionally, an Adonis analysis showed that there was a significant difference between the two groups ($P < 0.05$). Based on the unweighted and weighted UniFrac distances, a PCoA also showed that the microbial composition of the test group deviated from the control group (Adonis: $P = 0.0142$ and $P = 0.0467$, respectively).

Flora composition in the two sample groups

A total of 19 phyla were detected by classifying the species of all OTUs in the terminal ileal mucosa. At the phylum level, the gut microbiota of both groups was dominated by Bacteroidetes and Firmicutes, followed by (on average) Proteobacteria. The proportions of dominant flora in the test group were 35.19%, 39.29%, and 14.31% respectively, while those of the control group were 46.11%, 36.68%, and 8.34%, respectively. The average relative abundance of the microbiome at the phylum level is shown in Figure 4A.

At the genus level, the gut microbiota was dominated by *Bacteroides* in the control group, followed by *Faecalibacterium*, *Fusobacterium*, *Escherichia-Shigella*, and *Prevotella* with proportions of 36.85%, 8.47%, 6.93%, 4.96%, and 4.33%, respectively. Correspondingly, *Bacteroides* was the most dominant bacteria in the test group, followed by *Prevotella*,*Faecalibacterium*, *Fusobacterium*, and Escherichia−Shigella, with proportions of 19.63%, 10.31%, 8.39%, 8.33%, and 7.43%, respectively. The average relative abundance of the microbiome at the genus level is shown in Figure 4B.

Differences in microbiome compositions between the groups

There were significant differences in the microbial composition between the groups, as analyzed by the Wilcoxon rank-sum test. At the phylum level, Bacteroidetes were significantly lower in the test group than in the control group. In contrast, Patescibacteria and Campilobacterota were significantly higher in the test group than in the control group. At the genus level, the number of *Bacteroides* was significantly

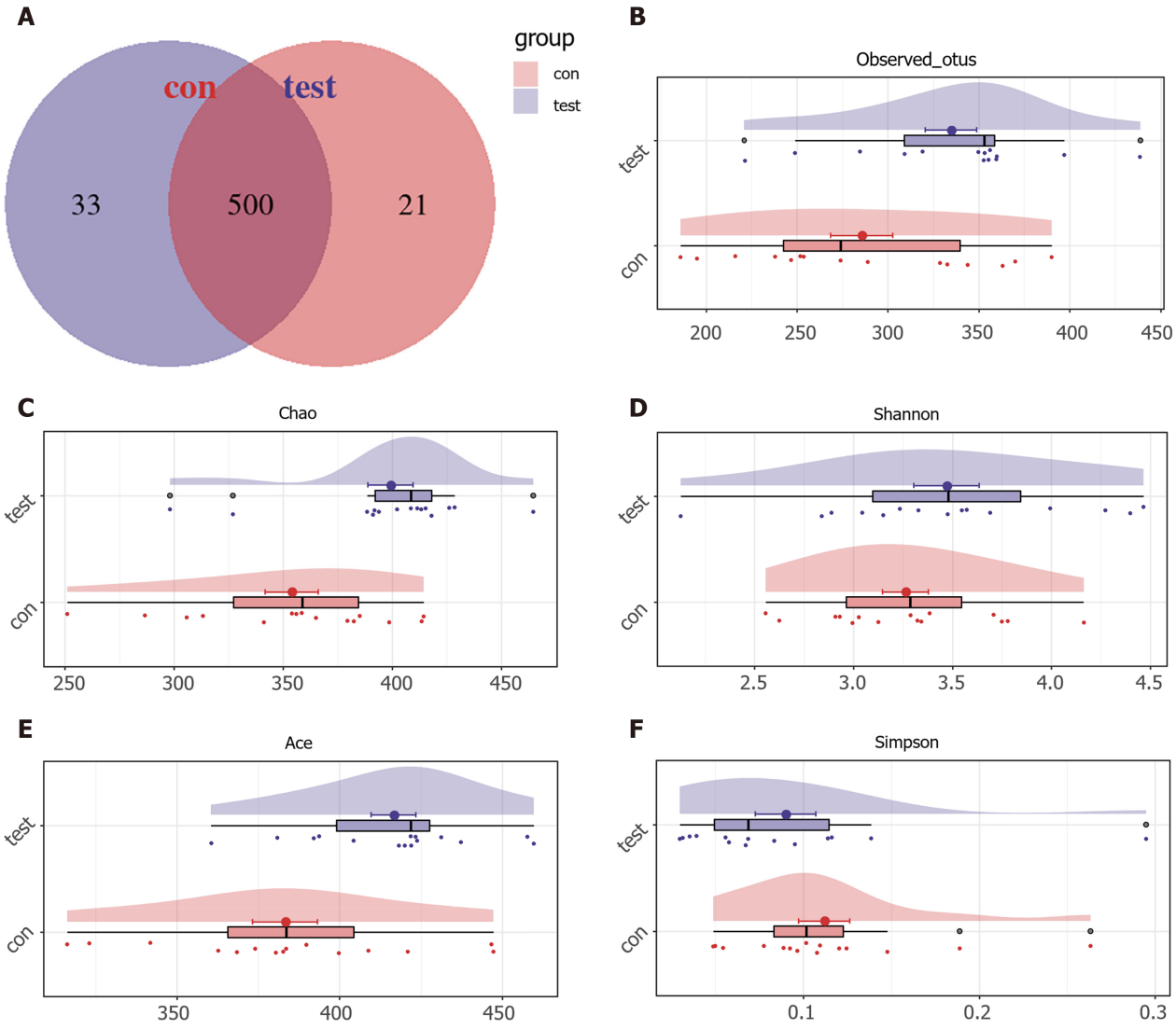

Figure 2 Operational Taxonomic Units clustering and alpha diversity analysis of mucosal intestinal microflora. A: Venn diagram demonstrates the shared and unique Operational Taxonomic Units (OTUs) in both groups; B: The OTUs in the single sample from each group; C: Chao index; D: Shannon-Wiener diversity index; E: ACE estimator; F: Simpson diversity index.

lower in the test group than in the control group. Conversely, the abundances of *Haemophilus*, *Streptococcus*, *Pseudomonas*, *Actinomyces*, *TM7X*, *Parvimonas*, *Granulicatella*, *Helicobacter* and the *[Eubacterium] nodatum group*, among others, were significantly higher in the test group than in the control group (Figures 4C and D).

A LEfSe was used to show the maximum difference in microbial structures between the groups to determine the specific bacterial taxa and predominant bacteria in the patients with terminal ileal NLH. This analysis showed that the abundances of various genera, including *Haemophilus*, *Streptococcus*, *Phocea*, *Candidatus_saccharimonas*, *Pseudomonas*, *Vagococcus*, *Cutibacterium*, *Actinomyces*, the *Eubacterium nodatum group*, *TM7X*, *Delftia*, *Chryseobacterium*, *Peptostreptococcus*, *Helicobacter*, *Parvimonas*, *Solobacterium*, and *Peptococcus*, among others, were significantly higher in the test group than in the control group. Conversely, the abundances of *Bacteroides*, *Bilophila*, and the *Eubacterium hallii group* were significantly higher in the control group than in the test group (Figure 4E).

The results of the heatmap analysis of the microbiomes using a random forest model revealed a discriminatory intestinal microbiome between both groups. A total of 28 OTUs were found to be different between the sample groups. Among these OTUs, 24 were more abundant in the test group than in the control group; these OTUs belonged to the genera of *Rothia*, *Roseburia*, *Cutibacterium*, *Peptococcus*, *Parabacteroides*, *Lachnoanaerobaculum*, *Actinomyces*, *Streptococcus* and *SaccharimonAdales*, *Solobacterium*, *Peptostreptococcus*, *Granulicatella*, *Parvimonas*, *Lachnoclostridium*, *Pseudomonas*, *Fusobacterium* , *Haemophilus*, *Prevotella*, and *Clostridia UCG-014*. *Bacteroides*, *Bilophila*, and the [*Eubacterium*] *Siraeum group* were more abundant in the control group than in the test group (Figure 4F).

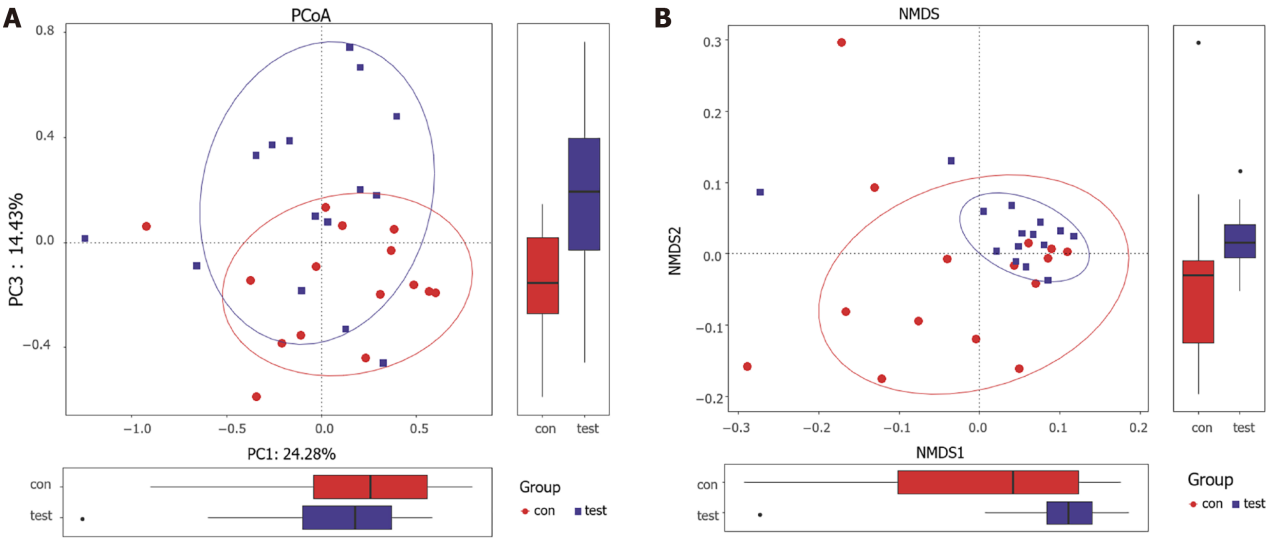

Figure 3 Analysis of beta diversity based on Operational Taxonomic Units levels. A: Principal coordinates analysis plots based on the Bray-Curtis distances shows the differences in the bacterial flora between the terminal ileal nodular lymphoid hyperplasia and control groups; B: Nonmetric multidimensional scaling analysis based on the Bray-Curtis distances shows the differences between both groups. Each symbol represents one sample.

Functional alterations of gut microbiomes in both groups

PICRUSt2 version 2.4.1 was used to predict metabolic pathways and orthologous groups according to the KEGG database, and a LEfSe was subsequently used to sort the different metabolic pathways and orthologous groups between the two groups. The results demonstrated that Photosynthesis, D Alanine metabolism, C5 Branched dibasic acid metabolism, Peptidoglycan biosynthesis, Aminoacyl tRNA biosynthesis, Bacterial chemotaxis, ABC transporters, D glutamine and D glutamate metabolism, synthesis and degradation of ketone bodies, ulfur relay system, ribosome, mismatch repair, homologous recombination, Glycerophospholipid metabolism, base excision repair, DNA replication, butanoate metabolism, bacterial secretion system, terpenoid backbone biosynthesis, and styrene degradation were significantly higher in test group compared to the control group. However, glycosaminoglycan degradation, secondary bile acid biosynthesis, sphingolipid metabolism, biotin metabolism, pentose and glucuronate interconversions, galactose metabolism, streptomycin biosynthesis, cyanoamino acid metabolism, and alanine aspartate and glutamate metabolism pathways were all significantly higher in the control group than in the test group. Altered metabolic pathways, orthologous groups, and modules in both groups are presented in Figure 5.

DISCUSSION

Terminal ileal NLH is commonly detected through colonoscopy. The pathogenesis of terminal ileal NLH remains unclear, it is generally regarded that infection-induced immune responses play a key role. Alterations of the gut microbiota are closely related to immune-related diseases. However, whether the gut flora plays a role in terminal ileal NLH is unclear. Currently, there are no studies that have reported on the relationship between the intestinal flora and terminal ileal NLH. In this study, alpha diversity was higher in the test group than in the control group. *Helicobacter, Fusobacterium nucleatum, Actinomyces, TM7X*, and *Peptostreptococcus* were significantly more abundant in the test group than in the control group. Additionally, the Peptidoglycan and Aminoacyl tRNA biosynthesis pathways were significantly more abundant in the test group than in the control group.

In this study, we found that the bacterial diversity was significantly higher in the terminal ileal NLH group than in the control group, suggesting the presence of a small intestinal bacterial overgrowth (SIBO). SIBO is defined as bacterial overgrowth in the small intestine caused by an abnormally high number of bacteria and/or changes in the kinds of bacteria; it is accompanied by an overgrowth of bacteria in the small bowel in excess of 10^5 colony forming units per milliliter in upper gut aspirate culture[14]. This is due to the bacteria migrating into the small intestine from distal intestinal tract, resulting in intestinal mucosal inflammation and permeability and villi damage, which mainly manifests as nutrient malabsorption, abdominal pain and distension, diarrhea, and intestinal motility abnormity[15]. SIBO is closely associated with many diseases, including colorectal cancer, irritable bowel syndrome, inflammatory bowel disease, and non-alcoholic fatty liver disease[16-19]. In this study, we found an increased alpha diversity of intestinal flora in the test group than in the control group. This observation may be related to the local inflammatory response caused by the overgrowth of intestinal

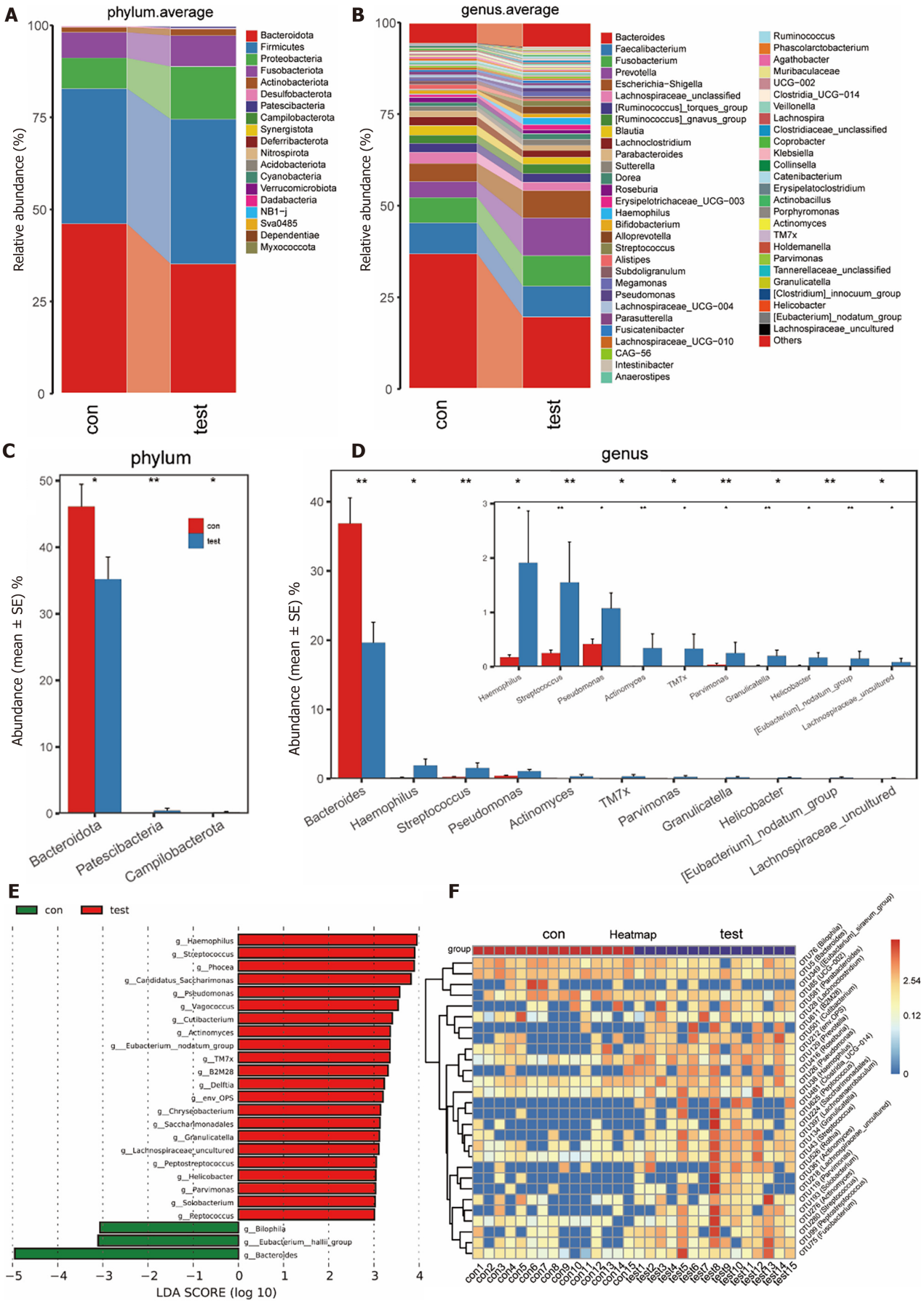

Figure 4 Flora composition and comparison of microbiome composition in both groups. A: Flora composition at the phylum level; B: Flora composition at the genus level; C: The abundance of Bacteroidetes was significantly lower at the phylum level in the test group, as assessed by a Wilcoxon rank-sum test; D: Differences in the flora at the genus level between the groups, as assessed by a Wilcoxon rank-sum test; E: Flora differences at the genus level, as assessed

by Linear discriminant analysis effect size; F: A heatmap analysis of the microbiomes using a random forest model.

bacteria, which results in NLH.

Bacteroidetes and Firmicutes make up most of the human intestinal flora, with a higher abundance of Bacteroidetes. In this study, Bacteroidetes and Firmicutes were the dominant bacteria in both groups, followed by Proteobacteria. Similarly, Bacteroides was the most dominant bacteria in both groups at the genus level. However, Bacteroidetes and Bacteroides were significantly less abundant in the test group than in the control groups, suggesting that Bacteroides may play a protective role in the development of terminal ileal inflammation and NLH.

In our research, *H.pylori* was higher in abundance in the test group. *H.pylori*, which is a proteobacteria, is considered to be a carcinogenic factor of gastric cancer. Gastric mucosa-associated lymphoid tissue (MALT) lymphoma is related to *H.pylori*[20,21], as most patients with MALT can achieve long-term clinical remission after *H.pylori* eradication[22,23]. *H.pylori* is thought to be an antigenic stimulant that can activate the NF-κB pathway[24] and induce pro-inflammatory cytokines expression [25,26]. Persistent inflammation promotes the formation of mucosal lymphoid follicles, typically consisting of B lymphocytes, which might contribute to the genesis of gastric MALT lymphoma once the inflammatory courses are uncontrolled[27]. Khuroo *et al* studied a large cohort of patients (n = 40) with NLH that was etiologically related to *H.pylori* infection. Compared with patients with consistent *H.pylori* infection, patients with eradicated *H.pylori* showed a significant clinical response and lesion regression/resolution [28]. However, the location was limited to the postbulbar duodenum (second and third parts) and duodenojejunal junction in these cases. In our study, the *H.pylori* abundance increased in test group. Moreover, it has been reported that NLH may be associated with an increased risk of parenteral lymphoma. However, currently, there are no relevant reports on the correlation and causal relationship between terminal ileal NLH and *H.pylori*, which is worthy of studying. When treating patients with *H.pylori*, we suggest that endoscopists should routinely observe the terminal ileum.

In this study, the abundance of Actinomycetes and TM7x increased in the test group. Actinomycetes mostly reside in the oral cavity, upper respiratory tract, digestive tract, and urogenital tract in humans and animals, as part of the normal flora. Actinomycetes are recognized to be a cause of chronic appendicitis. Actinomycetes are also associated with Crohn-like appendicitis with significant fibrosis, transmural inflammation, lymphoid hyperplasia, and granuloma[29]. TM7x is a saccharifying bacteria, which is a parasitic bacterium that interacts with core members of Actinomycetes. A previous study suggested that ultra-small bacteria may have the ability to regulate the immune response of normal hosts, and there was a signal overlap between TM7x and basibiont Actinomyces odontolyticus species (XH001) by metabolic pathway prediction[30]. Moreover, through *in vitro* experiments, the authors demonstrated that TM7x inhibited TNF-α expression in XH001-induced macrophages[31]. Consequently, the interaction between Actinomycetes and TM7x may promote terminal ileal NLH.

In this study, we found that the abundance of Fusobacterium nucleatum increased in the test group, and this increase is possibly related to terminal ileal NLH. Fusobacterium nucleatum has been reported to be enriched in colorectal cancer tissues and played a crucial role in the occurrence and development of colorectal cancer[32]. It can adhere to and invade intestinal epithelial cells and activate the β-catenin pathway by releasing FadA adhesin and binding with cadherin E-cadherin, thus promoting inflammation and tumor response[33]. Recently, it has been reported that Fusobacterium nucleatum macromolecules (> 50 KDA) have a proinflammatory effect on human intestinal epithelium, and the outer membrane vesicles can promote the secretion of proinflammatory cytokines, including IL-8 and TNFα by epithelial cells[34]. Further animal experiments also verified the proinflammatory effect of Fusobacterium nucleatum on intestinal epithelium. Thus, Fusobacterium nucleatum may be associated with the development of terminal NLH.

In our study, the abundance of Peptostreptococcus increased in the test group. Anaerobic peptostreptococcus and Peptostreptococcus magnus are most commonly in the genus of Peptostreptococcus, among which Anaerobic peptostreptococcus is the most common pathogen. Anaerobic peptostreptococcus is a gram-positive anaerobic bacteria commonly residing in the oral cavity and digestive tract. The abundance of Anaerobic peptostreptococcus in the stool samples of patients with colorectal cancer was reported to be higher than healthy volunteers. In vitro studies have shown that Anaerobic digestion streptococcus interacts with TLR2 and TLR4 in colon cells and increases the level of active oxides, thereby promoting cholesterol synthesis and cell proliferation[35]. The PCWBR2 integrin α2/β1-PI3K-Akt-NF-κB signal axis has been reported to be involved in the development of colorectal cancer[36]. Peptostreptococcus may be related to terminal ileal inflammation and NLH, although further confirmation of this is needed in the future.

Metabolic pathways, such as the Peptidoglycan biosynthesis and Aminoacyl tRNA biosynthesis, increased in the test group in the current study. Peptidoglycan, a bacterial cell wall component, is a conserved pathogen-associated molecule that is involved in the innate immune system because it recognizes pattern recognition receptors that are secreted and expressed in or on the cell surface[37]. Transfer RNAs (tRNAs) mainly participates in protein translation by transporting amino acids to the ribosome. Nevertheless, accumulating evidence has shown that tRNAs are closely associated with

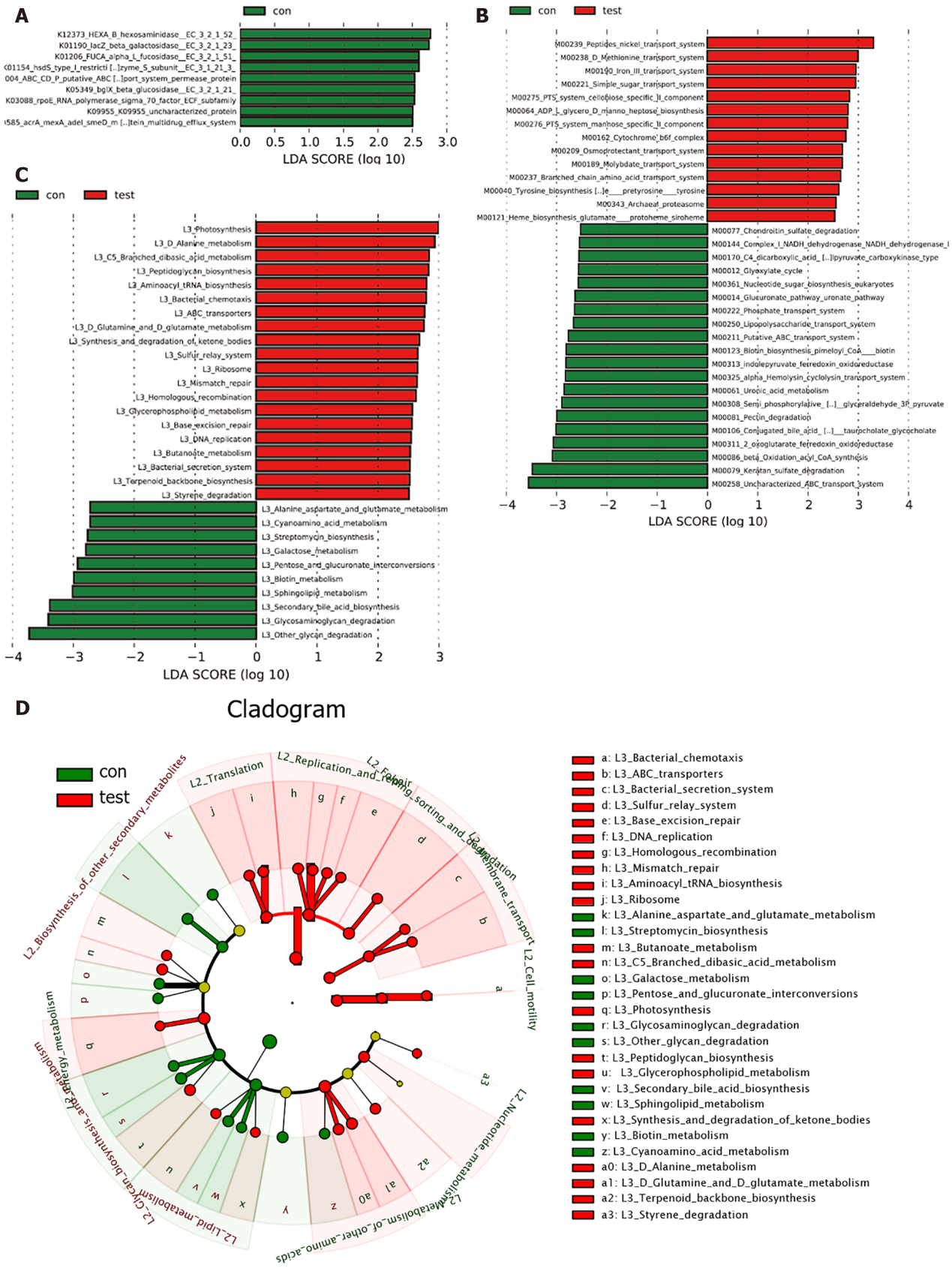

Figure 5 Predictions of the functional alteration to the gut microbiomes in patients with nodular lymphoid hyperplasia in the terminal ileum. A: Decreased the yoto Encyclopedia of Genes and Genomes orthologous groups in patients with terminal ileal nodular lymphoid hyperplasia (NLH) as shown by the histogram of the linear discriminant analysis (LDA) scores; B: Altered modules in patients with terminal ileal NLH (red, terminal ileal NLH tissue; green, control); C: Altered metabolic pathways in patients with terminal ileal NLH as shown by the histogram of the LDA scores (red, terminal ileal NLH tissue; green, control); D: Altered metabolic pathways in patients with terminal ileal NLH (red, terminal ileal NLH tissue; yellow, insignificant; green, control) as shown by a cladogram.

various physiological and pathological processes such as immune regulation. Aminoacyl-tRNA synthetases (ARSs) are essential components of translation in all living species, and it has taken scientists decades to confirm that eukaryotic ARSs act as global cell signaling mediators to regulate cell homeostasis beyond their intrinsic function as protein synthesis enzymes. Recent discoveries have revealed that ubiquitously expressed standby cytoplasmic ARSs sense and respond to danger signals and regulate immunity against infections, indicating their potential as therapeutic targets for infectious diseases[38,39]. Perhaps Peptidoglycan biosynthesis and Aminoacyl-tRNA biosynthesis might act as the targeted intervention sites.

The etiology of terminal ileal NLH has not been fully elucidated yet. In this study, we firstly analyzed the diversity and composition of intestinal flora in the mucosal tissues of the patients with terminal ileal NLH and predicted the metabolic pathways using 16S-rRNA technology. We subsequently found that terminal ileal NLH was related to the disturbance of the intestinal flora and certain microflora might act on the small intestinal mucosa thereby causing terminal ileal NLH. Moreover, the metabolic pathways that were predicted using PICRUSTs are possibly involved in terminal ileal NLH, which provides novel ideas for further exploration of potential molecular mechanisms. Diarrhea was frequently commonly found in patients with terminal ileal NLH, and some patients may get a satisfactory effect through probiotic supplementation. Therefore, exploring intestinal flora changes, seeking related bacteria genera in patients with terminal ileal NLH, and supplementing targeted protective bacteria or clearing targeted bacteria may reduce the risk of lymphoma and improve patient symptoms.

This study has some limitations. First, the sample size was relatively small. Although our preliminary results reveal that there was a significant difference between the two groups, studies with larger sample sizes covering different regions and populations are necessary to confirm the findings. Second, the male-female ratio in the NLH group was not balanced in this study due to the nature of terminal ileal NLH, which is thought to be significantly more common in men than women. Although the incidence of terminal ileal NLH in both men and women has not been investigated through a large-scale study, Lin *et al* observed that males outnumbered females by approximately four to one in a small-scale study[3]; their results support, to a certain degree, the suggestion that there is a higher frequence of terminal ileal NLH in males than females. We also performed a correlation analysis to compare the intestinal flora with gender using the Multivariable Association with Linear Models2, and we found that there was no correlation between the intestinal floras and gender at the phylum, genus, or OTU levels. To obtain more rigorous results, we will perform a large-scale study and ensure that there is an equal gender ratio among groups. Third, this study was only a preliminary correlation analysis between the intestinal flora and terminal ileal NLH, and no further research on the related mechanisms was performed. Further studies using animal testing *in vivo* and *in vitro* cellular experiments can be developed once our findings are verified in larger populations.

CONCLUSION

Intestinal flora disturbances are related to terminal ileal NLH, and our results show that certain microflora may act on the small intestinal mucosa and cause terminal ileal NLH. Maintaining the intestinal flora balance and supplementing targeted protective bacteria could improve terminal ileal NLH symptoms and potentially reduce the risk of lymphoma transformation.

ARTICLE HIGHLIGHTS

Research background

Nodular lymphoid hyperplasia (NLH) in the small intestine is a rare benign lesion characterized by multiple small nodules on the intestinal surface. NLH is linked to the immune system, and it may result from accumulation of plasma-cell precursors due to a maturational defect during the development of B lymphocytes. The intestinal microbiome plays an essential role in the immune system. However, whether the gut flora plays a role in NLH is unclear.

Research motivation

To explore the correlation between intestinal flora and terminal ileal NLH and predict the metabolic pathways that are involved in terminal ileal NLH.

Research objectives

To investigate the characteristics of the mucosal microbiata in patients with terminal ileal NLH for seeking related bacteria genera and bringing a new idea for related mechanisms.

Research methods

A total of 30 patients who underwent a colonoscopy were recruited for this study. A total of 15 Patients with terminal ileal NLH were assigned to the test group, while 15 healthy volunteers were assigned to the control group after undergoing a routine physical examination. We collected mucosal biopsy samples that were obtained *via* colonoscopy from both groups. We subsequently performed 16S-rRNA gene amplicon sequencing of these samples, and the results were evaluated using alpha diversity, beta diversity and microbial composition analyses. The Phylogenetic Investigation of Communities by Reconstruction of Unobserved States was used to predict the metabolic pathways and orthologous groups according to the Kyoto Encyclopedia of Genes and Genomes database.

Research results

The terminal ileal NLH group showed an increased alpha diversity.The overall intestinal microbiota in the NLH group was significantly different from that of the control group. The relative abundance of phylum Bacteroidetes was significantly lower in the NLH group, while that of Patescibacteria and Campilobacterota was significantly higher. The abundance of the genus *Bacteroides* was significantly lower in the test group. Conversely, the relative abundances of *Haemophilus, Streptococcus, Pseudomonas, Actinomyces, TM7X, Fusobacterium nucleatum, Parvimonas, Granulicatella, Helicobacter, and* the *[Eubacterium] nodatum group* were significantly higher in the test group. Metabolic pathways such as Peptidoglycan biosynthesis and Aminoacyl tRNA biosynthesis were both increased in the test group.

Research conclusions

Maintaining the microbial balance and supplementing targeted protective bacteria could improve symptoms and potentially reduce the risk of lymphoma transformation in patients with terminal ileal NLH.

Research perspectives

Further research on the related mechanisms was needed to be performed in future. Further studies using animal testing *in vivo* and *in vitro* cellular experiments can be developed once our findings are verified in larger populations.

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FOOTNOTES

Author contributions: Jiang QL and Lu Y equally contributed on this manuscript, designed and conceived the experiments; Lu Y and Li WH collected patient samples and data; Jiang QL, Zhang MJ, Cui ZY and Pei ZM performed the 16S rRNA sequencing data analysis; Jiang QL and Lu YY acquired and analyzed data, wrote the manuscript; Lu YY and Wang JJ provided guidance for sample processing methodology; Lu LG reviewed the manuscript; all authors approved the final version of the article.

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ORIGINAL ARTICLE

Retrospective Cohort Study

Differential DNA methylation analysis of *SUMF2*, *ADAMTS5*, and *PXDN* provides novel insights into colorectal cancer prognosis prediction in Taiwan

Jing-Quan Su, Pin-Yu Lai, Pei-Hsuan Hu, Je-Ming Hu, Pi-Kai Chang, Chao-Yang Chen, Jia-Jheng Wu, Yu-Jyun Lin, Chien-An Sun, Tsan Yang, Chih-Hsiung Hsu, Hua-Ching Lin, Yu-Ching Chou

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Abstract

BACKGROUND

Patients with colorectal cancer (CRC) undergo surgery, as well as perioperative chemoradiation or adjuvant chemotherapy primarily based on the tumor–node–metastasis (TNM) cancer staging system. However, treatment responses and prognostic outcomes of patients within the same stage vary markedly. The potential use of novel biomarkers can improve prognostication and shared decision making before implementation into certain therapies.

AIM
To investigate whether *SUMF2*, *ADAMTS5*, and *PXDN* methylation status could be associated with CRC prognosis.

METHODS
We conducted a Taiwan region cohort study involving 208 patients with CRC recruited from Tri-Service General Hospital and applied the candidate gene approach to identify three genes involved in oncogenesis pathways. A methylation-specific polymerase chain reaction (MS-PCR) and EpiTYPER DNA methylation analysis were employed to detect methylation status and to quantify the methylation level of candidate genes in tumor tissue and adjacent normal tissue from participants. We evaluated *SUMF2*, *ADAMTS5*, and *PXDN* methylation as predictors of prognosis, including recurrence-free survival (RFS), progression-free survival (PFS), and overall survival (OS), using a Cox regression model and Kaplan–Meier analysis.

RESULTS
We revealed various outcomes related to methylation and prognosis. Significantly shorter PFS and OS were associated with the CpG_3+CpG_7 hypermethylation of *SUMF2* from tumor tissue compared with CpG_3+CpG_7 hypomethylation [hazard ratio (HR) = 2.24, 95% confidence interval (CI) = 1.03-4.85 for PFS, HR = 2.56 and 95%CI = 1.08-6.04 for OS]. By contrast, a significantly longer RFS was associated with CpG_2 and CpG_13 hypermethylation of *ADAMTS5* from normal tissue compared with CpG_2 and CpG_13 hypomethylation [HR (95%CI) = 0.15 (0.03-0.71) for CpG_2 and 0.20 (0.04-0.97) for CpG_13]. The relationship between the methylation status of *PXDN* and the prognosis of CRC did not reach statistical significance.

CONCLUSION
Our study found that CpG_3+CpG_7 hypermethylation of *SUMF2* from tumor tissue was associated with significantly shorter PFS and OS compared with CpG_3+CpG_7 hypomethylation. CpG_2 and CpG_13 hypermethylation of *ADAMTS5* from normal tissue was associated with a significantly longer RFS compared with CpG_2 and CpG_13 hypomethylation. These methylation-related biomarkers which have implications for CRC prognosis prediction may aid physicians in clinical decision-making.

Key Words: DNA methylation; Biomarkers; Tumor tissue; Adjacent normal tissue; Prognosis prediction; Colorectal cancer

Core Tip: Our research revealed that differential DNA methylation of candidate genes in tumor tissue and adjacent normal tissue can be used to evaluate colorectal cancer prognosis. Certain CpG sites and the methylation status of *SUMF2* and *ADAMTS5* were significantly associated with colorectal cancer recurrence, progression, and survival. We recommend using our findings to investigate prognostic biomarkers applicable to patients with colorectal cancer.

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INTRODUCTION

Colorectal cancer (CRC) is the third most commonly diagnosed malignancy and the fourth leading cause of cancer-related deaths worldwide[1]. According to global estimations, in 2018, 1.8 million new cases of CRC were diagnosed, and 0.8 million people died from CRC that year[2]. By 2030, the worldwide CRC burden is predicted to increase by 60%; in 2030, it is expected that 2.2 million patients will be newly diagnosed as having CRC and 1.1 million CRC-related deaths will occur worldwide[1]. The treatment and survival of patients with CRC are closely related to cancer staging systems. The classification of CRC stages is based on the tumor– node–metastasis (TNM; T, size of the primary tumor; N, nearby affected lymph nodes; M, distant metastasis) staging system from the Eighth Edition of the

American Joint Committee on Cancer Staging Manual[3]. However, the heterogeneity of CRC means that patients with the same CRC stage may have different treatment responses and survival times[4]. Although breakthroughs in surgery, chemotherapy, radiotherapy, targeted therapy, and immunotherapy have improved the survival of patients with CRC, the 5-year survival of patients with stage IV disease is as low as 14% owing to CRC's heterogeneous nature and association with diverse molecular alterations, which are involved in cancer cell progression[5]. Intensive postoperative surveillance programs are proposed after tumor resection to detect asymptomatic recurrence in advance and prolong the survival patients who may be suited to further curative therapy[6]. Therefore, identifying molecular biomarkers for predicting patient prognoses or monitoring cancer relapse is crucial.

Several studies have focused on identifying new prognostic indicators for CRC[7]. Prognostic biomarkers can be employed as personalized indicators to predict disease progression, such as early recurrence, metastasis, and mortality[8]. These biomarkers are associated with molecular patterns of genomic mutations and epigenetic alterations that lead to CRC carcinogenesis[9]. DNA methylation alterations play a pivotal role in CRC progression and metastasis[10]. Promoter DNA hypermethylation silences genes such as *MLH1*, *CDKN2A*, *MGMT*, *RUNX3*, *TPEF*, *VIM*, and *SFRP1/2/4/5*, which play crucial roles in the cell cycle, DNA repair, and signal transduction[11]. Consequently, DNA methylation of genes may be a novel epigenetic indicator of patient prognosis.

SUMF2 was regarded as one of the frequently mutated genes in CRC, and *SUMF2* mutation frequently altered pathways in the tumorigenesis of CRC[12]. *ADAMTS5* was found upregulated in CRC, which was associated with tumor progression and even unfavorable clinical outcomes[13]. To determine the effect of the DNA methylation of selected genes on CRC prognosis over 5 years, we examined methylation status and extent in tumor tissue and tumor-free areas adjacent to such tissue. We propose that the differential DNA methylation of candidate genes in tumor samples and in matched adjacent normal tissue could assist in prognosis prediction and the optimization of CRC treatment.

MATERIALS AND METHODS

Patient and specimen collection

In this retrospective cohort study, we analyzed the data of patients diagnosed as having CRC from 2006 to 2010 and who underwent surgical treatment at Tri-Service General Hospital (TSGH), Taiwan, to assess their 5-year prognosis. All participants signed informed consent forms before their involvement in this research. Then surgeons gathered specimens in patients including colorectal cancer tissue and adjacent normal regions during surgery. The tissue were deposited at -80 ℃ ultra-low temperature freezers for further analysis. The study was approved by the TSGH Institutional Review Board (TSGHIRB approval numbers 098-05-292 and 2-105-05-129). According to the clinical practice guidelines of the Division of Colon and Rectal Surgery of TSGH, patients with CRC should return to the outpatient department for a follow-up every 3 mo in the first year after surgery and once every 3 to 6 mo afterward. The clinical and demographic characteristics of enrolled patients, including sex, age at surgery, clinical staging, tumor size, histological grade, lymph node count, tumor location, and adjuvant chemotherapy as well as follow-up information on recurrence, metastasis, and survival were acquired from the cancer registration database of TSGH.

Recurrence-free survival (RFS), progression-free survival (PFS), and overall survival (OS) were calculated from the date of surgery to disease progression (inclusive of cancer recurrence or metastasis), death from any cause, or until the final follow-up date before December 31, 2010. In total, 208 patients who met the inclusion criteria were enrolled. A flow diagram of the study's design is presented in Figure 1.

Gene selection and DNA extraction

The candidate gene approach was applied for genetic association studies, which has been widely used to investigate novel prognostic biomarkers related to CRC[14]. First, we searched for genes whose expression might influence CRC prognosis by browsing PubMed (https://pubmed.ncbi.nlm.nih.gov/), University of California, Santa Cruz Genome Browser (https://genome.ucsc.edu/) and Prediction of Clinical Outcomes from Genomic Profiles (https://precog.stanford.edu/). Subsequently, we confirmed gene methylation differences in CRC tissue and normal tissue using the Shiny Methylation Analysis Resource Tool website (http://bioinfo-zs.com/smartapp/). We then searched PubMed (https://pubmed.ncbi.nlm.nih.gov/) to review related literature with the keyword "gene name + colorectal cancer". If the number of results was less than 30, it was thought that the gene had been rarely studied for colorectal cancer. Those genes which met the above three conditions were included in this study. Therefore, we chose 16 candidate genes include *CFLAR*, *RBM44*, *ABCG1*, *WDR74*, *ZNF292*, *EFHA2*, *PXDN*, *TEC*, *CDH2*, *ADAMTS5*, *COL4A2*, *PCGF2*, *EMID2*, *GRPEL2*, *DKK2* and *SUMF2*. Because of the limited resources and experimental results, we narrowed down to *SUMF2*, *ADAMTS5*, and *PXDN*. These three candidate genes involved in the pathways associated with cancer stages and prognosis, such as inflammation, epithelial–mesenchymal transition, tumor migration, and angiogenesis.

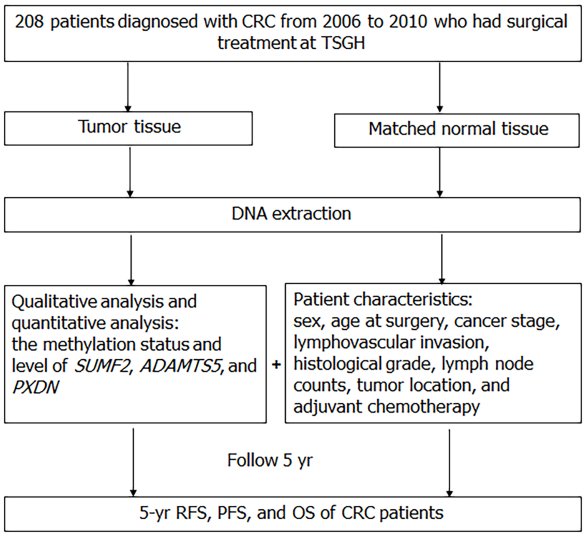

Figure 1 The study design flow-diagram. CRC: Colorectal cancer; TSGH: Tri-Service General Hospital; *SUMF2*: Sulfatase modifying factor 2; *ADAMTS5*: ADAM metallopeptidase with thrombospondin type 1 motif 5; *PXDN*: Peroxidasin; RFS: Recurrence-free survival; PFS: Progression-free survival; OS: Overall survival.

In accordance with the manufacturer's instructions, cellulose-coated magnetic beads were used to extract genomic DNA from the samples by using the Genomic DNA Tissue Kit (Catalog No. 69504; Qiagen, Taipei, Taiwan). We used sterile blades to cut about 10 mg tissue minced into the 1.5 mL microcentrifuge tube, and 200 μL Lysis Buffer and 6 μL Proteinase K were prepared and added. The 1.5 mL microcentrifuge tube was placed at 56°C water bath machine for 8-10 h after orbital shaker and centrifuge use. We confirmed that tissues were dissolved as clarified liquid, and then the tissue would have a vortex and centrifugation. Next, we used pipette to put tissue lysate into the 96 Deep Well Plate of the MagCore Compact Automated Nucleic Acid Extractor (Catalog No. MCA0801; RBC Bioscience, Taipei, Taiwan). Subsequently, we used the EZ DNA Methylation Kit (Zymo Research Corporation, Orange, CA, United States) to modify isolated DNA using sodium bisulfite.

Methylation-specific polymerase chain reaction and EpiTYPER DNA methylation analysis

The gene methylation statuses of *SUMF2*, *ADAMTS5*, and *PXDN* were evaluated through a methylation-specific polymerase chain reaction (MS-PCR). The total volume of the reaction solution was 20 μL, and it contained HotStart Taq Premix (RBC Bioscience, Taipei, Taiwan) (9 μL for *SUMF2* and *PXDN*; 10 μL for *ADAMTS5*), 0.5 μL of forward and reverse primers, 1 μL of bisulfite-modified DNA, and pure water (9 μL for *SUMF2* and *PXDN*; 8 μL for *ADAMTS5*).

For MS-PCR, the oligonucleotide primers, annealing temperature of each primer used for amplification, and PCR product sizes were described in Table 1. PCR cycling was performed as follows: 10 min at 95 °C, 38 cycles of denaturation for 30 s at 95 °C, 30-s annealing at a gene-appropriate temperature, 30-s elongation at 72 °C, final extension for 7 min at 72 °C, and holding at 4 °C. After amplification, PCR products were mixed with a loading buffer, electrophoresed (100 V for 28–30 min) on 2.75%-4% agarose gels using 1–2 μL of gel-stained dye, and visualized using an ultraviolet transilluminator. To confirm our experiment results were without error, we used SssI-treated DNA as positive control, and sterile water as negative control. Figure 2 showed that the methylation-specific polymerase chain reaction (MS-PCR) results of *PXDN* gene in CRC patients. Negative control, positive control and sterile water represent unmethylation-specific reaction, methylation-specific reaction and no contaminant reaction for PCR, respectively.

We further identified the CpG sites of *SUMF2* and *ADAMTS5* (Figure 3) and analyzed DNA methylation changes by using an Agena Bioscience MassARRAY system with EpiTYPER biochemistry (Agena Bioscience, San Diego, CA), an advanced method for quantitative DNA analysis.

The oligonucleotide primers, annealing temperature of each primer used for amplification, and PCR product sizes used in the EpiTyper assay were described in Table 1. The PCR cycling protocols were as follows: 15 min at 95 °C, 38 cycles of denaturation for 30 s at 95 °C, 30-s annealing at a gene-appropriate temperature, 30-s elongation at 72 °C, final extension for 10 min at 72 °C, and holding at 4 °C. In vitro transcription and base-specific cleavage were performed using the MassCLEAVE kit (Agena Bioscience). In total, 0.22 μL of T cleavage mix, 3.14 mmol/L DTT, 20 U of T7 RNA and DNA polymerase, and 0.09 mg/mL RNase A were prepared and added to a 7 μL reaction solution with shrimp alkaline phosphatase inactive PCR product. The final product was stored at 37 °C for 3 h. After the addition of a cation exchange resin to remove salt that remained after the reactions, the EpiTYPER reaction products were loaded onto the matrix pad of a SpectroCHIP Array (Agena Bioscience). The size

Table 1 Primer sequences, annealing temperature and product size for MS-PCR and EpiTYPER DNA methylation analysis of target genes

Genes		Forward primer (5' → 3')	Annealing temperature (°C)	Product size (bp)
SUMF2	M	F:TTTGATTATGGTCGGTTTTGC	59.4	191
		R:GACTACTTACAACTCCCCTAACGAC		
	U	F:TTTTTTGATTATGGTTGGTTTTGTG	60.6	198
		R:CCCAACTACTTACAACTCCCCTAACA		
	Q	F:TTTGTTATAGAGGGATGGGAGATAG aggaagagag	60	232
		R:CAAAATAAACAACACTCCAAATTCA cagtaatacgactcactatagggagaaggct		
ADAMTS5	M	F:GTTATTGTCGTGGAGCGTTAGC	59.4	170
		R:CCTACCTCCCGTACTTCCCG		
	U	F:TTATTGTTGTGGAGTGTTAGTGTTT	59.4	169
		R:CCTACCTCCCATACTTCCCACAT		
	Q	F:aggaagagagTTGAAATTGTTATTGTAGGATGGTATG	61.3	245
		R:cagtaatacgactcactatagggagaaggctAATTAAAACAAAAATACAAAAAAACAACC		
PXDN	M	F:TATGCGGGACGAGAACGAGA	61.6	137
		R:ACTTAAACAACTCCGTAACAATACGAT		
	U	F:GTGTATGTGGGATGAGAATGAGAG	60.4	142
		R:CAACTTAAACAACTCCATAACAATACAA		

MS-PCR: Methylation-specific polymerase chain reaction; SUMF2: Sulfatase modifying factor 2; ADAMTS5: ADAM metallopeptidase with thrombospondin type 1 motif 5; PXDN: Peroxidasin; M: Methylation; U: Unmethylation; Q: Quantitative analysis.

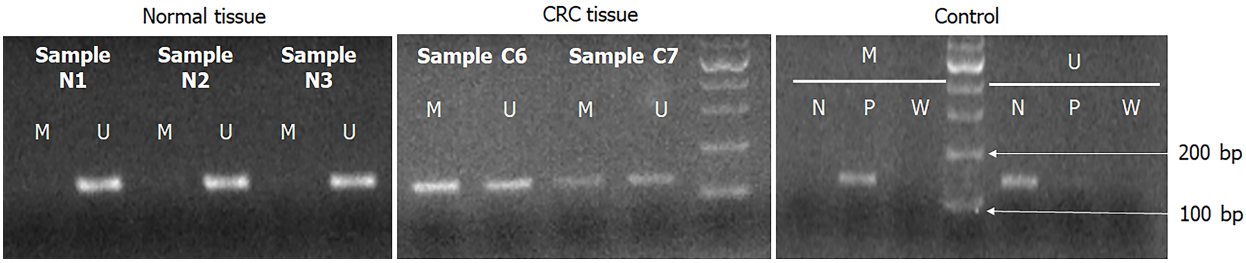

Figure 2 The methylation-specific polymerase chain reaction results of *PXDN* gene in colorectal cancer patients. Negative control, positive control and sterile water represent unmethylation-specific reaction, methylation-specific reaction and no contaminant reaction for polymerase chain reaction, respectively. CRC: Colorectal cancer.

and mass of the resulting cleavage products varied based on sequence changes. The fragment contained CpG sites had two kinds of molecular weight (GC or AC) because of the methylation status. There are 16 Daltons of signal divergence between the methylated and unmethylated sequence, which was observed by the mass spectrometry. These differences provided quantitative information on each target fragment, which was acquired using the MassARRAY Analyzer 4 (Agena Bioscience). Signal shifts of fragments indicated methylation events at single CpGs or small groups of CpGs (CpG units), and signal intensity was linked to DNA methylation extent, which was analyzed using EpiTYPER software (Agena Bioscience).

Statistical analysis

For each candidate gene, different DNA methylation statuses related to RFS, PFS, and OS were investigated using univariate Cox proportional hazards analyses. The resultant multivariate Cox proportional hazards regression model was then used to verify the independent prognostic effects of gene methylation status after adjustment for various clinical variables (sex, age at surgery, stage, and lymph node counts). The 5-year RFS, PFS, and OS curves for the methylation status of the selected genes were presented using a Kaplan–Meier survival analysis and compared using a log-rank test. We used SPSS

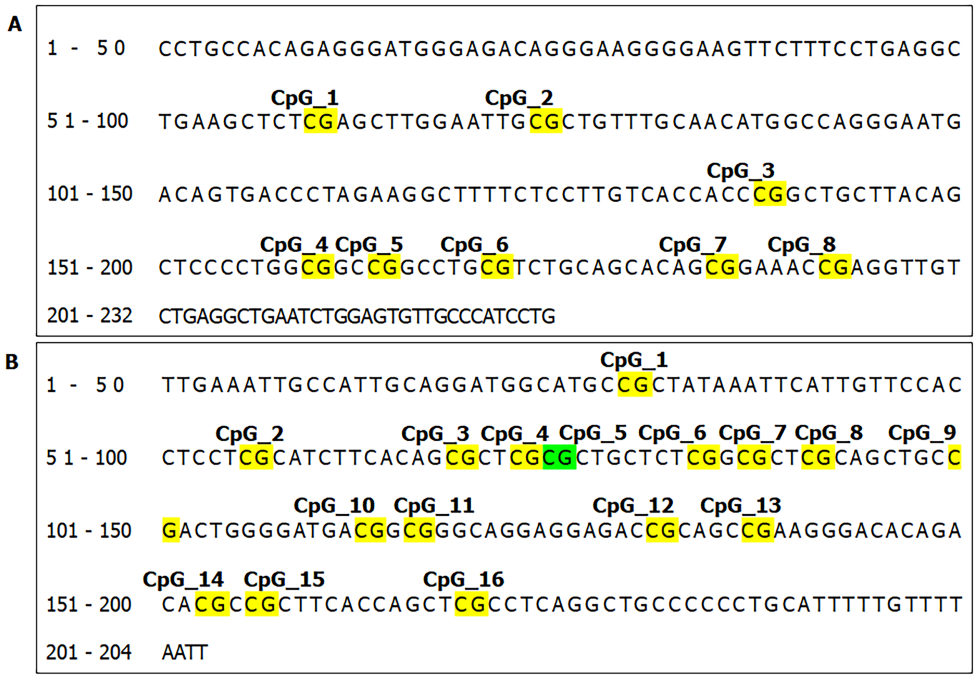

Figure 3 The location of informative CpG sites in the (A) *SUMF2* and (B) *ADAMTS5* promoter region.

version 23 (IBM SPSS Statistics 23) for statistical analyses. All *P* values were calculated from two - sided tests, and *P* value below 0.05 represented statistical significance.

RESULTS

Patient characteristics

In total, 208 patients diagnosed as having CRC at TSGH were recruited to participate. We analyzed tumor samples and adjacent nontumorous tissue of enrollees. The correlation of the candidate genes' methylation status and extent with the demographic and clinicopathological characteristics of patients was assessed, and the results are presented in Table 2. Normal tissue with *SUMF2* methylation significantly belonged to poor or undifferentiated histological grade. Tumor tissue with *PXDN* methylation significantly belonged to lower stages and had less lymphovascular invasion. No other associations were found between the methylation statuses of these three genes and the demographic or clinicopathological variables of the participants.

Quantitative analysis of genes

Although the selected genes were methylated in both tumor tissue and matched normal tissue (*SUMF2*, 50% *vs* 52.9%; *ADAMTS5*, 75.0% *vs* 62.9%; *PXDN*, 78.2% *vs* 72.4%), we observed that the differential DNA methylation of candidate genes, especially *SUMF2* and *ADAMTS5*, tended to affect patient prognosis according to the Kaplan–Meier method. To further investigate the extent and patterns of gene methylation in patients with CRC, we analyzed *SUMF2* and *ADAMTS5* methylation levels in primary CRC tissue samples and in adjacent normal samples. A higher percentage of *SUMF2* genes methylation at loci CpG_1, CpG_2, CpG_3 and CpG_7 was detected in primary CRC samples compared with adjacent normal tissue. We also found a higher level of *ADAMTS5* genes methylation at loci CpG_1, CpG_2, CpG_9, CpG_10.11, CpG_12, CpG_13, CpG_14.15, and CpG_16 in CRC tissue than in adjacent normal samples. Both findings reached statistical significance (Table 3).

Relationship between gene methylation and prognosis

The correlation between the methylation status of each gene and the 5-year RFS, PFS, and OS of patients with CRC was analyzed. We did not observe a significant correlation of the *SUMF2*, *ADAMTS5*, and *PXDN* methylation statuses of normal and tumor tissue and with patients' 5-year RFS, PFS, and OS in univariate and multivariate Cox proportional hazards regression analyses. The methylation of *SUMF2* in normal tissue was associated with poorer 5-year PFS and OS in a Kaplan–Meier survival analyses (*P* = 0.500 and 0.260, respectively). In a univariate analysis, we observed that patients with a stage III+IV disease had a poor RFS, PFS, and OS [hazard ratio (HR) = 4.68 and 95% confidence interval (95%CI) = 2.09-10.46 for RFS, 2.21 (1.25-3.91) for PFS, and 1.90 (1.00-3.61) for OS], even after adjustment for

Table 2 Characteristics and distribution of methylation status in patients with colorectal cancer (*n* = 208)

Characteristics	Total	Methylation status					
		SUMF2		*ADAMTS5*		*PXDN*	
		Normal	Tumor	Normal	Tumor	Normal	Tumor
Sex							
Male	103 (49.5)	20 (60.6)	27 (55.1)	28 (60.9)	44 (77.2)	35 (76.1)	44 (77.2)
Female	105 (50.5)	17 (45.9)	25 (45.5)	38 (64.4)	49 (73.1)	41 (69.5)	53 (79.1)
χ^2 (*P* value)		0.97 (0.324)	0.62 (0.432)	0.03 (0.866)	0.1 (0.755)	0.28 (0.596)	< 0.01 (0.969)
Age at surgery							
mean ± SD	64.3 ± 14.6	64.9 ± 14.2	66.9 ± 15.8	66.4 ± 14.8	67.7 ± 15.5	65.0 ± 14.4	66.2 ± 15.0
< 65	103 (49.5)	17 (51.5)	26 (53.1)	28 (58.3)	43 (72.9)	34 (70.8)	46 (78.0)
≥ 65	105 (50.5)	20 (54.1)	26 (47.3)	38 (66.7)	50 (76.9)	42 (73.7)	51 (78.5)
χ^2 (*P* value)		< 0.01 (1.00)	0.15 (0.694)	0.46 (0.498)	0.1 (0.755)	0.01 (0.915)	< 0.01(1.00)
Stage							
I	29 (13.9)	7 (58.3)	7 (50.0)	9 (50.0)	12 (63.2)	12 (66.7)	17 (89.5)
II	77 (37.0)	13 (48.1)	17 (45.9)	21 (56.8)	33 (75.0)	29 (78.4)	39 (88.6)
III	68 (32.7)	13 (65.0)	21 (63.6)	22 (68.8)	31 (75.6)	22 (68.8)	27 (65.9)
IV	34 (16.3)	4 (36.4)	7 (35.0)	14 (77.8)	17 (85.0)	13 (72.2)	14 (70.0)
χ^2 (*P* value)		2.77 (0.429)	4.5 (0.212)	4.06 (0.255)	2.5 (0.476)	1.17 (0.760)	8.69[a] (0.034)
5-yr recurrence[1]							
No	141 (82.0)	28 (51.9)	35 (49.3)	43 (56.6)	61 (70.9)	54 (71.1)	71 (82.6)
Yes	31 (18.0)	4 (40.0)	9 (60.0)	12 (80.0)	14 (82.4)	11 (73.3)	12 (70.6)
χ^2 (*P* value)		0.12 (0.731)	0.22 (0.639)	1.98 (0.160)	0.45 (0.504)	< 0.01 (1.00)	0.65 (0.421)
5-yr all-cause death							
No	168 (80.8)	29 (54.7)	43 (51.8)	50 (61.0)	79 (78.2)	59 (72.0)	77 (76.2)
Yes	40 (19.2)	8 (47.1)	9 (42.9)	16 (69.6)	14 (60.9)	17 (73.9)	20 (87.0)
χ^2 (*P* value)		0.07 (0.786)	0.24 (0.625)	0.26 (0.611)	2.15 (0.142)	< 0.01 (1.00)	0.71 (0.399)
5-yr progression							
No	155 (74.5)	28 (56.0)	39 (50.0)	45 (58.4)	73 (76.8)	56 (72.7)	74 (77.9)
Yes	53 (25.5)	9 (45.0)	13 (50.0)	21 (75.0)	20 (69.0)	20 (71.4)	23 (79.3)
χ^2 (*P* value)		0.32 (0.570)	< 0.01(1.00)	1.75 (0.185)	0.38 (0.540)	< 0.01 (1.00)	< 0.01 (1.00)
Lymphovascular invasion[1]							
No	106 (52.5)	20 (48.8)	25 (48.1)	32 (56.1)	46 (73.0)	41 (71.9)	55 (87.3)
Yes	96 (47.5)	16 (57.1)	27 (51.9)	34 (72.3)	47 (78.3)	34 (72.3)	41 (68.3)
χ^2 (*P* value)		0.19 (0.662)	0.04 (0.845)	2.26 (0.133)	0.23 (0.634)	< 0.01 (1.00)	5.44[a] (0.02)
Histological grade[1]							
Well or moderately	156 (89.7)	27 (48.2)	35 (47.3)	52 (64.2)	67 (74.4)	58 (71.6)	67 (74.4)
Poor or undifferentiated	18 (10.3)	6 (100.0)	9 (64.3)	7 (70.0)	14 (82.4)	9 (90.0)	13 (76.5)
χ^2 (*P* value)		3.94[a] (0.047)	0.76 (0.382)	< 0.01 (0.99)	0.15 (0.697)	0.75 (0.387)	< 0.01 (1.00)
Lymph node counts[1]							
0-11	34 (18.4)	7 (63.6)	7 (50.0)	12 (80.0)	15 (83.3)	13 (86.7)	16 (88.9)
≥ 12	151 (81.6)	29 (52.7)	40 (50.0)	50 (61.7)	70 (73.7)	58 (71.6)	70 (73.7)
χ^2 (*P* value)		0.07 (0.786)	< 0.01 (1.00)	1.14 (0.287)	0.33 (0.568)	0.81 (0.368)	1.18 (0.278)

Tumor location[1]							
Colon	147 (79.9)	28 (50.0)	38 (50.7)	53 (67.9)	62 (70.5)	60 (76.9)	68 (77.3)
Rectum	37 (20.1)	8 (80.0)	9 (47.4)	9 (50.0)	23 (92.0)	11 (61.1)	18 (72.0)
χ^2 (*P* value)		1.99 (0.158)	< 0.01(1.00)	1.35 (0.245)	3.76 (0.052)	1.17 (0.280)	0.08 (0.780)
Adjuvant chemotherapy[1]							
No	54 (29.3)	10 (58.8)	12 (42.9)	15 (55.6)	25 (73.5)	20 (74.1)	29 (85.3)
Yes	130 (70.7)	26 (53.1)	35 (53.0)	47 (68.1)	60 (75.9)	51 (73.9)	57 (72.2)
χ^2 (*P* value)		0.02 (0.898)	0.46 (0.499)	0.85 (0.358)	0.01 (0.971)	< 0.01(1.00)	1.59 (0.207)

[1]The total number of colorectal cancer patients does not correspond because of missing data.
[a]*P* < 0.05. *SUMF2*: Sulfatase modifying factor 2; *ADAMTS5*: ADAM metallopeptidase with thrombospondin type 1 motif 5; *PXDN*: Peroxidasin; SD: Standard deviation.

Table 3 Methylation level of sulfatase modifying factor 2 and ADAM metallopeptidase with thrombospondin type 1 motif 5 in normal tissue and tumor tissue (*n*= 208)

	Normal			Tumor			*P* value
	n[1]	Median	mean ± SD[2]	*n*[1]	Median	mean ± SD[2]	
SUMF2							
CpG_1	69	0.40	0.43 ± 0.15	104	0.53	0.55 ± 0.17	< 0.001
CpG_2	70	0.56	0.56 ± 0.11	104	0.76	0.73 ± 0.13	< 0.001
CpG_3	70	0.38	0.39 ± 0.11	104	0.54	0.54 ± 0.17	< 0.001
CpG_7	48	0.64	0.64 ± 0.15	80	0.87	0.81 ± 0.20	0.001
ADAMTS5							
CpG_1	66	0.06	0.08 ± 0.07	95	0.19	0.25 ± 0.20	< 0.001
CpG_2	70	0.06	0.08 ± 0.06	105	0.20	0.24 ± 0.18	< 0.001
CpG_9	69	0.06	0.07 ± 0.06	91	0.21	0.25 ± 0.18	< 0.001
CpG_10.11	69	0.08	0.10 ± 0.09	103	0.30	0.34 ± 0.22	< 0.001
CpG_12	65	0.09	0.10 ± 0.08	98	0.19	0.24 ± 0.21	0.001
CpG_13	63	0.07	0.12 ± 0.14	94	0.15	0.22 ± 0.22	0.009
CpG_14.15	71	0.32	0.33 ± 0.05	105	0.43	0.44 ± 0.11	< 0.001
CpG_16	71	0.10	0.11 ± 0.04	105	0.19	0.22 ± 0.12	< 0.001

[1]The total number of colorectal cancer patients does not correspond because of missing data.
[2]Represent the ratio of DNA methylation.
SD: Standard deviation; *SUMF2*: Sulfatase modifying factor 2; *ADAMTS5*: ADAM metallopeptidase with thrombospondin type 1 motif 5.

confounding factors in the multivariable analysis of tumor tissue [HR (95%CI) = 9.85 (2.20-44.15) for RFS, 4.08 (1.49-11.14) for PFS, and 4.70 (1.50-14.74) for OS]. Methylation of *ADAMTS5* in normal or tumor tissue was associated with a poor 5-year RFS (*P* = 0.144 for normal tissue and 0.332 for tumor tissue), and *ADAMTS5* methylation in normal tissue was associated with a poor 5-year PFS (*P* = 0.176). Patients with a stage III+IV disease had a poor RFS, PFS, and OS even after adjustment for confounding factors in the multivariable analysis [HR (95%CI) = 4.82 (1.33-17.48) for RFS (normal tissue), 7.37 (2.11-25.83) for RFS (tumor tissue), 2.83 (1.19-6.74) for PFS (tumor tissue), and 2.63 (1.02-6.81) for OS (tumor tissue)]. Finally, no relationship was observed between *PXDN* methylation status and CRC prognosis.

We assessed the associations of *SUMF2* and *ADAMTS5* with prognosis using a quantitative analysis (Table 4). To enhance statistical power, we classified the methylation level of candidate genes into hypermethylation and hypomethylation groups according to the median value shown in Table 3, which functioned as the threshold value. On the basis of consecutive 5-year RFS, PFS, and OS Kaplan–Meier plot validation, a clear trend of differences was observed between hypermethylation and hypomethylation of the *SUMF2* gene at CpG_3 and CpG_7 Loci from tumor tissue. We further focused

Table 4 Multivariate 5-year progression and survival analysis of *SUMF2* and *ADAMTS5* gene

	RFS		PFS		OS	
	cHR (95%CI)	aHR (95%CI)[1]	cHR (95%CI)	aHR (95%CI)[1]	cHR (95%CI)	aHR (95%CI)[2]
SUMF2 in tumor tissue						
CpG_3+CpG_7						
Hypomethylation	1.00 (Reference)	1.00 (Reference)	1.00 (Reference)	1.00 (Reference)	1.00 (Reference)	1.00 (Reference)
Hypermethylation	2.37 (0.86-6.55)	1.64 (0.55-4.89)	2.24 (1.03-4.85)[a]	2.05 (0.91-4.62)	2.56 (1.08-6.04)[a]	3.53 (1.35-9.26)[a]
ADAMTS5 in normal tissue						
CpG_2						
Hypomethylation	1.00 (Reference)	1.00 (Reference)	1.00 (Reference)	1.00 (Reference)	1.00 (Reference)	1.00 (Reference)
Hypermethylation	0.15 (0.03-0.71)[a]	0.17 (0.03-0.95)[a]	0.57 (0.24-1.37)	0.54 (0.21-1.41)	0.82 (0.31-2.16)	0.94 (0.31-2.85)
CpG_13						
Hypomethylation	1.00 (Reference)	1.00 (Reference)	1.00 (Reference)	1.00 (Reference)	1.00 (Reference)	1.00 (Reference)
Hypermethylation	0.20 (0.04-0.97)[a]	0.16 (0.03-0.85)[a]	0.48 (0.19-1.19)	0.45 (0.17-1.18)	0.50 (0.19-1.30)	0.72 (0.24-2.15)

[1]Adjusted for sex, age, and stage.

[2]Adjusted for sex, age, stage, and lymph node counts.

[a]$P < 0.05$. RFS: Recurrence-free survival; PFS: Progression-free survival; OS: Overall survival; cHR: Crude hazard ratio; aHR: Adjusted hazard ratio; CI: Confidence interval; *SUMF2*: Sulfatase modifying factor 2; *ADAMTS5*: ADAM metallopeptidase with thrombospondin type 1 motif 5.

on the methylation statuses at CpG_3+CpG_7 to evaluate their relationship with patient prognosis. The 5-year PFS and OS curves revealed a significant difference between the hypermethylation and hypomethylation of the *SUMF2* gene at CpG_3+CpG_7 Loci from tumor tissue ($P = 0.026$ for PFS and 0.036 for OS; Figure 4). Compared with the CpG_3+CpG_7 hypomethylation of tumor tissue, a significantly shorter PFS and OS were observed for CpG_3+CpG_7 hypermethylation [HR (95%CI) = 2.24 (1.03-4.85) for PFS and 2.56 (1.08-6.04) for OS]. After adjustment for confounders in the multivariable analysis, the shorter OS associated with CpG_3+CpG_7 hypermethylation remained significant [HR (95%CI) = 3.53 (1.35-9.26)], whereas the shorter PFS associated with CpG_3+CpG_7 hypermethylation did not [HR (95%CI) = 2.05 (0.91-4.62)] (Table 4).

The 5-year RFS curves showed a significant difference between hypermethylation and hypomethylation of the *ADAMTS5* gene at CpG_2 and CpG_13 from normal tissue ($P = 0.006$ for CpG_2 and 0.026 for CpG_13; Figure 5). Compared with CpG_2 and CpG_13 hypomethylation of normal tissue, a significantly longer RFS was observed for CpG_2 and CpG_13 hypermethylation [HR (95%CI) = 0.15 (0.03-0.71) for CpG_2 and 0.20 (0.04-0.97) for CpG_13]. After adjustment for confounders in the multivariable analysis, the longer RFS associated with CpG_2 and CpG_13 hypermethylation remained significant [HR (95%CI) = 0.17 (0.03-0.95) for CpG_2 and 0.16 (0.03-0.85) for CpG_13] (Table 4).

DISCUSSION

The TNM staging system based on tumor depth, nodal status, and metastasis guides treatment strategies and improves the accuracy of predicting prognosis. However, the etiologically heterogeneous characteristics of CRC contributes to differences in survival between patients with the same TNM stage of CRC. Because of this heterogeneity, TNM staging of CRC requires further classification for better disease management. Therefore, combining other classifications related to several biologic mechanisms with TNM staging is required[15].

Aberrant DNA methylation of certain loci is involved in the aberrant expression of oncogenes through the hypomethylation of CpG islands in promoters. On the other hand, transcriptional silencing by the hypermethylation of CpG islands in promoters is observed in tumor-suppressor genes[16]. Aberrant DNA methylation elicits apoptosis[17], metastasis[18], cell adherence[19], tumor progression [18,20], and resistance to current anticancer therapies[21]. Hypermethylation at CpG islands of genes has promise as a robust and valid diagnostic method for CRC[22]. Additionally, methylation-based molecular markers can be employed as prognostic or predictive biomarkers for CRC to improve clinical management; such markers can predict malignant tumor potential and survival outcomes[23].

Globally acquired DNA hypomethylation profiles represent a key step in the development and advancement of CRC because hypomethylation triggers genomic instability and the global loss of imprinting[24]. This epigenetic change contributes to the activation of oncogenes and mainly affects

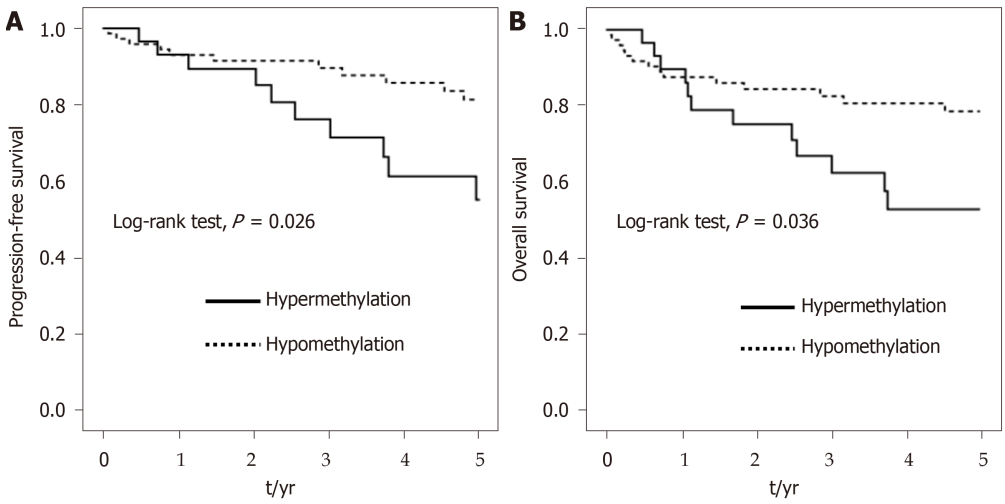

Figure 4 Kaplan–Meier survival curves depicting the effect of hypermethylation and hypomethylation of *SUMF2* at CpG_3+CpG_7 from tumor tissue on 5-year (A) progression-free survival and (B) overall survival of colorectal cancer patients.

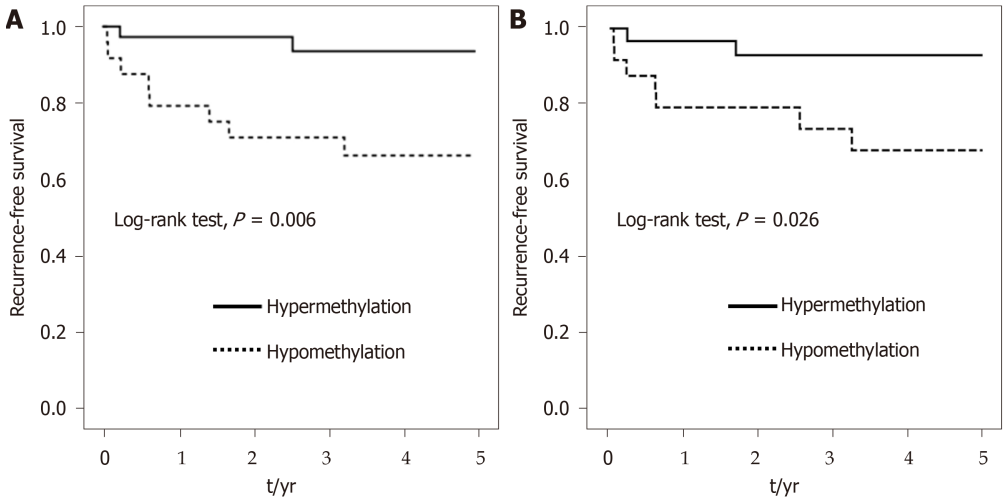

Figure 5 Kaplan–Meier survival curves depicting the effect of hypermethylation and hypomethylation of *ADAMTS5* at (A) CpG_2 and (B) CpG_13 from normal tissue on 5-year recurrence-free survival of colorectal cancer patients.

repetitive transposable elements, such as long interspersed nuclear element-1 (LINE-1 or L1), which represent 17% of the human genome[25]. LINE-1 is more hypomethylated in CRC tumors compared with adjacent normal tissue, and this is linked to metastasis. Moreover, LINE-1 is more hypermethylated in the neoplastic tissue of patients treated for CRC compared with untreated patients, and after neoadjuvant treatment, poor survival was observed in patients with tumor LINE-1 hypomethylation[26]. The results of a meta-analysis also support the idea that LINE-1 hypomethylation is considerably associated with the shortened OS and disease-free survival (DFS) of patients with CRC [27].

In this study, we analyzed 208 of each tumor tissue samples and normal-appearing tissue samples from patients with CRC. CpG_3+CpG_7 hypermethylation of *SUMF2* in tumor tissue was strongly related to poorer 5-year PFS and OS according to a Cox proportional hazards regression model and Kaplan–Meier curves. By contrast, CpG_2 and CpG_13 hypermethylation of *ADAMTS5* in adjacent normal tissue was significantly associated with better 5-year RFS than CpG_2 and CpG_13 hypomethylation of *ADAMTS5* in adjacent normal tissue. Finally, no correlation was observed between the promoter methylation status of *PXDN* and patient prognosis regardless of the tissue type analyzed.

Therefore, our findings indicate that *SUMF2* could be a tumor-suppressor gene, whereas *ADAMTS5* may be classified as a CRC oncogene. These findings can be applied along with current staging to modify the treatment of patients with CRC, and the genes discussed identified herein can serve as appropriate biomarkers to identify patients at a higher risk of having a poor prognosis and to indicate requirements for intensive follow-up.

We revealed that *ADAMTS5* methylation status in tumor-free areas adjacent to tumors was significantly associated with the prognosis of patients with CRC. The progressive accumulation of genetic mutations and DNA methylation changes in normal tissue around tumors lead to the development and progression of adenomas, which might become adenocarcinomas. Such alterations are a result of field cancerization[28]. Studies have demonstrated that the aberrant methylation of cancer-related genes could serve as epigenetic markers for CRC risk owing to the field of susceptibility[29], and such findings are consistent with our finding that compared with abnormal DNA methylation in tumor tissue, abnormal DNA methylation in adjacent normal tissue is highly correlated with an unfavorable prognosis after surgical resection.

SUMF2, located in the luminal space of the endoplasmic reticulum, is a member of the formylglycine-generating enzyme family and regulates the activity of sulfatase and the formation of formylglycine [30]. According to a whole-genome microarray expression study, Ala62Thr changes in ZNF365 isoform D are related to the altered expression of *SUMF2* in patients with Crohn disease[31]. According to Liang *et al*[32], *SUMF2* was one of the main genes with significant mutation in CRC. No report has examined the association between *SUMF2* methylation status and the prognosis of patients with CRC. *ADAMTS* family members containing disintegrin domains, metalloproteinases, and thrombospondin motifs likely contribute to malignant transformation such as cancer cell adhesion, fusion, migration, proliferation, and metastasis in CRC through their modification of the structure and function of the extracellular matrix (ECM)[33] and desmoplastic reactions (the overgrowth of fibrous connective tissue around carcinoma cells)[34]. The expression level of *ADAMTS5* was increased in late CRC stages, and *ADAMTS5* may have served as the fundamental component of tumor invasion through degrading ECM so as to promote tumor progression to more advanced stages of CRC[35]. The ratios of lymphatic invasion and lymph node metastasis were significantly higher in patients with CRC and high *ADAMTS5* expression, but such expression did not affect OS and DFS[36]. However, Li *et al*[37] suggested that *ADAMTS5* overexpression inhibited the invasion and migration of CRC, and *ADAMTS5* was more hypermethylated in tumor tissue compared with normal tissue, corresponding to poor OS and DFS. This result was not observed in our research. *PXDN*, which regulates cell plasticity and remodels the ECM by encoding ECM protein with peroxidase activity[38], engages in epithelial mesenchymal transition(EMT)[39]. EMT is a process by which epithelial cells lose cell polarity and cell–cell adhesion and acquire migratory and invasive capabilities to become mesenchymal cells; this activity has shown to occur during the initiation of metastasis[40]. A few studies have studied *PXDN* in the context of cancer; for example, the increased expression of *PXDN* has been shown in patients with melanoma[41] as well as in patients with brain[42], breast[43], ovarian[44] or prostate[45] cancer. No reports have examined the correlation between *PXDN* expression and the prognosis of patients with CRC. Thus, we investigated whether *PXDN* methylation status had an impact on CRC. We did not identify any correlation of *PXDN* methylation status in tumor tissue or adjacent normal tissue with CRC prognosis.

Our study has several advantages. The candidate gene approach was used in a rigorous approach comprising three steps. We further conducted quantitative analysis determined by what we observed from an MS-PCR. EpiTYPER DNA methylation analysis is a quantitative method of assessing methylation status. It also can confirm the degree of methylation at different CpG sites, which might be conductive to accurately identifying sites that affect CRC prognosis. This study was not without limitations. The results should be interpreted with caution on account of the small sample size. In the future, a larger prospective cohort study should be conducted to confirm our results. Furthermore, the participants were recruited from a single medical center in Taiwan. The effectiveness of the candidate genes as prognostic biomarkers for CRC should be validated in other ethnic populations. Finally, we did not detect the expression levels of selected genes, meaning we could not prove that the status or extent of methylation directly influenced gene expression.

CONCLUSION

Current clinical staging systems do not allow physicians to precisely evaluate the prognosis and outcomes of patients with CRC. Novel methylation biomarkers in tumor tissue and adjacent normal samples were identified in our study, providing a novel insight into how these markers could be used for CRC prognosis estimations. We anticipate that large-scale and independent cohort studies will clarify the utility of these novel markers and address whether clinical management can be adjusted based on supplementary information on the RFS, PFS, and OS of patients with CRC. We propose investigating these new biomarkers in patients with CRC to assist with clinical decision-making.

ARTICLE HIGHLIGHTS

Research background

The tumor–node–metastasis (TNM) cancer staging system provides clinical guidelines for the classification of tumors and prediction of outcomes. However, patients within the same stage can have markedly different outcomes. For example, some patients with an early disease stage of colorectal cancer (CRC) experience relapse after surgical treatment. Prognostic factors related to relapse or progression should be considered to improve treatment selection. The combination of several novel prognostic biomarkers involving epigenetic changes may aid CRC prognosis predictions.

Research motivation

To investigate the impact of the differential DNA methylation of novel candidate genes on CRC prognosis.

Research objectives

This study focused on the association between CRC prognosis and the status and level of differential DNA methylation of candidate genes.

Research methods

In total, 208 patients with CRC were recruited to assess the relationship between the methylation status of selected genes and clinical outcomes after surgical resection. The methylation statuses of *SUMF2*, *ADAMTS5*, and *PXDN* in tumor tissue and tumor-free adjacent areas were evaluated through a methylation-specific polymerase chain reaction (MS-PCR), and the methylation degrees of *SUMF2* and *ADAMTS5* were assessed using EpiTYPER DNA methylation analysis. The relationships of gene methylation with recurrence-free survival (RFS), progression-free survival (PFS), and overall survival (OS) were evaluated using a Cox proportional hazards model and Kaplan–Meier survival curves.

Research results

CpG_3+CpG_7 hypermethylation of *SUMF2* from tumor tissue was associated with significantly shorter PFS and OS compared with CpG_3+CpG_7 hypomethylation. CpG_2 and CpG_13 hypermethylation of *ADAMTS5* from normal tissue was associated with a significantly longer RFS compared with CpG_2 and CpG_13 hypomethylation. No significant difference was noted in the association between the methylation status of *PXDN* in both tissue types and CRC prognosis.

Research conclusions

These results can be applied to develop useful prognostic biomarkers of CRC, especially the methylation of certain CpG islands of candidate genes. The results can add value to current cancer staging systems.

Research perspectives

Examining the differential DNA methylation of candidate genes could aid in clinical decision-making related to CRC. Further validation and investigations involving larger cohorts are required to confirm the utility of these new epigenetic biomarkers and determine whether they can be used to improve the RFS, PFS, and OS of patients with CRC.

FOOTNOTES

Author contributions: Hsu CH, Lin HC and Chou YC contributed equally to this work; Su JQ, Hsu CH, Lin HC and Chou YC designed the research; Sun CA and Yang T performed the research; Hu JM, Chang PK and Chen CY collected the data; Su JQ, Lai PY, Hu PH, Wu JJ and Lin YJ analyzed the data; Su JQ wrote the original draft; Hsu CH, Lin HC and Chou YC wrote the review and editing; Chou YC was the project administration.

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Informed consent statement: Written informed consent was obtained from all patients before enrollment into the study to evaluate their prognosis.

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

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ORIGINAL ARTICLE

Retrospective Study

Long-term outcomes of endoscopic submucosal dissection and surgery for undifferentiated intramucosal gastric cancer regardless of size

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Abstract

BACKGROUND

The clinical outcomes of endoscopic submucosal dissection (ESD) for undifferentiated (UD) intramucosal early gastric cancer (EGC) compared with those of surgery, regardless of lesion size, are not well known. Furthermore, there is a concern regarding the treatment plan before and after ESD in cases of UD intramucosal EGC within expanded indications.

AIM

To evaluate clinical outcomes of ESD compared with those of surgery in UD intramucosal EGC patients regardless of tumor size.

METHODS

We enrolled patients with UD intramucosal EGC after ESD with complete resection or surgery from January 2005 to August 2020 who met the within or beyond expanded indications with lesion size > 2 cm (the only non-curative factor). Overall, 123 and 562 patients underwent ESD and surgery, respectively. After propensity-score matching, clinical and long-term outcomes, *i.e.*, recurrence-free survival (RFS) and overall survival (OS), were analyzed. The multivariable Cox proportional hazard model with treatment modality and ESD indication was used to evaluate the recurrence risk.

RESULTS

After matching, 119 patients each were finally enrolled in the ESD and surgery groups. The median length of hospital stay was shorter in the ESD group than surgery group (4.0 *vs* 9.0 days, $P < 0.001$). Four cases of recurrence after ESD were local recurrences, all of which occurred within 1 year. Total recurrence was seven (5.9%) and two (1.7%) in the ESD and surgery groups, respectively. No difference was observed between the two groups with respect to OS ($P = 0.948$). However, the ESD group had inferior RFS compared with the surgery group ($P = 0.031$). ESD was associated with the risk of recurrence after initial treatment in all enrolled patients (hazard ratio, 5.2; 95% confidence interval: 1.0-25.8, $P = 0.045$).

CONCLUSION

Although OS was similar between the two groups, surveillance endoscopy was important for the ESD than for the surgery group because RFS was inferior and local recurrence was an issue.

Key Words: Early gastric cancer; Undifferentiated cancer; Expanded indication; Endoscopic submucosal dissection; Surgery

Core Tip: This retrospective study evaluated the clinical outcomes of endoscopic submucosal dissection (ESD) compared with those of surgery in patients with undifferentiated (UD) intramucosal early gastric cancer (EGC) after propensity-score matching. No difference in overall survival was observed between two groups, although recurrence-free survival was inferior in the ESD group. Lymph node metastasis was not observed after ESD; however, local recurrence was higher after ESD than surgery. Surveillance endoscopy is important in ESD, even if complete resection is performed for UD intramucosal EGC. A short interval endoscopic follow-up is necessary when observing lesion sizes > 2 cm as the only non-curative factor.

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INTRODUCTION

Endoscopic submucosal dissection (ESD) is recommended as a treatment modality for early gastric cancer (EGC) because it allows curative *en bloc* resection and complete histopathological evaluation[1,2]. With the development of endoscopic instruments and techniques, the indication for ESD has expanded, and short- and long-term outcomes of ESD have been favorably reported in various studies[3-8]. Accordingly, ESD can be performed for patients with undifferentiated (UD) intramucosal EGC without lymphovascular invasion when the lesion size is ≤ 2 cm and there is no ulceration. Compared with surgery, ESD may be an alternative treatment option for UD intramucosal EGC within expanded indications[9-12]; however, concerns regarding lymph node (LN) metastasis in patients with UD intramucosal EGC remain[12].

Even if UD intramucosal EGC meets the criteria of expanded indications, additional surgical treatment is recommended if the lesion size alone is a non-curative factor (lesion diameter > 2 cm)[2]. In this case, physicians are concerned about determining the appropriate treatment modality and whether additional treatment should be performed. Various risk factors for LN metastasis have been reported in several surgical reports based on a lesion size of 2 cm[13-16]. Therefore, the role of ESD is limited in patients with UD intramucosal EGC because of the lesion size. However, patients may choose ESD for several reasons such as refusal of surgical treatment or older age. A recent multicenter study reported that mortality was not significantly higher in patients who underwent endoscopic resection for UD intramucosal EGC with tumor size > 2 cm as the only non-curative factor than in those who underwent additional surgery[17].

Compared with surgery, ESD can reduce the period of hospital stay after treatment and improve quality of life. To date, no study has compared long-term outcomes between ESD and surgical treatment based on propensity-score matching in patients with UD intramucosal EGC who are within or beyond expanded indications but satisfy the criteria of curative resection except for lesion size. Thus, this study

aimed to evaluate the clinical outcomes and adverse events of ESD compared with those of surgery in patients with UD intramucosal EGC using propensity-score matching analysis. Furthermore, we compared long-term clinical outcomes of ESD and surgery after matching for patients with UD intramucosal EGC who are beyond the expanded indication but meet the criteria of curative resection, except for lesion sizes > 2 cm.

MATERIALS AND METHODS

Patients

We retrospectively analyzed patients who underwent ESD (n = 212) or surgery (n = 1373) for UD intramucosal EGC at the Ajou University Medical Center (Suwon, Republic of Korea) between January 1, 2005 and August 31, 2020. Among those patients, patients with included expanded indications and curative resection[2] were enrolled. Patients who satisfied the condition of curative resection but had a lesion size > 2 cm (beyond expanded indications, tumor size > 2 cm as the only non-curative factor) were also included. The expanded indications with curative resection for UD intramucosal EGC were described as follows: intramucosal tumor; UD type; without ulceration; *en bloc* resection; tumor-free lateral and deep resection margin; without lymphovascular (or LN) invasion; and lesion size ≤ 2 cm[2]. Exclusion criteria were as follows: previous history of gastric cancer; previous history of other malignancy; or initial multiple gastric cancers. Additionally, we excluded patients who underwent additional surgery after ESD. The study protocol was approved by Ajou University Hospital Institutional Review Board and Ethics Committee (Approval No. AJIRB-MED-MDB-21-101). The requirement for informed patient consent was waived owing to the retrospective nature of the study. All co-authors had access to study data and reviewed and approved the final manuscript.

ESD procedure and surgery

All ESD procedures were performed by expert endoscopists using single-channel (GIF-Q260J; Olympus, Tokyo, Japan) or two-channel (GIF-2TQ260M; Olympus) endoscopy. After identifying the lesion, circumferential marking was done 5 mm outside the tumor margin using a needle knife (Dual knife; Olympus) or argon plasma coagulation (Erbe Elektromedizin, Tübingen, Germany). Epinephrine mixed fluid (0.01 mg/mL) was injected into the submucosal layer to lift the lesion from the muscle layer, and dissection was performed using an insulated-tip knife (IT knife; Olympus). The resected specimen was retrieved using a Swirl Net (Olympus), and all samples were fixed in 10% buffered formalin solution and embedded in paraffin.

Patients underwent total or subtotal gastrectomy with LN dissection according to the treatment guidelines of the Japanese Gastric Cancer Association (2). Therefore, patients who were enrolled in the surgery group underwent laparoscopy-assisted or open gastrectomy with D1 or D1+β LN dissection. The surgeons decided on the extent of gastric resection according to the tumor location.

Gross and histopathologic evaluations

Tumor locations were categorized into upper, middle, or lower third of the stomach based on the longitudinal axis of the stomach. Endoscopic findings were classified into elevated, flat, and depressed according to the predominant type based on the Japanese Research Society for Gastric Cancer classification system[18]. A standard histopathological examination, including hematoxylin and eosin staining, was conducted. Tumor size, presence of ulceration, histologic type, depth of invasion, lymphatic and vascular invasions, and presence of tumor cells in the resection margin were assessed. Pathological diagnoses were made according to the Japanese Classification of Gastric Cancer[1].

Follow-up schedules after gastric cancer resection

Follow-up endoscopy was performed 3 mo after ESD. A subsequent endoscopy with abdominal computed tomography (CT) was performed every 6-12 mo for 2 years and annually thereafter for 5 years after the treatment. In surgically resected patients, follow-up endoscopy and abdominal CT scans were performed every 6 mo for the first 2-3 years and then annually until 5 years after the initial treatment.

Clinical outcomes

The primary outcome of this study was overall survival (OS). The secondary outcomes were recurrence-free survival (RFS) and adverse events of short-term clinical outcomes. In this study, we defined OS as the duration between treatment and death owing to any cause; RFS was defined as the duration between treatment and first recurrence or death with evidence of recurrence. We collected data regarding survival status from the National Cancer Center (Goyang, South Korea); however, cause of death was not obtained for privacy after follow-up loss.

We defined local recurrence as a recurrence at the resection site after ESD or a recurrence at the anastomosis site after surgery. A synchronous lesion was defined as the occurrence of a new lesion

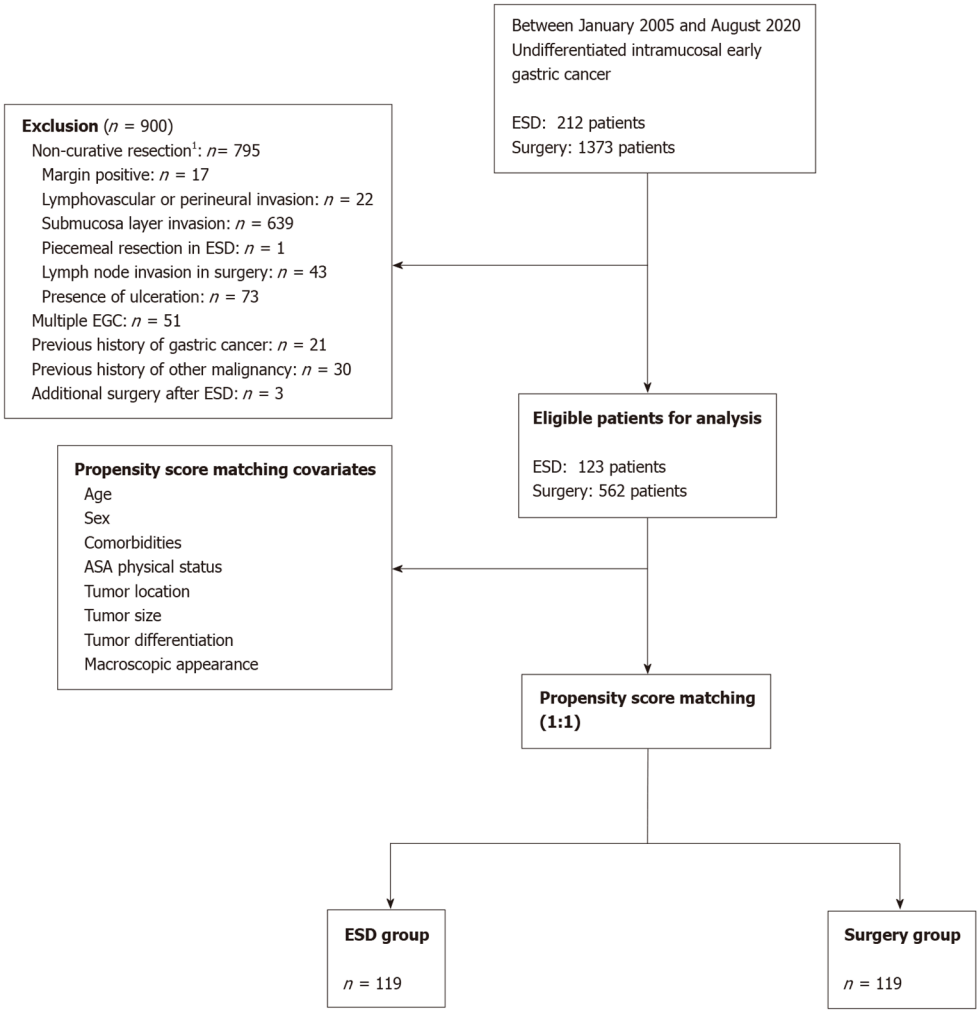

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Figure 1 Flow diagram of study population. [1]If the patient met within expanded indications except for lesion size factor > 2 cm (the only non-curative factor), we enrolled that patient for analysis. ASA: American Society of Anesthesiologists; EGC: Early gastric cancer; ESD: Endoscopic submucosal dissection.

detected at a different site from the previous treatment site within 1 year after gastric cancer resection. A metachronous lesion was defined as the occurrence of a new lesion detected at a different site from the previous treatment site more than 1 year after initial treatment. Distant metastasis was defined as a tumor metastasis in another organ.

Statistical analysis

We performed propensity-score matching analysis using the radius method to balance covariates across groups and reduce selection bias in the observational study. The propensity score was estimated using a logistic regression model with seven matching variables such as age, sex, comorbidities, lesion size, tumor location, gross morphology, histology appearance, and American Society of Anesthesiologists (ASA) physical status classification system score. Based on these propensity scores, the ESD and surgery groups were matched in a 1:1 ratio on an allowable absolute difference between exact propensity scores. The standardized mean differences were computed to measure the balance of covariates between groups before and after propensity-score matching.

We compared demographics and clinical characteristics, clinical outcomes, and adverse events between the ESD and surgery groups using the independent *t*-test or Wilcoxon rank-sum test for continuous variables and Pearson's chi-square test or Fisher's exact test for categorical variables, as appropriate. Survival curves were plotted, and 5-year survival rates with 95% confidence intervals (CIs) were estimated using the Kaplan-Meier method. Differences in OS and RFS were examined using the log-rank test between the ESD and surgery groups and within and beyond the expanded indication and between the ESD and surgery groups among patients beyond the expanded indication separately. The multivariable Cox proportional hazard model with treatment modality and ESD indication was used to estimate the hazard ratio (HR) with 95%CIs to assess the recurrence risk.

All statistical analyses were performed using SAS software, version 9.4 (SAS Institute Inc., Cary, NC, United States), and R software, version 3.6.2 (R Project for Statistical Computing), and all P values < 0.05 were two sided and considered statistically significant.

RESULTS

Study population

In our center, 212 and 1373 patients with UD intramucosal EGC underwent cancer resection *via* ESD and surgery, respectively. Patients who failed to meet the expanded ESD indication and curative resection criteria, except lesion size, were excluded. For long-term outcome analysis, we finally enrolled 123 and 562 patients in the ESD and surgery groups, respectively. Before matching, the mean age ± SD of the ESD group was older than that of the surgery group (55.3 ± 12.4 *vs* 53.0 ± 11.8, P < 0.001). The proportions of male patients were 55.3% and 50.2% in the ESD and surgery groups respectively (P = 0.305). These two groups showed differences with respect to hypertension history, ASA physical status, tumor location, and histology type. In particular, the proportion of patients with signet ring cell carcinoma was higher in the surgery group than in the ESD group (73.1% *vs* 54.5%, P < 0.001); however, there was no significant difference between the two groups with respect to lesion size and ESD indication. There was also no significant difference between the ESD and surgery groups [43 (35.0%) *vs* 233 (41.5%), P = 0.183] with respect to the proportion of beyond expanded indications with lesion size > 2 cm (the only non-curative factor). We performed propensity-score matching to compare long-term outcomes of the ESD and surgery groups on a one-to-one basis, and all differences in baseline characteristics after matching were eliminated (Table 1). The flow diagram of enrolled patients is shown in Figure 1, and the distribution of propensity scores is shown in Supplementary Figure 1.

Clinical outcomes and adverse events

The median hospital stay [interquartile range (IQR)] was shorter in the ESD group than in the surgery group [4.0 (4.0-5.0) *vs* 9.0 (8.0-10.0) d, P < 0.001]. Regarding intensive care unit admission related to treatment complications, treatment complications caused by severe bleeding was noted one patient in the ESD group, who eventually died. Although the incidence of all adverse events was not different between the two groups, five cases (4.2%) of perforations and eight cases (6.7%) of bleeding occurred in the ESD group, all of which were early complications within 30 days. Surgical complications, including anastomotic leakage (n = 1, 0.8%), bowel obstruction (n = 3, 2.5%), and hernia (n = 3, 2.5%), were mostly successfully treated with conservative treatment; however, three hernia cases required additional surgery. Late complications were not observed in the ESD group; however, four cases (3.4%) were observed in the surgery group (Table 2).

Long-term outcomes

The median follow-up period was 45 mo (IQR, 21-65 mo) and 59 mo (IQR, 36-81 mo) in the ESD and surgery groups, respectively. During the follow-up period, three (2.5%) and five (4.2%) patients died in the ESD and surgery groups, respectively. Among those patients, gastric cancer-related death was identified in one patient (0.8%) in each group. The incidence of total recurrence was higher in the ESD group (n = 7, 5.3%) than in the surgery group (n = 2, 1.7%) (Table 3). Local recurrence was identified in four patients (3.4%) who underwent ESD, and all whom had EGC. A synchronous lesion was identified in one patient (0.8%), who had EGC and was treated with surgery. A metachronous lesion was identified in three patients (2.5%) of the ESD group. Distant metastasis was identified in one patient (0.8%) who underwent surgery with peritoneal metastasis. The number of patients whose tumor size was ≤ 2 cm before ESD or surgery and therefore, satisfied the criteria for expanded-indication lesions but had a size of > 2 cm in the final pathology analysis was 22 (18.5%) and 20 (16.8%) in the ESD and surgery groups, respectively. Of these patients, one patient in the ESD group had a recurrence but no mortality in both groups. Clinical and tumor data for all recurrent patients are given in Table 4.

We analyzed RFS and OS using Kaplan-Meier survival plots (Figure 2). Regarding RFS, according to the treatment modality, the ESD group had inferior results compared with the surgery group (P = 0.031) (Figure 2A). The 5-year RFS rates were 93.3% (95%CI: 85.1-97.0) and 99.2% (95%CI: 94.2-99.9) in the ESD and surgery groups, respectively. However, there was no significant difference between the two groups with respect to OS (ESD *vs* surgery, 5-year OS, 97.2%; 95%CI: 91.6-99.1 *vs* 99.0%; 95%CI: 93.0-99.9, P = 0.948) (Figure 2B). Among non-curative factors, we analyzed RFS (within *vs* expanded, 5-year RFS, 97.7%; 95%CI: 93.0-99.3 *vs* 94.6%; 95%CI: 85.6-98.0, P = 0.777) and OS (within *vs* expanded, 5-year OS, 98.5%; 95%CI: 94.1-99.6 *vs* 97.4%; 95%CI: 90.1-99.4, P = 0.698) for patients with lesion size > 2 cm (beyond the expanded indication but meeting the criteria of curative resection except for lesion size > 2 cm) and within expanded indication, in which no difference according to the indication was observed (Figure 2C and D). While there was no difference in OS (ESD *vs* surgery, 5-year OS, 97.5%; 95%CI: 83.5-99.6 *vs* 97.7%; 95%CI: 84.9-99.7, P = 0.610) according to the treatment modality in patients with beyond expanded indication with lesion size > 2 cm only, the ESD group had a significantly lower RFS than the surgery group (5-year RFS, 86.2%; 95%CI: 64.9-95.0 *vs* 100.0%; 95%CI: 100.0-100.0, P = 0.013) (Figure 2E

Table 1 Baseline characteristics of the study population

Variables	Before matching			After matching		
	ESD (*n* = 123)	Surgery (*n* = 562)	*P* value	ESD (*n* = 119)	Surgery (*n* = 119)	*P* value
Age, yr, mean SD	55.3 12.4	53.0 11.8	< 0.001	56.6 11.9	55.6 11.9	0.546
Male, *n* (%)	68 (55.3)	282 (50.2)	0.305	67 (56.3)	57 (47.9)	0.194
Comorbidity, *n* (%)						
Hypertension	44 (35.8)	143 (25.4)	0.020	41 (34.5)	40 (33.6)	0.891
Diabetes	21 (17.1)	65 (11.6)	0.095	19 (16.0)	15 (12.6)	0.459
Cerebrovascular disease	8 (6.5)	18 (3.2)	0.113	7 (5.9)	2 (1.7)	0.171
Respiratory disease	7 (5.7)	29 (5.2)	0.811	6 (5.0)	6 (5.0)	-
Liver disease	3 (2.4)	26 (4.6)	0.275	3 (2.5)	3 (2.5)	-
Renal disease	2 (1.6)	4 (0.7)	0.294	1 (0.8)	1 (0.8)	-
ASA physical status[1], *n* (%)			0.022			0.254
1	101 (82.1)	503 (89.5)		100 (84.0)	106 (89.1)	
2	22 (17.9)	59 (10.5)		19 (16.0)	13 (10.9)	
Tumor location, *n* (%)			< 0.001			0.822
Upper third	8 (6.5)	55 (9.8)		8 (6.7)	6 (5.0)	
Middle third	97 (78.9)	321 (57.1)		93 (78.2)	93 (78.2)	
Lower third	18 (14.6)	186 (33.1)		18 (15.1)	20 (16.8)	
Lesion size, mm, *n* (%)			0.430			0.418
10	30 (24.4)	105 (18.7)		28 (23.5)	19 (16.0)	
10-20	50 (40.7)	224 (39.9)		50 (42.0)	49 (41.2)	
20-30	24 (19.5)	133 (23.7)		22 (18.5)	28 (23.5)	
> 30	19 (15.4)	100 (17.8)		19 (16.0)	23 (19.3)	
Gross morphology type[1], *n* (%)			0.315			0.760
Elevated	33 (26.8)	127 (22.6)		29 (24.4)	27 (22.7)	
Flat or depressed	90 (73.2)	435 (77.4)		90 (75.6)	92 (77.3)	
Helicobacter pylori infection, *n* (%)	64 (51.2)	316 (56.2)	0.397	61 (51.3)	60 (50.4)	0.897
ESD indication, *n* (%)			0.183			0.183
Within expanded indication	80 (65.0)	329 (58.5)		78 (65.5)	68 (57.1)	
Beyond expanded indication	43 (35.0)	233 (41.5)		41 (34.5)	51 (42.9)	
Histology appearance, *n* (%)			< 0.001			0.794
Poorly differentiated carcinoma	56 (45.5)	151 (26.9)		52 (43.7)	54 (45.4)	
Signet ring cell carcinoma	67 (54.5)	411 (73.1)		67 (56.3)	65 (54.6)	

[1]Physical status classification of the American Society of Anesthesiologists.
ESD: Endoscopic submucosal dissection; SD: Standard deviation.

and F). In the multivariable analysis, ESD was a significant risk factor for recurrence after cancer resection in patients with UD intramucosal EGC (HR, 5.2; 95%CI: 1.0-25.8, *P* = 0.045). The lesion included in the beyond expanded indication was not associated with recurrence risk. Moreover, ESD as a treatment modality increased the HR for recurrence in the beyond expanded indication with lesion size > 2 cm compared with the within expanded indication; however, this result was not statistically significant (Table 5).

Table 2 Clinical outcomes and adverse events of early gastric cancer resection according to treatment modality

Variables	ESD (*n* =119)	Surgery (*n* = 119)	*P* value
Median hospital stay, d (IQR)	4.0 (4.0-5.0)	9.0 (8.0-9.0)	< 0.001
ICU admission, *n* (%)	1 (0.8)	0 (0.0)	-
30-d readmission, *n* (%)	3 (2.5)[1]	2 (1.7)[2]	-
Operation-related death, *n* (%)	1 (0.8)	0 (0.0)	-
Complication, *n* (%)	14 (11.8)	7 (5.9)	0.110
Bleeding (early/late)	8/0	0/0	
Perforation (early/late)	5/0	N/A	
Pneumonia (early/late)	1/0	0/0	
Anastomosis site leakage (early/late)	N/A	1/0	
Adhesion or bowel obstruction (early/late)	0/0	1/2	
Hernia (early/late)	N/A	1/2	

[1]All cases were bleeding.
[2]Vomiting owing to anastomosis site stricture, and pain owing to hernia.
Early and late complications occurred within or later than 30 days after operation or procedure, respectively. ESD: Endoscopic submucosal dissection; ICU: Intensive care unit; IQR: Interquartile range; N/A: Not applicable.

Table 3 Incidence and characteristics of recurrent tumors after initial treatment (endoscopic submucosal dissection or surgery)

Variables	ESD (*n* = 119)	Surgery (*n* = 119)	*P* value
Recurrence, *n* (%)	7 (5.9)	2 (1.7)	0.171
Local recurrence	4 (3.4)	N/A	
Adenoma	0 (0.0)	N/A	
Cancer	4 (3.4)	N/A	
Differentiated	3 (2.5)	N/A	
Undifferentiated	1 (0.8)	N/A	
Synchronous lesion	0 (0.0)	1 (0.8)	
Adenoma	0 (0.0)	0 (0.0)	
Cancer	0 (0.0)	1 (0.8)	
Differentiated	0 (0.0)	0 (0.0)	
Undifferentiated	0 (0.0)	1 (0.8)	
Metachronous lesion	3 (2.5)	0 (0.0)	
Adenoma	1 (0.8)	0 (0.0)	
Cancer	2 (1.7)	0 (0.0)	
Differentiated	1 (0.8)	0 (0.0)	
Undifferentiated	1 (0.8)	0 (0.0)	
Distant metastasis	0 (0.0)	1 (0.8)	

ESD: Endoscopic submucosal dissection; N/A: Not applicable.

DISCUSSION

We comparatively analyzed the long-term outcomes of patients who underwent ESD and surgery for UD intramucosal EGC with complete resection regardless of lesion size using propensity score-matched analysis. ESD was similar to surgery in terms of OS; however, RFS in the ESD group was lower than that in the surgery group. In both the groups, LN metastasis was absent during the follow-up period, and

Table 4 Clinical and tumor information for recurrent patients

Age	Sex	Location	Size (mm)	Morphology	Histology	Initial treatment	Recurrence type	Pathology of recurred lesion	Recurrence location	Recurrence time (mo)	Treatment for recurred lesion
61	F	Middle 1/3	6	Flat	SRC	ESD	Metachronous lesion	Undifferentiated cancer	Lower 1/3	70	ESD
62	M	Middle 1/3	22	Flat	SRC	ESD	Local recurrence	Undifferentiated cancer	Middle 1/3	6	Surgery
68	F	Middle 1/3	68	Flat	PDA	ESD	Metachronous lesion	Differentiated cancer	Upper 1/3	50	ESD
46	M	Middle 1/3	60	Flat	PDA	ESD	Local recurrence	Differentiated cancer	Middle 1/3	3	Surgery
50	F	Middle 1/3	25	Depressed	PDA	ESD	Local recurrence	Differentiated cancer	Middl1 1/3	12	ESD
62	M	Middle 1/3	40	Elevated	PDA	ESD	Local recurrence	Differentiated cancer	Middle 1/3	6	Surgery
56	F	Lower 1/3	8	Flat	PDA	ESD	Metachronous lesion	Adenoma	Middle 1/3	23	ESD
66	M	Lower 1/3	15	Flat	PDA	Surgery	Synchronous lesion	Undifferentiated cancer	Upper 1/3	5	Surgery
64	M	Middle 1/3	10	Elevated	SRC	Surgery	Distant metastasis	Undifferentiated cancer	Peritoneum	122	Conservative care

ESD: Endoscopic submucosal dissection; PDA: Poorly differentiated adenocarcinoma; SRC: Signet ring cell carcinoma.

Table 5 Cox proportional hazard model for risk of recurrence after initial treatment

Variables	Adjusted hazard ratio (95% confidence interval)	*P* value
Treatment modality		
Surgery	1.0	
ESD	5.2 (1.0-25.8)	0.045
Indication with any treatment modality		
Within expanded indication	1.0	
Beyond expanded indication	1.4 (0.4-5.4)	0.585
Indication with ESD		
ESD for the lesion within expanded indication	1.0	
ESD for the lesion beyond expanded indication	2.8 (0.6-12.4)	0.183

ESD: Endoscopic submucosal dissection.

only one case of distant metastasis was observed in the surgery group. ESD was advantageous because of shorter hospital stays and fewer late complications compared with surgery. Although complete resection was performed for patients with UD intramucosal EGC, ESD was identified as a recurrence risk factor in terms of treatment modality compared with surgery. In patients with lesion size > 2 cm as the only non-curative factor, there was a difference in RFS depending on the treatment modality, with ESD having an inferior outcome.

In our study, there was a clear bias in the patient population depending on treatment modality. Before matching, patients in the ESD group were older and had a higher ASA physical status score, fewer lesions located in the upper third of the stomach, and lower signet ring cell carcinoma rate than those in the surgery group. While the expanded indication proposed by Gotoda *et al*[19] is based on the pathology of specimens, clinicians should select the treatment modality for EGC based on gross and pathological findings. However, this method sometimes makes it difficult to determine a treatment modality in clinical practice. Besides, there are cases in which patients beyond the expanded indication of ESD refuse surgery and thus undergo ESD in hopes of endoscopic resection instead of surgery. In

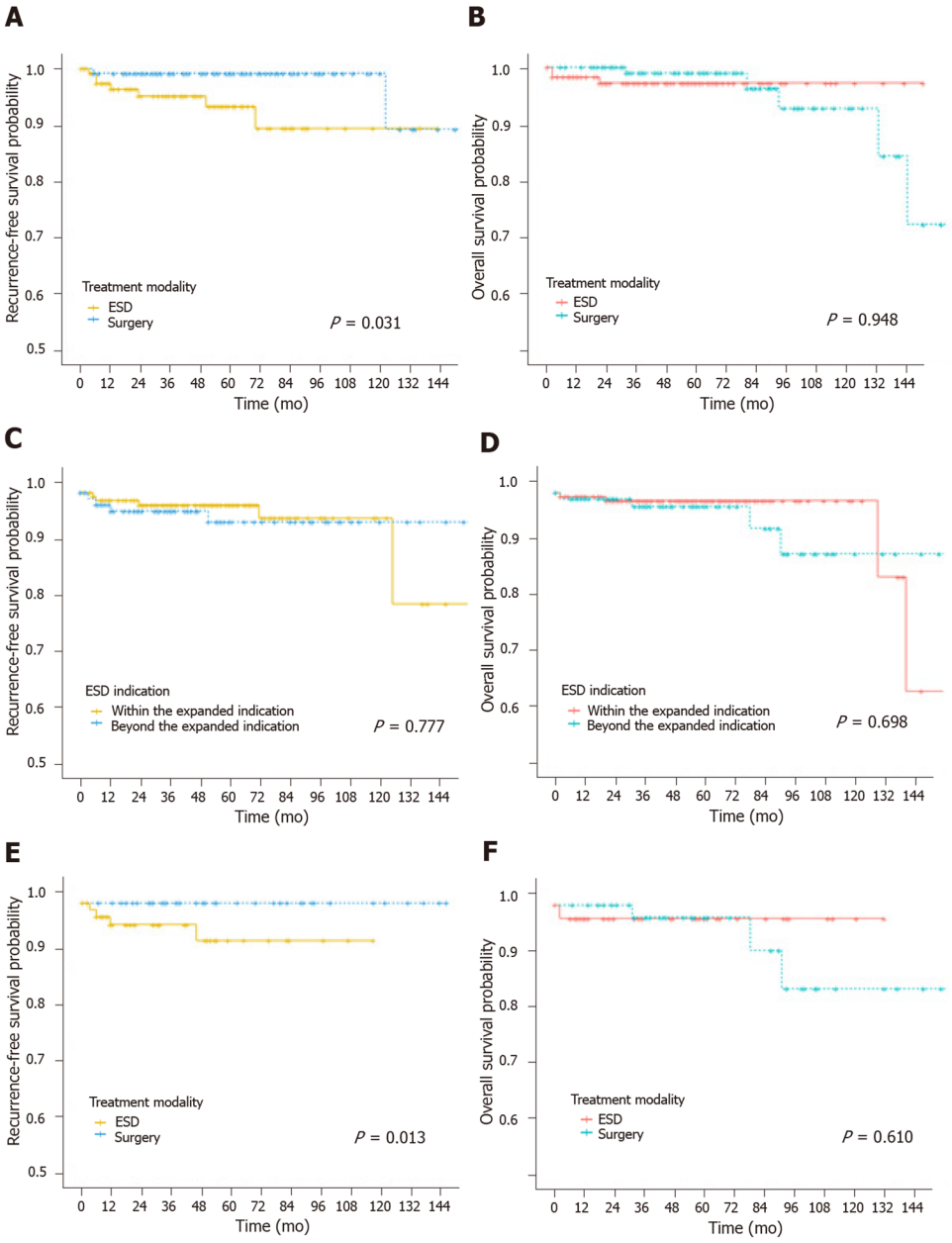

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Figure 2 Kaplan-Meier survival plots for recurrence-free survival and overall survival according to treatment modality for gastric cancer.
A: Recurrence-free survival (RFS), endoscopic submucosal dissection (ESD) *vs* surgery; B: Overall survival (OS), ESD *vs* surgery; C: RFS, within the expanded indication *vs* beyond expanded indication; D: OS, within the expanded indication *vs* beyond expanded indication; E: RFS, ESD *vs* surgery in patients with beyond expanded indication; F: OS, ESD *vs* surgery in patients with beyond expanded indication. ESD: Endoscopic submucosal dissection.

contrast, there are a significant number of patients who meet the expanded indications and undergo surgery. As a result, patients and lesion characteristics are not the same between patients who undergo ESD and those who undergo surgery. Our results suggested that patients were hesitant to undergo surgery if they were older or if their ASA physical status score was high. For this reason, selection bias was inevitable in this study group. Therefore, we performed propensity-score matching analysis to reduce selection bias owing to treatment modality.

With the development of ESD techniques and instruments and the accumulation of various clinical outcomes, the scope of ESD has been gradually expanding. However, issues regarding the role of ESD in patients with UD intramucosal EGC are continuously raised. Currently, the ESD criteria for UD intramucosal EGC are strict. ESD is performed only when the lesion has no ulceration and is ≤ 2 cm and when it satisfies the complete resection conditions of negative resection margins and the absence of lymphovascular and perineural invasion in the final pathological examination[2]. Lesion size > 2 cm is a non-curative factor, and various studies have reported a difference in LN metastasis based on lesion size of 2 cm[13,14,15,20]. However, in cases of UD-EGC with a lesion size > 2 cm, LN metastasis was reported to be 0% (0/54) when neither ulceration nor lymphovascular invasion was present[16], all of

which were from surgical studies. To our knowledge, only one study analyzed LN metastasis in patients who underwent endoscopic resection for UD intramucosal EGC with lesion size > 2 cm. Yang *et al*[17] reported an incidence of 1.1% (2/176), which showed no increase in mortality during observation after ESD based on Cox regression analysis. Similar results were also obtained in our study. LN metastasis was not observed among 119 patients who underwent ESD. However, 71.4% (5/7) of the total recurrence cases had a lesion size > 2 cm, which revealed a higher recurrence rate than that in patients with a lesion size ≤ 2 cm (11.9% *vs* 2.6%). Furthermore, patients with lesion size > 2 cm in the ESD group had a lower RFS than those in the surgery group.

The results of our analysis after matching for UD intramucosal EGC patients with complete resection, irrespective of lesion size, showed that RFS was inferior in the ESD group compared with the surgery group. However, no LN metastasis or distant metastasis was identified during the follow-up period. In other words, all recurrence cases occurred in the stomach, all were treated successfully after recurrence, and no further recurrences occurred during the follow-up period. However, taking ESD into account over surgery as the preferred treatment for UD intramucosal EGC irrespective of lesion size should be carefully considered. LN metastasis was not observed; however, local recurrence was found in four patients (4/119, 3.4%), all of whom had an initial tumor size > 2 cm that occurred less than a year after initial treatment, and surgery as a rescue treatment was performed because endoscopic treatment was impossible. In the study by Yang *et al*[17], the local recurrence rate was 2.3% (4/176), and one case had LN metastasis. Therefore, ESD must overcome the problem of local recurrence, which does not need to be considered in surgery. Furthermore, we should consider the why local recurrence occurs even after complete resection. The initial pathologic evaluation could have been incorrect or a new cancer may have occurred. We repeated the pathologic evaluation for these cases; however, the initial diagnosis did not change. Additional studies and data accumulation are required to examine why local recurrence occurs within a short period, although complete or curative resection is performed after the initial pathologic evaluation. In this study, in the 212 patients considered, the margin negative resection rate in the ESD group before matching was 92.0% (195/212). This was similar to previous studies where endoscopic resection was performed in UD EGC[21-26].

Therapeutic endoscopists always consider that a sufficient lateral margin can reduce the possibility of local recurrence. While it would be best to secure as much safety margin as possible, the operator should consider the duration of intervention, acute complications (bleeding or perforation), or delayed complications (bleeding, stricture). These considerations may be more prominent in UD EGC. A recently published study mentioned that local recurrence may be related to sequential molecular changes in various cancer-related proteins in histological margin-free endoscopically resected EGCs[27]. In this study, a tumor-free distance of 5.5 mm was considered insufficient as a safety margin. Besides, a subepithelial spread beneath the normal mucosa may exist in UD EGC, especially in signet ring cell cancer, and this subepithelial spread could reach up to 6 mm[28]. These studies suggest that securing sufficient margin in the endoscopic resection of UD EGC using the ESD method might reduce the rate of local recurrence. In the endoscopic resection of UD EGC using the ESD method, the endoscopically predicted and the actual size of the lesion is often different. In the Japanese algorithm, an additional biopsy was recommended from the surrounding mucosa of UD EGC to accurately evaluate the margin of the lesion[29]. In addition, other studies have reported that narrow-band imaging with magnifying endoscopy may help in accurately predicting the tumor extent in UD EGC[30]. Prospective randomized studies are required to evaluate whether the various attempts to accurately determine the tumor margin and resection with sufficient margin can reduce the rate of local recurrence.

In our study, all recurrence cases in the ESD group occurred in the lower or middle third of the stomach, except for one case (6/7, 85.7%). Although it is difficult to judge because of only a small number of recurrence cases, there is a report that incidence increased in the middle and lower third of the stomach in metachronous cancer after ESD[31]. This may be why fewer synchronous or metachronous recurrences were observed in the surgery group. In our study, 16.8% of patients underwent total gastrectomy after matching (20/119). Since the portion of the remnant stomach is small, even in patients who have undergone subtotal gastrectomy, surgery can be advantageous in terms of recurrence. In our study, only one recurrence occurred in the upper third of the stomach in the ESD and surgery groups. It is challenging to select ESD as the initial treatment option if surgical treatment is performed as a rescue treatment in a short period, given that ESD has a probability of local recurrence. Therefore, our results suggest that ESD should not be actively recommended to patients with UD intramucosal EGC with lesion size > 2 cm even without ulceration on preoperative workup.

This study had some limitations. First, this was a retrospective single-center study. A randomized study is required to compare long-term outcomes of ESD and surgery, but this is difficult to perform for UD intramucosal EGC. Second, baseline characteristics and tumor information were different between the groups; however, we analyzed the data after propensity-score matching to minimize the difference between baseline characteristics and reduce selection bias of treatment modality. Third, the number of patients with UD intramucosal EGC with lesion size > 2 cm was significantly lower in the pre-matching ESD group than in the surgery group (43 *vs* 233 patients). Although the data were corrected as much as possible with propensity-score matching, most ESD patients were assigned after matching; thus, selection bias could exist. Finally, although we confirmed survival based on data from the National Cancer Center registry, we did not check the cause of the death in all patients who died. Therefore, we

did not evaluate gastric cancer-related deaths in both groups after follow-up loss.

CONCLUSION

In conclusion, ESD is a treatment modality for stomach preservation with fewer late complications and shorter hospital stays than surgery. For patients with UD intramucosal EGC, if the lesion size is the only non-curative factor, ESD may be an alternative treatment option when surgery is not possible. However, all cases of local recurrence were identified within 1 year in our study, all of which were cancer, although LN metastasis was not observed after ESD. Therefore, even for complete resection, endoscopic surveillance is essential. Especially in cases with lesion sizes > 2 cm, endoscopic surveillance should be more thoroughly performed.

ARTICLE HIGHLIGHTS

Research background

Endoscopic submucosal dissection (ESD) is performed as an alternative treatment modality for undifferentiated (UD) intramucosal early gastric cancer (EGC) who are within the expanded indication. However, the ESD role for UD intramucosal EGC with lesion size > 2 cm (the only non-curative factor) is still controversial compared with surgery.

Research motivation

Several studies showed ESD could be performed for patients with UD intramucosal EGC within the expanded indication. However, the role of ESD is limited in these patients because of the lesion size. Even if UD intramucosal EGC meets the criteria of expanded indications, additional surgical treatment is recommended if the lesion size alone is a non-curative factor (lesion diameter > 2 cm).

Research objectives

In this study, the authors compared ESD with surgery in patients with UD intramucosal EGC who meet both the within expanded indications or beyond expanded indications with lesion size > 2 cm (the only non-curative factor).

Research methods

The authors retrospectively analyzed patients with UD intramucosal EGC after ESD with complete resection or surgery. After propensity-score matching, clinical outcomes and long-term outcomes, *i.e.*, recurrence-free survival (RFS) and overall survival (OS), were analyzed.

Research results

After propensity-scored matching, although ESD with complete resection was performed in UD intramucosal EGC regardless of lesion size, RFS increased, while there was no difference in OS compared to surgery. Especially, all cases of local recurrence were identified within 1 year in our study in the ESD group.

Research conclusions

Although ESD may be an alternative treatment option when surgery is not possible for UD intramucosal EGC with lesion sizes > 2 cm, endoscopic surveillance should be carefully performed within one year for local recurrence.

Research perspectives

Multicenter randomized studies with large cohorts are expected to evaluate ESD in patients with UD intramucosal EGC regardless of tumor size.

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FOOTNOTES

Author contributions: Lee GH and Lee E contributed equally to this work; Lee GH planned the study design, reviewed the data, analyzed the data and drafted the manuscript; Lee E and Park B analyzed and reviewed the statistical data; Roh J reviewed the pathologic data; Lim SG planned the study design and collected the data; Shin SJ and Lee KM interpreted the data and supervised the report; Noh CK conceptualized, drafted the manuscript and critically revised the manuscript; all the authors approved the final version of the article and agree to be accountable for all aspects of the work.

Institutional review board statement: The study protocol was approved by Ajou University Hospital Institution's Review Board and Ethics Committee (Approval No. AJIRB-MED-MDB-21-101).

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ORIGINAL ARTICLE

Retrospective Study

Inverse correlation between gastroesophageal reflux disease and atrophic gastritis assessed by endoscopy and serology

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Abstract

BACKGROUND

Helicobacter pylori (*H. pylori*) infection is known to prevent the occurrence of gastroesophageal reflux disease (GERD) by inducing gastric mucosal atrophy. However, little is known about the relationship between atrophic gastritis (AG) and GERD.

AIM

To confirm the inverse correlation between AG and the occurrence and severity of GERD.

METHODS

Individuals receiving health checkups who underwent upper gastrointestinal endoscopy at Seoul National University Healthcare System Gangnam Center were included. The grade of reflux esophagitis was evaluated according to the Los Angeles classification. Endoscopic AG (EAG) was categorized into six grades. Serologic AG (SAG) was defined as pepsinogen I ≤ 70 ng/mL and pepsinogen I/II ratio ≤ 3.0. The association between the extent of EAG and SAG and the occurrence and severity of GERD was evaluated using multivariate logistic regression analysis.

RESULTS

In total, 4684 individuals with GERD were compared with 21901 healthy controls.

In multivariate logistic regression analysis, advanced age, male sex, body mass index > 23 kg/m^2, presence of metabolic syndrome, current smoking, and alcohol consumption were associated with an increased risk of GERD. Seropositivity for *H. pylori* immunoglobulin G antibodies was associated with a decreased risk of GERD. There was an inverse correlation between the extent of EAG and occurrence of GERD: Odds ratio (OR), 1.01 [95% confidence interval (CI): 0.90-1.14] in C1, 0.87 (0.78-0.97) in C2, 0.71 (0.62-0.80) in C3, 0.52 (0.44-0.61) in O1, 0.37 (0.29-0.48) in O2, and 0.28 (0.18-0.43) in O3. Additionally, the extent of EAG showed an inverse correlation with the severity of GERD. The presence of SAG was correlated with a reduced risk of GERD (OR = 0.49, 95%CI: 0.28-0.87, P = 0.014).

CONCLUSION

The extent of EAG and SAG exhibited strong inverse relationships with the occurrence and severity of GERD. AG followed by *H. pylori* infection may be independently protect against GERD.

Key Words: Gastroesophageal reflux disease; Reflux esophagitis; *Helicobacter pylori*; Atrophic gastritis; Pepsinogen

Core Tip: This is a retrospective study to evaluate the inverse correlation of atrophic gastritis and the occurrence and severity of gastroesophageal reflux disease (GERD). Old age, male sex, body mass index over 23 kg/m^2, metabolic syndrome, current smoking, and alcohol intake increased the risk of GERD. Seropositivity for *Helicobacter pylori* (*H. pylori*) immunoglobulin G antibody decreased the risk of GERD. There was an inverse correlation between extent of endoscopic atrophic gastritis (EAG) and occurrence of GERD. Additionally, extent of EAG showed inverse correlation with severity of GERD. Presence of serologic atrophic gastritis was correlated with reduced risk of GERD. Atrophic gastritis followed by *H. pylori* infection could be an inde-pendent protective factor against GERD.

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INTRODUCTION

Gastroesophageal reflux disease (GERD) is a disorder with specific symptoms, such as acid regurgitation and heartburn, caused by the recurrent reflux of gastric contents into the esophagus due to transient relaxation or low pressure of the lower esophageal sphincter[1,2]. A condition with troublesome symptoms accompanied by esophageal structural changes, such as mucosal breaks, erosion, or ulcers (*i.e.*, reflux esophagitis) is defined as erosive reflux disease (ERD); however, only about one-third to one-half of patients with GERD exhibit positive findings on endoscopic examinations[1]. Meanwhile, the presentation of typical esophageal symptoms in the absence of endoscopic changes is classified as non-ERD (NERD).

GERD is a common disease with a prevalence ranging from 18.1% to 27.8% in North America, 8.8% to 25.9% in Europe, and 2.5% to 7.8% in East Asia[3,4]. According to a systematic review conducted in 2014, the prevalence of GERD is significantly increased when comparing studies conducted before and after 1995[3]. Several factors such as a prolonged life expectancy, westernized lifestyle, and increasing prevalence of obesity might influence the increase in GERD prevalence[5]. GERD and its complications, such as reflux esophagitis and Barrett's esophagus, cause a considerable socioeconomic burden in terms of hospital visits, incur significant medical costs for diagnosis and treatment, and induce several problems related to quality of life. A number of epidemiological studies have evaluated the risk factors for GERD. Male sex, caffeine intake, smoking, alcohol consumption, dietary factors (especially the consumption of large meals and a high-fat diet), a low education level, and obesity are known risk factors for GERD[2,6-8].

Interestingly, an inverse correlation between GERD and *Helicobacter pylori* (*H. pylori*) infection has been demonstrated in several studies. In a case-control study performed in Japan, most cases of reflux esophagitis occurred in the absence of *H. pylori* and atrophic gastritis or in milder cases of gastritis with *H. pylori* infection[9]. *H. pylori* infection has been shown to reduce the risk and severity of GERD in

several epidemiological studies and systematic reviews[10-12]. Furthermore, the grade of reflux esophagitis and GERD symptoms are aggravated after *H. pylori* eradication therapy[2,13]. These findings suggest that *H. pylori* infection might be a protective factor against GERD as a result of a decreased acid secretory capacity induced by gastric mucosal atrophy.

As gastric mucosal atrophy is considered a key mechanism by which *H. pylori* infection prevents the occurrence or aggravation of GERD, several studies have assessed the relationship between atrophic gastritis and GERD. According to a few studies grading the severity of atrophic gastritis using endoscopic biopsies according to a modified updated Sydney classification, *H. pylori* infection alone may not influence the occurrence of reflux esophagitis. Meanwhile, the involvement and degree of atrophy in the gastric corpus are independent protective factors against GERD[9,14]. However, the histological diagnosis of atrophic gastritis based on endoscopic forcep biopsy is not always feasible in daily clinical settings because of its invasive nature. Pepsinogen is a validated serologic marker reflecting the acid secretory ability of the gastric gland; thus, it may predict the presence or absence of gastric atrophy[19]. In general, pepsinogen I levels ≤ 70 ng/mL and pepsinogen I/II ratios ≤ 3.0 are considered positive results for gastric atrophy[15]. A prospective case-control study in Korea found that the pepsinogen I/II ratio was higher in patients with ERD than in those without ERD[16]. Furthermore, the prevalence of reflux esophagitis decreased as the stage of *H. pylori*-related chronic gastritis assessed based on serum *H. pylori* antibody and pepsinogen levels progressed[17]. These studies showed that atrophic gastritis is a major preventive factor for GERD; however, the diagnosis of atrophic gastritis based on histological or serologic methods is not intuitive. In addition, these methods have limitations in terms of medical expenses, which cannot be easily applied in clinical trials.

Endoscopically, gastric atrophy is defined as a visible submucosal pattern in a non-overdistended stomach[18,19]. Previous epidemiological studies have revealed that the prevalence of endoscopic atrophic gastritis (EAG) and incidence of GERD show an inverse correlation[20-23]. However, these studies had several limitations; the extent of EAG was simplified into two types (closed and open types), and the definition of GERD was based solely on the presence of symptoms. Furthermore, these studies did not evaluate various confounding variables, such as demographic, metabolic, and lifestyle factors, which might interfere with the prevalence and grade of GERD.

In this study, we aimed to evaluate whether there is a quantitative correlation between the extent of atrophic gastritis and severity of GERD using endoscopic grading and serologic markers considering a variety of confounding variables in a large Korean population.

MATERIALS AND METHODS

Study population

Individuals receiving health checkups who underwent upper gastrointestinal (GI) endoscopy at Seoul National University Healthcare System Gangnam Center between January 2015 and December 2016 were screened for inclusion in the present study. We excluded those who had prior history of esophageal or gastric cancer and who performed esophagectomy or gastrectomy, active or healing stage of benign gastric or duodenal ulcer, and recent proton pump inhibitor (PPI) medication within one month. The study protocol conformed to the ethical guidelines of the 1975 Declaration of Helsinki and was approved by the International Review Board of Seoul National University Hospital (IRB No. H-1701-028-655).

Clinical and laboratory assessment

Demographic data such as age, sex, height, weight, and waist circumference were collected. Physical examinations were performed using a written, systematic protocol with standardized instruments by trained personnel: The waist circumference were measured at midpoint between the lower border of rib cage and iliac crest[24]. Body mass index (BMI) was calculated using height and weight according to the formula: BMI = weight (kg)/height^2 (m^2). According to the modified World Health Organi-zation criteria from the Asia-Pacific guideline, BMI was categorized as follows: Normal (< 23 kg/m^2), overweight (23-24.9 kg/m^2) and obese (≥ 25 kg/m^2)[25].

Structured self-administered questionnaires were used to collect information including alcohol consumption (≥ 140 g/wk), current smoking (at least one cigarette per day for the previous 12 mo), current use of PPI (history of taking PPI within the last month), medication of sedatives or hypnotics and prior eradication therapy for *H. pylori* infection. We also assessed the types of physical activity and the time spent for each exercise. The MET values were assigned to physical activity data: 3.3 for walking at a moderate pace, 4.0 for moderate intensity, and 8.0 for vigorous intensity. The MET-minutes per week were estimated by multiplying the reported time spent at each activity by the corresponding MET value. Insufficient physical activity was defined when the MET value was under 3500 per week**.** Pattern of daily diet was also evaluated for having large meal, high fat diet, high salt diet or high caffeine intake.

Blood samples were drawn for the measurement of serum glucose, triglyceride, high-density lipoprotein cholesterol (HDL-C), *H. pylori* immunoglobulin G (IgG) antibody and pepsinogen I and II, in the morning after participants had fasted for at least 8 h.

Blood pressure was measured using an automated blood pressure monitor (TM-2655P, A&D Company, Saitama, Japan) twice after at least 5 min of rest in a seated position, and the mean value was used. High blood pressure was defined as a systolic blood pressure ≥ 130 mmHg or a diastolic blood pressure ≥ 85mmHg or taking anti-hypertensive medication. Hyperglycemia was defined as a fasting glucose ≥ 100 mg/dL or taking glucose lowering agents. Metabolic syndrome was diagnosed according to the National Cholesterol Education Program Adult Treatment Panel III criteria[26], if there are three or more of the following characteristics: High blood pressure, hyperglycemia, abdominal obesity (waist circumference ≥ 90 cm in men and ≥ 80 cm in women), hypertriglyceridemia (serum triglyceride level ≥ 150 mg/dL), and low HDL-C (HDL-C level < 40 mg/dL in men and < 50 mg/dL in women).

Endoscopic assessment

Seventeen experienced board-certified gastroenterologists performed all endoscopic examinations using conventional white light videoscopes (GIFH260 or GIFH290; Olympus, Aizu, Japan/EG-450WR5). All the gastroenterologists had more than 5 years of endoscopy experience (mean 12.1 years, range 5-27 years) and performed at least 2000 cases esophagogastroduodenoscopies each year.

Definition of GERD

The reflux esophagitis was defined if mucosal breaks or mucosal change such as erythema and/or discoloration were present. The grade of reflux esophagitis was categorized from M to D according to the Los Angeles (LA) classification system with Japanese modification which is based on the length of the longest mucosal break, and confluence of erosions[27,28]: N, normal mucosa; M, minimal changes to mucosa, such as erythema and/or whitish turbidity; A, non-confluent mucosal breaks no longer than 5 mm in length; B, non-confluent mucosal breaks more than 5 mm in length; C, confluent mucosal breaks less than 75% circumferential; D, confluent mucosal breaks at least 75% circumferential. NERD was diagnosed when a subject had the symptom of acid regurgitation or heartburn at a frequency of at least once per week in the absence of reflux esophagitis.

Definition of EAG

Gastric atrophy was evaluated endoscopically according to the location of endoscopic atrophy border (EAB), which is characterized by differences in color, visible capillary network, and height of the gastric mucosa[18,29]: "Closed type" indicates that the EAB is on the lesser curvature of stomach. On the other hand, in "open type", that is parallel to the vertical axis of stomach and extends along the anterior and posterior walls. The extent of EAG was categorized into six grades (C1 to O3): C0, no visible atrophy; C1, closed atrophy confined to the antrum; C2, closed atrophy confined to the antrum and lesser curvature of the distal gastric body; C3, closed atrophy involving the antrum and lesser curvature of the proximal gastric body; O1, open atrophy with the EAB placing between the lesser curvature and the anterior wall; O2, open atrophy with the EAB placing in the middle of the anterior wall; O3, open atrophy widely spread with the EAB between the anterior wall and the greater curvature.

Definition of serologic atrophic gastritis and H. pylori infection

A pepsinogen test was conducted for serological diagnosis of atrophic gastritis. Serum levels of pepsinogen I and pepsinogen II were measured by Latex Turbid immunoassay kits (HiSens Pepsinogen kit, HBI, Korea). Presence of serologic atrophic gastritis (SAG) was defined when pepsinogen I ≤ 70 ng/mL and pepsinogen I/II ratio ≤ 3[15].

H. pylori infection was measured by serum IgG antibodies using chemiluminescent microparticle immunoassay kits (Siemens, Germany). The values higher than 1.10 IU/mL were considered as a positive test for *H. pylori* infection.

Statistical analysis

For demographic and clinical characteristics of the study population, results were expressed as mean ± SD, median with interquartile range, or as counts with percentage. Pearson's chi-square test was used to compare categorical variables and an independent *t*-test was performed for continuous variables.

The association between EAG and GERD was evaluated using univariate and multivariate logistic regression analyses. First, the extent of EAG was categorized into 7 groups (normal to O3) and multivariate logistic regression analysis was performed to evaluate the risk of GERD according to the extent of EAG. It could show whether the extent of EAG influence the occurrence of GERD. Second, the risk of each grade of GERD (normal to LA-C/D) was independently analyzed according to the extent of EAG (normal to O3). It could show the relationship between extent of EAG and severity of GERD.

Pepsinogen I, II, and I/II ratio were compared using Kruskal-Wallis test due to their non-normal distributions, and post-hoc analysis was done with Wilcoxon rank sum test. In post-hoc analysis, *P*-value less than 0.0033 was considered statistically significant according to Bonferroni correction. To figure out the impact of SAG on the risk of GERD, multivariate logistic regression analysis was done.

The data were analyzed using R software version 4.0.5 (R Foundation for Statistical Computing, Vienna, Austria). *P*-values less than 0.05 were considered statistically significant.

RESULTS

Demographic and clinical characteristics of the study population

A total of 27764 individuals underwent health checkup including screening upper GI endoscopy at Seoul National University Healthcare System Gangnam Center between January 2015 and December 2016. Among them, a total of 1179 were excluded from the study; 127 with a history of gastrectomy, 831 with current use of PPI, 157 with active or healing stage of gastric ulcer, 56 with active or healing stage of duodenal ulcer and 8 with esophageal cancer. Finally, a total of 26585 subjects were enrolled in this study and were divided into two groups [4684 (17.6%) in GERD group and 21901 (82.4%) in healthy group] according to the presence or absence of reflux symptom and esophageal structural change. The severity of GERD was graded as follows: NERD (1149 patients, 24.5%), LA-M (1229 patients, 26.2%), LA-A (1804 patients, 38.5%), LA-B (484 patients, 10.3%), LA-C (17 patients, 0.4%) and LA-D (1 patient, < 0.1%) (Figure 1).

Demographic and clinical characteristics of the study population were compared between the GERD and healthy groups (Table 1). Higher proportion of male was observed in GERD group (72.1% *vs* 53.1%, $P < 0.001$). Patients with general obesity (BMI > 25 kg/m^2) were more prevalent in GERD group (37.3% *vs* 25.0%, $P < 0.001$). Also, abdominal obesity (waist circumference ≥ 90 cm in men and ≥ 80 cm in women) were more common in GERD group (45.0% *vs* 35.6%, $P < 0.001$). Patients with GERD showed higher prevalence for high blood pressure (43.3% *vs* 35.6, $P < 0.001$), hyperglycemia (46.3% *vs* 38.6%, $P < 0.001$), hypertriglyceridemia (27.0% *vs* 18.9%, $P < 0.001$) and metabolic syndrome (30.7% *vs* 22.5%, $P < 0.001$). Excessive alcohol consumption and current smoking were more frequently observed in GERD group (21.6% *vs* 15.7%, $P < 0.001$ and 22.5% *vs* 15.4%, $P < 0.001$, respectively). Several life-style factors such as insufficient physical activity (33.7% *vs* 32.0%, $P = 0.022$), having large meal (12.3% *vs* 10.3%, $P < 0.001$), high fat diet (34.8% *vs* 31.3%, $P < 0.001$), high salt diet (13.3% *vs* 11.0%, $P < 0.001$), and high caffeine intake (30.5% *vs* 28.3%, $P = 0.003$) were more frequently accompanied in patients with GERD. Meanwhile, *H. pylori* antibody sero-positivity was less frequent in GERD group (25.3% *vs* 43.3%, $P < 0.001$).

Univariate and multivariate analysis on the risk for occurrence of GERD

Several variables on the risk for GERD were analyzed by univariate and multivariate logistic regression analysis (Table 2). All the variables included in the univariate analysis were used in multivariate analysis. Advanced age and male sex were significant risk factors for GERD in multivariate analysis [Odds ratio (OR) = 1.10, 95% confidence interval (CI): 1.06-1.14, $P < 0.001$ and OR = 1.96, 95%CI: 1.79-2.15, $P < 0.001$, respectively]. As BMI increased, the risk of GERD also increased (OR = 1.27, 95%CI: 1.16-1.39, $P < 0.001$ for BMI 23-25; and OR = 1.51, 95%CI: 1.37-1.67, $P < 0.001$ for BMI > 25). Metabolic syndrome was a significant risk factor for GERD (OR = 1.12, 95%CI: 1.03-1.22, $P = 0.008$). Excessive alcohol intake and current smoking also significantly increased risk of GERD (OR = 1.10, 95%CI: 1.00-1.21, $P = 0.043$; and OR = 1.26, 95%CI: 1.14-1.39, $P < 0.001$, respectively). Among various life-style factors including exercise and dietary habits, there were no significant risk factors for GERD occurrence. On the other hand, sero-positivity for *H. pylori* IgG antibody lowered the risk for GERD by approximately half (OR = 0.49, 95%CI: 0.45-0.54, $P < 0.001$).

Interestingly, there was an inverse correlation between extent of EAG and occurrence of GERD: As the extent of EAG progressed from normal to O3, the prevalence of GERD decreased gradually. In the multivariate logistic regression analysis, EAG was a significant protective factor for GERD: OR, 1.01 (95%CI: 0.90-1.14) in C1, 0.87 (0.78-0.97) in C2, 0.71 (0.62-0.80) in C3, 0.52 (0.44-0.61) in O1, 0.37 (0.29-0.48) in O2, and 0.28 (0.18-0.43) in O3. (Figure 2).

Correlation of EAG and severity of GERD

The grade of GERD in relation to the extent of EAG was evaluated and showed in Figure 3. The prevalence of GERD was highest in C1 and showed decreased tendency as extent of EAG progressed; 19.7% in normal, 22.1% in C1, 17.9% in C2, 13.3% in C3, 10.8% in O1, 8.2% in O2 and 8.1% in O3 (Figure 3A). As the extent of EAG progressed, the proportion of NERD gradually increased and proportion of ERD progressively decreased (Figure 3B).

To evaluate the impact of extent of EAG on the severity of GERD, subgroup analysis was performed (Figure 4). Grossly, as extent of EAG advanced, the risk of GERD decreased. This decreased tendency of OR for GERD intensified as the severity of GERD progressed from NERD to LA-B. Because the number of patients with LA-C/D was too small, it was impossible to calculate OR for LA-C/D.

Correlation of SAG and GERD

Among 26585 individuals included in this study, 2857 individuals (10.7%) underwent blood

Table 1 Demographic and clinical characteristics of the study population

	GERD group (*n* = 4684)	Healthy group (*n* = 21901)	*P* value
Age	50.9 ± 11.5	50.7 ± 11.1	0.330
Sex			< 0.001
Female	1305 (27.9%)	10265 (46.9%)	
Male	3379 (72.1%)	11636 (53.1%)	
BMI			< 0.001
< 23	1657 (35.4%)	11079 (50.6%)	
23-25	1278 (27.3%)	5346 (24.4%)	
≥ 25	1748 (37.3%)	5476 (25.0%)	
Abdominal obesity	2109 (45.0%)	7802 (35.6%)	< 0.001
High B	2028 (43.3%)	7795 (35.6%)	< 0.001
Hyperglycemia	2168 (46.3%)	8451 (38.6%)	< 0.001
Hypertriglycemia	1267 (27.0%)	4136 (18.9%)	< 0.001
Low-HDL	853 (18.2%)	4214 (19.2%)	0.108
Metabolic syndrome	1437 (30.7%)	4920 (22.5%)	< 0.001
Medication of sedatives or hypnotics	149 (3.2%)	704 (3.2%)	0.942
Alcohol consumption	1010 (21.6%)	3449 (15.7%)	< 0.001
Smoking history			< 0.001
Never smoker	2188 (46.7%)	12759 (58.3%)	
Ex-smoker	1444 (30.8%)	5764 (26.3%)	
Current smoker	1052 (22.5%)	3378 (15.4%)	
Insufficient physical activity	1578 (33.7%)	6998 (32.0%)	0.022
Having large meal	577 (12.3%)	2250 (10.3%)	< 0.001
High fat diet	1631 (34.8%)	6847 (31.3%)	< 0.001
High salt diet	624 (13.3%)	2414 (11.0%)	< 0.001
High caffeine intake	1427 (30.5%)	6200 (28.3%)	0.003
Seropositivity for *H. Pylori* IgG Ab	1068 (25.3%)	8453 (43.4%)	< 0.001

Ab: Antibody; BMI: Body mass index; GERD: Gastroesophageal reflux disease; HDL: High-density lipoprotein cholesterol; *H. Pylori*: *Helicobacter pylori*; IgG: Immunoglobulin G.

examination for pepsinogen test. There were minor and no significant differences in clinical characteristics including the prevalence of GERD between individuals with and without blood examination for pepsinogen test. Among them, 703 patients who underwent *H. pylori* eradication therapy were excluded and finally 2154 patients were included in the analysis. 358 patients (16.6%) had GERD and the severity of GERD was graded as follows: 78 patients (21.8%) with NERD, 79 patients (22.1%) with LA-M, 154 patients (43.0%) with LA-A, 46 patients (12.8%) with LA-B, and 1 patient (0.3%) with LA-C. Pepsinogen I level showed no significant association with the severity of GERD (P = 0.802). On the other hand, pepsinogen II level was significantly different according to GERD severity (P < 0.001). Post-hoc analysis revealed that LA-A showed significant lower level of pepsinogen II compared with normal group (normal *vs* LA-A, P < 0.001). In addition, pepsinogen I/II ratio was significantly higher in GERD group compared with normal group (normal *vs* LA-M, P =0.002; normal *vs* LA-A, P < 0.001; and normal *vs* LA-B, P = 0.002). LA-A group also showed significant higher pepsinogen I/II ratio than NERD (NERD *vs* LA-A, P < 0.001) (Figure 5).

SAG group showed lower prevalence of GERD than normal group (Figure 6). Total 343 out of 1889 individuals without SAG (18.2%) had GERD, in other hands, 15 out of 265 individuals with SAG (5.7%) had GERD. In the multivariate logistic regression analysis, the risk of GERD was adjusted for age, sex, BMI, metabolic syndrome, medication of sedatives or hypnotics, alcohol intake, smoking history, physical activity, dietary factor, and *H. pylori* IgG. Presence of SAG was correlated with reduced risk of

Table 2 Univariate and multivariate analysis on the risk for gastroesophageal reflux disease

	Univariate analysis			Multivariate analysis		
	OR	95%CI	*P* value	OR	95%CI	*P* value
Age (10 yr)	1.01	0.99-1.04	0.319	1.10	1.06-1.14	< 0.001
Male sex	2.28	2.13-2.45	< 0.001	1.96	1.79-2.15	< 0.001
BMI						
< 23	Reference			Reference		
23-25	1.60	1.48-1.73	< 0.001	1.27	1.16-1.39	< 0.001
> 25	2.13	1.98-2.30	< 0.001	1.51	1.37-1.67	< 0.001
Metabolic syndrome	1.53	1.42-1.64	< 0.001	1.12	1.03-1.22	0.008
Medication of sedatives or hypnotics	0.99	0.83-1.18	0.906	1.01	0.83-1.22	0.919
Alcohol intake	1.47	1.36-1.59	< 0.001	1.10	1.00-1.21	0.043
Smoking history						
Never smoker	Reference			Reference		
Ex-smoker	1.46	1.36-1.57	< 0.001	0.97	0.88-1.06	0.439
Current smoker	1.82	1.67-1.97	< 0.001	1.26	1.14-1.39	< 0.001
Low level of physical activity	1.08	1.01-1.16	0.021	1.06	0.98-1.14	0.132
Having large meal	1.23	1.11-1.35	< 0.001	1.06	0.95-1.18	0.304
High fat diet	1.17	1.10-1.26	< 0.001	1.00	0.93-1.08	0.992
High salt diet	1.24	1.13-1.36	< 0.001	1.07	0.96-1.19	0.195
High caffeine intake	1.11	1.04-1.19	0.003	0.97	0.90-1.05	0.464
Seropositivity for *H. Pylori* IgG Ab	0.44	0.41-0.47	< 0.001	0.49	0.45-0.54	< 0.001

Ab: Antibody; BMI: Body mass index; CI: Confidence interval; OR: Odds ratio; *H. Pylori*: *Helicobacter pylori*.

GERD (OR = 0.49, 95%CI: 0.28-0.87, P = 0.014) (Figure 7).

DISCUSSION

In this study, we aimed to evaluate the association between atrophic gastritis assessed using endoscopy and the occurrence and severity of GERD adjusting for many confounding variables in a large population of individuals receiving health checkups. As a result, the severity of GERD was inversely correlated with the extent of EAG. After adjusting for several known risk factors for GERD, EAG remained an independent protective factor for the occurrence of GERD. Furthermore, GERD also exhibited a negative association with SAG based on measurements of serum pepsinogen I and II levels. It has been postulated that a decreased acid secretory ability induced by gastric mucosal atrophy lowers the risk of GERD development and progression.

Several previous studies have evaluated the negative association between *H. pylori* infection and GERD[9-12]. Meanwhile, our study directly focused on atrophic gastritis as a protective factor for GERD, which is a more fundamental and systematic approach to show how *H. pylori* infection affects GERD. Furthermore, we performed stratified analysis based on the extent of EAG and endoscopic grade of GERD, while previous studies only provided limited information and simplified analyses based on the presence or absence of atrophic gastritis or GERD. We assessed atrophic gastritis using two independent methods: Endoscopy and serology. Both methods showed consistent protective effects of atrophic gastritis on the occurrence of GERD. We graded atrophic gastritis endoscopically, which is a non-invasive and relatively reliable method to evaluate the extent of gastric mucosal atrophy[29-33]. Meanwhile, histology-based assessment of gastric mucosal atrophy requires tissue sampling with biopsy forceps, which may induce complications such as bleeding, pain, and inflammation. Endoscopic diagnosis of atrophic gastritis is intuitive and applicable in daily clinical settings. We also assessed SAG by measuring serum pepsinogen I and II levels. We excluded patients who underwent *H. pylori* eradication therapy because pepsinogens normalize after successful *H. pylori* eradication. It would be incorrect to evaluate atrophic gastritis merely by pepsinogens in cases after *H. pylori* eradication

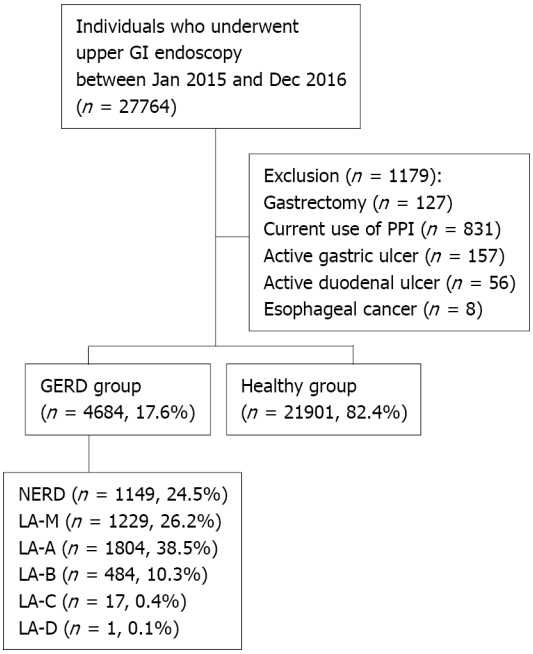

Figure 1 Flow diagram of the study enrollment. GERD: Gastroesphageal reflux disease; GI: Gastrointestinal; NERD: Non-erosive reflux disease; PPI: Proton pump inhibitor; LA: Los Angeles.

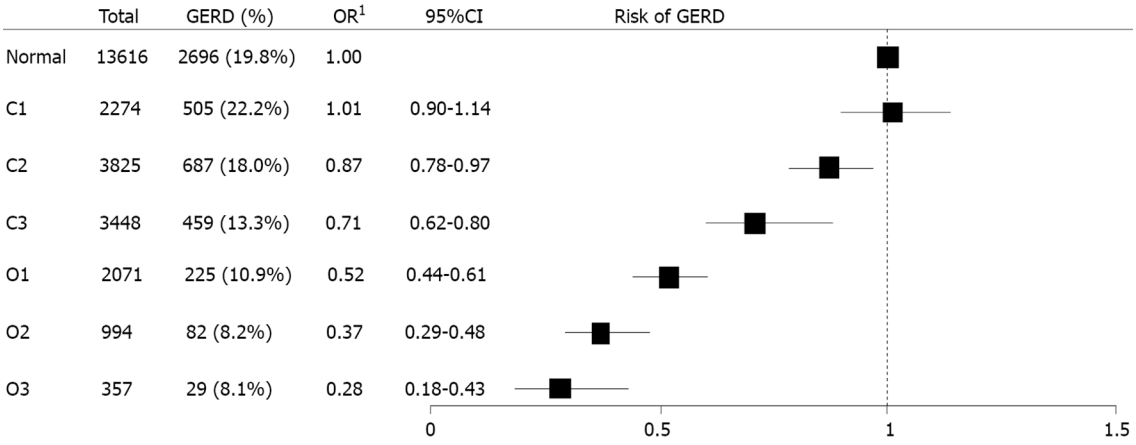

Figure 2 Multivariate analysis on the risk for occurrence of gastroesphageal reflux disease according to the extent of endoscopic atrophic gastritis. [1]Adjusted for age, sex, body mass index, metabolic syndrome, medication of sedatives or hypnotics, alcohol consumption, smoking history, physical activity, dietary factor, and *Helicobacter pylori* immunoglobulin G. CI: Confidence interval; GERD: Gastroesophageal reflux disease; OR: Odds ratio.

therapy. We found that the risk of GERD decreased as SAG progressed. This suggests that not only endoscopy but also simple blood tests may provide information about the risk and severity of GERD. However, interpretation of our findings requires careful consideration from several aspects. Since GERD can only be diagnosed endoscopically, picking up patients without SAG as GERD high risk would only increase the burden of excessive endoscopy and seems unnecessary. It may be sufficient to recommend endoscopy to patients with GERD-related symptoms. In order to prove the clinical usefulness of serological tests in GERD risk assistance, more research data is needed in future studies.

We investigated a variety of possible risk factors for GERD based on structured questionnaires. According to our study, several factors also influence the occurrence of GERD. Advanced age, the male sex, a high BMI, and the presence of metabolic syndrome were shown to be significant risk factors for GERD. Alcohol consumption and smoking were also associated with an increased GERD risk. A recent meta-analysis revealed that advanced age, a high BMI, a low education level, living in an urban area, current smoking, a low income, the consumption of carbonated drinks, and coffee/tea intake increase the risk of GERD, which is comparable with our results[4].

Interestingly, the seropositivity of *H. pylori* IgG was negatively correlated with GERD. Several previous studies have revealed that the prevalence of *H. pylori* infection is significantly lower in patients with GERD[10,11]. It is assumed that chronic inflammation induced by *H. pylori* results in gastric

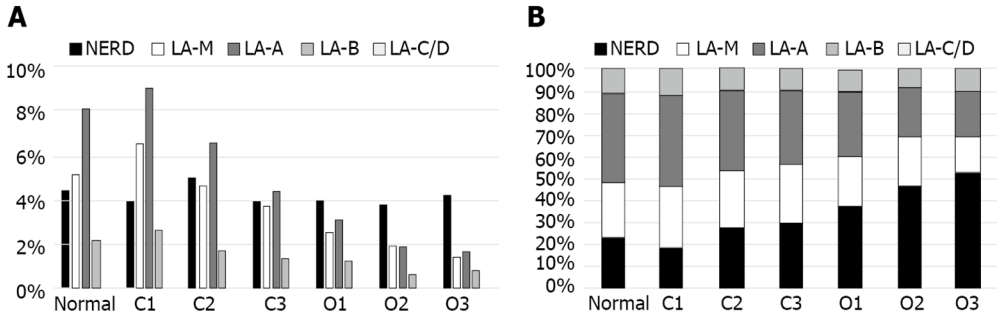

Figure 3 The grade of gastroesophageal reflux disease in relation to the extent of endoscopic atrophic gastritis. A: Prevalence of gastroesophageal reflux disease (GERD) according to the extent of endoscopic atrophic gastritis (EAG); B: Distribution of the severity of GERD according to the extent of EAG. NERD: Non-erosive reflux disease; LA: Los Angeles.

atrophy, which further decreases the acid secretory capacity of gastric mucosa. Therefore, the distribution and type of gastritis related to *H. pylori* are more important than the *H. pylori* infection itself[34]. Antrum-predominant gastritis induces hypergastrinemia and increased acidity; consequently, the risk of GERD increases in patients with antral gastritis[35]. In contrast, in cases of severe corpus gastritis, decreased gastric acid production is considered the main pathogenesis by which *H. pylori* infection protects against GERD[36]. Based on this, we evaluated the risk of GERD according to the extent of EAG. Interestingly, the risk of GERD was highest in association with C1 and gradually decreased as the extent of EAG progressed. This supports that atrophic gastritis rather than *H. pylori* infection itself is a key risk factor for GERD.

Our study has several advantages over other studies. First, we defined the extent and severity of atrophic gastritis using endoscopic and serologic methods, which enabled us to evaluate the effect of atrophic gastritis on the occurrence and severity of GERD. Second, we enrolled participants from a health screening cohort that represented the general population. For this reason, there was a minimal risk of selection or referral bias. The subjects were limited to individuals who had no specific disease and underwent regular endoscopic screening, as these conditions facilitated the assessment of the true impact of the disease. Third, high-quality data were obtained based on structured questionnaires and fully computerized electronic medical records, including data on demographics, laboratory examinations, family history, lifestyle factors, and most importantly, disease-specific symptoms. Healthwatch version 2.0, the large database in our center, fully computerizes a broad range of exposures including a comprehensive drug history and makes it possible to control for potential confounders, thereby permitting a less biased estimate of the association.

The interpretation of our findings requires careful consideration from several perspectives. First, the cross-sectional design prevents any conclusions regarding causality among *H. pylori* infection, atrophic gastritis, and GERD. Second, this study was conducted in subjects at a single healthcare center; therefore, the pool of subjects might represent a relatively high socioeconomic status, and it is possible that the enrolled individuals were more concerned about health. However, this study design is more favorable to other studies performed in tertiary hospitals, and our study population approached the general population. Third, *H. pylori* infection status was evaluated solely using a serologic test. There was no clinical information available regarding current or past *H. pylori* infection status in the serologic test. Nevertheless, serology is the most frequently used method for epidemiological research and has been used to predict the prevalence of *H. pylori* infection in various populations with approved sensitivity and specificity. Fourth, it would be better if we had performed 24-h esophageal pH monitoring tests for the diagnosis of GERD, which is considered the gold-standard method. Twenty-four-hour esophageal pH monitoring tests require specific medical devices, and it is an uncomfortable and time-consuming procedure for patients. At our institute, 24-h esophageal pH monitoring is not generally available in most clinics; thus, the diagnosis of GERD is based on subjective reports of symptoms and endoscopic examination results. Therefore, we believe our data reflect real-world practice. Finally, gastric atrophy was assessed using conventional white-light endoscopy. The diagnostic accuracy of conventional white-light endoscopy is reported to be relatively low compared with that of autofluorescence imaging[37,38].

The primary goal of *H. pylori* eradication therapy is to improve atrophic gastritis and to reduce carcinogenic risk and associated mortality, and it is clear from previous studies that eradication therapy can reduce cancer deaths[39,40]. Even if eradication therapy is likely to exacerbate the symptoms and clinical course of GERD, it seems clear that eradication treatment should be prioritized over GERD prevention. Another implication of our study is that clinicians should pay attention to asymptomatic patients with atrophic gastritis. Patients with GERD may experience diverse symptoms such as heartburn or acid reflux; therefore, they might have a high chance of visiting clinics to manage the symptoms and undergo thorough testing, such as upper GI endoscopy. However, patients with severe atrophic gastritis, even with a higher risk of gastric cancer[8,30], have a lower risk of developing GERD.

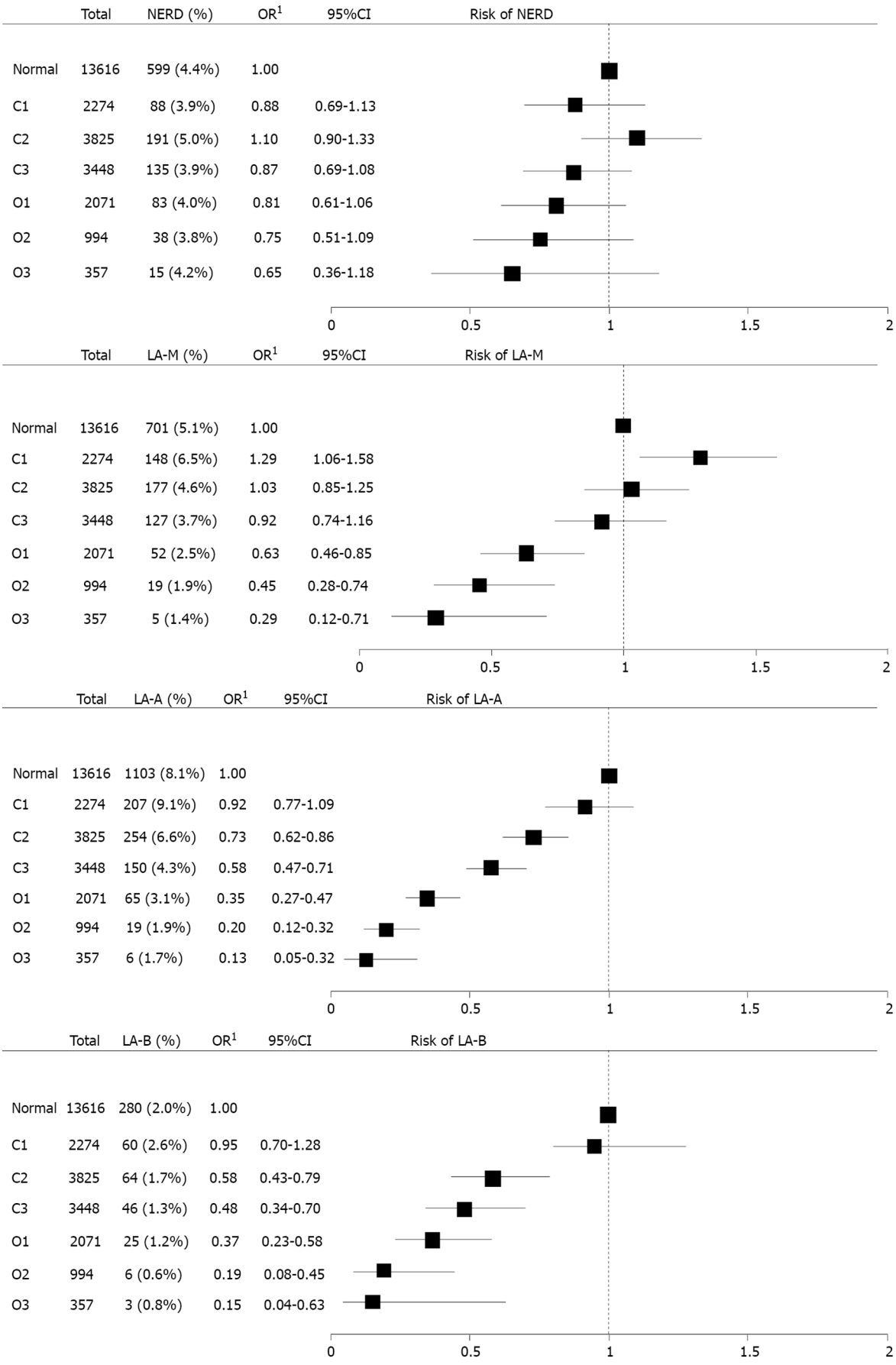

Figure 4 Impact of the extent of endoscopic atrophic gastritis on the risk for severity of gastroesophageal reflux disease. [1]Adjusted for age, sex, body mass index, metabolic syndrome, medication of sedatives or hypnotics, alcohol consumption, smoking history, physical activity, dietary factor, and *Helicobacter pylori* immunoglobulin G. NERD: Non-erosive reflux disease; CI: Confidence interval; OR: Odds ratio; LA: Los Angeles.

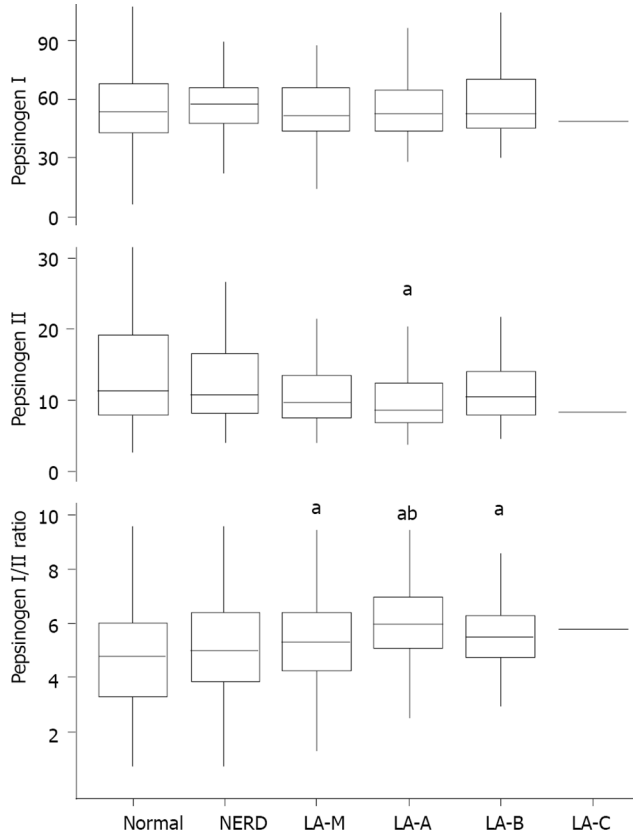

Figure 5 Pepsinogen I, II and I/II ratio according to the severity of gastroesophageal reflux disease. [a]*P* < 0.0033 compared to normal; [b]*P* < 0.001 compared to non-erosive reflux disease. NERD: Non-erosive reflux disease; LA: Los Angeles.

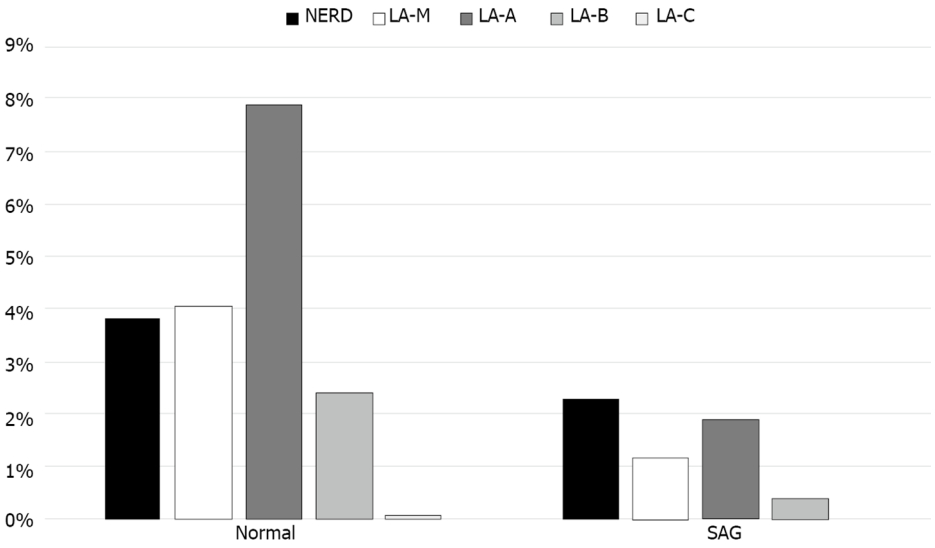

Figure 6 Prevalence of gastroesophageal reflux disease according to serologic atrophic gastritis. NERD: Non-erosive reflux disease; LA: Los Angeles; SAG: Serologic atrophic gastritis.

These individuals are less likely to visit a medical center on their own because they are free of GERD-related symptoms. Therefore, it is important to perform screening endoscopy in individuals without symptoms, especially in cases of extended EAG or SAG presented in previous examinations. Furthermore, if a patient with severe EAG complains of GERD-related symptoms such as heartburn or acid reflux, the possibility of other upper GI diseases including non-acid reflux or esophageal hypersensitivity should be considered because GERD is rarely accompanied by severe gastric atrophy. In the present patient, we considered a different approach than the routine prescrip-tion of PPIs.

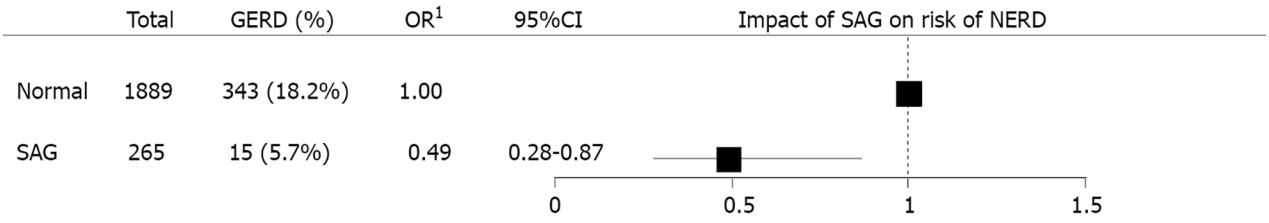

Figure 7 Multivariate analysis on the risk for occurrence of gastroesophgeal reflux disease according to serologic atrophic gastritis. [1] Adjusted for age, sex, body mass index, metabolic syndrome, medication of sedatives or hypnotics, alcohol consumption, smoking history, physical activity, dietary factor, and *Helicobacter pylori* immunoglobulin G Ab. NERD: Non-erosive reflux disease; CI: Confidence interval; SAG: Serologic atrophic gastritis; OR: Odds ratio.

Our findings must be confirmed through prospective clinical trials. The acid secretory activity of the gastric mucosa according to *H. pylori* status and extent of EAG should be further evaluated to determine the pathogenesis of GERD. In addition, an analysis of gastroesophageal motility based on *H. pylori* status and the extent of EAG would provide a better understanding of the mechanisms of GERD. Moreover, long-term interventional studies examining the effect of treating *H. pylori* infection on esophageal disease will provide clear information on which to base clinical decisions.

CONCLUSION

In summary, we found that the extent of EAG and SAG had strong inverse relationships with the occurrence and severity of GERD in a large sample of the Korean population after adjusting for multiple confounding factors. These data support the hypothesis that atrophic gastritis followed by *H. pylori* infection is an independent protective factor against GERD. As the number of patients with gastric atrophy may decrease based on the reduced *H. pylori* infection rate and broad application of *H. pylori* eradication therapy, the prevalence of GERD may increase. Appropriate diagnosis and treatment strategies are required based on the endoscopic findings and symptoms of patients.

ARTICLE HIGHLIGHTS

Research background

Helicobacter pylori (*H. pylori*) infection is known to prevent the occurrence of gastroesophageal reflux disease (GERD) by inducing gastric mucosal atrophy. However, little is known about the relationship between atrophic gastritis (AG) and GERD.

Research motivation

Our study directly focused on AG as a protective factor of GERD, which was more fundamental and systematic approach to show how *H. pylori* infection affects GERD.

Research objectives

We aimed to confirm the inverse correlation between AG and the occurrence and severity of GERD.

Research methods

We assessed AG in two independent methods, endoscopy and serology, furthermore, investigated variety of possible risk factors for GERD based on laboratory examination and structured questionnaires.

Research results

Advanced age, male sex, body mass index > 23 kg/m^2, presence of metabolic synd-rome, current smoking, and alcohol consumption were associated with an increased risk of GERD. Seropositivity for *H. pylori* immunoglobulin G antibodies was associated with a decreased risk of GERD. There was an inverse correlation between the extent of endoscopic AG (EAG) and occurrence of GERD. Additionally, the extent of EAG showed an inverse correlation with the severity of GERD. The presence of serologic AG (SAG) was correlated with a reduced risk of GERD.

Research conclusions

The extent of EAG and SAG exhibited strong inverse relationships with the occurrence and severity of GERD. AG followed by *H. pylori* infection may be independently protect against GERD.

Research perspectives

As the Kimura-Takemoto visual endoscopic method used in our study might be subjective, it would be better to continue further study using the endoscopic morphological method - Updated Kimura-Takemoto classification of AG. Furthermore, to clarify the causality between AG and GERD, prospective studies are warranted to follow how the prevalence and severity of GERD change according to the progression or regression of AG.

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FOOTNOTES

Author contributions: Han YM, Chung SJ designed and performed the research and wrote the paper; Yoo S designed the research and contributed to the analysis; Yang JI, Choi JM, Lee J collected the patients' clinical data and provided clinical advice; Kim JS provided clinical advice and supervised the report; and all authors have read and approve the final manuscript.

Institutional review board statement: This study was approved by the International Review Board of Seoul National University Hospital (IRB No. H-1701-028-655).

Informed consent statement: Patients were not required to give informed consent to the study because the analysis used anonymous clinical data. Written informed consent was waived by the Ethics Committee of the designated hospital.

Conflict-of-interest statement: The authors declare that they have no conflicts of interest to disclose.

Data sharing statement: Some or all data and code generated or used during the study are available from the corresponding author by request.

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CASE REPORT

Treatment strategy for pancreatic head cancer with celiac axis stenosis in pancreaticoduodenectomy: A case report and review of literature

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Abstract

BACKGROUND

During pancreaticoduodenectomy in patients with celiac axis (CA) stenosis due to compression by the median arcuate ligament (MAL), the MAL has to be divided to maintain hepatic blood flow in many cases. However, MAL division often fails, and success can only be determined intraoperatively. To overcome this problem, we performed endovascular CA stenting preoperatively, and thereafter safely performed pancreaticoduodenectomy. We present this case as a new preoperative treatment strategy that was successful.

CASE SUMMARY

A 77-year-old man with a diagnosis of pancreatic head cancer presented to our department for surgery. Preoperative assessment revealed CA stenosis caused by MAL. We performed endovascular stenting in the CA preoperatively because we knew that going into the operation without a strategy could lead to ischemic complications. Double-antiplatelet therapy (DAPT) – which is needed when a stent is inserted – was then administered in parallel with neoadjuvant chemotherapy (NAC). This allowed us to administer DAPT for a sufficient period before the main pancreaticoduodenectomy procedure while obtaining therapeutic effects from NAC. Subtotal stomach-preserving pancreaticoduodenectomy was then

performed. The operation did not require any unusual techniques and was performed safely. Postoperatively, the patient progressed well, without any ischemic complications. Histopathologically, curative resection was confirmed, and the patient had no recurrence or complications due to ischemia up to six months postoperatively.

CONCLUSION
Preoperative endovascular stenting, with NAC and DAPT, is effective and safe prior to pancreaticoduodenectomy in potentially resectable pancreatic cancer.

Key Words: Pancreaticoduodenectomy; Celiac axis stenosis; Median arcuate ligament; Endovascular stenting; Pancreatic head cancer; Case report

Core Tip: Celiac axis stenosis (CAS), caused by the median arcuate ligament, is an anatomical anomaly that should be noted when performing pancreaticoduodenectomy. In this case, an endovascular stent was placed preoperatively to recanalize the stenotic celiac axis, allowing the patient to safely undergo radical surgery without concern for intraoperative organ perfusion. The point to be highlighted with this preoperative strategy, was the use of double-antiplatelet therapy following endovascular stenting, during the interval when for neoadjuvant chemotherapy for pancreatic cancer (PC), thus providing a rational and effective combination of the two preoperative treatments for patients with PC and CAS.

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INTRODUCTION

Celiac axis stenosis (CAS) – caused by the median arcuate ligament (MAL) – often hinders blood flow to the upper abdominal organs, particularly the liver, during pancreaticoduodenectomy[1-4]. This is because the gastroduodenal artery (GDA), which is responsible for maintaining hepatic blood flow during the procedure, needs to be dissected. The most common intervention to maintain hepatic arterial flow is intraoperative MAL division[1,5,6], which fails to recanalize the arterial flow in 30%-40% of cases [1,4,7]. Additionally, the success of MAL division can only be determined intraoperatively and, in case of failure, surgeons must choose to either undertake a complex revascularization or abandon the resection intraoperatively.

Recently, neoadjuvant chemotherapy (NAC) has become a common preoperative treatment for pancreatic cancer (PC)[8,9]. NAC improves the overall survival of patients with PC and is usually required for 2-3 mo, or more, depending on the necessary treatment regimen. To eliminate concerns about MAL compression prior to the scheduled pancreaticoduodenectomy, we undertook preoperative endovascular celiac axis (CA) stenting. Double-antiplatelet therapy (DAPT) was subsequently administered to avoid stent obstruction, with the NAC for PC continuing concurrently. Herein, we report on a reasonable and effective preoperative strategy that combines NAC for PC and endovascular stenting for MAL compression.

CASE PRESENTATION

Chief complaints

A 77-year-old man was referred to our department with a progressively enlarging pancreatic head mass.

History of present illness

The patient had been followed-up for intraductal papillary mucinous neoplasm of the pancreas for 20 years, with no significant changes in the periodically scheduled examinations until half a year ago.

In the last six months, a tumor of approximately 30 mm became visible on the pancreatic head and diagnosed as cancer after further investigation.

History of past illness

The patient had undergone robot-assisted laparoscopic prostatectomy for prostate cancer six years prior, as well as endoscopic submucosal dissection for early gastric cancer two years prior. He had a pacemaker implanted for atrioventricular block two years prior.

Personal and family history

The patient had no family history of PC or genetic disorders.

Physical examination

The patient's appetite was normal, and he had no weight loss or abdominal pain. Stool was normal with no history of constipation or diarrhea. Furthermore, jaundice was not observed.

Laboratory examinations

Laboratory results included an elevated carbohydrate antigen 19-9 (CA19-9) level of 308.5 U/mL, but the carcinoembryonic antigen level was normal. Other results, including diabetes indices such as HbA1c and glycoalbumin levels, were within normal limits. Bilirubin levels were also normal.

Imaging examinations

Computed tomography (CT) revealed a 30 mm pancreatic head tumor with poor enhancement in the early phase (Figure 1A). The tumor had not invaded the major vessels including the common hepatic artery, CA, and the superior mesenteric artery (SMA) and superior mesenteric vein. No metastatic lymph nodes and metastases to distant organs were apparent. Three-dimensional reconstruction images showed developed collateral pathways around the pancreatic head (Figure 1B and C). In the sagittal view, the CA was compressed by the MAL, which developed caudally (Figure 1D). Endoscopic retrograde cholangiopancreatography revealed stenosis in the main pancreatic duct (MPD) and biopsy from the stenotic site of the MPD detected adenocarcinoma.

FINAL DIAGNOSIS

He was diagnosed with cT2N0M0 (8th edition of the UICC-TNM classification) pancreatic head cancer comorbid with CAS caused by MAL.

TREATMENT

A pancreaticoduodenectomy was planned due to the diagnosis of pancreatic head cancer. To secure hepatic blood flow, endovascular stenting of the CA was undertaken prior to the surgery. After successful endovascular stenting, DAPT was administered to avoid stent obstruction, with NAC for PC continuing concurrently.

On aortography, the CA could not be visualized, and severe stenosis of the CA was suggested (Figure 2A). It was difficult to achieve an antegrade approach to the CA directly from the aorta because the root of the CA was severely stenotic, and the root was strongly bent. Therefore, we planned to approach the CA retrogradely from the SMA *via* the collateral pathway.

The procedure was initiated by placing sheaths in the right and left common femoral arteries. A 4Fr shepherd hook catheter (Angiomaster^{R}, Terumo, Tokyo, Japan) was selectively inserted into the SMA through the left sheath. Next, we used a triple-coaxial system to reach the CA[10]. In the triple coaxial system, a 2.7 Fr high flow catheter (Bishop HF, Piolax Medical Devices, Yokohama, Japan), a 1.9 Fr micro catheter (Carnelian^{R} Marvel^{R}, Tokai Medical Products, Aichi, Japan), and a 0.014-inch micro guidewire (Jupiter™ FC, Boston Scientific, Marlborough, MA, United States) were used. Because the guidewire was as fine as 0.014-inch, it was possible to break through the stenosis of the CA (Figure 2B). The microguidewire that reached the aorta was grasped with a snare (Atrieve™ Vascular Snare Kit, Argon Medical Devices, TX, United States) inserted through the right sheath and pulled out of the body. Then, a guiding sheath (Parent Plus^{R}60, Medikit, Tokyo, Japan) was inserted into the CA using the pull-through technique. A balloon expandable stent (Express™ LD vascular stent, Boston Scientific, Massachusetts, United States) was successfully deployed in the CA and an additional stent was used to reinforce the stenotic lesion. Finally, stent dilation was performed with a balloon (Mustang™ Balloon Dilatation Catheter, Boston Scientific, Massachusetts, United States). Aortography demonstrated CA patency and antegrade flow, and the procedure was completed (Figure 2C).

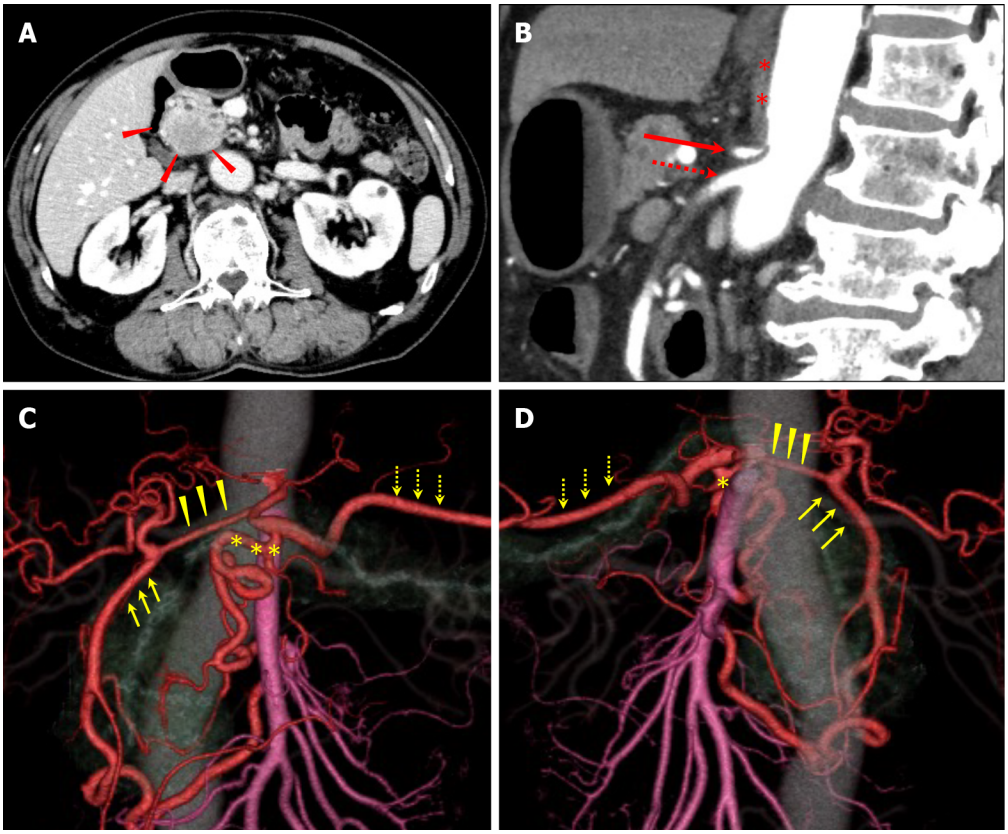

Figure 1 Pre-treatment imaging findings. A: There was a tumor, with poor contrast, in the pancreatic head on computed tomography (CT) imaging. Red arrowheads: the tumor; B and C: Three-dimensional reconstruction imaging showed developed collateral pathways around the pancreatic head. One connected the superior mesenteric artery (SMA) and common hepatic artery (CHA) *via* the gastroduodenal artery (GDA) and another connected the SMA and splenic artery (SPA) *via* the dorsal pancreatic artery (DPA); D: The sagittal view of the CT showed celiac axis (CA) stenosis due to compression by MAL which developed caudally. Yellow arrows: GDA; yellow arrowheads: CHA; yellow asterisks: DPA; yellow dotted arrows: SPA; red asterisks: MAL; red arrow: CA; red dotted arrow: SMA.

DAPT was started the day after stent insertion, and NAC was introduced on day 5. DAPT consisted of oral 100 mg aspirin and 75 mg $P2Y_{12}$ inhibitor once daily. NAC regimen was gemcitabine plus S-1 therapy, which consisted of intravenous gemcitabine at a dose of 1000 mg/m^2 on days 1 and 15 and S-1 orally at a dose of 60 mg twice daily on days 1-14 of a 28-d cycle. DAPT continued for 12 wk and two courses of NAC were administered. The preoperative course is shown in Figure 3.

After completing preoperative treatment, subtotal stomach-preserving pancreaticoduodenectomy was performed (Figure 4A). Since the CA flow was sufficient before the surgery, the surgical procedure was routinely performed, without any special intraoperative techniques. After clamping the GDA, we confirmed good blood flow in the proper hepatic artery (PHA) by palpation, then dissected the GDA. There was no need to preserve the collateral pathways that continued to the DPA or GDA, so we dissected them and obtained an adequate SMA-margin. When dissecting these collateral pathways, we confirmed that the PHA flow remained pulsatile as an indicator that the blood flow in the CA was adequate. We did not apply the MAL division due to concerns regarding stent dislocation induced by dissection procedures near the stent.

OUTCOME AND FOLLOW-UP

Postoperatively, the transaminase level was transiently elevated which quickly improved, and there were no complications related to liver ischemia. No delayed gastric emptying due to decreased gastric blood flow occured, and the patient was able to resume eating four days post-surgery. The patient developed a Grade B postoperative pancreatic fistula (POPF), based on the International Study Group of Pancreatic Surgery grading, but no specific treatment other than drainage was needed[11]. The POPF did not cause any complications, such as hemorrhage and abscess, and the patient was discharged on postoperative day 39. Both the patient and his family were satisfied.

The pathological diagnosis was pancreatic adenocarcinoma, pT1cN0M0, the cancer was StageIA based on the Eighth edition of the UICC-TNM classification, and the pathological treatment effect was graded as IIb based on the Evans classification[12,13]. Since no viable cancer cells were observed in the resected margin, R0 resection was achieved (Figure 5).

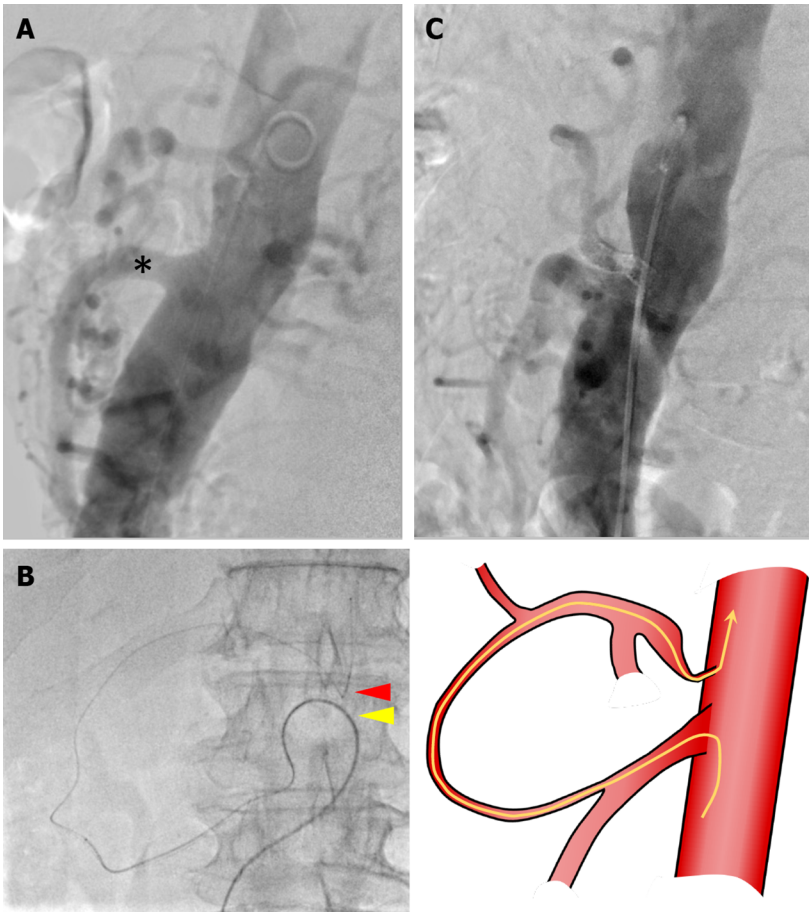

Figure 2 Preoperative endovascular stenting. A: In preoperative aortography, the superior mesenteric artery (SMA) was visualized immediately, but the celiac axis (CA) was not visualized. Black asterisks: SMA; B: The microguidewire reached the CA *via* a collateral pathway from the SMA using a triple coaxial system; C: Final aortography confirmed CA patency and antegrade blood flow. Red arrowhead: Root of the CA; yellow arrowhead: Root of the SMA; yellow line: Running of wire.

Stent patency was confirmed by CT imaging up to six months postoperatively, and the patient had no complications related to liver ischemia (Figure 4) and no recurrence.

DISCUSSION

This case report introduces the procedure and outcome of a preoperative treatment strategy for pancreatic head cancer comorbid with CA stenosis due to compression by the MAL, which combined NAC for cancer and endovascular stenting for MAL compression.

CAS is often observed and reportedly present in approximately 4% to 10.5% of patients undergoing pancreaticoduodenectomy[7,14]. If the GDA is dissected while performing pancreaticoduodenectomy, hepatic blood flow disappears and hepatic ischemia may occur, leading to complications such as liver failure, liver abscess, and bile leakage[1-4]. There are various causes of stenosis, such as MAL compression, atherosclerosis, and arthritis; however, the most common cause is MAL compression[1, 15]. In this case, MAL compression was diagnosed preoperatively, allowing us to intervene preoperatively to maintain hepatic blood flow.

In MAL compression, MAL division is often selected because it is easy and quick. However, while MAL division is simple, it is not reliable, because the CA often does not recanalize and hepatic blood flow is not secured even if the MAL is divided.

Through a comprehensive literature review using the MESH term "pancreat(ic)oduodenectomy and celiac axis stenosis", we identified 108 cases in which the patients underwent pancreaticoduodenectomy and had MAL compression (Table 1). In a literature review of cases in which an intervention was performed for MAL compression, 90.4% underwent MAL division. However, hepatic arterial flow could not be restored in 21.2% of patients despite MAL division. The reason for the failure of MAL division in recanalizing the CA was scarring of the arterial wall caused by longstanding stenosis[16]. In this case, the CT images from approximately 10 years ago already showed signs of MAL compression, which suggested that blood flow might not resume even if the MAL was divided. Additionally, according to a

Table 1 Literature review of previous cases which had median arcuate ligament compression and underwent pancreaticoduodenectomy[24-37]

Factor	Subject	No. of cases (*n* = 108)
Age	yr, median (range)	61 (38-91)[1]
Sex	Male/female	30/19[1]
Diagnosis	Ampullary cancer	4
	Bile duct cancer	10
	Pancreatic head cancer	33
	Others	4
	None described	57
Preoperative detection of CAS	Yes	97
	No	9
	None described	2
Procedure	MAL division	66
	Revascularization	5
	Stenting	1
	Preservation of collateral pathway	1
	No	35
Outcome	Success	91
	Especially MAL division	52
	Failure	17
	Especially MAL division	14
Additional procedures	Revascularization	7
	Stenting	5
	Reoperation	3
	No	3[2]
Complications related to CAS	Liver abscess	3
	Organ ischemia	2
	Anastomotic leakage	3[2]

[1]Among those described.
[2]Indicates some duplication.
CAS: Celiac axis stenosis; MAL: Median arcuate ligament.

previous report which classified MAL compression morphologically by stenosis rate, length of stenosis, and distance between the stenosis and the aorta, these factors were useful in predicting procedures that would be required during the operation. The authors found that MAL division was often ineffective, and revascularization or preservation of the collateral pathways were required, in cases of severe stenosis (stenosis rate of 80% or more) and with a small distance between the stenosis and the aorta[4].

The failure of MAL division can only be determined intraoperatively, and in these situations surgeons have to make a choice that is detrimental to the patient i.e., complex revascularization or abandonment of the surgery. Hepatic arterial revascularization during pancreaticoduodenectomy may result in substantial risk of postoperative hemorrhage – possibly related to POPF – and should be avoided. In previous reports, some cases of CAS had postoperative hemorrhage in the revascularized area[17,18].

NAC has become a common treatment for PC in recent years and is recommended by the NCCN guidelines. NAC is usually administered at an interval of 2-3 mo before radical surgery. With this background, we developed a preoperative strategy to eliminate concerns regarding MAL compression.

In our case, we applied stenting to the CA preoperatively, allowing CA recanalization and hepatic blood flow to be confirmed prior to the operation. Furthermore, DAPT was required to avoid stent

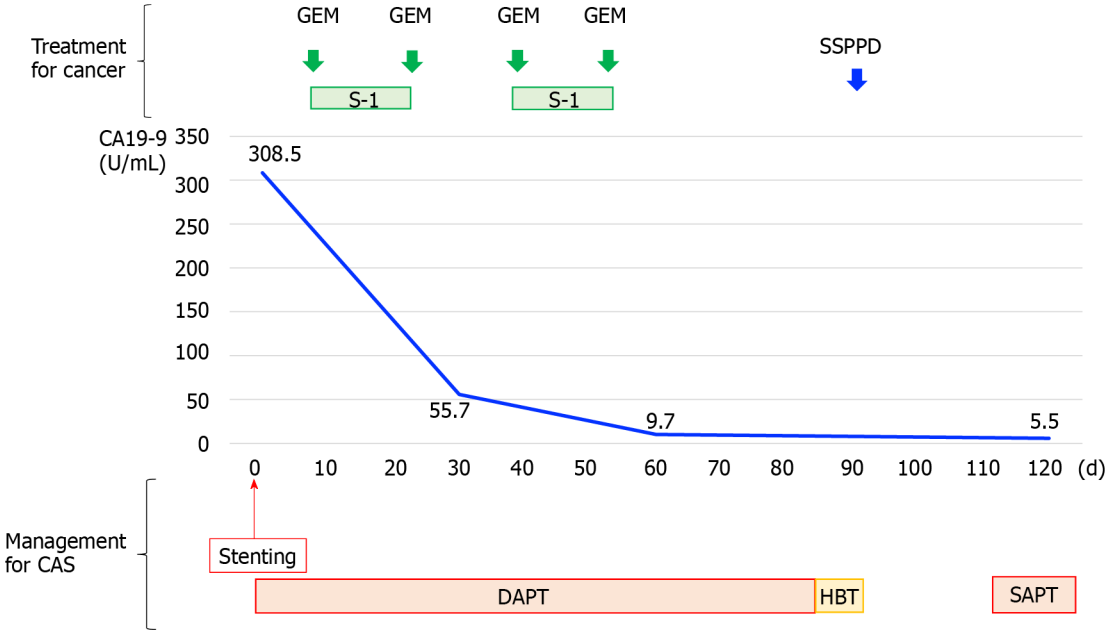

Figure 3 Clinical course timeline. DAPT: Double-antiplatelet therapy; HBT: Heparin-bridging therapy; SAPT: Single-antiplatelet therapy; CA19-9: Carbohydrate antigen 19-9; GEM: Gemcitabine; SSPPD: Subtotal stomach preserving pancreaticoduodenectomy.

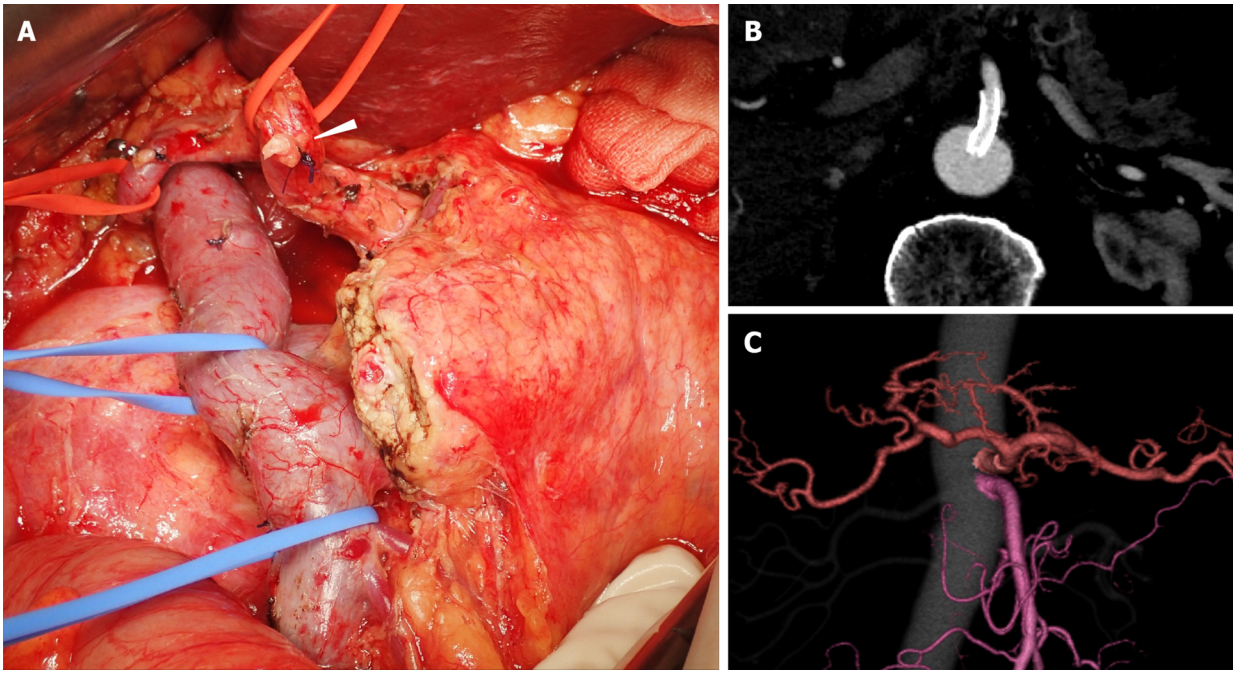

Figure 4 Intraoperative view and postoperative computed tomography images. A: Subtotal stomach preserving pancreaticoduodenectomy was performed. White arrowhead: stump of gastroduodenal artery; B and C: Postoperative computed tomography imaging confirmed patency of the celiac axis.

obstruction, and could also be performed during the NAC treatment period. Clearly, the combination of these two treatments was reasonable. One of the aims in administering NAC is, primarily, to reduce postoperative recurrence. A recent study found that failure of CA19-9 to normalize from preoperatively elevated levels, by the time of surgery, is a predictor of early postoperative recurrence[19]. For our patient, the normalization of the initially very high levels of CA19-9 upon NAC during DAPT, and the preservation of stent patency during the same period, were helpful. The duration of DAPT was determined by referring to the antithrombotic therapy guidelines for patients with coronary artery disease, specifically antithrombotic therapy after percutaneous coronary intervention[20]. MAL division may also be an option to avoid stent obstruction, even after the CA stenting. However, we did not perform MAL division in this case because the risk of dislocating the stent during this procedure defeats the purpose of stenting.

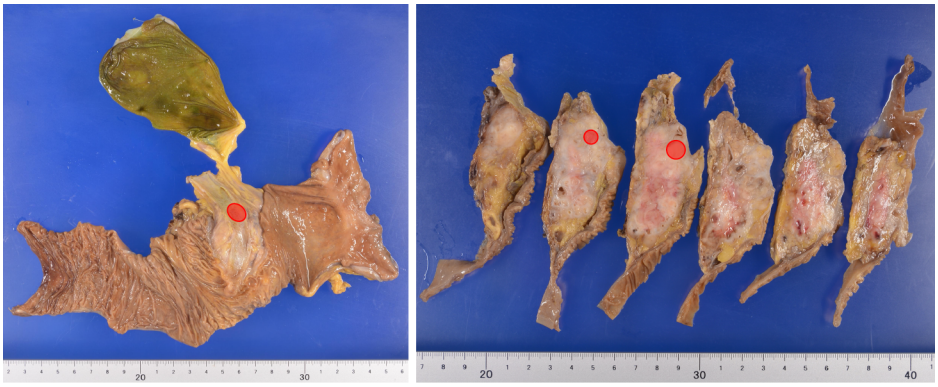

Figure 5 Pathological findings. Tumor mapping on the divided surface of specimens. The resected specimen showed a shrunken invasive tumor with a 12-mm diameter in the pancreatic head. Red circle: Viable tumor site.

Interventional radiology (IVR) for vascular stenosis is performed in various diseases. However, there are few reports regarding the adaptation of IVR for MAL compression, since MAL division is often performed. This case is the first report of the use of the combination of NAC, stent, and DAPT. There is no reason to perform MAL division in addition to this method, and there are no reports of MAL division performed after stenting in existing literature.

Clinical diagnosis of MAL compression is essential for preoperative intervention. In the literature review, a preoperative diagnosis was made in most of the cases (91.5%), indicating that preoperative diagnosis is relatively easy. This high proportion of preoperative diagnosis is thought to be due to the widespread use of multidetector CT and the high recognition of MAL compression[1]. Although the definitive diagnosis of blood flow disturbance by MAL compression can only be made intraoperatively, color doppler ultrasound is a useful diagnostic technique because it is easy and non-invasive[1,3,5].

Intraoperative procedures other than MAL division are used to preserve the collateral pathway, but these surgical techniques are complicated. Additionally, MAL division may impair the curativeness of cancer, so its indications are limited to benign tumors and inflammatory diseases[5,7], and it is not recommended for malignant PC, such as in our case. We were able to dissect the collateral pathway around the SMA and ensure the SMA-margin.

This method has two limitations. First, it requires a high level of skill for stenting in the case of MAL compression. There are previous reports of failure in cases of stenting[21-23]. It is hoped that IVR techniques will become more widespread and improve in the future. Second, the optimal time between stent insertion and radical surgery has not yet been investigated. In this case, since the patient had PC, NAC was administered and sufficient DAPT could be administered during the same period. However, in diseases where NAC is not a common treatment, such as biliary tract cancer, it may worsen the primary disease.

Thus, preoperative simulation and maintenance of skills, such as MAL division and revascularization, remain important for surgeons.

CONCLUSION

Preoperative stent insertion followed by a combination of NAC and DAPT is a safe way to perform pancreaticoduodenectomy; therefore, this procedure may be an effective preoperative treatment strategy.

FOOTNOTES

Author contributions: Yoshida E was the patient's surgeon, reviewed the literature, and contributed to drafting the manuscript; Yoshida E and Kimura Y wrote the paper; Kawagishi R, Sato K, Chiba T, Kimura T, Yonezawa H, and Kobayashi M were involved in the clinical management; Kyuno T, Kono T, and Funato O were assistants at the radical surgery; Murakami K was the radiologist who performed the endovascular stenting; Takagane A and Takemasa I were responsible for the conceptualization and supervision; all authors issued final approval for the version to be submitted.

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LETTER TO THE EDITOR

"Role of exercise in preventing and restoring gut dysbiosis in patients with inflammatory bowel disease": A letter to the editor

Neasa Mc Gettigan, Aoibhlinn O'Toole, Karen Boland

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Abstract

Exercise-induced changes of the microbiome in inflammatory bowel diseases (IBD) is a promising field of research with the potential for personalized exercise regimes as a promising therapeutic adjunct for restoring gut dysbiosis and additionally for regulating immunometabolic pathways in the management of IBD patients. Structured exercise programmes in IBD patients of at least 12 wk duration are more likely to result in disease-altering changes in the gut microbiome and to harness potential anti-inflammatory effects through these changes along with immunometabolic pathways.

Key Words: Inflammatory bowel diseases; Microbiota; Dysbiosis; Metabolism; Exercise; Cytokines

Core Tip: Exercise-induced changes of the microbiome in inflammatory bowel diseases (IBD) is a promising field of research with the potential for personalized exercise regimes as a promising therapeutic adjunct for restoring gut dysbiosis and additionally for regulating immunometabolic pathways in the management of IBD patients. We have observed that exercise programmes of at least 12 wk duration are required to exert any meaningful effects on gut dysbiosis restoration and suggest that the positive effects of a more prolonged programme may extend to inflammatory mediation through regulation of immunometabolism.

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TO THE EDITOR

We read with interest a review article by Koutouratsas *et al*[1] on the "Role of exercise in preventing and restoring gut dysbiosis in patients with inflammatory bowel diseases: A review".

We agree with the authors conclusion that the effects of prescribed exercise on the microbiome is a promising area for further research and that the potential for personalized exercise regimes is a promising therapeutic adjunct when considering the restoration of gut dysbiosis in the management of inflammatory bowel diseases (IBD) patients.

With personalization of exercise regimes in mind, we find it is pertinent to consider the duration of any given exercise programme prescribed for IBD patients. This review article presents the findings of a number of clinical trials in humans examining the effect of various forms of exercise on gut microbiome composition, functionality and diversity. Interestingly, we would like to remark on the duration of exercise programmes and to highlight that the studies of short-term exercise programmes (6 wk duration or less) did not show any clinically significant effect on gut microbiome diversity or composition[2,3] in comparison to studies of at least 12-wk duration which showed changes in gut microbiome composition, diversity and functionality[4,5]. A study of IBD patients not included in the review of 8 wk duration of a prescribed aerobic exercise programme also did not show any significant difference in gut microbiome composition/diversity in response to the exercise programme but other benefits were demonstrated including an improvement in muscle mass and body fat %[6]. Furthermore, two studies of elite athletes, one of rugby players and the other of rowers showed significant differences in microbiome with exercise which likely reflects the habitual nature of the exercise in addition to other factors such as diet[7,8].

A range of exercises have been shown to be safe in patients with IBD including moderate intensity aerobic exercise, resistance training and high intensity interval training[6,9,10]. We suggest that any future studies examining the effects of exercise on changes in the gut microbiome should be of at least 12 wk duration with consideration given to the recommended physical activity guidelines to avoid potential harmful effects of excessive vigorous exercise whilst also being mindful of disease activity (*i.e.*, a personalized approach would be the optimum)[11-13].

Exercise has been shown as a promising therapeutic intervention or adjunct to influence metabolism in disorders including multiple sclerosis through regulation of immune cells[14]. This is mediated through cytokine secretion, and modulation of metabolic regulators including tryptophan[15,16]. Therefore, we suggest that future studies on the effects of structured exercise programmes in IBD patients should be at least 12 wk duration to promote disease-altering changes in the gut microbiome and harness potential anti-inflammatory effects through these changes along with immunometabolic pathways. These benefits would be in addition to promoting sustained exercise behavioral patterns.

FOOTNOTES

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