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Clinical epidemiology and disease burden of nonalcoholic fatty liver disease

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Abstract

Nonalcoholic fatty liver disease (NAFLD) is defined as the presence of hepatic fat accumulation after the exclusion of other causes of hepatic steatosis, including other causes of liver disease, excessive alcohol consumption, and other conditions that may lead to hepatic steatosis. NAFLD encompasses a broad clinical spectrum ranging from nonalcoholic fatty liver to nonalcoholic steatohepatitis (NASH), advanced fibrosis, cirrhosis, and finally hepatocellular carcinoma (HCC). NAFLD is the most common liver disease in the world and NASH may soon become the most common indication for liver transplantation. Ongoing persistence of obesity with increasing rate of diabetes will increase the prevalence of NAFLD, and as this population ages, many will develop cirrhosis and end-stage liver disease. There has been a general increase in the prevalence of NAFLD, with Asia leading the rise, yet the United States is following closely behind with a rising prevalence from 15% in 2005 to 25% within 5 years. NAFLD is commonly associated with metabolic comorbidities, including obesity, type II diabetes, dyslipidemia, and metabolic syndrome. Our understanding of the pathophysiology of NAFLD is constantly evolving. Based on NAFLD subtypes, it has the potential to progress into advanced fibrosis, end-stage liver disease and HCC. The increasing prevalence of NAFLD with advanced fibrosis, is concerning because patients appear to

experience higher liver-related and non-liver-related mortality than the general population. The increased morbidity and mortality, healthcare costs and declining health related quality of life associated with NAFLD makes it a formidable disease, and one that requires more in-depth analysis.

Key words: Nonalcoholic fatty liver disease; Hepatic steatosis; Fatty liver; Prevalence; Incidence; Fibrosis; Risk factor; Epidemiology; Outcomes; Nonalcoholic steatohepatitis

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Core tip: Nonalcoholic fatty liver disease (NAFLD) is a term for a host of histological findings stemming from hepatic steatosis and remains the most common liver disease globally with increasing prevalence. The vast variation in disease presentation complicates diagnosis, leading to an underestimate of actual disease occurrence. NAFLD is associated with many metabolic comorbidities, including obesity, type II diabetes, dyslipidemia, and metabolic syndrome. Its potential to develop into more severe liver conditions, such as nonalcoholic steatohepatitis, advanced fibrosis, cirrhosis and hepatocellular carcinoma, can lead to a state in which liver transplantation is the only treatment option available. The population at risk of developing progressive liver disease creates a challenge to the healthcare system in terms of screening for this evolving epidemic of liver disease.

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INTRODUCTION

Nonalcoholic fatty liver disease (NAFLD) has become a common cause of chronic liver disease in the world^[1] since its first description in 1980 as the “unnamed disease”^[2]. It has been studied in-depth subsequently with continuous myriad of further investigations being carried into this soon to be common indication for liver transplantation (LT). Figure 1 summarizes some of the most landmark studies in the current literature on NAFLD.

NAFLD CLASSIFICATION

NAFLD encompasses a wide histological variety: Nonalcoholic fatty liver (NAFL), nonalcoholic steatohepatitis (NASH), fibrosis, NASH cirrhosis, and

NASH-related hepatocellular carcinoma (HCC) (Figure 2). NAFLD is characterized by $\geq 5\%$ of hepatic fat accumulation in the absence of any secondary causes and is a diagnosis of exclusion. Therefore, other etiologies leading to similar hepatic histology must be ruled out including excessive alcohol consumption; viral hepatitis; other chronic liver disease such as, Wilson’s disease, hemochromatosis, viral hepatitis, autoimmune hepatitis, cholestatic liver disease and other chronic liver diseases; starvation; lipodystrophy; celiac disease; Cushing’s disease; and medications (corticosteroids, methotrexate, diltiazem, oxaliplatin, amiodarone, isoniazid, highly active anti-retroviral therapy, etc.). Current guidelines recommend utilizing criteria requiring an alcohol exposure of less than 30 g/d for men and less than 20 g/d for women as a component of NAFLD diagnosis^[1].

EPIDEMIOLOGY

NAFLD has diverse manifestations described in all ethnicities all over the world and present in both sexes^[3]. The variable presentations probably contribute to the underreported new and existing cases of NAFLD as well as the limited studies undertaken to elucidate the exact incidence and prevalence of NAFLD.

Disease burden

It is currently estimated that the global prevalence of NAFLD is as high as one billion^[4]. In the United States, NAFLD is estimated to be the most common cause of chronic liver disease, affecting between 80 and 100 million individuals, among whom nearly 25% progress to NASH.

Incidence of NAFLD

A study from Japan which followed 3147 patients over 414 d found a 10% annual incidence rate^[5]. Another Japanese study evaluated elevated aminotransferase levels, weight gain and insulin resistance development over 5 years to classify patients with NAFLD and their incidence was reported as 31 per 1000 person-years^[6]. A retrospective study done in England later demonstrated a much lower incidence of 29 per 100000 person-years^[7]. A recent extensive meta-analysis described a pooled regional incidence of NAFLD in Asia and Israel to be 52 [95% confidence interval (CI): 28-97] per 1000 person-years and 28 (95%CI: 19-41) per 1000 person-years, respectively^[3]. Current data on incidence for NAFLD are limited in some regions of the world due to the limited number of studies. Further studies seem warranted to determine the true incidence in general population.

Prevalence of NAFLD

In general, the prevalence of NAFLD has increased over the last 20 years. In addition to the gold standard

Hamaguchi *et al*^[5] The metabolic syndrome as a predictor of nonalcoholic fatty liver disease. *Ann Intern Med* 2005; 143: 722-728

- **Study design:** A prospective cohort study done over 414 d to investigate the effect of metabolic syndrome on pathogenesis of non-alcoholic fatty liver disease.
- **Summary results:** Participants with metabolic syndrome had 4 to 11 times higher risk of future non-alcoholic fatty liver disease.
- **Limitations:** Abdominal ultrasonography, which is not the gold standard, was used to classify non-alcoholic fatty liver disease.

Szczepaniak *et al*^[14] Magnetic resonance spectroscopy to measure hepatic triglyceride content: prevalence of hepatic steatosis in the general population. *Am J Physiol Endocrinol Metab* 2005; 288: E462-E468

- **Study design:** Randomized controlled clinical trial to measure hepatic triglyceride content (HTGC) using magnetic resonance spectroscopy (MRS).
- **Summary results:** A value of 5.56% or greater of HTGC defined as abnormal in patients with no risk factors. Estimated prevalence of NAFLD as 33.6% in the Dallas heart study cohort.
- **Limitations:** 43% of the study population was obese contributing to the higher prevalence reported in comparison to general population.

Younossi *et al*^[16] Changes in the prevalence of the most common causes of chronic liver diseases in the United States from 1988 to 2008. *Clin Gastroenterol Hepatol* 2011; 9: 524-530

- **Study design:** A retrospective analysis of National health and nutrition examination surveys used to estimate changes in the prevalence and predictors of chronic liver disease (CLD).
- **Summary results:** Prevalence of CLD is increasing: 11.78% \pm 0.48% (1988-1994), to 14.78% \pm 0.58% (2005-2008) ($P < 0.0001$). Prevalence of NAFLD has increased steadily as well: 5.51% \pm 0.31% (1988-1994) to 11.01% \pm 0.51% (2005-2008) ($P < 0.0001$).
- **Limitations:** The analysis and results are limited to adults only. There was no histological definition of NAFLD or NASH used to account for prevalence.

Younossi *et al*^[3] Global epidemiology of nonalcoholic fatty liver disease- Meta-analytic assessment of prevalence, incidence, and outcomes. *Hepatology* 2016; 64: 73-84

- **Study design:** A systematic review and meta-analytic approach to report the incidence, prevalence, disease progression and burden of NAFLD.
- **Summary results:** Pooled incidence rate from Asia and Israel were 52 and 28 per 1000 person-year respectively. Prevalence of NAFLD in US has increased from 15% to 25% between 2005 and 2010. Prevalence of NASH is between 1.5% to 6.45%. 9% of NASH patients had advancements in their fibrosis.
- **Limitations:** High unexplained heterogeneity of included studies. Under representation of under-developed countries and besides two studies all others were from countries with high human development index.

Schwimmer *et al*^[21] Prevalence of fatty liver in children and adolescents. *Pediatrics* 2006; 118: 1388-1393

- **Study design:** A retrospective review to determine the prevalence of pediatric fatty liver as diagnosed by histology in a population-based sample.
- **Summary results:** Prevalence of fatty liver in pediatric age group 2-19 yr old was 9.6% (95%CI: 7.4 - 11.7). Prevalence increases with increasing age. Ages 2-4: 0.7 (95%CI: 0.0-2.0), ages 15-19: 17.3 (95%CI: 13.8-20.8).
- **Limitations:** A specific cause of fatty liver disease could not be determined.

Wong *et al*^[98] Disease progression of non-alcoholic fatty liver disease: a prospective study with paired liver biopsies at 3 years. *Gut* 2010; 59: 969-974

- **Study design:** Prospective longitudinal hospital based cohort study to investigate disease progression over 36 months of different degrees of NAFLD.
- **Summary results:** 13 patients with simple steatosis at baseline, three (23%) continued to have simple steatosis at month 36, five (39%) developed borderline NASH and three (23%) developed NASH. Among 17 patients with NASH at baseline, 10 (59%) continued to have NASH and six (35%) had borderline NASH at month 36. Only one (6%) patient regressed to simple steatosis.
- **Limitations:** All patients received lifestyle advice and regular monitoring of metabolic factors. This might have altered the natural history of the disease. Patients with NAFLD in a hospital clinic may have more advanced disease than those in the community. Small Sample size precluded more detailed analysis of factors associated with disease progression. Liver biopsy might be limited by sampling bias.

Angulo *et al*^[113] Liver Fibrosis, but no other Histologic Features, Associated with Long-term Outcomes of Patients With Nonalcoholic Fatty Liver Disease. *Gastroenterology* 2015; 149: 389-397

- **Study design:** A retrospective analysis of 619 patients diagnosed with NAFLD from 1975 through 2005 underwent analysis of their laboratory and biopsies results.
- **Summary results:** Features associated with death or liver transplantation included fibrosis stage 1 (HR = 1.88; 95%CI: 1.28-2.77), stage 2 (HR = 2.89, 95%CI: 1.93-4.33), stage 3 (HR = 3.76, 95%CI: 2.40-5.89), and stage 4 (HR = 10.9, 95%CI: 6.06-19.62) compared with stage 0. Survival free of liver transplantation in patients with non-NASH was significantly lower in those with fibrosis as compared to those without fibrosis ($P < 0.001$).
- **Limitations:** Lack of a specific protocol for patient follow-up with regards to endoscopy and imaging procedures in non-cirrhotic patients, and thus it is possible that the number of liver-related events was underestimated. Over-representation of the white population.

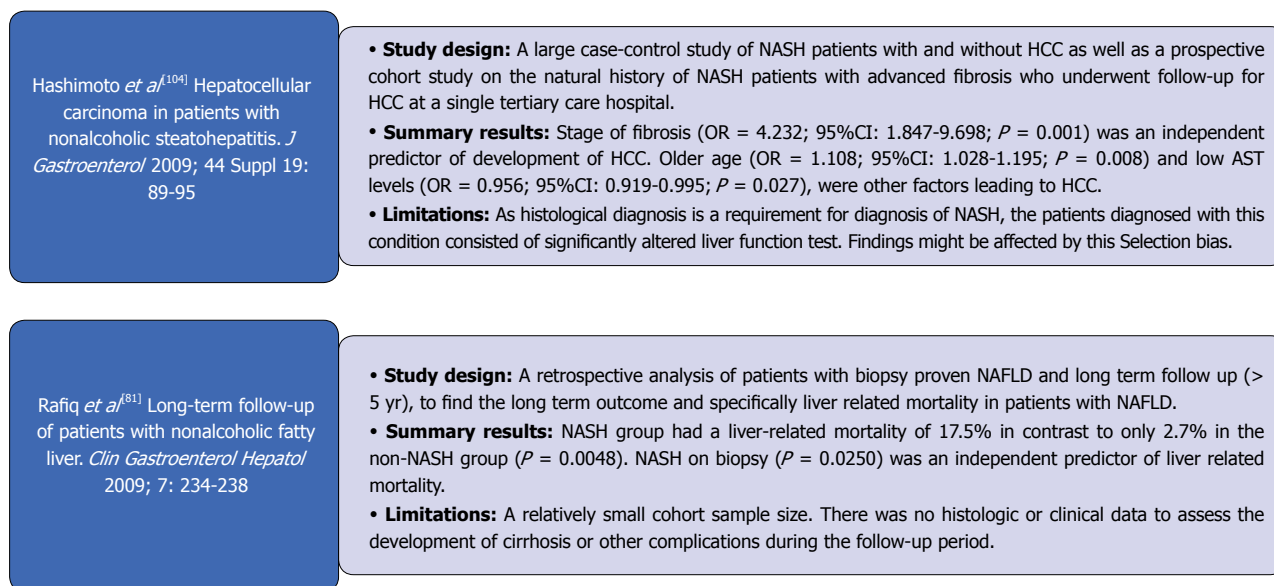


Figure 1 Summary of landmark literature.

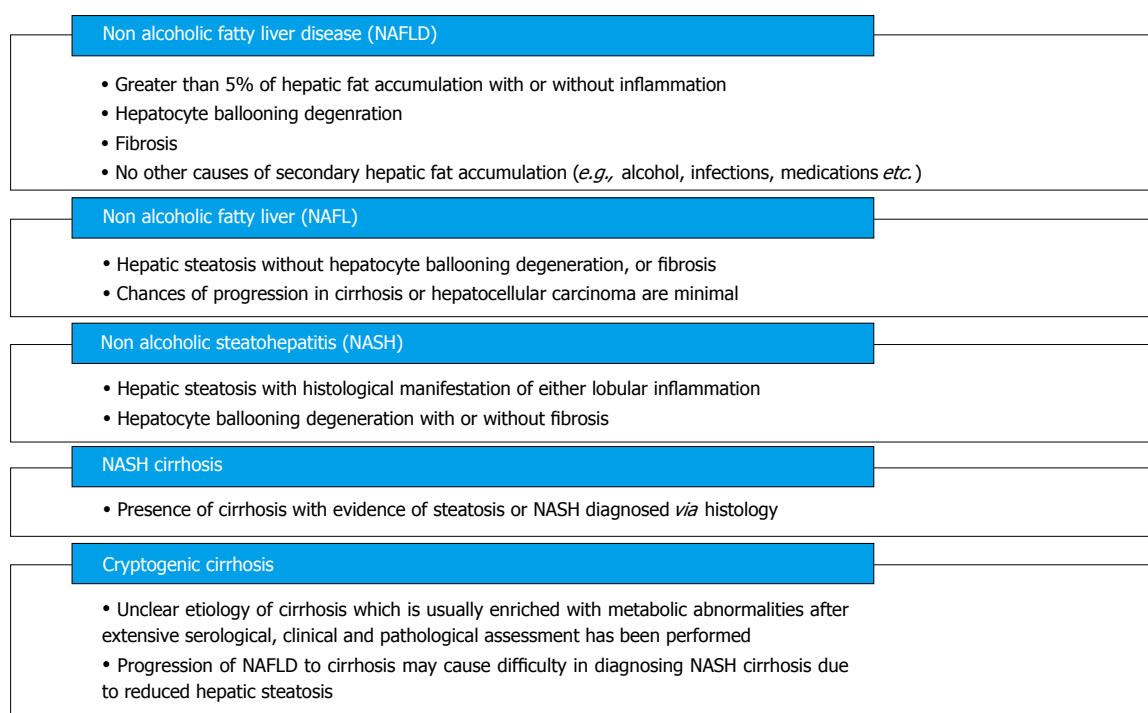


Figure 2 Definitions of nonalcoholic fatty liver disease and its subsets.

diagnostic test of liver biopsy, there are some non-invasive modalities available to diagnose NAFLD. Hepatic ultrasonography, computed tomography (CT), and MRI are accepted modalities for detecting hepatic fatty infiltration. The difference in sensitivity of diagnostic modalities may account for the discrepancy in prevalence data for NAFLD. Using aminotransferase levels as a screening laboratory test for liver disease, prevalence of elevated aminotransferases was 7.9% in the United States general population (1988-1992) with unexplained liver disease in 69% of these subjects^[8,9].

In a recent meta-analysis, hepatic ultrasonography allowed for the reliable and accurate detection of moderate-to-severe fatty liver and is now considered the screening modality of choice^[10]. Prevalence of ultrasonographic diagnosis of NAFLD ranged between 17% in India to 46% in the United States^[8,11,12]. MRS remains one of most sensitive and accurate noninvasive tests available with a NAFLD prevalence of 33% reported in the Dallas Heart Study^[13,14]. The Middle East and South America have the highest NAFLD prevalence at 31% and 32% respectively with

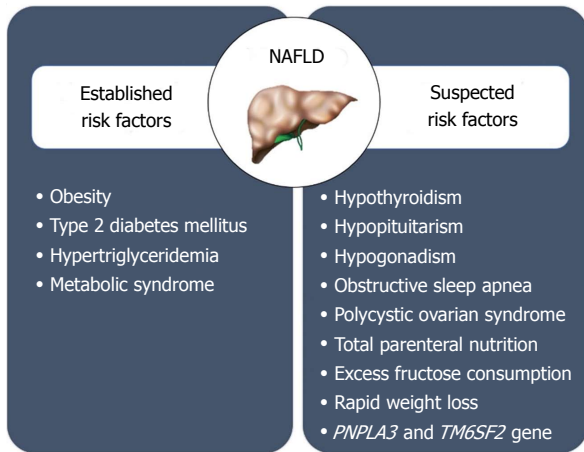


Figure 3 Established and suspected risk factors for nonalcoholic fatty liver disease. NAFLD: Nonalcoholic fatty liver disease.

the lowest prevalence in Africa at 13.5%^[3]. Recently, Asia has been facing the highest obesity epidemic and thus not surprisingly has been experiencing a rapid rate of increase in the prevalence of NAFLD. Chinese adolescents on a “westernized” diet have a greater than 25% prevalence of NAFLD. Studies from Korea, China, Japan and Taiwan have all reported a prevalence ranging from 11%-45%^[15]. Along with the global drift, United States has not been immune to the uptrend in NAFLD. A recent United States-based study using the National Health and Nutrition Examination Surveys (NHANES) conducted between 1988 and 2008 found that the prevalence of NAFLD using elevated alanine aminotransferase (ALT) doubled in the United States during this time period (5.5% to 11.0%)^[16]. Based on the NHANES-III data collected between 1988 and 1994, the prevalence of ultrasonography-diagnosed NAFLD was 34%^[17]. Meta-regression of studies done globally also displayed an increased prevalence of NAFLD from 15% in 2005 to 25% in 2010^[3]. The discrepancy in the prevalence of NAFLD among studies is most likely due to differences in sample selection, diagnostic modalities, dietary and lifestyle habits.

Economic impact

The current annual medical and societal costs of NAFLD are estimated at \$292 billion in the United States^[18]. The projected cost of caring for patients is expected to increase by 18% from 2000 to 2035 and health-related quality of life of NAFLD patients is described as declining^[19,20].

HOST AND ENVIRONMENTAL RISK FACTORS FOR NAFLD

Based on our current knowledge, it appears that a combination of genetic, demographic, clinical and environmental factors may play a role in determining the likelihood of NAFLD in a given individual (Figure 3).

Therefore, the pathogenesis of NAFLD is a multifactorial and multi-step process.

Genetic predisposition to NAFLD

Although obesity, lifestyle variation, and insulin resistance are the most prevalent risk factors leading to the development of NAFLD in a person, NAFLD varies substantially among subjects with comparable lifestyle, environmental impact, and metabolic abnormalities, indicating that other factors contribute to pathogenesis. The heritability^[21] and interethnic variations in susceptibility^[13] suggest that genetic factors may play an important role in determining the phenotypic manifestation and overall risk for NAFLD. NAFLD clusters in families with certain genetic variants on or near *TM6SF2*, *PNPLA3*, *NCAN*, and *PPP1R3B* genes that increase the heritability of NAFLD by up to 27% within families^[22,23]. One genetic variant that is associated with NAFLD is a missense mutation [Ile148 - > Met148 (I148M)] in the palatin-like phospholipase domain-containing 3 gene (*PNPLA3*)^[24]. A recent meta-analysis showed that *PNPLA3* exerts a strong influence not only on hepatic fat accumulation (GG homozygous individuals showed a 73% higher hepatic fat content compared with CC homozygous individuals, $P < 1 \times 10^{-9}$) but also on the susceptibility to develop more severe histologic liver damage (GG homozygous individuals had a 3.24-fold greater risk of higher necro-inflammatory scores and a 3.2-fold greater risk of developing fibrosis compared with CC homozygous individuals, $P < 1 \times 10^{-9}$, respectively)^[22]. These associations were maintained irrespective of the degree of obesity or the presence of diabetes^[23,25,26]. A single variant in *PNPLA3* gene (I148M) has been observed in highest frequency in Hispanics, followed by non-Hispanic whites and least in African Americans^[24]. A minor allele in transmembrane 5 superfamily member 2 (*TM6SF2*) was associated with MRS-measured hepatic triglyceride content from the Dallas Heart Study^[27]. In addition, a minor allele of *TM6SF2* was noted to increase the risk for hepatic fibrosis independent of age, obesity, diabetes, and *PNPLA3* genotype^[28].

Gender and age-related risk for NAFLD

Generally, gender differences exist in NAFLD. Prevalence of NAFLD and NASH was higher in men^[12]. Women are at a reduced risk of NAFLD compared with men at their reproductive period, whereas after menopause women lose the protective effect and have a comparable prevalence of NAFLD as men^[29]. These associations were consistent with children^[30]. Superseding gender, age trends have been associated with NAFLD. Based on the NHANES data, suspected NAFLD prevalence defined as elevated ALT rose from 3.9% in 1988-1994 to 10.7% in 2007-2010, with increases among all race/ethnic subgroups, males and females ranging 12-19 years in age^[30]. These trends

were also consistent among adolescent and young adults aged 15-39 years^[31]. Although the majority of studies are among people aged 30 to 70 years, the general trend of increased prevalence is observed with age with peak prevalence of NAFLD noted between age 50-60 in men^[32]; with 16.1% in ages 30 to 40 years old, 22.3% in 41 to 50 years old, 29.3% in 51 to 60 years old, and 27.6% in over 60 years old based on NHANES III^[33]. In women, prevalence of NAFLD increased with age especially after menopause; with 12.5% in ages 30 to 40 years old, 16.1% in 41 to 50 years old, 21.6% in 51 to 60 years old, and 25.4% in over 60 years old^[33]. A study with octogenarians admitted in a geriatric hospital showed a higher than usual prevalence of 46%^[34].

Differences in NAFLD from race/ethnicity

Race/ethnicity is another variable affecting the prevalence of NAFLD, with the highest prevalence among Hispanics followed by non-Hispanic whites, and lowest prevalence in African Americans^[12,13,35]. The numbers cited are at times double for Hispanics (45%-58%) in comparison to African Americans (24%-35%), with Latinos of Mexican origin having the highest prevalence in a subgroup analysis of the Latino population^[13,36]. These findings hold true even in studies in the pediatric population^[30]. Underlying genetic and lifestyle variations amongst these ethnicities could further account for the skewed prevalence of NAFLD.

Linking obesity and NAFLD

The prevalence of NAFLD among the obese population ranges from 30% to 37%^[8]. Abdominal obesity with increased waist circumference is specifically more strongly correlated with NAFLD^[37]. In a recent cohort study of 2017 subjects during a median 4.4 year follow-up, the visceral adiposity was associated with incident NAFLD in a dose-dependent manner, with an adjusted hazard ratio [HR, per 1-standard deviation (SD) increase] for incident NAFLD of 1.36 (1.16-1.59)^[38]. In addition, this study found significant relationships with subcutaneous adiposity for regressed NAFLD of HR = 1.36 (95%CI: 1.08-1.72) independent of visceral adiposity^[38]. Furthermore, a recent study reported that visceral adiposity increased the risk for NAFLD without significant fibrosis and NAFLD with significant fibrosis after adjusting for known risk factors^[39]. Multivariate analysis showed that the visceral adipose tissue area was independently associated with increased risks of NASH and significant fibrosis^[39]. These studies suggest that certain types of abdominal fat are risk factors for NAFLD and more advanced NAFLD-related fibrosis, whereas other types could reduce risk for NAFLD. In recent years, several cohort studies demonstrated an association between body weight change and incident NAFLD^[40-43]. Even a modest gain in body weight of 2 kg within the normal range has been shown to increase the risk of developing NAFLD^[41]. Obesity has also

been noted to be an additive factor causing a two-fold increase in steatosis in the setting significant alcohol use^[28]. While it is common to have NAFLD in obese population, it is even more common to have obesity in patients with NAFLD. The pooled prevalence of obesity in NAFLD globally is reported to be 51%^[3].

Contribution of diet composition to NAFLD

Due to the evidence supporting that obesity is associated with NAFLD, some macro- and micro-nutrients contribute more to the epidemic of NAFLD. Fructose is a major player, either from sucrose or high fructose corn syrup found in beverages. Consumption of such beverages has increased five-fold in the United States since 1950, and drinking two average size sugar containing beverage servings for 6 mo ends up mirroring many features of NAFLD^[44]. It is hypothesized that sugars promote de novo lipogenesis and trigger inflammatory response leading to hepatocyte apoptosis via the c-Jun-N-Terminal pathway^[45].

Diabetes as a risk factor for NAFLD

Pre-existing metabolic disorders, specifically type 2 diabetes mellitus (T2DM), have a close association with NAFLD, with more than three-quarters of diabetic patients reportedly having NAFLD^[46]. T2DM and insulin resistance promote lipolysis of the adipose tissue leading to release of free fatty acids and their deposition in the liver leading to steatosis^[45]. T2DM is a significant risk factor to cause progressive NASH, fibrosis, cirrhosis and an independent risk factor of mortality in addition to liver-related mortality^[47].

Sleep deprivation as a risk factor for NAFLD

Sleep disturbances and disorders are common medical problems in the current era. Epidemiological studies^[48,49] have provided evidence that poor sleep quality and sleep deprivation is associated with obesity which plays a key role in the pathogenesis of NAFLD. Recently, population cohort studies^[50-52] reported that sleep deprivation may be independently associated with NAFLD with odds ratio 1.28 (1.13-1.44) in men and 1.71 (1.38-2.13) in women. Further, poor quality sleep was found to be a positive predictor of NAFLD in men and women 1.10 (1.02-1.19) and 1.36 (1.17-1.59) respectively^[52]. Biologic plausibility for this independent association has been explored by evaluating the role of inflammatory cytokines interleukin 6 and TNF- α ^[53,54]. These cytokines are increased by sleep disturbances and play a role in pathogenesis of NAFLD by increasing adipocyte lipolysis which in turn can cause hepatic overflow of free fatty acids^[55]. Further, sleep deprivation can affect hypothalamus pituitary adrenal axis, which in turn affects cortisol metabolism leading to hepatic fat accumulation^[56,57].

Medical conditions associated with NAFLD

In addition to the above listed risk factors, other

emerging contributors such as hypothyroidism, hypopituitarism, polycystic ovarian disease and obstructive sleep apnea (Figure 3) should be kept in mind^[1].

METHODOLOGY FOR NAFLD DIAGNOSIS

NAFLD is diagnosed based on clinical history, laboratory and radiographic studies which are further complemented by histologic information. Abdominal imaging revealing hepatic steatosis may be sufficient for diagnosis of NAFLD and liver biopsy may not be required if clinical and laboratory data have ruled out other causes of liver disease. However, role of liver biopsy is important in differentiating NASH from simple steatosis and this may have implications in management as NASH has a higher risk of disease progression as compared to simple steatosis^[58]. NASH is confirmed when all four features viz. steatosis, inflammation, cellular ballooning and fibrosis are present on histology^[58,59]. Apart from imaging and liver biopsy, certain non-invasive tests can help in clinical decision making regarding the presence of advanced fibrosis in NAFLD patients. NAFLD fibrosis score (NFS) is one of the most commonly employed non-invasive tests to assess severity of hepatic fibrosis by utilizing six variables: age, BMI, hyperglycemia, platelet count, albumin and aspartate aminotransferase (AST)/ALT ratio. It is calculated using the published formula available at (*Hepatology* 2007; 45: 846-854 DOI: 10.1002/hep.21496). A meta-analysis of 3064 patients reported that NFS has an area under the receiver operating curve (AUROC) of 0.85 for predicting bridging fibrosis with nodularity or cirrhosis. A score < -1.45 had 90% sensitivity to exclude advanced fibrosis, whereas a score > 0.67 had a 97% specificity to identify presence of advanced fibrosis^[60]. FIB-4 index is another algorithmic score utilized in studies to predict advanced fibrosis. It is based on age, platelet count, AST and ALT and is calculated using published formula (*Hepatology* 2006; 43: 1317-1325 DOI: 10.1002/hep.21178). Using this formula, patients with score > 3.25 are likely to have advanced fibrosis whereas, those with score < 1.45 are unlikely to have advanced fibrosis. Imajo *et al.*^[61] compared various risk scores and elastography against liver histology and showed that NFS and FIB-4 were better than other non-invasive scoring indices like AST to platelet ratio index and AST/ALT ratio. Further, NFS and FIB-4 were as good as MR elastography (MRE) in predicting advanced fibrosis in patients with biopsy-proven NAFLD.

Abdominal imaging as a means of measuring hepatic steatosis

A variety of imaging tools can be utilized for the diagnosis of NAFLD. Abdominal ultrasound is limited by low sensitivity in patients with less than 30% steatosis on histology^[62]. However, it is noninvasive,

widely available and does not require contrast. On the other hand, CT can be associated with radiation hazard and contrast linked nephropathy. It is also limited by low sensitivity hepatic mapping and is expensive^[62]. Magnetic resonance imaging (MRI) and magnetic resonance spectroscopy (MRS) provide the highest precision (sensitivity and specificity) in quantifying steatosis and liver fat mapping^[63] and may become the test of choice in management of NAFLD^[64,65]. Hepatic stiffness measurement with MRE is superior to MRI for the non-invasive diagnosis of significant liver fibrosis and cirrhosis^[66], but the role of transient elastography may be limited in subjects with high body mass indices^[67]. Further, MRE has the advantage of identifying individuals with steatohepatitis, even before the onset of significant fibrosis^[68]. NAFLD with inflammation but without fibrosis demonstrates greater hepatic stiffness than simple steatosis and lower mean stiffness than NAFLD with fibrosis^[68]. Despite this, abdominal imaging studies are currently unable to accurately diagnose NASH.

Role of liver biopsy in the diagnosis of NAFLD

Liver biopsy with key histologic features is essential for confirmation of NASH. However, due to its invasive nature experts recommend selective use in NAFLD patients who have a higher probability of progressing to NASH. An individualized assessment is needed with discussion of risks and benefits of a diagnostic liver biopsy. Early diagnosis of NASH has crucial management implications and these patients can benefit from newly approved medications, off-label therapy with promising agents and treatment in the setting of a clinical trial in an attempt to retard the progression of liver disease^[69-74]. Steatosis may be absent in the setting of advanced fibrosis or cirrhosis^[58,69]. Inter-observer variability among experienced pathologists can occur during the histologic evaluation of hepatic balloon degeneration on a liver biopsy sample^[58,59,75,76]. Poor inter-observer agreement among pathologists regarding sampling error or identification of hepatic ballooning may have resulted in a lower number of patients meeting the entry criteria in clinical trials^[69]. Therefore, liver biopsy although considered as a gold standard for diagnosis of NASH may have several limitations. Patients with isolated hepatic steatosis with any degree of necroinflammation on an index liver biopsy are at risk for progressive histologic damage^[77,78]. In addition, patients with metabolic syndrome or those with individual components of metabolic syndrome coupled with isolated hepatic steatosis on liver biopsy may be at risk for more rapidly worsening histologic damage^[77,78]. Figure 2 organizes the predictors of histologic evidence of NASH on an index liver biopsy in patients with NAFLD. Liver biopsy is indicated in NAFLD patients who have persistently elevated ALT and/or AST levels with abdominal imaging consistent with fatty liver age

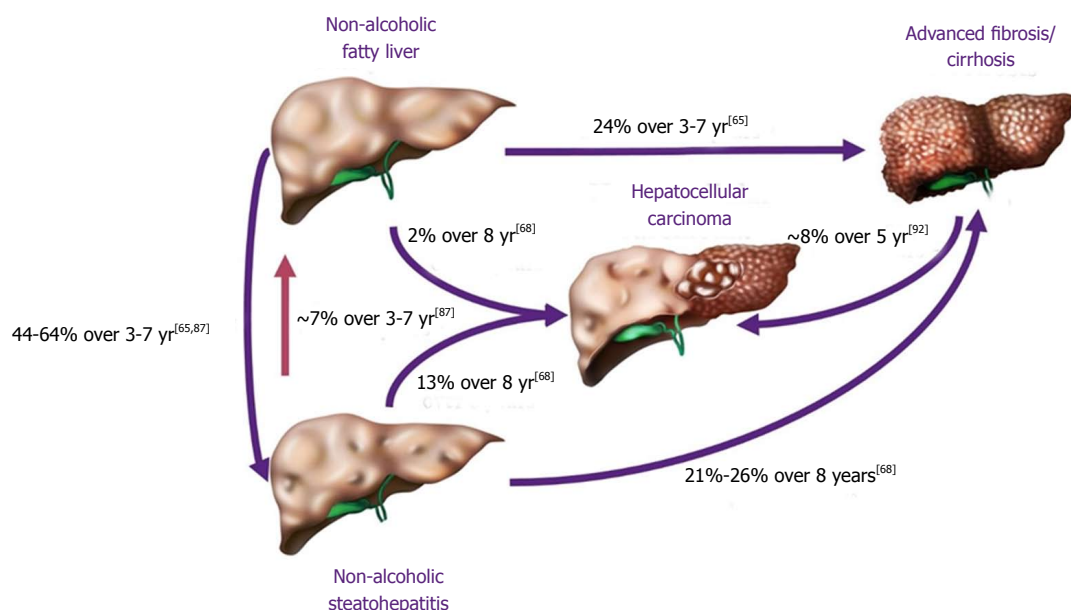


Figure 4 The natural history of nonalcoholic fatty liver disease.

65 years or older, suspicion of other coexisting liver disease, suspicion that another liver disease has been misdiagnosed as NAFLD and those with metabolic syndrome or its components^[1,79-84].

Alternative methods to differentiate NAFLD and NASH

Due to high prevalence of NAFLD along with limitations of liver biopsy and clinical predictors of NASH, there has been a need to develop next generation of noninvasive biomarkers for early diagnosis of NASH^[85]. These noninvasive markers may be able to differentiate lack of fibrosis or mild fibrosis from advanced bridging fibrosis or cirrhosis^[85,86]. However, they are limited in their ability to consistently detect intermediate grade and stage of hepatic fibrosis^[85,86]. Further, abdominal ultrasound have low sensitivity to diagnose NAFLD with less than 30% steatosis^[87]. Keratin 8/18 immunostaining and other next generation noninvasive biomarkers may become available in the near future^[88]. Based on preliminary data, levels of cytokeratin 18 are associated with the presence of NASH, but lacks sensitivity and the histologic details provided by a liver biopsy^[89,90]. Several panels have been developed and studied to predict the presence of advanced fibrosis in patients with NASH^[91]. NAFLD fibrosis score^[92] and FIB-4 are derived from readily available clinical markers for the assessment of advanced fibrosis^[93]. The Enhanced Liver Fibrosis panel utilizes an extracellular matrix marker panel to predict the stage of fibrosis in patients with chronic liver disease^[94].

NAFLD PROGRESSION FROM SIMPLE STEATOSIS TO NASH AND HCC

In terms of progression of NAFLD, the cohort of

patients falls in two broad categories, NASH and NAFL (Figure 4). They are primarily divided by the likelihood of progression; NAFL which represents simple steatosis and steatosis with non-specific inflammatory changes, following a more indolent course of progression, while NASH may progress more rapidly to end-stage liver disease.

Clinical assessment of NAFLD

NAFLD activity score (NAS) has gained popularity in defining NASH, yet histology is still the gold standard. As NASH advances to cirrhosis, it loses its characteristic histologic features, including inflammation and steatosis. Thus, it is increasingly being recognized as "cryptogenic cirrhosis" which essentially means cirrhosis of unclear etiology. Cryptogenic cirrhosis is referred to as 'burnt out' NASH by experts in the medical literature^[8,95]. Patients with cryptogenic cirrhosis have clinical manifestations commonly observed in patients with NASH, such as obesity, dyslipidemia, insulin resistance, T2DM and metabolic syndrome.

Histologic progression and risk factor for NAFLD

NAFL is more readily reversible if lifestyle modifications are implemented in a timely fashion. The benign progression of NAFL and rapid progression of NASH has also been supported by earlier cohort studies from United Kingdom^[96] and Denmark^[97]. In one of the earliest histology-based studies, biopsy-proven NAFLD was divided into 4 types with type 3 (fatty liver and ballooning degeneration) and type 4 (fatty liver, ballooning degeneration, and either Mallory bodies or fibrosis) representing the modern-day definition of NASH^[80]. Over follow up periods of 8 years, 21% to 26% of patients with histological type 3 and type 4 developed cirrhosis compared to only 3% of patients

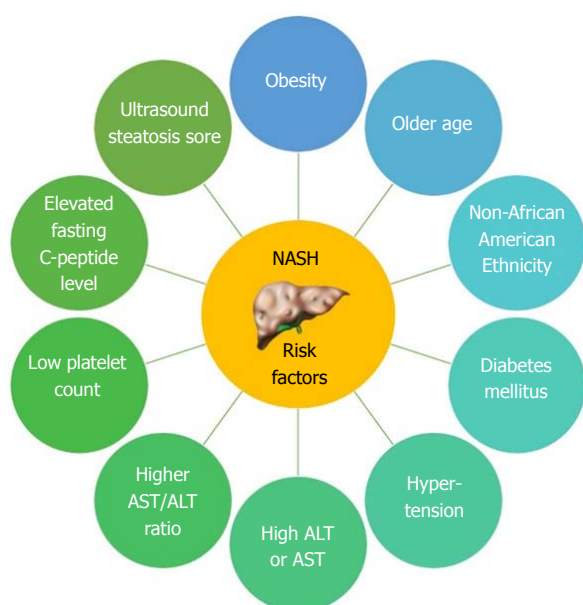


Figure 5 Risk factors for nonalcoholic steatohepatitis subset. NASH: nonalcoholic steatohepatitis.

with type 1 (fatty liver alone) and type 2 (fatty liver and lobular inflammation)^[80]. Recent studies are challenging the widespread belief that non-NASH (simple steatosis) has a benign course. Based on histological diagnosis and follow up biopsies of 52 patients, NAFL advanced to NASH in 23% of cases over a period of 3 years^[98]. The evolution into NASH can be as high as 44%-64% and progression of simple steatosis into advanced fibrosis was reported in up to 24% of the patients with NAFL^[77,99] (Figure 4). Risk factors causing increasing NASH likelihood include obesity, older age, female sex, non-African American race/ethnicity, diabetes mellitus, and hypertension (Figure 5)^[100]. With fibrosis staging and its progression from one stage to another being an important marker of mortality, recent studies reported around 9% to 25% of the patients developed NASH^[101].

Risk of progression from NASH to NASH-related cirrhosis

The risk of progression of NASH into cirrhosis has been delineated in previous studies, and is estimated to be between 21% and 26% in 8 years^[80,102]. Although development of cirrhosis further increases the risk of progression to HCC and/or hepatic decompensation, the stage of fibrosis is also an excellent predictor of outcome.

Risk of HCC development from NAFLD

The incidence of HCC has been increasing in parallel with the rise in NAFLD and its subsets. HCC incidence has grown four-fold from 1973 to 2011^[103]. Advanced fibrosis is a reliable risk factor for HCC with 8% 5-year cumulative incidence rate of developing HCC in patients with advanced fibrosis^[104]. The annual incidence of NAFLD-related HCC (0.44 per 1000

person-years) is rare at this moment and 15-35 times lower than the incidence of HCC in chronic hepatitis B^[3]. In comparison, the annual incidence rate of NASH-related HCC was a significant 5.29 cases per 1000 person-years^[3]. This highlights the increased need of preventative measures that should be adopted; as the prevalence of NAFLD increases so will the incidence of NASH-related HCC. Younossi *et al.*^[105] described a 9% annual increase of HCC cases related to NAFLD over a period of six years from 2004 to 2009. While previous studies have described progression of advanced fibrosis and cirrhosis as a major link between NAFLD and HCC, the latest studies are describing 35% to 50% of HCC without cirrhosis^[106,107]. Understanding of underlying pathogenetic pathways remains unclear at best. A few potential mechanisms to explain the link between NAFLD and HCC include hyperinsulinemia or metabolic syndrome, functioning of hepatic progenitor cells activated by hepatocyte damage, activation of CD8+/CD4+ T lymphocyte and natural killer cells activation causing self-damage and *PNPLA3*-related pathways^[108].

NAFLD OUTCOMES

Liver transplantation in NAFLD patients

NASH is characterized by histologic evidence of progressive hepatocellular injury (ballooning) which can progress to cirrhosis and its complications including HCC with eventual need for liver transplant^[1,109,110]. During last decade, NASH-related LT increased from 1.2% in 2001 to 9.7% in 2009 to become the third most common indication for LT in the United States^[110]. A 2013 population cohort study based on data from the United Network for Organ Sharing/Organ Procurement Transplant Network revealed that NASH has become the second leading etiology of liver disease among adults awaiting LT in the United States and is predicted to become the leading indication in the near future^[110,111]. In addition, NASH is also the second leading etiology for HCC in adults requiring LT in the United States^[112].

Mortality rates associated with NAFLD

A retrospective longitudinal study during 12.6 years showed that increasing fibrosis stage from 1 (HR = 1.88) to stage 4 (HR = 10.49) increased mortality, liver-related events and need for LT^[113]. Over a 8 years follow-up period, liver-related mortality increased in NASH and NASH-related cirrhosis compared to NAFL (11% vs 2%)^[80]. A more recent study using follow-up data from the same cohort reported 18% liver-related mortality in NASH patients compared to 3% in non-NASH patients during 18.5 years^[81].

Predictors of mortality in NAFLD

Previous studies comparing NAFLD to the general

population have consistently shown increased mortality in NAFLD. However, these studies did not adjust for metabolic confounders in the setting of NAFLD. Data from NHANES III revealed no significant difference in the overall survival of ultrasonography-diagnosed subjects with NAFLD compared with the non-NAFLD population after adjusting for multiple metabolic factors^[17]. These results suggest that NASH and/or fibrosis may be the major driver contributing to significant long-term outcomes^[17].

Causes of mortality in NAFLD

NAFLD is associated with increased overall mortality, with ranges for the standardized mortality ratio (SMR) of 1.34-2.6 compared to the general population^[114]. An early landmark study by Adams *et al.*^[82] documented that patients with NAFLD ($n = 435$) from Olmsted County, diagnosed histologically or by ultrasonography demonstrated a significantly higher risk of mortality during 7.6 years of follow-up (SMR = 1.34, 95%CI: 1.00-1.76). In this study, liver-related mortality was the third most common cause of death, after malignancy and cardiovascular disease^[82]. This is in contrast to the general population where liver-related mortality is reported 12th most common cause of death^[115]. NASH cirrhosis has been compared to hepatitis C-related cirrhosis in multiple studies with majority of the studies showing decreased or comparable mortality and lower or similar cirrhosis-related complications and/or HCC^[101,114]. However, the cardiovascular mortality was higher in NASH cirrhosis^[100]. The increased risk for cardiovascular mortality can be explained by the decreased morbidity when compared to chronic hepatitis C-related cirrhosis. Thus, most patients may outlive their liver disease but develop fatal complications from cardiovascular disease and malignancies.

CONCLUSION

NAFLD is a term for a host of histological findings stemming from hepatic steatosis and remains the most common liver disease globally with increasing prevalence. The vast variation in disease presentation complicates diagnosis, leading to an underestimate of actual disease occurrence. NAFLD is associated with many metabolic comorbidities, including obesity, type II diabetes, dyslipidemia, and metabolic syndrome. Its potential to develop into more severe liver conditions, such as NASH, advanced fibrosis, cirrhosis and HCC, can lead to a state in which LT is the only treatment option available. The population at risk of developing progressive liver disease creates a challenge to the healthcare system in terms of screening for this evolving epidemic of liver disease. Further research must be conducted to understand NAFLD pathophysiology and its treatment, as well as, define accurate incidence, current disease burden, and

socioeconomic effects of this disease.

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Obese children with fatty liver: Between reality and disease mongering

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Abstract

Following the current epidemic of obesity, the worldwide prevalence of nonalcoholic fatty liver disease (NAFLD)

has increased with potential serious health implications. While it is established that in adults NAFLD can progress to end-stage liver disease in many cases, the risk of progression during childhood is less well defined. Since most obese children are not adherent to lifestyle modifications and hypocaloric diets, there is a growing number of studies on pharmacological interventions with the risk of disease mongering, the practice of widening the boundaries of illness in order to expand the markets for treatment. Here, we propose a critical appraisal of the best available evidence about long-term course of pediatric NAFLD and efficacy of treatments other than hypocaloric diet and physical exercise. As a result, the number of NAFLD children with a poor outcome is small in spite of the alarming tones used in some papers; large-scale longitudinal studies with long-term follow-up of pediatric NAFLD patients are lacking; the studies on ancillary pharmacological interventions have been performed in few patients with inconclusive and conflicting results.

Key words: Obesity; Children; Non alcoholic fatty liver disease; Non alcoholic steatohepatitis; Cirrhosis; Liver transplant; Disease mongering

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Core tip: The number of obese children with nonalcoholic fatty liver with a documented poor outcome is small in spite of the alarming tones used in some papers. The available studies are insufficient to determine whether or not children with nonalcoholic fatty liver have an elevated risk of developing detrimental health conditions. Large-scale longitudinal studies with long-term follow-up of children with nonalcoholic fatty liver are desirable. Since most obese children are not adherent to lifestyle modifications and hypocaloric diets, there is a growing number of studies on pharmacological interventions with the risk of disease mongering, the practice of widening the

boundaries of illness in order to expand the markets for treatment. The studies on ancillary pharmacological interventions, in addition to diet and exercise, have been performed in few children with inconclusive and conflicting results. The proposal to the obese patient of an ancillary drug may divert his attention from the diet and exercise.

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INTRODUCTION

Childhood obesity can adversely affect nearly every organ system with increased mortality in adult life from a wide variety of systemic diseases^[1]. Following the current epidemic of obesity, the worldwide prevalence of nonalcoholic fatty liver disease (NAFLD) has doubled during the last 20 years with consequent potential serious health implications^[2].

In adults NAFLD has been reported to progress to fibrosis and end-stage liver disease in some 15%-20% of cases, sometimes with need of liver transplantation^[1,3]. Data on fibrosis, evolution to cirrhosis and/or liver cancer in large cohorts of children with NAFLD followed up long-term are scarce^[1]. Despite this lack of information, there is a widespread tendency to draw alarming scenarios also for childhood NAFLD^[4-6], bordering on the phenomenon of so called "disease mongering", i.e. the practice of widening the diagnostic boundaries of illnesses and aggressively promoting their public awareness in order to expand the markets for treatment^[7-9]. This concept is strictly related to medicalization, which implies an extension of medicine domain on three possible ways: qualitative (disease or not disease), quantitative (lowering threshold), temporal (antedating a diagnosis)^[7-9]. It has been reported that the phenomenon of disease mongering is supported by informal alliances comprising drug company staff, physicians and consumer groups, which tend to promote a view of their particular condition as widespread, serious, and treatable^[8]. In many cases these alliances are not maliciously preconceived and simply reflect the fear towards some conditions deemed dangerous to health^[10]. Given the severe well-documented impact of obesity on health, for which it has been stated that we may see the first generation that will be less healthy and have a shorter life expectancy than their parents^[11], it seems paradoxical to invoke the phenomenon of "disease mongering" for obesity-related liver disease. Nevertheless, in the case of obesity there are many myths and presumptions not scientifically supported^[12]. Therefore, we think that the

impact of pediatric NAFLD on morbidity and mortality must be critically evaluated.

Risk of progression of pediatric NAFLD toward end-stage liver disease

As recently reported^[1], there is only one long-term outcome study on the natural history of NAFLD in children, which emphasizes the risk of an unfavorable evolution^[4]. In this paper 66 children with NAFLD were enrolled and only 29 patients underwent a baseline liver biopsy, 5 of whom also had a follow-up histology. Moreover, a substantial proportion of the patients enrolled in this study, considered the reference paper for the natural history of children with obesity-related liver disease^[1], were not obese (34%) and did not have metabolic syndrome (17%). Anyway, only two patients required liver transplantation: an 11-year-old Hispanic female, with a body mass index (BMI) of 26.9 kg/m², dyslipidemia, cirrhosis and esophageal varices at onset, transplanted at the age of 20 years for hepatopulmonary syndrome, with recurrence of NAFLD after 9 mo; and a 18.9-year-old female with a BMI of 33.6 kg/m², low HDL level and hepatopulmonary syndrome, transplanted at the age of 25 years, re-transplanted for recurrence of NAFLD 2.3 years after, who died from multiple organ failure at the age of 27 years.

Both cases had a very severe and atypical clinical course with early recurrence of NAFLD after liver transplantation, suggesting that they might have been affected by an unrecognized genetic metabolic disorder other than NAFLD. In this respect, very little information is provided in the paper on what investigations were done to exclude underlying chronic liver disease. It is to note that hypothalamic-pituitary axis dysfunction and lysosomal acid lipase deficiency (in which the recurrence of non alcoholic steatohepatitis (NASH) following liver transplantation is common) were not ruled out^[13].

In Feldstein's study there were only two children with cirrhosis and these were the same two who required liver transplantation^[4]. Overall, four children were included in the poor prognosis group: the two transplanted and two who died for complications related to bariatric surgery and whose death was not liver related. On the basis of the outcome of these four "atypical" patients with NAFLD, a standardized mortality risk of 13.6 was assigned to the category of the children with NAFLD in comparison with general population.

In the introduction of Feldstein's report^[4], particular emphasis is attributed to some cases of cirrhotic stage disease in children with NAFLD previously reported in literature. If we analyze the relative references, we realize that overall a total of only 5 cases were reported. These 5 cases included a 12-year-old boy with craniopharyngioma with secondary obesity^[14], and a patient who developed at the age of 30 years

hypertransaminasemia without evidence of metabolic syndrome with hepatic decompensation at 32 years^[15]. Interestingly, though this patient had a low ceruloplasmin, Wilson disease was excluded only on the basis of urinary copper excretion^[15]. The other two patients were drawn out of two case studies: one reported in 2003 by Schwimmer including 43 obese children^[16] and the other reported in 1984 including 299 patients^[17]. In these two studies further details about the two patients with cirrhosis were not provided.

Therefore, the critical analysis of the study^[4] and its references shows that progressive liver disease is not a common complication of pediatric NAFLD^[14-17].

Among the other reports of cirrhosis in children with NAFLD not cited in Feldstein's study^[4], one showed 3 cases of cirrhosis and 8 cases of advanced fibrosis among 100 children with histologically documented NAFLD^[18]. Unfortunately, further details about these patients with severe histology were not provided also in this study which, however, documented fibrosis absent or mild in about two thirds of cases^[18]. Furthermore, an Italian study evaluating liver histology on a large sample of 203 children with NAFLD showed no case of stage 4 fibrosis and/or cirrhosis^[19].

So far, the histologic evolution of children with NAFLD has been evaluated in few longitudinal studies^[20,21]. In a cohort of one-hundred six children, 7 cases (6.6%) had a stage 3-4 fibrosis^[21]. Paradoxically, these patients were significantly younger compared with those with mild or no fibrosis. Although the enrolled patients had an accurate histological evaluation, only 46 patients (43%) were investigated for metabolic syndrome^[21].

At the present time, severe cases seem to be too few to refute the arguments on the generally favorable course of pediatric NAFLD as supported from the literature analysis performed here and elsewhere^[1].

Table 1^[4,14-19,21-24] summarizes pediatric studies on NAFLD with indication of the cases of end stage-liver disease. Unfortunately, none of them provided long enough follow-up to assess long-term cumulative risk of severe outcomes. It is to note that almost all the evaluations were assessed in individuals under 20 years of age.

Risk of liver transplant for pediatric NAFLD

While NASH has become the second leading etiology of liver disease among adults awaiting liver transplantation, little information is available for children^[1]. A recent paper from the States reports that NASH may be an important cause of transplant also in children and young adults^[5]. The study included United States patients under 40 years of age transplanted for NASH (no information about the etiology of NASH was provided in the paper) and for cryptogenic cirrhosis associated with a BMI > 30 kg/m². The overall frequency of transplantation for NASH and cryptogenic cirrhosis

associated with obesity was only 1.67% (330/19904), though this low percentage was not emphasized in the conclusions. Of interest, among these patients only 4.2% were < 18 years old, while 16.4% were between 18 and 29 years and 79.4 % between 30 and 40 years of age, suggesting that NAFLD is not a frequent indication for transplantation in children. Moreover, some 15% of the patients had a BMI < 25 kg/m² and therefore were not obese.

Despite this, the study is frequently cited to stress the high risk for liver transplantation in obese children^[1]. To reinforce the concept that fatty liver due to obesity is rarely leading to liver transplantation is the observation that no children with NAFLD required liver transplant in large pediatric series in Europe and United States^[25-28].

Risk of hepatocellular carcinoma among children with NAFLD

Though it has been frequently stated that NAFLD can progress to hepatocellular carcinoma in children, because of the role of obesity and insulin resistance in carcinogenesis, Nobili *et al.*^[1] reported that "only two cases have been described to date, in both cirrhotic and non-cirrhotic background". Is it reasonable to conclude that these cases of HCC are causally associated with obesity? Or, more likely, was it just a fortuity? In brief, given the paucity of data showing a direct correlation between the progression of NAFLD and hepatocellular carcinoma, currently, the risk estimates are not clear and NAFLD can be considered a risk factor likely but not certain. However, what is proved by the evidence is that childhood obesity by itself increases the risk of liver cancer in adulthood, as well as other carcinomas^[12,29]. Therefore it appears more important to focus on the systemic impact of obesity in general rather than on the fatty liver.

Treatment of NAFLD in children

All studies accept the premise that the most effective treatment for patients with NAFLD, both adults and children, is lifestyle optimization, with a focus on nutrition and exercise. These measures have been proven to be able to revert liver damage^[1]. Unfortunately, the majority of obese children are not adherent to lifestyle modifications and hypocaloric diets^[30]. Therefore, there is a growing number of studies focused on pharmacological interventions, based on proven or perceived mechanisms involved in the pathogenesis of NAFLD. In children, most of these studies have been generally performed in small series of patients with conflicting and sometimes inconclusive results^[31,32]. The evaluation of the effectiveness of the various drugs is based in most cases on serum levels of transaminases with few determinations after a short-term intervention^[31,32]. Long-term results of these treatments and their ability to modify the natural course of NAFLD are not available.

Table 1 Studies with histologically documented cases of advanced liver disease in pediatric nonalcoholic fatty liver disease

Study	Yr	No. of patients	Age (yr)	Follow-up (yr)	Case of cirrhosis (n)	Progression of fibrosis (n)	Case of liver transplantation (n)
Cross-sectional studies							
Kinugasa <i>et al</i> ^[17]	1984	299	N/A	N/A	1	N/A	N/A
Schwimmer <i>et al</i> ^[16]	2003	43	N/A	N/A	1	N/A	N/A
Suzuki <i>et al</i> ^[15]	2005	1	12	N/A	1	N/A	N/A
Schwimmer <i>et al</i> ^[18]	2005	100	Range 2-18	N/A	3	N/A	N/A
Alkhouiri <i>et al</i> ^[19]	2012	203	Mean 12.4	N/A	0	N/A	N/A
Longitudinal studies							
Molleston <i>et al</i> ^[14]	2002	2	10 and 14	N/A	2	2/2	None
Feldstein <i>et al</i> ^[14]	2009	66	Mean 13.9	6.4	2	4/5	2
(5 followed longitudinally)							
A-Kader <i>et al</i> ^[21]	2008	106	Range 7-19	2.3	2	7/18	N/A
(18 followed longitudinally)							
Lavine <i>et al</i> ^[22]	2012 (preliminary report)	58	Range 8-17	1.8	N/A	15/58	N/A
Brunt <i>et al</i> ^[23]	2014 (preliminary report)	102	Range 11-17	2.2	N/A	20/102	N/A
Alkhouiri <i>et al</i> ^[24]	2015 (preliminary report)	330	4-40	N/A	N/A	N/A	14/330

N/A: Not available.

Since many studies in humans have shown a relationship between gut bacterial overgrowth, enhanced gut permeability, increased paracellular leakage of gut luminal antigens and liver disease progression through an increased exposure of the liver to gut-derived bacterial products^[33,34], modulating gut microbiota with probiotics, prebiotics, and synbiotics has become an attractive, safe and well tolerated treatment strategy of obesity and NAFLD. Nevertheless, also in adults, their therapeutic use is not supported by high-quality clinical studies^[34,35]. Unfortunately, the only two pediatric RCTs, evaluating the influence of either single strain (*Lactobacillus rhamnosus* strain GG)^[36] or multistrain VSL#3^[37] probiotic supplementation on hepatic biomarkers in small groups of patients (20 and 40, respectively), gave different results. Vajro *et al*^[36] reported no effect of *L. rhamnosus* strain GG on liver echogenicity, but a decrease in serum alanine aminotransferase levels in children treated with *L. rhamnosus* strain GG as compared to placebo. Conversely, Alisi *et al*^[37] found that VSL#3 supplementation reduced the severity of steatosis as assessed by ultrasound. These findings were observed in short periods (2 and 4 mo, respectively) and with a single evaluation at the end of the study. From a pathophysiological point of view, it is difficult to understand how a short term intervention, as administration of probiotic for few months, could have such a long term impact on the composition of the intestinal microbiota (which is highly mutable and related to prenatal, perinatal and environmental factors)^[38,39] to the point of affecting liver health. In particular, the problem is to hypothesize a lasting effect over time, given that the complications of NAFLD are expected in the long term.

Another critical point is the risk of stressing the beneficial effect of a drug on a limited aspect, albeit important, of a disease. This could be the case of

vitamin E on ballooning degeneration, documented in TONIC trial, one of the best designed pediatric studies in a large sample of NAFLD patients^[40]. This finding, although the Authors clearly stated that neither vitamin E nor metformin were superior to a placebo in attaining sustained reduction in ALT level (primary outcome) or improvement in fibrosis (secondary outcome) in patients with pediatric NAFLD, can encourage the use of vitamin E in patients with NAFLD. As stated before, it is important to understand if a therapeutic agent has an impact on a single parameter (liver enzymes) in a limited time interval or an impact on the long term course of disease. If we accept the hypothesis that a treatment with probiotics can really have a favorable impact on liver injury, as a result, probiotics should be prescribed, on a regular basis, to the patient in addition to the recommendation of reducing caloric intake and increasing physical activity. Given the long life expectancy of pediatric patients and the need of preserving obesity-related liver damage in the long term, for how many years (decades?) probiotics should be prescribed in addition to lifestyle modification? and with what economic cost? Furthermore, we must consider that the proposal to the obese patient of an ancillary drug, in addition to diet and exercise, may divert his attention from the diet and exercise.

Despite the absence of strong evidence and although the majority of the Authors is cautious in recommending the extensive use of these drugs^[1], it is reasonable to fear a strong demand from parents who see the drug as a potential remedy for the liver disease of their child. Furthermore, it creates a favorable environment for the development of the phenomenon of disease mongering. Of course, with these considerations we do not deny the usefulness of research on the potential role of drugs and food

supplements in the therapy of this condition. What we hope however is that their effectiveness is documented with a robust methodology and on large series, that are actually missing.

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Procalcitonin in inflammatory bowel disease: Drawbacks and opportunities

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Abstract

The measurement of procalcitonin has recently become a mainstay for the diagnosis and therapeutic management of severe bacterial infections, especially those sustained by Gram-negative bacteria. Therefore, the aim of this article is to provide a narrative overview on the potential role of procalcitonin measurement in patients with inflammatory bowel disease (IBD). According to the available scientific literature, the clinical significance of procalcitonin for diagnosing IBD or monitoring disease activity remains elusive, and its association with disease severity is confined to a limited number of case-control studies, with low sample size. Nevertheless, literature data also suggests that a supranormal procalcitonin serum concentration (*i.e.*, > 0.5 ng/mL) may reflect the presence of a number of infective complications in IBD, especially bacterial enterocolitis, bacterial gastroenteritis, intraabdominal abscess, postsurgical infection and sepsis. Rather than for diagnosing or assessing disease activity, the measurement of this biomarker may hence retain practical clinical significance for early prediction, timely diagnosis and therapeutic monitoring of many IBD-associated infections and complications.

Key words: Intestinal bowel disease; Chron's disease; Ulcerative colitis; Procalcitonin

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Core tip: According to current evidence, the clinical significance of measuring procalcitonin for diagnosing intestinal bowel disease (IBD) or monitoring disease activity remains elusive. Nevertheless, literature data

suggests that supranormal procalcitonin concentrations may reflect the presence of a number of infective complications in IBD, including bacterial enterocolitis, bacterial gastroenteritis, intraabdominal abscess, postsurgical infection and sepsis. Rather than for assessing disease activity, the measurement of this biomarker may hence retain clinical significance for predicting or timely diagnosing of many IBD-associated infections and complications.

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INTRODUCTION

Structure and metabolism of procalcitonin

Procalcitonin is the precursor of calcitonin, an essential hormone involved in calcium homeostasis. In physiological conditions, thyroid C cells synthesize pre-procalcitonin, a 141 amino acids precursor of calcitonin, which is then rapidly converted into procalcitonin (116 amino acids) by endopeptidases-catalyzed removal of the 25-amino acid signal sequence^[1]. Procalcitonin is then converted into the circulation by the enzyme prohormone convertase (PC) in the mature hormone calcitonin (32 amino acids), N-terminal procalcitonin (57 amino acids) and katacalcin (21 amino acids) (Figure 1)^[1]. In physiological conditions, procalcitonin has a very low blood concentration (typically < 0.05 ng/mL) (Figure 2). Nevertheless, in patients with severe bacterial infections, especially in those with systemic infections and sepsis, an extra-thyroid synthesis of procalcitonin occurs in several organs, such as liver, lung, pancreas, kidney and intestine, as well as in leukocytes (Figure 2)^[2]. Consequently, its circulating concentration can be enhanced from 100-fold to 10000-fold over. For this reason, finding blood levels of procalcitonin beyond 100 ng/mL is commonplace in patients with sepsis, with the magnitude of such increase often correlating with both the severity of infections and prognosis.

The mechanisms leading to an enhanced extra-thyroid production is prevalently attributable to both direct and indirect bacterial stimulation of the calcitonin gene *CALC-1* (directly triggered by endotoxin and other bacterial toxins, or indirectly caused by the metabolic reaction of the organism in response to infection), but is also due to reduced cleavage of the protein into calcitonin, N-terminal procalcitonin and katacalcin (Figure 2)^[3]. Notably, procalcitonin synthesis is mostly inhibited (blocked) by interferon- γ in viral infections, so that its concentration remains usually

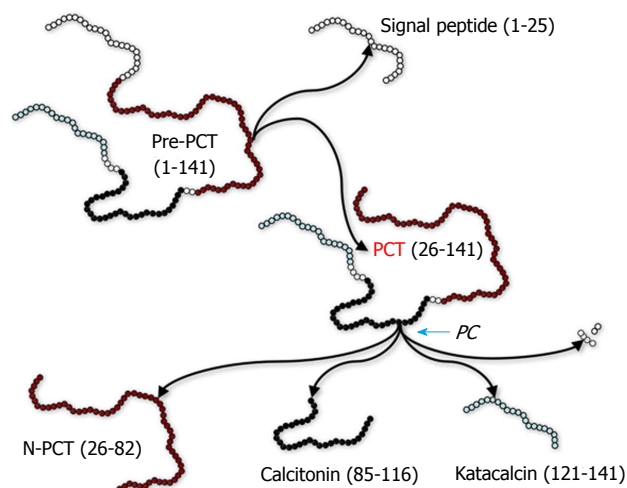


Figure 1 Biochemistry and biology of procalcitonin. N-PCT: N-terminal procalcitonin; PC: Prohormone convertase; pre-PCT: Pre-procalcitonin; PCT: Procalcitonin.

low^[4].

In patients with severe bacterial infections, increased synthesis of procalcitonin typically occurs within 2-4 h from the onset of sepsis, reaching peak blood values 6 to 8 h afterward and persisting as long as the inflammatory process continues, regardless of preserved or impaired renal function^[5]. The half-life of procalcitonin is usually comprised between 20-24 h. Several lines of evidence suggest that procalcitonin kinetics in the blood may provide more useful clinical information than its absolute value. An increase of serum or plasma procalcitonin values over time is associated with worse prognosis, whilst decreasing levels mirrors improved clinical outcome and/or therapeutic effectiveness.

Procalcitonin as a biomarker of infection

Procalcitonin was originally identified as a useful marker of severe systemic infections in 1993 by Assicot *et al*^[6], who studied 79 children with suspected infections and showed that procalcitonin value was substantially increased in those with sepsis and procalcitonin serum concentration was also strongly correlated with disease severity and complications. Since then, many other clinical studies and meta-analyses have confirmed the considerable value of this biomarker for early diagnosis, prognostication and even therapeutic management of patients with sepsis and septic shock^[7]. Albeit its consolidated role in severe systemic infections, the role of procalcitonin in localized infections has remained less conclusive^[8,9]. Nevertheless, recent data suggest that procalcitonin measurement may be clinically useful in patients with bacterial meningitis^[10], community-acquired pneumonia^[11], erysipelas^[12] and arthritis^[13].

In all these conditions procalcitonin measurement is now regarded as a first-line screening test for timely

identification of bacterial infections and to facilitate rapid establishment of an antibiotic treatment. Notably, the results of the vast majority of microbiological tests cannot be immediately available, so that the severity of the infection may progress unless a final diagnosis can be made. Procalcitonin has many advantages in this respect, since its measurement may allow for identifying infections with minimal host response, is sufficiently specific for discriminating bacterial infections from other severe stimuli that may also induce systemic inflammatory response syndrome, is present early during the course of disease, can be timely and conveniently assayed and, finally, may also provide prognostic information^[4,8].

With regards to gastrointestinal infectious disorders, the combination of procalcitonin with symptoms and conventional laboratory tests yielded an improved diagnostic or prognostic accuracy in patients with bacterial pancreatitis^[14], acute bacterial appendicitis^[15], gastroenteritis^[16], ascites^[17], intestinal ischemia^[18], bacterial peritonitis^[19], and other intraabdominal bacterial infections^[20]. Controversial evidence has been published about the role of procalcitonin measurement in patients with inflammatory bowel disease (IBD), as thoughtfully discussed in the next section of this narrative review.

LITERATURE SEARCH

An electronic literature search was performed in Embase, MEDLINE (PubMed interface) and Web of Science to identify eligible literature from the earliest available date to October 24, 2017. The following search terms were used: "inflammatory bowel disease" OR "Crohn's disease" OR "ulcerative colitis" AND "procalcitonin" in title, abstract and keywords, with no language restriction. Review articles, letters to the editor, editorials and original articles were evaluated, and their list of references was also hand-searched to identify additional articles about this topic. The electronic searches returned 29 documents, from which 10 original articles and 1 meeting abstract were finally selected according to their clinical relevance.

EPIDEMIOLOGICAL STUDIES ON PROCALCITONIN IN IBD

The first study assessing the role of procalcitonin in IBD was published by Korczowski *et al.*^[21] in 2004. The serum concentration of procalcitonin and C-reactive protein (CRP) was measured in 30 healthy controls and 129 children hospitalized with diarrhea of various origin, which also included 13 children with IBD. Procalcitonin values were found to be higher than the diagnostic cutoff (*i.e.*, 0.5 ng/mL) in 23% children (3/13) with IBD *versus* 0% of healthy controls ($P = 0.019$). Moreover, the percentage of children in the overall cohort with bacterial enterocolitis displaying increased serum procalcitonin concentration was as

high as 61%.

In the same year, Herrlinger *et al.*^[22] published another study including 51 IBD patients [26 with Crohn's disease (CD), 25 with ulcerative colitis (UC)], along with 25 patients with self-limited enterocolitis. The concentration of procalcitonin was found to be considerably higher in patients with self-limited enterocolitis compared to those with IBD (0.36 ng/mL vs 0.10 ng/mL, $P < 0.001$). Interestingly, although the procalcitonin concentration was in the normal range in all IBD patients (*i.e.*, < 0.5 ng/mL), those with active disease [*i.e.*, Clinical Disease Activity Index (CDAI) score > 150 or Truelove severity index moderate or severe] had a nearly 40% higher procalcitonin value than those with inactive disease (0.13 ng/mL vs 0.09 ng/mL, $P < 0.001$).

Thia *et al.*^[23] carried out a prospective single-center study, including 81 patients with bacterial gastroenteritis and 71 with IBD (27 with CD, 44 with UC). Procalcitonin displayed good performance for discriminating bacterial gastroenteritis from IBD [area under the curve (AUC), 0.727; $P < 0.001$], and its serum levels were higher between patients with active or inactive IBD, although such difference did not reach statistical significance (0.052 ng/mL vs 0.003 ng/mL, $P = 0.416$).

These results were confirmed by Oruç *et al.*^[24], who also measured serum procalcitonin in 50 healthy volunteers and 45 patients with IBD (9 with CD, 36 with UC). Significantly higher procalcitonin values were observed in CD patients (0.14 ng/mL; $P < 0.05$) but not in UC patients (0.10 ng/mL; $P = \text{ns}$) compared to controls (0.06 ng/mL). A procalcitonin threshold of 0.05 ng/mL had modest sensitivity (*i.e.*, 0.67) and very poor specificity (*i.e.*, 0.42) for distinguishing between active and inactive IBD (AUC, 0.57; $P = \text{ns}$).

Oussalah *et al.*^[25] carried out a prospective observational study which included 30 patients with CD and 27 with UC. These authors measured serum procalcitonin values and found they were correlated with several demographic and clinical features. The serum concentration of procalcitonin was found to be significantly higher in patients with active IBD than in those with inactive IBD (0.10 ng/mL vs 0.07 ng/mL, $P = 0.02$). Serum procalcitonin value was also significantly associated with both endoscopic and radiologic indices of activity in CD patients, and with radiologic indices of activity in UC. Interestingly, a serum procalcitonin value > 0.14 ng/mL was found to have optimal diagnostic sensitivity (*i.e.*, 1.00), combined with remarkable diagnostic specificity (*i.e.*, 0.96), for identifying CD patients with more severe disease (AUC, 0.963; $P < 0.001$). However, its diagnostic accuracy was apparently inadequate for identifying UC patients with more severe disease (AUC, 0.736; $P = 0.08$).

Koido *et al.*^[26] analyzed serum procalcitonin concentrations in 11 healthy volunteers and 18 patients

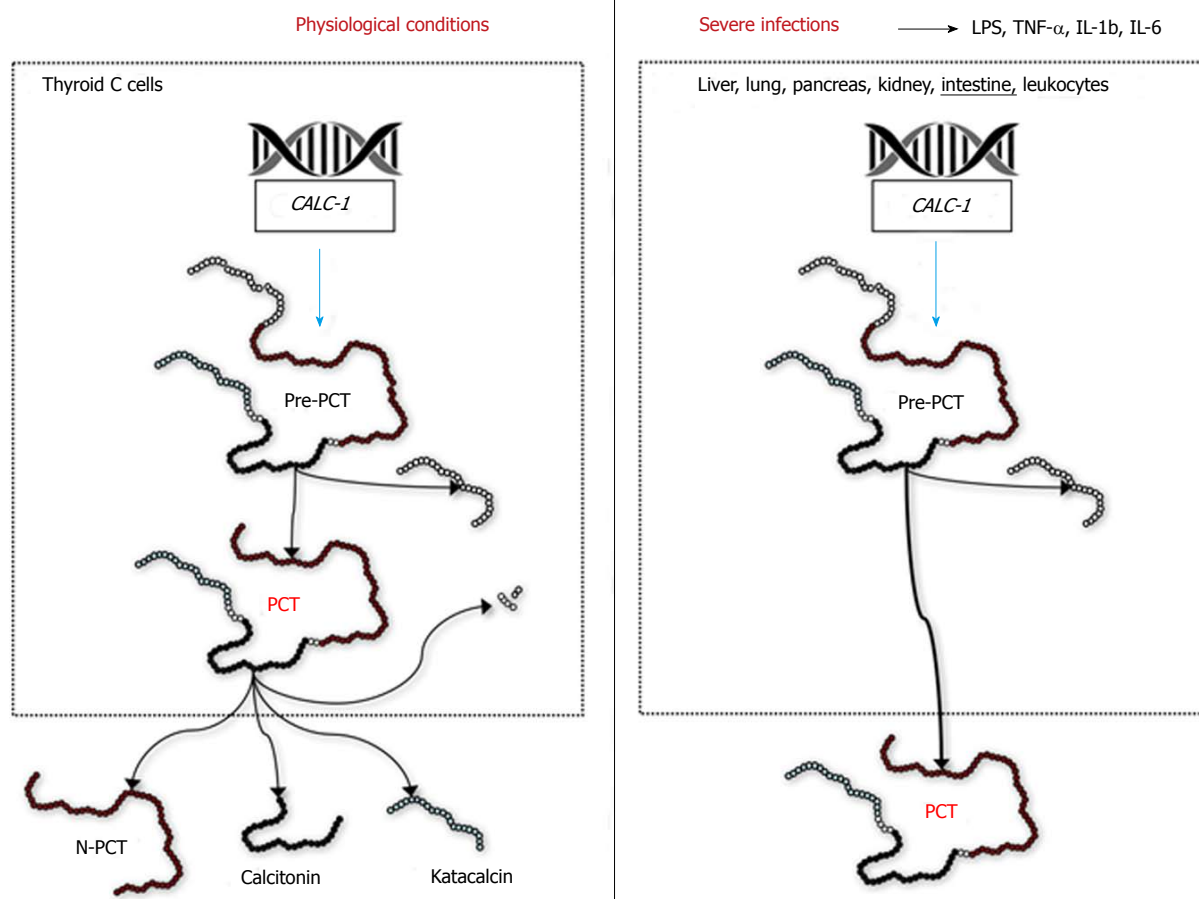


Figure 2 Biology of procalcitonin in normal and infective conditions. CALC-1: Calcitonin-related polypeptide gene 1; LPC: Lipopolysaccharide; IL-1b: Interleukin-1b; IL-6: Interleukin-6; N-PCT: N-terminal procalcitonin; pre-PCT: Pre-procalcitonin; PCT: Procalcitonin; TNF- α : Tumor necrosis factor- α .

with UC. Disease severity was assessed according to Mayo endoscopic subscore and Truelove and Witts' severity index. Interestingly, serum procalcitonin values were significantly higher in patients with severe UC (0.096 ng/mL) than in those with mild-to-moderate UC (0.033 ng/mL; $P < 0.001$) or in healthy controls (0.035 ng/mL; $P < 0.001$). No difference was found between patients with mild-to-moderate UC and healthy controls ($P = 0.311$). Notably, a procalcitonin value > 0.055 ng/mL displayed 1.00 sensitivity and 1.00 specificity for identification of severe UC.

Chung *et al.*^[27] performed a retrospective study including 58 patients with IBD (38 with CD, 20 with UC) and 71 with intestinal Behçet's disease. Interestingly, procalcitonin values were not different in patients with active/inactive CD (0.11 ng/mL vs 0.07 ng/mL, $P = 0.521$) nor in patients with active/inactive UC (0.15 ng/mL vs 0.05 ng/mL, $P = 0.553$). Nonetheless, the procalcitonin values progressively increased as follows: patients with no infection (0.07 ng/mL), with localized bacterial infection (0.22 ng/mL), and with septic shock or sepsis (3.46 ng/mL; $P = 0.001$). Overall, procalcitonin displayed 0.83 positive predictive value and 0.84 negative predictive value (AUC, 0.636; $P < 0.01$) for predicting the infection status.

In a subsequent study, Ge *et al.*^[28] studied 80 patients with CD, 16 of whom developed an intra-abdominal abscess. The serum concentration of procalcitonin was found to be higher in CD patients with an intraabdominal abscess than in those without (0.505 ng/mL vs 0.112 ng/mL, $P < 0.01$). A diagnostic threshold of 0.35 ng/mL for procalcitonin displayed 0.81 sensitivity and 0.97 specificity (AUC, 0.954; $P < 0.001$) for differentiating patients with or without intraabdominal abscess. A significant correlation was also observed between CDAI score and serum procalcitonin value ($r = 0.575$; $P < 0.001$).

Nishio *et al.*^[29] studied 55 IBD patients (18 with CD, 37 with UC), showing that serum procalcitonin values were significantly correlated with active disease expressed as CDAI index in CD ($r = 0.7$; $P < 0.001$), but not with active disease expressed as Mayo score in UC ($r = -0.2$; $P = \text{ns}$). In particular, patients with severe active to fulminant CD had serum procalcitonin values approximately 3-fold higher than those with non-severe active CD (0.14 ng/mL vs 0.04 ng/mL, $P < 0.001$).

More recently, Hosomi *et al.*^[30] measured serum procalcitonin values in 101 patients with IBD (33 with CD, 68 with UC). No significant correlation was observed between serum procalcitonin values and

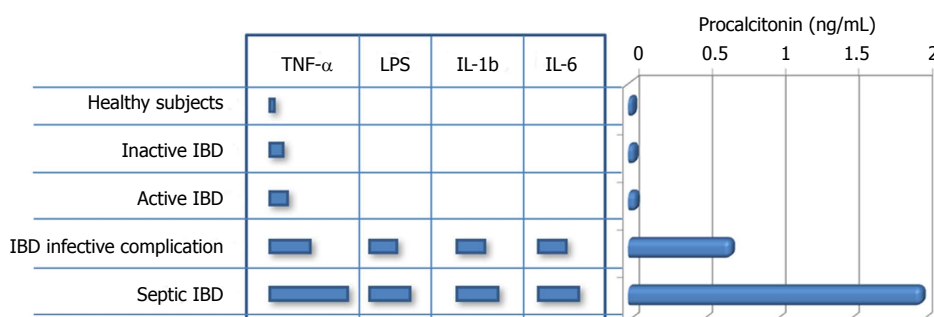


Figure 3 Procalcitonin in inflammatory bowel disease. CALC-1: Calcitonin-related polypeptide gene 1; LPC: Lipopolysaccharide; IL-1b: Interleukin-1b; IL-6: Interleukin-6; N-PCT: N-terminal procalcitonin; pre-PCT: Pre-procalcitonin; PCT: Procalcitonin; TNF- α : Tumor necrosis factor- α .

disease extension, location, perianal involvement, partial Mayo score, and Mayo endoscopic subscore, whilst a weak correlation was observed with Harvey-Bradshaw index ($r = 0.353$; $P = 0.044$). In both groups of IBD patients, serum procalcitonin value was not associated with complete mucosal healing and complete clinical remission. Accordingly, the sensitivity and specificity of serum procalcitonin for predicting complete mucosal healing was poor, being 0.86 and 0.35 in CD (cutoff, 0.04 ng/mL; AUC, 0.49), 0.60 and 0.53 in UC (cutoff, 0.03 ng/mL; AUC, 0.57), respectively. The sensitivity and specificity of serum procalcitonin for predicting complete clinical remission was even poorer, being 0.57 and 0.30 in CD (cutoff, 0.03 ng/mL; AUC, 0.35), and 0.21 and 0.46 in UC (cutoff, 0.03 ng/mL; AUC, 0.43), respectively.

Finally, Zielińska-Borkowska *et al.*^[31] carried out an observational study including 154 patients undergoing major elective colorectal surgery for cancer ($n = 95$), IBD ($n = 38$), and other conditions ($n = 21$). Overall, 16 patients (10%) developed postsurgical infections due to anastomotic leakage, in whom the frequency of serum procalcitonin concentration > 0.5 ng/mL was significantly higher than those who did not develop complications (31% vs 4%, $P < 0.001$). A serum procalcitonin value > 1.09 ng/mL displayed 0.87 sensitivity and 0.87 specificity for predicting postsurgical infection (AUC, 0.88; $P < 0.01$).

Taken together, the available published studies suggest that procalcitonin is probably unwarranted for the diagnosis of IBD and/or assessing disease severity, whilst its measurement in patients with suspected infections may enable a timely diagnosis as well as an effective therapeutic monitoring of infective complications in IBD.

BIOLOGICAL ROLE OF PROCALCITONIN IN IBD

The diagnostic role of procalcitonin for predicting bacterial complications in IBD is supported by reliable biological evidence. It has now been clearly established that bacterial endotoxin and a wide range of cytokines

[especially interleukin-1b (IL-1b), interleukin-6 (IL-6) and tumor necrosis factor- α (TNF- α)] are actively released in response to both systemic and localized bacterial infections. This process strongly interplays with *CALC1*, eliciting abundant extra-thyroid synthesis of pre-procalcitonin, which is in turn rapidly converted to procalcitonin (Figure 2). This extra-thyroid production is magnified in patients with Gram-negative infections, which are associated with the highest circulating values of TNF- α ^[32].

Although the precise mechanism is still unclear, the ensuing PC-mediated cleavage of procalcitonin does not occur efficiently in patients with severe infections, finally contributing to further increase in the circulating concentration of this biomarker^[33]. Therefore, it is not surprising that procalcitonin values may be increased in IBD patients with bacterial complications, whilst its concentration remains virtually unchanged in those without infections, irrespective of disease severity (Figure 3). Albeit a modest increase of TNF- α can be frequently observed in patients with IBD (especially in those with CD), which in turn may explain the occasional association with increased values of serum procalcitonin in IBD patients with more active disease (Table 1), its effect on the pathogenesis of IBD seems mostly mediated by altered expression of TNF receptors^[34]. On the other hand, TNF- α is more strongly up-regulated by a number of proinflammatory factors, such as endotoxin and other bacterial products. This fact would lead to substantial enhancement of intestinal procalcitonin synthesis and also explains its potential clinical usefulness for predicting bacterial complications in IBD (Figure 3).

CONCLUSION

Recent data attest that the rate of *Clostridium difficile* infection is constantly increasing and is now responsible for a remarkable number of IBD hospitalizations^[35]. Unfortunately, the diagnosis of local infective complications is challenging in patients with IBD, since the symptoms are nonspecific or often overlap with those of the underlying pathology. The suggestive

Table 1 Procalcitonin in inflammatory bowel disease

Ref.	Type of study	Study population	PCT		
			IBD <i>vs</i> HCs	Active <i>vs</i> inactive IBD	Predicting complications
Korczowski <i>et al</i> ^[21] , 2003	Cross-sectional	129 children with diarrhea and 30 HCs	Non significantly different	Non assessed	PCT predicted bacterial enterocolitis
Herrlinger <i>et al</i> ^[22] , 2004	Cross-sectional	51 IBD patients (26 with CD and 25 with UC) and 25 patients with self-limited enterocolitis	Nonassessed	PCT ~40% higher in patients with active disease	Nonassessed
Thia <i>et al</i> ^[23] , 2008	Cross-sectional	71 IBD patients (27 with CD and 44 with UC) and 81 with bacterial gastroenteritis	Nonassessed	PCT non significantly higher in patients with active disease	PCT predicted bacterial gastroenteritis
Oruç <i>et al</i> ^[24] , 2009	Cross-sectional	45 patients with IBD (9 with CD and 36 with UC) and 50 HCs	PCT higher in CD (but not in UC) than in HCs	PCT nonsignificantly higher in patients with active disease	Nonassessed
Oussalah <i>et al</i> ^[25] , 2010	Prospective observational	57 IBD patients (30 with CD and 27 with UC)	Nonassessed	PCT ~40% higher in patients with active disease; PCT predicted disease severity in CD but not in UC	Nonassessed
Koido <i>et al</i> ^[26] , 2013	Cross-sectional	18 patients with UC and 11 HCs	Nonsignificantly different between inactive UC and HCs, higher in active UC than in HCs	PCT ~3-fold higher in patients with active disease	Nonassessed
Chung <i>et al</i> ^[27] , 2016	Cross-sectional	58 IBD patients (38 with CD and 20 with UC)	Nonassessed	PCT nonsignificantly higher in patients with active disease	PCT predicted bacterial infection and sepsis
Ge <i>et al</i> ^[28] , 2016	Cross-sectional	80 CD patients (16 with intraabdominal abscess)	Nonassessed	PCT nonsignificantly higher in patients with active disease	PCT predicted intraabdominal abscess
Nishio <i>et al</i> ^[29] , 2016	Cross-sectional	55 IBD patients (18 with CD and 37 with UC)	Nonassessed	PCT ~3-fold higher in patients with active CD, but not in those with active UC	Nonassessed
Hosomi <i>et al</i> ^[30] , 2017	Cross-sectional	101 IBD patients (33 with CD and 68 with UC).	Nonassessed	PCT nonsignificantly higher in patients with active disease	Nonassessed
Zielińska-Borkowska <i>et al</i> ^[31] , 2017	Observational	154 patients undergoing major elective colorectal surgery (38 with IBD)	Nonassessed	Nonassessed	PCT predicted postsurgical infection

CD: Crohn's disease; HCs: Healthy controls; IBD: Intestinal bowel disease; PCT: Procalcitonin; UC: Ulcerative colitis.

endoscopic findings (*e.g.*, pseudomembranous exudates in *Clostridium difficile* infection) are lacking in the vast majority of IBD patients, whilst stool culture is characterized by long turn-around time (usually around 48 h), high cost, and considerably low specificity^[36]. Therefore, the availability of alternative diagnostic biomarkers may be seen as a valuable perspective to follow-up of IBD patients.

According to current evidence in the scientific literature, the clinical significance of measuring procalcitonin for diagnosing and monitoring IBD disease is rather elusive, and its association with disease severity is still confined to a limited number of studies (Table 1). Nevertheless, though procalcitonin values do not seemingly provide clinically useful information as serological marker of disease activity and inflammatory status, literature data suggest that supranormal procalcitonin concentrations may reflect the presence of a number of infective complications in IBD, thus

including bacterial enterocolitis, bacterial gastroenteritis, intraabdominal abscess, postsurgical infection and sepsis (Table 1). To conclude, the measurement of this biomarker may retain clinical significance for predicting or timely diagnosing many IBD-associated infections and complications rather than for assessing the disease activity.

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Basic Study

Gene mutations in stool from gastric and colorectal neoplasia patients by next-generation sequencing

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Abstract

AIM

To study cancer hotspot mutations by next-generation sequencing (NGS) in stool DNA from patients with different gastrointestinal tract (GIT) neoplasms.

METHODS

Stool samples were collected from 87 Finnish patients diagnosed with various gastric and colorectal neoplasms, including benign tumors, and from 14 healthy controls. DNA was isolated from stools by using

the PSP® Spin Stool DNA Plus Kit. For each sample, 20 ng of DNA was used to construct sequencing libraries using the Ion AmpliSeq Cancer Hotspot Panel v2 or Ion AmpliSeq Colon and Lung Cancer panel v2. Sequencing was performed on Ion PGM. Torrent Suite Software v.5.2.2 was used for variant calling and data analysis.

RESULTS

NGS was successful in assaying 72 GIT samples and 13 healthy controls, with success rates of the assay being 78% for stomach neoplasia and 87% for colorectal tumors. In stool specimens from patients with gastric neoplasia, five hotspot mutations were found in *APC*, *CDKN2A* and *EGFR* genes, in addition to seven novel mutations. From colorectal patients, 20 mutations were detected in *AKT1*, *APC*, *ERBB2*, *FBXW7*, *KIT*, *KRAS*, *NRAS*, *SMARCB1*, *SMO*, *STK11* and *TP53*. Healthy controls did not exhibit any hotspot mutations, except for two novel ones. *APC* and *TP53* were the most frequently mutated genes in colorectal neoplasms, with five mutations, followed by *KRAS* with two mutations. *APC* was the most commonly mutated gene in stools of patients with premalignant/benign GIT lesions.

CONCLUSION

Our results show that in addition to colorectal neoplasms, mutations can also be assayed from stool specimens of patients with gastric neoplasms.

Key words: Stool DNA; Next-generation sequencing; Mutations; Gastric neoplasia; Colorectal neoplasia

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Core tip: Next-generation sequencing (NGS) was successfully applied for detecting cancer gene mutations in stool DNA of patients with different gastrointestinal neoplasms. Using a gene panel, comprising up to 50 cancer genes, it was found that mutations not only could be detected in stool DNA from colorectal cancer patients but also in patients with stomach cancer and those with benign or premalignant lesions. No hotspot mutations were detected in healthy controls. Our results show that NGS could be useful in screening for neoplastic changes of the gastrointestinal tract.

Youssef O, Sarhadi V, Ehsan H, Böhling T, Carpelan-Holmström M, Koskensalo S, Puolakkainen P, Kokkola A, Knuutila S. Gene mutations in stool from gastric and colorectal neoplasia patients by next-generation sequencing. *World J Gastroenterol* 2017; 23(47): 8291-8299 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v23/i47/8291.htm> DOI: <http://dx.doi.org/10.3748/wjg.v23.i47.8291>

INTRODUCTION

Gastrointestinal tract (GIT) malignancies are a diverse

group of neoplasms with diverse epidemiology and incidences that affect different regions of the GIT, from the stomach to the large intestine. The value of somatic mutations in GIT malignancies is recognized, (1) as markers for early detection; (2) as markers that predict drug resistance, and (3) for follow up of cancer treatment^[1-6]. In global terms, gastric carcinoma is the fifth most common cancer and the third most common cause of cancer-related mortality. Adenocarcinoma is its most typical subtype and present in 90% of all cases^[7]. *TP53*, *PIK3CA*, *ARID1A* and cell adhesion pathway genes have been found to be the most frequently mutated genes in gastric adenocarcinomas^[8]. The *CDH1* gene is described as being involved in the pathogenesis of diffuse gastric carcinoma^[9]. In sporadic colorectal cancer (CRC), *APC*, *TP53*, *KRAS*, *PI3CA*, *FBXW7*, *SMAD4* and *BRAF* are the most commonly mutated genes^[10].

One of the major issues in GIT malignancies, specifically gastric carcinoma, is that they are usually detected at an advanced stage, due to late diagnosis^[11]. Moreover, recent studies have demonstrated the diversity of morphological (intestinal and diffuse subtypes) and molecular subtypes (mesenchymal-like type, microsatellite-unstable tumor type, and *TP53* tumor type) of gastric carcinoma, which contributes to the challenge of optimizing proper diagnosis and treatment^[12]. The principal problem hindering early detection of gastric and colorectal neoplasia is the lack of symptoms; even when symptoms are present, they tend to be mild and nonspecific, which may delay subjecting the patient for endoscopic examination. Exfoliation of cells, whether premalignant or malignant, is continuously occurring from epithelial layer into the digestive lumen^[13,14]; these display various genetic changes that have occurred in these cells, and can provide evidence of tumor pathogenesis^[15]. Testing DNA abnormalities in stool specimens from GIT carcinoma patients represents a promising noninvasive approach for early cancer detection and for treatment follow-up. Multi-target stool DNA test is currently being used for CRC screening^[16].

We have previously shown that next-generation sequencing (NGS) can be successfully applied for investigating mutations in stool DNA obtained from patients with CRC^[13]. In the current study, we applied the NGS method to determine whether cancer mutations could also be detected in stool samples from patients with other GIT tumors, including both diffuse and intestinal subtypes of gastric adenocarcinoma, gastric dysplasia, colorectal adenocarcinoma and adenoma, and colorectal leiomyoma.

MATERIALS AND METHODS

Patients

During the period from April 2015 to May 2017, 79 gastric cancer, 38 gastrointestinal stromal tumor

Table 1 Summary of stool samples collected and analyzed

Tumor location	Total collected samples	Successful DNA extraction	Successful sequencing	Number of cases with only known mutations	Number of cases with Mutations, all
Stomach	41	35	32 (78)	5 (15.6)	8 (25)
Carcinoma	38	32	29	3	6
Intestinal type	18	17	15	1	1
Diffuse type	20	15	14	2	5
Benign	3	3	3	2	2
Hamartoma	1	1	1	0	0
Dysplasia	1	1	1	1	1
NET	1	1	1	1	1
Colorectal	46	42	40 (87)	12 (30)	12 (30)
Carcinoma	40	37	35	9	9
Benign	6	5	5	3	3
Adenoma	4	3	3	2	2
Dysplasia	1	1	1	0	0
Leiomyoma	1	1	1	1	1
Healthy controls	14	13	13 (92.9)	0	2 (15)
Total	101	90	85	17	22

Data are presented as *n* or *n* (%). NET: Neuroendocrine tumor.

(GIST), 669 colon cancer and 271 primary rectal cancer patients were referred to three hospitals in Finland: Kirurgi, Meilahti and Jorvi. Three authors (Kokkola A, Carpelan-Holmström M and Koskensalo S) collected stool samples from patients who were referred to them for surgery. Stool specimens were collected from 87 patients with stomach or colorectal neoplasia, representing 41 stomach neoplasia (18 intestinal type, 20 diffuse type, 1 neuroendocrine tumor, 1 gastric dysplasia and 1 hamartoma) and 46 colorectal lesions (40 adenocarcinoma, 4 adenoma, 1 dysplasia and 1 colorectal leiomyoma), as well as from 14 healthy individuals (Table 1). A total number of 21 patients had received treatment before the time of sampling (10 patients with stomach neoplasia and 11 with colorectal lesions). Treatments were in the form of chemotherapy (17 patients), radiotherapy (1 patient), or antibiotics for *Helicobacter pylori* infection (3 patients).

Patients were diagnosed and treated in Meilahti Hospital in Helsinki. The study was approved by the Hospital District of Helsinki and Uusimaa (HUS) Review Board (Ethical permission number 351/13/03/02/2014). Written informed consent was obtained from all subjects.

Stool specimens

Stool specimens were collected in special collection tubes provided in the extraction kit (PSP® Spin Stool DNA Plus Kit; Stratec Biomedical, Berlin, Germany). These tubes are prefilled with 8 mL of stool DNA stabilizer to allow collection, transport and storage of the samples without DNA degradation. One spoon of stool specimen (spoon provided with the collection tubes) was transferred to the tube and mixed thoroughly to obtain a stool homogenate. The samples

were stored at -20 °C until analysis, for an average of 7 d before extraction.

DNA extraction

Before starting the DNA isolation, each stool specimen tube was vortexed vigorously to ensure proper mixing of the contents with the stabilizer liquid provided in each collection tube. A volume of 1.4 mL of the stabilized stool specimens was transferred to 2 mL tubes. Then, DNA was extracted from each stool specimen using the PSP® Spin Stool DNA Plus Kit (Stratec Biomedical) according to the manufacturer's instructions. Extracted DNA was eluted in 50 µL of elution buffer, and then DNA was quantified by a Qubit® 2.0 Fluorometer (Life Technologies, Carlsbad, CA, United States) using the Qubit® dsDNA BR Assay Kit. The extracted DNA was stored at -20 °C.

NGS

Library preparation: Twenty nanograms of stool DNA was used for preparing amplicon libraries using Ion AmpliSeq™ Library Kit 2.0 (Life Technologies) according to the manufacturer's guidelines. Gene panels comprising pools of primer mixes were used to amplify templates. The gene panels used were one of the following: (1) Ion AmpliSeq Cancer Hotspot Panel v2 (Life Technologies), consisting of a primer pool for 207 amplicons from an average of 2800 mutational hotspot regions in 50 genes, including *KIT* and *PDGFRA* mutations. The genes included in the panel are *ABL1*, *AKT1*, *ALK*, *APC*, *ATM*, *BRAF*, *CDH1*, *CDKN2A*, *CSF1R*, *CTNNB1*, *EGFR*, *ERBB2*, *ERBB4*, *EZH2*, *FBXW7*, *FGFR1*, *FGFR2*, *FGFR3*, *FLT3*, *GNA11*, *GNAS*, *GNAQ*, *HNF1A*, *HRAS*, *IDH1*, *JAK2*, *JAK3*, *IDH2*, *KDR*, *KIT*, *KRAS*, *MET*, *MLH1*, *MPL*, *NOTCH1*, *NMP1*, *NRAS*, *PDGFRA*, *PIK3CA*, *PTEN*, *PTPN11*, *RB1*, *RET*, *SMAD4*, *SMARCB1*,

SMO, *SRC*, *STK11*, *TP53*, and *VHL*; (2) Ion AmpliSeq Colon and Lung Cancer panel v2 (Life Technologies), consisting of a primer pool for 92 amplicons from 504 hotspot regions in 22 genes frequently mutated in CRC. The genes included in this panel are *AKT1*, *ALK*, *BRAF*, *CTNNB1*, *DDR2*, *EGFR*, *ERBB2*, *ERBB4*, *FBX7*, *FGFR1*, *FGFR2*, *FGFR3*, *KRAS*, *MAP2K1*, *MET*, *NOTCH1*, *NRAS*, *PIK3CA*, *PTEN*, *SMAD4*, *STK11*, and *TP53*.

All specimens from patients with gastric neoplasms and 19 specimens from patients with colorectal neoplasia were assayed using Ion AmpliSeq Cancer Hotspot Panel v2, while the remaining specimens from colorectal neoplasia patients were studied by Ion AmpliSeq Colon and Lung Cancer panel v2. The major reason for using two different platforms was that Ion AmpliSeq Cancer Hotspot Panel v2 contains several genes that are commonly mutated in gastric neoplasia.

The amplified libraries were purified using Agencourt AMPure XP beads (Beckman Coulter Genomics, High Wycombe, United Kingdom). The concentration of the purified libraries was measured on the Qubit® 2.0 Fluorometer, using the Qubit® dsDNA HS Assay Kit. The DNA libraries were stored at -20 °C until further use.

Template preparation and sequencing: The amplified and purified libraries were diluted to 100 pmol/L, and the templates were prepared and enriched using the Ion OneTouch™ 2 System (Life Technologies), an automated emulsion PCR system. Finally, sequencing was carried out on the Ion Personal Genome Machine System (PGM™; Life Technologies) using Ion 316™ Chips and the Ion PGM™ Sequencing Hi-Q Kit v2.

Data analysis

The Torrent Suite Software v.5.2.2 (Life Technologies) was used to assess run performance and data analysis, and Integrative Genomics Viewer (IGV v 2.2; Broad Institute, Cambridge, MA, United States) was used for visual inspection of the aligned reads.

Variants were further filtered based on quality score (score of 15 or more) and mutant allele frequency (more than 3%). Only single nucleotide variants (SNVs) resulting in a nonsynonymous amino acid change, or a premature stop codon, and all short indels resulting in either a frameshift or insertion/deletion of amino acids were selected. All SNVs were analyzed for previously reported hotspot mutations (somatic mutations reported in the COSMIC database) and novel variations, *i.e.* new mutations detected by NGS but not reported in either COSMIC or dbSNP (build 150) databases.

RESULTS

Success rate

Successful DNA extraction was performed on 77/87

patient stool samples, while NGS assay was successfully carried out on 72 patient stool DNA samples. Five samples were removed from NGS assay due to poor DNA quality (too little or degraded DNA). Of the 14 controls, DNA could be isolated from 13 samples and all were successfully sequenced. The success rates of sequencing stool samples for stomach and colorectal neoplasia were rather similar (78% and 87% respectively) (Table 1).

Hotspot (COSMIC) and novel mutations

In patients' stool samples, a total of 25 hotspot mutations were found (20 in patients with colorectal neoplasia and 5 in patients with stomach neoplasia), while 9 novel mutations were detected (7 in patients with stomach neoplasia and 2 in control samples).

Thirteen control samples from healthy individuals did not reveal any hotspot mutations, but two novel mutations were observed in *ALK*, and *STK11* genes in two subjects.

Mutations in patients with stomach neoplasms

A total number of five hotspot mutations that had been reported earlier in the COSMIC database were detected in *APC*, *CDKN2A* and *EGFR* genes in stool specimens from 3 gastric adenocarcinoma patients, 1 patient with neuroendocrine tumor, and 1 patient with gastric dysplasia (Table 2). Four samples from patients with adenocarcinoma (diffuse type) revealed a total of seven novel mutations that led to an amino acid change and which had not been reported previously in either the COSMIC or dbSNP databases. The detected novel mutations were found in seven genes, that included *APC*, *CDH1*, *DDR2*, *HRAS*, *NRAS*, *PTEN*, and *SMARCB1* (Table 2).

Mutations in patients with colorectal tumors

Twenty hotspot mutations in *AKT1*, *APC*, *ERBB2*, *FBXW7*, *KIT*, *KRAS*, *NRAS*, *SMARCB1*, *SMO*, *STK11*, and *TP53* were seen in 9 patients with adenocarcinomas, 2 with benign adenoma, and 1 with leiomyoma. *APC* was the most frequently mutated gene with five mutations, followed by *TP53* (five mutations), and *KRAS* (two mutations) (Table 2). One case of benign leiomyoma revealed a *TP53* mutation (R306*) mutation, which had been reported as a germline mutation associated with the hereditary cancer predisposing syndrome. Additionally, this mutation has also been reported as a somatic pathogenic mutation in COSMIC in tumors of the colon and other parts of the digestive tract (Table 2). No novel mutations were found in our study in stool samples from colorectal cases.

DISCUSSION

We are one of the first groups that applied NGS to

Table 2 COSMIC and novel mutations detected by Ion Torrent sequencing

Histopathology	Cases ID	Age, yr	Sex	TNM staging	Gene	Mutation type	COSMIC No.
Stomach							
AC (D)	3	66	F	T2N0	<i>SMARCB1</i>	p.T72K	Novel
AC (D)	14	80	F	T4aNxM1	<i>APC</i>	p.D1570N	Novel
					<i>CDH1</i>	p.V82A	Novel
					<i>HRAS</i>	p.V44M	Novel
					<i>NRAS</i>	p.K135R	Novel
AC (D)	39	65	F	T1aN0M0	<i>EGFR</i>	p.A750T	COSM1651572
					<i>DDR2</i>	p.E523K	Novel
AC (D)	43	43	M	T4bNxM1	<i>PTEN</i>	p.E256G	Novel
AC (D)	77	78	M	TxNxM1	<i>APC</i>	p.A1582P	COSM4170230
AC (I)	100	69	M	T3N3M1	<i>APC</i>	p.A1582P	COSM4170230
NET	11	67	M	5% PR	<i>CDKN2A</i>	p.V126I	COSM13778
Dysplasia	78	77	M	T0N0M0	<i>APC</i>	p.A1582P	COSM4170230
Colorectal							
AC	20	70	M	T3N0M0	<i>KIT</i>	p.N564S	COSM30732
AC	12	71	F	T3N1bM0	<i>APC</i>	p.A1582P	COSM4170230
					<i>TP53</i>	p.P72A	COSM3738520
AC	21	79	M	T2N0	<i>APC</i>	p.A1582P	COSM4170230
AC	22	64	F	T2N0	<i>STK11</i>	p.F354L	COSM21360, COSM4169323
					<i>SMO</i>	p.N202S	COSM5979442
AC	23	53	M	TxNxM1	<i>APC</i>	p.A1582P	COSM4170230
AC	31	64	M	T2N0	<i>TP53</i>	p.Y205D	COSM43844
AC	55	69	M	T3N0M0	<i>KRAS</i>	p.G12V	COSM520
					<i>TP53</i>	p.T172fs	COSM44371
AC	68	63	M	T3N0M0	<i>TP53</i>	p.Y163H	COSM43846
AC	28	77	F	T1N0	<i>KRAS</i>	p.G12D	COSM521
					<i>AKT1</i>	p.E17K	COSM34142, COSM33765
Adenoma	24	81	M		<i>APC</i>	p.E1295*	COSM18961
Adenoma	19	64	F		<i>NRAS</i>	p.Q61R	COSM584, COSM28048
					<i>FBXW7</i>	p.R505C	COSM22975, COSM33844
					<i>APC</i>	p.S1465fs	COSM18873, COSM19688, COSM19332, COSM18931, COSM13864
					<i>ERBB2</i>	p.V842I	COSM14065, COSM1666633
					<i>SMARCB1</i>	p.R377C	COSM3972885
Leiomyoma	94	69	F		<i>TP53</i>	p.R306*	COSM10663, COSM145026
Healthy controls							
	C 2	F	27		<i>ALK</i>	p.L1190P	Novel
	C 8	F	33		<i>STK11</i>	p.Y36H	Novel

AC: Adenocarcinoma; D: Diffuse type; I: Intestinal type; NET: Neuroendocrine tumor; PR: Proliferative rate.

detect mutations in DNA isolated from stool samples of colorectal carcinoma patients^[13]. We have now applied NGS analysis on stool samples of not only malignant colorectal carcinoma but also demonstrated that it is possible to detect mutations in stool specimens from patients with gastric neoplasms, and also from patients with benign colorectal tumors. Moreover, we observed mutations in stool from patients with early tumor stages, with no hotspot mutations in stools of healthy subjects. As in our previous study, we set the threshold for variant quality score at 15 and the mutant allele frequency cutoff at 3%, and when using these threshold values, no COSMIC hotspot mutations were found in the 13 control specimens.

Mutations in patients with colorectal neoplasms

The overall success rate of NGS for colorectal neoplasm patients was 87%, which is similar to the 80% reported in our earlier study^[13]. In our previous

study, the patients were of Iranian origin, whereas in the current study, the patients were from Finland; nonetheless, the mutation types were similar in both of these ethnic groups. In the current series, the most common mutated genes were *APC*, *TP53* and *KRAS*, while in the earlier study, the top mutated genes were *TP53*, *KRAS*, *FBXW7*, *EGFR* and *SMAD4*.

The most recurrently occurring mutation in colorectal carcinoma cases was *APC* mutation (A1582P), which was found in 3 patients. In our series, four *TP53* mutations were seen in stool samples from CRC patients.

We detected *KRAS* codon 12 mutations (G12V and G12D) in two specimens and *NRAS* codon 61 (Q61R) in one specimen from colorectal carcinoma patients. Similar to our present results, codon 12 mutations were the most common *KRAS* mutations found in our previous study on Iranian samples, although other *KRAS* mutations at codons 12, 13, 20, 63, 117, 146 and 43 were also found previously^[13]. Additionally,

a recent study demonstrated the detection of *KRAS* G12D mutation in stool samples from patients with colorectal carcinoma by using droplet PCR^[17]. Clinical data available from those patients for who *KRAS* testing in tumor tissue was carried out correlated to *KRAS* mutation status in stool. In patient number 55, the presence of *KRAS* G12V mutation was confirmed in tumor tissue specimens with 20% mutant allele fraction. The same mutation (*KRAS* G12V) was detected in the stool DNA from the same patient with 13% mutant allele fraction. Moreover, tissue samples from patient number 23 revealed no *KRAS* or *NRAS* mutations, and the same negative findings for those two mutations were also observed in the stool DNA specimen from this case.

Among the patients with benign colorectal tumors, *APC* mutations were most common and found in two samples with colorectal benign adenoma. Adenomas with *APC* mutations have been reported more likely to progress into large adenomas and invasive carcinomas^[18,19]. Inactivation of the *APC* gene and the subsequent activation of Wnt signaling pathway are key factors in the initiation of tumorigenesis of CRC^[20,21]. The R505C mutation in *FBXW7* seen in a colorectal adenoma patient in the present study was also reported in our previous study in a colorectal carcinoma patient^[13].

TP53 is another gene commonly mutated in CRC, and plays a crucial role in the adenoma to carcinoma transition during carcinogenesis, and may have an impact on cancer prognosis^[22]. A patient with leiomyoma revealed a nonsense *TP53* mutation (R306*), which has been reported as a somatic mutation in colon tumors and also considered as a germline mutation associated with hereditary cancer-predisposing syndrome and Li-Fraumeni syndrome in CRC, although not in gastric carcinoma^[23]. In our case, this *TP53* mutation is apparently somatic, as the allele fraction was 5.5%. A meta-analysis of studies carried out on stool DNA testing has shown an overall sensitivity of 68% and 93% specificity in the diagnosis of advanced colorectal adenoma^[24].

Mutations in patients with stomach neoplasms

As far as we aware, this is the first study to have utilized stool samples from patients with stomach neoplasia. Eight out of 32 patients' samples (25%) with stomach neoplasia revealed 12 mutations (both hotspot COSMIC and novel).

In gastric neoplasia, the *APC* gene mutations were those most frequently encountered. Four *APC* mutations were detected in patients with gastric neoplasia (three mutations in gastric carcinoma, and one in benign gastric dysplasia). *APC* is a tumor suppressor gene that has a key role in several molecular processes, such as suppression of canonical

Wnt signaling^[25], and the presence of *APC* mutations have been demonstrated in gastric adenocarcinoma samples^[26,27]. *APC* gene mutations have been reported in both intestinal and diffuse types of gastric carcinoma with a higher frequency in the intestinal subtype of the disease^[28,29]. The adenoma to carcinoma transition pathway has a 20% *APC* mutation in the intestinal type of gastric carcinoma^[30]. In our study, an *APC* A1582P mutation was seen in both the intestinal and diffuse types, and an *APC* D1570N mutation was seen in the diffuse type. Furthermore, we detected the same *APC* mutation (A1582P) in a stool specimen from a patient with benign gastric dysplasia. This is in concordance with an earlier study that identified the presence of *APC* mutations in tumorous tissue in cases with gastric adenomas or flat dysplasia, and also in benign cases associated with adenocarcinoma^[5,31].

In the diffuse type of gastric carcinoma, *CDH1* is reported to be commonly mutated^[32], and *CDH1* germline mutations have also been reported to play an important role in diffuse gastric carcinoma development^[33]. *EGFR* mutations are also commonly encountered in the diffuse subtype^[34-36] of gastric neoplasia, although their role is still controversial. We found the E-cadherin gene (*CDH1*) V82A mutation in a diffuse gastric carcinoma patient, and also found the exon 19 *EGFR* (A750T) mutation in another case with diffuse gastric carcinoma.

Novel mutations were found in *NRAS* (K135R), *DDR2* (E523K) and in exon 7 of *PTEN* (E256). Codon 12 or 13 *NRAS* mutations in tumor tissues have been reported to be associated with a poor prognosis in metastatic stomach carcinoma^[37,38], whereas *DDR2* expression in gastric tumor tissues has been described to be associated with an increased risk of peritoneal dissemination^[39]. Despite the low frequency of *PTEN* mutations in gastric malignant tumors, they tend to be associated with poorly differentiated gastric carcinoma, TNM staging and resistance effect to chemotherapy^[40,41]. Interestingly, the novel *PTEN* (E256G) mutation seen in our study was found in a gastric cancer case with an advanced tumor stage (T4bNxM1).

In conclusion, our results demonstrate that NGS technology can be applied for detection of gene mutations in stool specimens from not only colorectal cancer patients but also from patients with stomach neoplasms, as well as those with benign tumors of the gastrointestinal tract.

ARTICLE HIGHLIGHTS

Research background

Stool DNA sample is a simple, noninvasive source for studying genetic markers of diagnostic/prognostic or predictive significance in colorectal cancer. The significance of stool DNA testing is, however, not well known for stomach cancers and for benign tumors. Current assays screen individual or few mutations only, and do not cover all important cancer mutations. Amplicon-

based NGS could, thus, provide a sensitive method for DNA testing from stool samples in gastrointestinal (GIT) malignancies.

Research motivation

The main challenge in stool DNA-based genetic testing is that only a small proportion of stool DNA is of human origin, thus requiring a very sensitive test. We, therefore, hypothesized that diagnostic value of stool-based DNA testing could be enhanced by applying sensitive amplicon-based next-generation sequencing (NGS) to stool DNA. With the application of NGS we could screen all important mutations in 50 genes from a small amount of input DNA in a single test.

Research objectives

The objective of the study was to apply NGS for screening hotspot mutations in commonly mutated genes in GIT malignancies from stool DNA. The aim was also to see if mutations could be detected in patients with gastric cancer and in patients with early neoplasms, in addition to those with colorectal cancer.

Research methods

Mutation detection was performed by amplicon-based NGS using the Ion AmpliSeq Cancer Hotspot Panel v2 and Ion AmpliSeq Colon and Lung Cancer panel v2. Template preparation was done using the Ion OneTouch™ 2 System and sequencing was performed on Ion PGM (Thermo-Fisher Scientific). Sequencing data analysis and variant calling was performed by using the Torrent Suite Software v.5.2.2 with variant caller plugin. All single nucleotide variants were analyzed for previously reported hotspot mutations (reported in the COSMIC database) and novel variations, *i.e.* not reported in either COSMIC or dbSNP databases.

Research results

Hotspot mutations in stool DNA were found in *APC*, *CDKN2A* and *EGFR* in patients with stomach neoplasms and in *AKT1*, *APC*, *ERBB2*, *FBXW7*, *KIT*, *KRAS*, *NRAS*, *SMARCB1*, *SMO*, *STK11* and *TP53* in patients with colorectal neoplasia. *APC* was the most commonly mutated gene in stools of patients with premalignant/benign GIT lesions.

Research conclusions

This study demonstrates that NGS-based mutation screening can be successfully applied to stool DNA from patients with GIT neoplasms. In addition to mutation detection in stool DNA from colorectal cancer patients, mutations can also be detected from gastric cancer patients, as well as from patients with premalignant or benign neoplasms.

Mutation testing from stool DNA is mainly carried out for individual gene mutations by PCR-based methods for colorectal cancer screening. Since the amount of DNA of human origin is very low in stool, it was hypothesized that amplicon-based NGS could be highly sensitive and suitable for studying a large number of mutations, which could greatly enhance the diagnostic value of stool DNA testing. The methods used in this study require low input of DNA, can amplify around 200 targeted regions of important cancer genes, and together with the high sensitivity of NGS provide a great advantage over prevailing methods for mutation detection from stool DNA.

This study showed that mutations can also be detected in stool DNA from patients with stomach neoplasms. Detection of mutations in stool DNA of patients with premalignant neoplasm, and also in patients with stage I and II of tumor, demonstrates its application for early detection of GIT neoplasms.

The results of this study could have implication in future NGS-based stool DNA diagnostic tests that could be useful for screening of GIT malignancies and for detection at the premalignant stage. It could also act as a guide in a targeted therapy regimen, and to ease follow up of the treatment.

Research perspectives

Genetic mutations can be detected by amplicon-based NGS in stool DNA from patients with GIT tumors other than colorectal cancer also. Moreover, early neoplastic changes in GIT can also be detected in stool DNA. These results open up possibilities of development of NGS-based stool DNA test. Further testing of this method on a larger number of samples from patients with different GIT malignancies, premalignant lesions and healthy individuals is needed to

fully assess its applicability in cancer diagnostics.

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Basic Study

Polymorphisms in oxidative pathway related genes and susceptibility to inflammatory bowel disease

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Abstract

AIM

To investigate whether common variants in the oxidative pathway genes influence inflammatory bowel disease (IBD) risk among Moroccan patients.

METHODS

The distribution of (TAAA)_n_rs12720460 and (CCTTT)_n_rs3833912 *NOS2A* microsatellite repeats, *HIF-1A*_rs11549467 and *NFKB1*-94ins/delATTG_rs28362491 was analyzed in 507 subjects grouped in 199 IBD and 308 healthy controls. Genotyping was performed with

polymerase chain reaction-fluorescent method and the TaqMan® allelic discrimination technology.

RESULTS

The allele and genotype frequencies of *HIF1A*_rs11549467, *NFKB1*_rs28362491 and *NOS2A*_ (TAAA)_n did not differ significantly between patients and controls. Analysis of *NOS2A*_ (CCTTT)_n markers evidenced differences between patients and healthy controls. A preferential presence of the (CCTTT)₈ ($P = 0.02$; OR = 1.71, 95%CI: 1.07-2.74), (CCTTT)₁₄ ($P = 0.02$; OR = 1.71, 95%CI: 1.06-2.76) alleles in IBD, (CCTTT)₈ ($P = 0.008$; OR = 1.95, 95%CI: 1.17-3.23) in CD and (CCTTT)₇ ($P = 0.009$; OR = 7.61, 95%CI: 1.25-46.08), (CCTTT)₁₁ ($P = 0.05$; OR = 0.51, 95%CI: 0.25-1.01), (CCTTT)₁₄ ($P = 0.02$; OR = 2.05, 95%CI: 1.07-3.94), (CCTTT)₁₅ ($P = 0.01$; OR = 2.25, 95%CI: 1.16-4.35) repeats in UC patients indicated its possible association with higher disease risk which need to be confirmed in a larger sample size.

CONCLUSION

Our results suggest that the *NOS2A*_ (CCTTT)_n gene variations may influence IBD susceptibility in the Moroccan population.

Key words: *HIF1A*; *NFKB1*; *NOS2A*; Inflammatory bowel disease; Moroccan patients

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Core tip: This is the first study to assess the involvement of oxidative pathway related genes in inflammatory bowel disease (IBD) development and to determine a possible effect of these variants on clinical course. We genotyped 507 subjects grouped in 308 healthy controls and 199 IBD patients for the (TAAA)_n_rs12720460 and (CCTTT)_n_rs3833912 *NOS2A* microsatellite repeats, *HIF-1A*_rs11549467 and *NFKB1*-94ins/delATTG_rs28362491 polymorphisms. The present study showed that *NOS2A*_ (CCTTT)_n gene variations may influence IBD susceptibility in the Moroccan population. However, our data do not support a role for the *NFKB1* and *HIF1A* polymorphisms in the pathogenesis of IBD.

Senhaji N, Nadifi S, Zaid Y, Serrano A, Rodriguez DAL, Serbati N, Karkouri M, Badre W, Martin J. Polymorphisms in oxidative pathway related genes and susceptibility to inflammatory bowel disease. *World J Gastroenterol* 2017; 23(47): 8300-8307 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v23/i47/8300.htm> DOI: <http://dx.doi.org/10.3748/wjg.v23.i47.8300>

INTRODUCTION

Inflammatory bowel disease (IBD), a chronic and

relapsing-remitting disorder of the gastrointestinal tract, encompasses Crohn's disease (CD) and ulcerative colitis (UC).

IBD was previously found mainly in Western countries, with higher prevalence reported in developed countries^[1]. However, during the last few decades, incidence rates of the two major forms of IBD have been increasing in developing countries^[2], including Morocco.

Chronic intestinal inflammation is a hallmark of both disorders, and is believed to result from a number of abnormal conditions. The involvement of oxidative damage in IBD development has been thoroughly documented. Oxidative stress mainly contributes to aberrant inflammatory responses of intestinal cells to commensal bacteria and dietary antigens. During IBD, activated leukocytes generate a wide spectrum of proinflammatory cytokines, in addition to excessive oxidative reactions that alter the redox equilibrium within the gut mucosa. Therefore, the capacity to maintain inflammation by induction of transcription factors and redox-sensitive signaling pathways may influence the occurrence and severity of the disease^[3]. Induction of inducible nitric oxide synthase (iNOS) was reported to play a key role in oxidative stress-induced inflammation^[4]. The genetic polymorphisms of the *NOS2A* (nitric oxide synthase) gene have been proposed to be involved in IBD aetiology^[5]. Two functionally relevant polymorphisms located at *NOS2A* gene promoter region were reported, the first one is a highly polymorphic pentanucleotide (CCTTT)_n microsatellite repeat which is important in the regulation of *NOS2A* transcription^[6]. The second one is located at the proximal promoter region and consists of an insertion/deletion of one TAAA repeat^[7].

Perpetuation of inflammation is also mediated by cellular stress responses of inflammatory cells that produce soluble mediators and reactive species which act by further inducing changes in transcription factors, among which hypoxia-inducible factor-1 α (HIF-1 α) and nuclear factor κ B (NF- κ B)^[8]. HIF-1- α is a key regulator of cellular response to hypoxia, the gene encoding the HIF-1 α subunit (HIF1A) carries a common missense mutation, A588T (G>A, rs11549467), that has been related to increased trans-activation capacity^[9]. The involvement of HIF-1- α in the enhancement of the inflammatory response was demonstrated; elevated levels of HIF-1- α in biopsies of primary lesions of patients confirmed its role in inflammatory diseases^[10]. HIF-1- α has been shown to induce the secretion of inflammatory mediators by indirect signaling through NF- κ B-mediated cytokine and chemokine secretion^[11].

NF- κ B is activated during inflammation, giving rise to induction of gene expression of several genes involved in mucosal inflammation such as cytokines (TNFA, IL6, IL1 β ...), Cox-2, and *NOS2A*^[12,13]. A functional *NFKB1* promoter polymorphism consisting of a common (-94ins/delATTG) insertion/deletion,

that seems to affect promoter activity of the *NFKB1* gene and differential nuclear protein binding^[14], was associated with the risk of CD and UC^[14,15].

In search of relevant gene polymorphisms related to oxidative stress signaling that are involved in IBD development, we explored the association of *HIF1A*_rs11549467, *NFKB1*_rs28362491 *NOS2A* (CCTTT)*n*_rs3833912 and *NOS2A* (TAAA)_rs12720460 polymorphisms with IBD (CD and UC) in a Moroccan population.

MATERIALS AND METHODS

Study population

Peripheral blood was obtained from 308 healthy unrelated blood donors. 199 patients diagnosed with IBD at the CHU Ibn Rochd Hospital (Casablanca, Morocco) were included in this study. The diagnosis of CD or UC was established according to conventional endoscopic, clinical, histological and radiological criteria as previously described^[16,17]. CD phenotype was classified according to the Montreal classification^[18]. UC anatomic location was subgrouped using Paris classification^[19]. Patient's clinical and demographic characteristics were collected in a case report form including questions on disease location and phenotype, age at diagnosis and other clinical features.

Ethics statement

The ethics committee of the Faculty of Medicine and Pharmacy of Casablanca approved the study in accordance with the declaration of Helsinki for experiments involving humans, and a written informed consent was obtained from all participants.

DNA analysis

Genomic DNA was extracted from peripheral blood using the salting out procedure and from Formalin Fixed Paraffin Embedded Tissues using the QIAamp® DNA FFPE Tissue Kit (Qiagen). DNA quality and quantity was determined with the NanoDrop™ 1000 Spectrophotometer (Thermo Fisher Scientific, Wilmington, DE) and the QuBit Quantification Platform (Invitrogen, Ltd., Paisley, United Kingdom) using the QuBit high-sensitivity assay reagents.

The *NOS2A* (TAAA)*n*_rs12720460 and (CCTTT)*n*_rs3833912 genotyping was performed using a polymerase chain reaction (PCR)-based method combined with fluorescent technology as previously described^[20]. Forward and reverse primers were. F: 5'-TGC CAC TCC GCT CCA G-3'; R: 5'-GGC CTC TGA GAT GTT GGT CTT-3' for (TAAA)*n*, and F: 5'-ACC CCT GGA AGC CTA CAA CTG CAT-3'; R: 5'-GCC ACT GCACCC TAG CCT GTC TCA-3' for (CCTTT)*n*. The forward primers were 5' labeled with the fluorescent dye 6-Carboxyfluorescein amino hexy FAM. The different alleles were resolved after capillary electrophoresis

on automated DNA sequencer (ABI 3130xl Genetic Analyzer, Applied Biosystems) and analyzed with the GeneMapper® 4.0 software (Applied Biosystems). Selected samples from each genotype were sequenced in order to confirm the length of each allele.

Genotyping of *HIF1A* (G/A) rs11549467 and *NFKB1*-94ins/delATTG (rs28362491) was performed on the Light Cycler 480 System (Roche, Barcelona, Spain) using a pre-designed TaqMan SNP genotyping assays (Applied Biosystems, Foster City, CA, United States) as previously described^[21,22]. PCR was carried out in a total reaction volume of 5 µL with the following amplification protocol: initial denaturation at 95 °C for 3 min followed by 50 cycles of denaturation at 95 °C for 3 s, and annealing/extension at 60 °C for 20 s. The primer sequences of *NFKB1* promoter polymorphism IN/DEL -94ATTG were: F: 5'-GCC TCC GTG CTG CCT-3' and R: 5'-AGG GAA GCC CCC AGGAA-3', and the probe sequences were *NFKB1*-INS: 5'-VIC-CCCGACCATGATTGG-NFQ-3' and *NFKB1*-DEL: 5'-FAM-TTCCCCGACCATGTTGG-NFQ-3'.

Statistical analysis

Genotype and allele distributions among patients with CD, UC and IBD versus healthy controls were compared using the χ^2 test or Fisher test as appropriate. Odds ratios (ORs) with a confidence interval (CI) of 95% were assessed to measure the strength of association. Statistical power was calculated using Power Calculator of Genetic Studies 2006 software (<http://www.sph.umich.edu/csg/abecasis/CaTS/>). A chi-square test was used to test for deviation from Hardy-Weinberg equilibrium (HWE). Statistical analyses were performed with Plink software V1.07 (<http://pngu.mgh.harvard.edu/purcell/plink/>). *P* value < 0.05 was considered to be statistically significant. Bonferroni correction was applied to significant *P* values of *NOS2A* polymorphisms to correct by the number of comparisons.

RESULTS

One hundred ninety-nine patients with IBD (136 CD; 63 UC) and 308 control subjects were included in this study. The success rates of genotyping assays ranged between 95% and 100%. Baseline demographic and clinical characteristics of cases were reported in a previous paper^[23] and are presented in Table 1. Differences in our cohort between CD and UC patients in terms of age at diagnosis and gender distribution are to be underlined.

HIF1A (G/A) rs11549467 and *NFKB1* -94ins/del ATTG (rs28362491) polymorphisms

In both patients and controls, the genotype distribution of examined polymorphisms complied with the Hardy-Weinberg expectations.

In order to study associations of *HIF1A* (rs11549467)

Table 1 Basic characteristics of inflammatory bowel disease patients *n* (%)

Patient characteristics		CD (<i>n</i> = 136)	UC (<i>n</i> = 63)
Gender	Male	96	34
	Female	40	29
Age at diagnosis	< 16	16	1
	17-40	79	34
	≥ 40	22	19
	Data not available	19	9
Location of CD	L1 ± L4	38 (28)	-
	L2 ± L4	30 (22)	-
	L3 ± L4	53 (39)	-
	L4	4 (3)	-
	Data not available	11 (8)	-
Behavior of CD	B1 ± p	35 (26)	-
	B2 ± p	47 (34)	-
	B3 ± p	39 (29)	-
	Data not available	15 (11)	-
Location of UC	E1	-	5 (8)
	E2	-	24 (38)
	E3	-	8 (12)
	E4	-	13 (21)
	Data not available	-	13 (21)
Smoking habits	Yes	35	11
	No	67	32
	Data not available	34	20

CD: Crohn's disease; UC: Ulcerative colitis.

and *NFKB1* (rs28362491) variants in IBD overall and in CD and UC in particular, the distribution of polymorphic alleles was assessed. Genotype and allele frequencies are given in Tables 2 and 3.

The analysis of both *HIF1A* (rs11549467) and *NFKB1* (rs28362491) polymorphisms distribution among patients and controls did not reveal any statistically significant association, both in terms of allele and genotype frequencies. Similarly, we did not observe any effect on disease risk when Genetic models were assessed (Table 3).

***NOS2A* (TAAA)*n* and (CCTTT)*n* repeat microsatellite repeat polymorphisms**

We explored the potential influence of *NOS2A* polymorphisms on the susceptibility to IBD in the Moroccan population. Tables 4 and 5 show the distribution of the pentanucleotide (CCTTT)*n* microsatellite alleles in controls, IBD, CD and UC cases. Ten different alleles, comprising of 7-16 repeats *i.e.* 171-216 bp, were observed in our population. (CCTTT)₁₂ was observed to be the most frequent allele in IBD (21.2%), CD (21.7%), UC cases (20.0%) and controls (22.0%). The overall (CCTTT)*n* distribution showed differences between cases and controls. The average amount of CCTTT tandem repeats was shown to be less in CD and more in UC patients compared to controls.

When individual CCTTT alleles were analyzed, a significant increase in frequency of the 8-repeat (10.2% vs 6.2%, *P* = 0.02, OR = 1.71, 95%CI: 1.07-2.74) and 14-repeat (9.9% vs 6.2%, *P* = 0.02, OR = 1.71,

95%CI: 1.06-2.76) alleles was observed in IBD cases compared with controls respectively.

Similarly the (CCTTT)₈ repeat/allele was found to be higher in CD patients cohort as compared to controls (11.4% vs 6.2%, *P* = 0.008, OR = 1.95, 95%CI: 1.17-3.23). Whereas the increased distribution of the (CCTTT)₁₄ repeat among CD cases compared to controls did not reach the significance level (9.1% vs 6.2%, *P* = 0.09, OR = 1.56, 95%CI: 0.91-2.68).

Furthermore, determination of allele frequencies in UC patients revealed significant association of the 7, 11, 14 and 15 repeats polymorphic forms of the microsatellite to disease risk. Similar trend, though non-significant, was observed for the 16 repeat (*P* = 0.06, OR = 3.79, 95%CI: 0.83-17.18).

However, it should be noted that after Bonferroni correction by the number of comparisons, none of the observed associations remained significant in all patient groups.

We further analyzed the distribution of TAAA insertion/deletion polymorphism of *NOS2A* gene in our population. In this regard, no statistically significant allele or genotype differences were observed between IBD patients and controls (Table 6). Nor were significant differences between stratified CD and UC patients when compared to controls.

DISCUSSION

IBD is an inflammatory disease resulting from a compound effect of a number of abnormal conditions. Genetic factors may play a pivotal role in the development of IBD. In this regard, we explored the potential contribution of genetic polymorphisms of oxidative pathway genes to the risk of IBD development. Our attention was focused on functionally relevant polymorphisms located in the *NOS2A* and *NFKB1* genes, and a common missense mutation of *HIF1A* gene.

We found differential distribution of (CCTTT)*n* microsatellite repeats between patients and controls; namely the (CCTTT)₈ and (CCTTT)₁₄ repeats for IBD, the (CCTTT)₈ for CD and the (CCTTT)₇, (CCTTT)₁₁, (CCTTT)₁₄ and (CCTTT)₁₅ repeats for UC patients. As for Caucasians, the most common allele observed in our population was the 12 repeats instead of 10 and 11 repeats for Northwestern Colombians^[20]. To our knowledge only two reports investigated the involvement of *NOS2A* gene polymorphisms in IBD etiology. Concordantly to our results, Martín *et al*^[5] evidenced the influence of the inducible nitric oxide synthase (CCTTT)*n* microsatellite repeats on UC risk. However, in contrast to our finding, Oliver *et al*^[24] demonstrated no tendency toward an association with IBD predisposition. This discrepant observation can be explained by geographic factors and ethnicity-related gene effect on disease susceptibility. Results from

Table 2 Minor Allele Frequencies of *HIF1A* and *NFKB1* genetic variants in inflammatory bowel disease patients and healthy controls from Morocco

Gene	SNP	Group	Number of alleles	MAF (%)	Allele test	
					OR (95%CI)	P value
<i>HIF1A</i>	rs11549467	Controls (<i>n</i> = 308)	74/542	12.01		
		IBD (<i>n</i> = 199)	50/348	12.56	1.05 (0.72-1.54)	0.79
		CD (<i>n</i> = 136)	36/236	13.24	1.12 (0.73-1.71)	0.61
		UC (<i>n</i> = 63)	14/112	11.11	0.92 (0.50-1.68)	0.77
<i>NFKB1</i>	rs28362491 -94ATTG ins/del	Controls (<i>n</i> = 308)	257/359	41.72		
		IBD (<i>n</i> = 199)	167/231	41.96	1.01 (0.78-1.30)	0.93
		CD (<i>n</i> = 136)	113/159	41.54	0.99 (0.74-1.33)	0.96
		UC (<i>n</i> = 63)	54/72	42.86	1.05 (0.71-1.54)	0.81

IBD: Inflammatory bowel disease; CD: Crohn's disease; UC: Ulcerative colitis; MAF: Minor allele frequencies.

Table 3 Genotype and genetic models distribution of *HIF1A* and *NFKB1* SNPs in inflammatory bowel disease patients and controls *n* (%)

SNP ID	1/2	Subgroup	Genotype			Genotype 11 + 12 vs 22 ¹		Genotype 11 vs 12 + 22 ²	
			AA	GA	GG	OR (95%CI)	P value	OR (95%CI)	P value
<i>HIF1A</i> rs11549467	A/G	Controls	4 (1.30)	66 (21.43)	238 (77.27)				
		IBD	2 (1.01)	46 (23.12)	151 (75.88)	1.08 (0.71-1.64)	0.71	0.77 (0.14-4.25)	0.76
		CD	2 (1.47)	32 (23.53)	102 (75.00)	1.13 (0.70-1.81)	0.60	1.13 (0.20-6.26)	0.88
		UC	0 (0.00)	14 (22.22)	49 (77.78)	0.97 (0.50-1.86)	0.93	NA	0.99
<i>NFKB1</i> rs28362491	Del/Ins	Controls	del/del	ins/del	ins/ins				
		IBD	58 (18.83)	141 (45.78)	109 (35.39)	1.03 (0.71-1.5)	0.86	0.98 (0.62-1.55)	0.94
		CD	37 (18.59)	93 (46.73)	69 (34.67)	1.00 (0.65-1.53)	0.98	0.97 (0.57-1.63)	0.91
		UC	25 (18.38)	63 (46.32)	48 (35.29)	1.09 (0.61-1.94)	0.75	1.01 (0.50-2.02)	0.96

¹Dominant model; ²Recessive model. IBD: Inflammatory bowel disease; CD: Crohn's disease; UC: Ulcerative colitis; NA : Not applicable.

Table 4 Allelic frequencies of (CCTTT)*n* microsatellite polymorphism of *NOS2A* gene for Moroccan inflammatory bowel disease patients and healthy controls *n* (%)

Repeat No.	Size (bp)	Controls <i>2n</i> = 596	IBD <i>2n</i> = 382	P value	OR (95%CI)
7	171	2 (0.3)	4 (1.04)	0.16	3.14 (0.57-17.24)
8	176	37 (6.2)	39 (10.2)	0.02 ¹	1.71 (1.07-2.74)
9	181	75 (12.5)	54 (14.1)	0.48	1.14 (0.78-1.66)
10	186	89 (15.0)	46 (12.04)	0.20	0.77 (0.53-1.14)
11	191	90 (15.1)	45 (11.8)	0.14	0.75 (0.51-1.10)
12	196	131 (22.0)	81 (21.2)	0.77	0.95 (0.69-1.30)
13	201	99 (16.6)	50 (13.9)	0.13	0.75 (0.52-1.09)
14	206	36 (6.2)	38 (9.9)	0.02 ²	1.71 (1.06-2.76)
15	211	33 (5.5)	22 (5.8)	0.88	1.04 (0.59-1.81)
16	216	4 (0.6)	3 (0.7)	0.83	1.17 (0.26-5.26)

¹*P* = 0.02, OR (95%CI): 1.71 (1.07-2.74); Bonferroni's corrected *P*_c = 0.2; ²*P* = 0.02, OR (95%CI): 1.71 (1.06-2.76); *P*_c = 0.2. IBD: Inflammatory bowel disease.

case-control studies may be influenced by population stratification, selection bias, phenotypic heterogeneity and low power to detect true associations. Thereby, it is likely that differences in the features related to the population investigated could be responsible in part for the controversy over the influence of *NOS2A* polymorphisms on IBD.

Polymorphisms of *NOS2A* have also been involved in other autoimmune diseases such multiple sclerosis and rheumatoid arthritis^[25,26]. In terms of functional relevance, CCTTT polymorphic markers have been

described to affect nitric oxide synthase (NOS) transcription^[6]. Another study has also reported that the number of CCTTT repeats was shown to influence transcription of *NOS2* gene in which the transcriptional activity was much greater in fibroblasts transfected by a vector with a long allele of the CCTTT repeat than in those transfected by a vector with a short allele^[27].

Considering the different contribution that short and long alleles seem to exert, tandem repeats variation within the promoter region of *NOS2* gene could explain the differences observed in our study

Table 5 Allelic frequencies of (CCTTT)*n* microsatellite polymorphism of *NOS2A* gene for Crohn's disease, ulcerative colitis patients and healthy controls *n*(%)

Repeat No.	Size (bp)	Controls <i>2n</i> = 596	CD <i>2n</i> = 262	<i>P</i> value	OR(95%CI)	UC <i>2n</i> = 120	<i>P</i> value	OR(95%CI)
7	171	2 (0.3)	1 (0.4)	0.91	1.13 (0.10-12.6)	3 (2.5)	0.009 ²	7.61 (1.25-46.08)
8	176	37 (6.2)	30 (11.4)	0.008 ¹	1.95 (1.17-3.23)	9 (7.5)	0.59	1.22 (0.57-2.61)
9	181	75 (12.5)	43 (16.4)	0.13	1.36 (0.90-2.04)	11 (9.1)	0.29	0.70 (0.36-1.36)
10	186	89 (15.0)	33 (12.6)	0.36	0.82 (0.53-1.26)	13 (10.8)	0.24	0.69 (0.37-1.28)
11	191	90 (15.1)	35 (13.3)	0.50	0.86 (0.56-1.32)	10 (8.3)	0.05 ³	0.51 (0.25-1.01)
12	196	131 (22.0)	57 (21.7)	0.94	0.98 (0.69-1.40)	24 (20.0)	0.63	0.88 (0.54-1.44)
13	201	99 (16.6)	31 (11.8)	0.07	0.67 (0.43-1.03)	19 (15.8)	0.83	0.94 (0.55-1.61)
14	206	36 (6.2)	24 (9.1)	0.09	1.56 (0.91-2.68)	14 (11.6)	0.02 ⁴	2.05 (1.07-3.94)
15	211	33 (5.5)	8 (3.0)	0.11	0.53 (0.24-1.18)	14 (11.6)	0.01 ⁵	2.25 (1.16-4.35)
16	216	4 (0.6)	0 (0)	0.18	0 (0)	3 (2.5)	0.06	3.79 (0.83-17.18)

¹*P* = 0.008, OR (95%CI): 1.95 (1.17-3.23); *P*_c = 0.08; ²*P* = 0.009; OR (95%CI): 7.61 (1.25-46.08); *P*_c = 0.09; ³*P* = 0.05; OR (95%CI): 0.51 (0.25-1.01); *P*_c = 0.5; ⁴*P* = 0.02; OR(95%CI): 2.05 (1.07-3.94); *P*_c = 0.2; ⁵*P* = 0.01; OR(95%CI): 2.25 (1.16-4.35); *P*_c = 0.1. CD: Crohn's disease; UC: Ulcerative colitis.

Table 6 Allele and genotype frequencies of *NOS2A* TAAA polymorphism in inflammatory bowel disease patients and controls *n* (%)

	Controls <i>n</i> = 295	IBD patients <i>n</i> = 190	CD patients <i>n</i> = 132	UC patients <i>n</i> = 58
Genotype				
220/220	189 (64.07)	132 (69.47)	90 (68.18)	42 (72.41)
220/224	97 (32.88)	49 (25.79)	34 (25.76)	15 (25.86)
224/224	9 (3.05)	9 (4.74)	8 (6.06)	1 (1.72)
Allele	<i>2n</i> = 590	<i>2n</i> = 380	<i>2n</i> = 264	<i>2n</i> = 116
220	475 (80.5)	313 (82.4)	214 (81)	99 (85.3)
224	115 (19.5)	67 (17.6)	50 (19)	17 (14.7)

No statistically significant differences were found in any of the comparisons. IBD: Inflammatory bowel disease; CD: Crohn's disease; UC: Ulcerative colitis.

between case and control subjects. Likewise, the cumulative effect of CCTTT repeats number, which is less in CD and more in UC compared to controls might yield a progressive increase in *NOS2* gene expression and excessive production of NO. Enhanced levels of NO can promote tissue injury and contribute to IBD activity and progression. Our results should however be interpreted with caution due to small sample size.

The involvement of the inducible (calcium-independent) isoform, iNOS in inflammation has been largely demonstrated^[28] and is directly related to the large amounts of NO produced by the enzyme after transcriptional induction and the injurious levels of RNS generated by activated leukocytes, macrophages and epithelial cells in the intestinal mucosa^[29]. The overexpression of iNOS during active IBD is characterized by elevated rectal NO levels^[30]. Biopsies of UC-active patients demonstrate higher iNOS transcripts and enzyme levels as compared to controls or healthy relatives^[31]. It was also demonstrated that in UC, greatly increased production of iNOS-derived NO reacts with tyrosine leading to production of nitrotyrosine which is associated with infiltration of neutrophils in the epithelium^[32].

On the other hand, the present study sought to assess the association of *HIF1A* (G/A) rs11549467 and *NFKB1*-94ins/del ATTG (rs28362491) polymorphisms with IBD among Moroccan patients. Data on asso-

ciation of these genes with IBD in the North African population are currently lacking. Our results suggest that the studied *NFKB1* gene variation do not influence susceptibility to IBD (CD and UC) in the cohort tested herein. Our findings are in accordance with previous investigations analyzing Spanish^[33] British^[34] and German populations^[35]. In contrast, an association of the -94ins/del ATTG polymorphism with UC was demonstrated in a North American population^[14]. These discrepant results may have been caused by clinical, population and genetic differences in addition to ethnic origin heterogeneity.

Moreover, the present case-control study could not establish a role for *HIF1A* (G/A) rs11549467 polymorphism in the pathogenesis of both CD and UC and also found no evidence for disease risk when evaluating genetic models. This later polymorphism was shown to be associated with autoimmune diseases such as systemic sclerosis^[36]; however no investigation has assessed its involvement in IBD etiology.

Our results suggest that, variation in the distribution of CCTTT repeats in the *NOS2A* gene may differentially contribute to CD and UC development in the Moroccan population. Additionally, contrary to what was initially expected, no significant differences were found between patients and healthy subjects in the frequency of the *NFKB1* and *HIF1A* polymorphisms. Thereby, our data do not support a role for these

polymorphisms in the pathogenesis of IBD in the Moroccan population.

COMMENTS

Background

Inflammatory bowel disease (IBD), a chronic and relapsing-remitting disorder of the gastrointestinal tract, encompasses Crohn's disease (CD) and ulcerative colitis (UC). Chronic intestinal inflammation is a hallmark of both disorders, and is believed to result from a number of abnormal conditions. The involvement of oxidative damage in IBD development has been thoroughly documented. However, the genetic factors involved in this process have not been elucidated in the Moroccan population.

Research frontiers

Oxidative stress was reported to play a key role in the induction and perpetuation of inflammation. In search of relevant gene polymorphisms related to oxidative stress signaling that are involved in IBD development, we explored the association of *HIF1A*_rs11549467, *NFKB1*_rs28362491 *NOS2A* (CCTTT)_n_rs3833912 and *NOS2A* (TAAA)_n_rs12720460 polymorphisms with IBD (CD and UC) in Moroccan patients.

Innovations and breakthroughs

This study found that variation in the distribution of CCTTT repeats in the *NOS2A* gene may contribute to IBD development in the Moroccan population. Additionally, no significant differences were found between patients and healthy subjects in the frequency of the *NFKB1* and *HIF1A* polymorphisms. Thereby, our data do not support a role for these polymorphisms in the pathogenesis of IBD in our study cohort.

Applications

By assessing and identifying the genetic polymorphisms associated with susceptibility to inflammatory bowel disease, this study could represent a future preliminary basis for personalized medicine and targeted therapy in disease management.

Terminology

Oxidative stress is identified as an imbalance between the prooxidants and antioxidants in the body. Oxidative stress produced due to unresolved and persistent inflammation can be a major factor involved in the change of the dynamics of immune responses.

The mechanism by which oxidative stress and redox signaling induces inflammation in IBD is demonstrated by an increase in the levels of reactive oxygen and nitrogen species (ROS/RNS) in both human subjects and experimental animals.

Peer-review

This is an interesting paper, concerning the role of genetic factors related to the oxidative pathway in the susceptibility of inflammatory bowel disease. There are some points that need to be addressed.

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Basic Study

Effects of initiating time and dosage of *Panax notoginseng* on mucosal microvascular injury in experimental colitis

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Abstract

AIM

To investigate the effects of *Panax notoginseng* (PN) on microvascular injury in colitis, its mechanisms, initial administration time and dosage.

METHODS

Dextran sodium sulfate (DSS)- or iodoacetamide (IA)-induced rat colitis models were used to evaluate and investigate the effects of ethanol extract of PN on microvascular injuries and their related mechanisms. PN administration was initiated at 3 and 7 d after the model was established at doses of 0.5, 1.0 and 2.0

g/kg for 7 d. The severity of colitis was evaluated by disease activity index (DAI). The pathological lesions were observed under a microscope. Microvessel density (MVD) was evaluated by immunohistochemistry. Vascular permeability was evaluated using the Evans blue method. The serum concentrations of cytokines, including vascular endothelial growth factor (VEGF)A121, VEGFA165, interleukin (IL)-4, IL-6, IL-10 and tumor necrosis factor (TNF)- α , were detected by enzyme-linked immunosorbent assay. Myeloperoxidase (MPO) and superoxide dismutase (SOD) were measured to evaluate the level of oxidative stress. Expression of hypoxia-inducible factor (HIF)-1 α protein was detected by western blotting.

RESULTS

Obvious colonic inflammation and injuries of mucosa and microvessels were observed in DSS- and IA-induced colitis groups. DAI scores, serum concentrations of VEGFA121, VEGFA165, VEGFA165/VEGFA121, IL-6 and TNF- α , and concentrations of MPO and HIF-1 α in the colon were significantly higher while serum concentrations of IL-4 and IL-10 and MVD in colon were significantly lower in the colitis model groups than in the normal control group. PN promoted repair of injuries of colonic mucosa and microvessels, attenuated inflammation, and decreased DAI scores in rats with colitis. PN also decreased the serum concentrations of VEGFA121, VEGFA165, VEGFA165/VEGFA121, IL-6 and TNF- α , and concentrations of MPO and HIF-1 α in the colon, and increased the serum concentrations of IL-4 and IL-10 as well as the concentration of SOD in the colon. The efficacy of PN was dosage dependent. In addition, DAI scores in the group administered PN on day 3 were significantly lower than in the group administered PN on day 7.

CONCLUSION

PN repairs vascular injury in experimental colitis *via* attenuating inflammation and oxidative stress in the colonic mucosa. Efficacy is related to initial administration time and dose.

Key words: Microvascular injury; *Panax notoginseng*; Ulcerative colitis; Oxidative stress

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Core tip: *Panax notoginseng* (PN) is a traditional Chinese medicine used to treat ulcerative colitis, but its mechanisms are unclear. In our study, we found that PN promoted repair of injuries of colonic mucosa and microvessels in rat colitis. PN decreased concentrations of vascular endothelial growth factor, interleukin (IL)-6 and tumor necrosis factor- α while it increased the concentrations of IL-4 and IL-10 in serum. It also decreased concentrations of myeloperoxidase and hypoxia-inducible factor-1 α while it increased the concentration of superoxide dismutase in colon. So it is concluded that PN repairs mucosal and vascular injuries

in rat colitis *via* attenuating inflammation and oxidative stress in the colonic mucosa.

Wang SY, Tao P, Hu HY, Yuan JY, Zhao L, Sun BY, Zhang WJ, Lin J. Effects of initiating time and dosage of *Panax notoginseng* on mucosal microvascular injury in experimental colitis. *World J Gastroenterol* 2017; 23(47): 8308-8320 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v23/i47/8308.htm> DOI: <http://dx.doi.org/10.3748/wjg.v23.i47.8308>

INTRODUCTION

Microvessels are a major component of the colonic mucosa that nourish the colonic tissue and clear metabolic waste *via* controlling the intestinal blood flow^[1]. They also play an important role in maintaining normal intestinal permeability^[2]. Recent studies have found that injury of the colonic microvessels precedes injury of the colonic mucosa in the development of experimental colitis^[3]. The increased vascular permeability aggravates the early endothelial injury^[4], which induces hypoxia of the colonic mucosa and further oxidative stress^[5,6].

Previous studies have demonstrated that colonic mucosal hypoxia induced by mucosal vascular injury plays an important role in the pathogenesis of ulcerative colitis (UC)^[7]. Our previous study found that colonic mucosal injury in rats with experimental colitis improved along with repair of the mucosal microvascular injury^[8]. Therefore, microvascular injury is essential to the development of UC and could be a new therapeutic target. However, there are no drugs that can promote effective microvascular repair.

Panax notoginseng (PN) is a common Chinese herbal medicine that has long been used to treat vascular lesions^[9]. Some studies have found that it promotes repair of vascular damage *via* proangiogenic and anti-apoptotic effects^[10,11]. Animal experiments have shown that PN attenuates colonic mucosal injury and promotes mucosal repair in mouse models of colitis^[12], but its mechanism of action is unclear. It is hypothesized that PN repair of mucosal injury could be related to its promotion of repair of microvascular injury. In this study, we investigated the mechanism of PN repair of colonic mucosal injury in rats with colitis from the aspect of promotion of vascular repair. We also investigated the relationship between dose and initial time of administration of PN and its efficacy.

MATERIALS AND METHODS

Animals

Sprague-Dawley rats (120-140 g) were from Shanghai SLAC Laboratory Animal Co. Ltd. (Shanghai, China). The Institutional Animal Care and Use Committee of Shanghai University of Traditional Chinese Medicine

approved all procedures. Rats were housed in a pathogen-free environment and allowed to acclimate to the environment for 7 d before inclusion in an experiment.

Reagents

Iodoacetamide (IA; purity > 99%), dextran sodium sulfate (DSS), formamide (purity > 99%) and Evans blue (dye content > 75%) were purchased from Sigma-Aldrich (St. Louis, MO, United States). Enzyme-linked immunosorbent assay (ELISA) kits for interleukin (IL)-4, IL-6, IL-10 and tumor necrosis factor (TNF)- α were purchased from R&D Systems (Minneapolis, MN, United States). ELISA kits for vascular endothelial growth factor (VEGF)A165 and VEGFA121 were purchased from Cloud-Clone Corp. (Katy, TX, United States). CD31 antibody (ab23680) and goat anti-mouse antibody were purchased from Abcam (Cambridge, United Kingdom). Myeloperoxidase (MPO) and superoxide dismutase (SOD) assay kits were purchased from Cell Signaling Technology (Danvers, MA, United States).

Preparation of PN

PN was purchased from Shanghai Huayu Traditional Chinese Medicine Co. Ltd. (Shanghai, China) and authenticated by Professor Yang Dong (Shanghai University of Traditional Chinese Medicine). PN (0.9 kg) was dissolved in tap water (9 L), boiled at 100 °C for 3 h, filtered through a sieve (150 μ m), extracted with absolute ethanol, and dried in a freeze dryer. The brown extract powder of PN was stored at -20 °C.

Animal models of colitis

Two rat models of colitis were used in this study. One was induced by 0.1 mL 6% IA dissolved in 1% methylcellulose given to rats once by enema (7 cm above the anus). The other was induced by DSS, in which rats were allowed free access to purified water containing 5% DSS (w/v) for 7 d. DSS solution was prepared daily.

PN intervention in vivo

Rats were divided into control, low-dose, medium-dose and high-dose groups ($n = 6$ each). For the intervention groups, PN was intragastrically administered to rats with IA-induced or DSS-induced colitis once daily for seven consecutive days at doses of 0.5, 1.0 or 2.0 g/kg. For the control groups, normal saline was given to the corresponding rats. The initial administration times of PN were 3 and 7 d after establishing the colitis models. Three independent experiments were performed in triplicate.

Disease activity index

Before, during and after treatment, the severity of colitis was evaluated with disease activity index (DAI),

as described previously^[13]. The parameters of DAI included weight loss (0, none; 1, 0%-5%; 2, 5%-10%; 3, 10%-20%; 4, > 20%), stool consistency (0, none; 2, loose stool; 4, watery), and bleeding (0, none; 1, trace; 2, mild occult blood; 3, obvious occult blood; 4, gross bleeding).

Histological assays

Segments of colon were fixed in formalin buffer and embedded in paraffin. Sections of 5 μ m thick were deparaffinized and stained with hematoxylin and eosin (HE). The histological changes were assessed by a pathologist.

Microvessel density analysis

Five-micrometer-thick paraffin-embedded sections of colon were deparaffinized, subjected to heat-mediated antigen retrieval, and blocked with goat serum. The tissues were incubated with the primary anti-CD31 antibody (1:200, v/v) overnight at 4 °C. After three 5-min washes, the horseradish peroxidase (HRP)-labeled secondary antibody (1:300) was added and the samples were incubated at 37 °C for 1 h. The sections were counterstained with hematoxylin for 1 min at room temperature to visualize the endothelial cell nuclei. Three fields with CD31-positive cells in each section were chosen to assess microvessel density (MVD). Areas of highest vascularization were selected by scanning the sections at low magnification. Stained microvessels were counted in a single 200 \times field within the selected field by three observers without previous knowledge of control groups. The following cellular structures were considered as countable microvessels: (1) stained lumen; (2) stained endothelial cell; and (3) stained endothelial cell cluster (1 and 2 were clearly separated from adjacent stained lumens, colonic mucosal cells and other connective tissue elements). The MVD value was calculated as the average vessel counts in three selected areas within a microscopic field.

Vascular permeability analysis

Vascular permeability (VP) of vessels in colonic mucosa was evaluated by the Evans blue method, as described previously^[14]. Rats were anesthetized with intraperitoneal injection of sodium pentobarbital. Evans blue (1 mg/100 g) was injected intravenously 15 min before autopsy. Evans blue was extracted from the 1-cm segment of colonic tissue using formamide and measured by spectrophotometry at 610 nm. Results were expressed as OD value per milligram of colon.

ELISA

Blood samples were collected from the abdominal aorta. The serum concentrations of VEGFA165 (Cloud-Clone Corp.), VEGFA121 (Cloud-Clone Corp.), IL-4 (R&D Systems), IL-6 (R&D Systems), IL-10 (R&D

Systems) and TNF- α (R&D Systems) were detected using the appropriate ELISA kits.

SOD and MPO activity assays

SOD activity was measured using the SOD assay kit (Cell Signaling Technology). Tissue homogenate was prepared by vortex homogenizer, heated at 95 °C for 40 min and centrifuged at $178 \times g$ at 4 °C for 10 min. Ethanol-chloroform mixture (5:3, v/v) was used to extract SOD in the homogenate for total SOD activity assay. MPO activity was measured using the MPO assay kit (Cell Signaling Technology). Tissue homogenate was prepared by vortex homogenizer, heated at 95 °C for 40 min and centrifuged at $714 \times g$ at 4 °C for 10 min. The samples were added to phosphate buffer containing 30 mM H₂O₂ (pH 7.0) and incubated for 10 min. The enzymatic activity of SOD and MPO was expressed by the decrease of OD₂₄₀.

Western blotting

Hypoxia-inducible factor (HIF)-1 α was measured by western blotting, as described previously^[15]. Colonic tissue was cut into pieces and homogenized in 5-fold volumes of ice-cold homogenizing buffer (0.1 mmol/L NaCl, 0.1 mol/L Tris-HCl, and 0.001 mol/L EDTA) containing 1 mmol/L phenylmethylsulfonyl fluoride, 1 mg/mL aprotinin and 0.1 mmol/L leupeptin at $3000 \times g$ and 4 °C for 1 h. Bovine serum albumin was used to estimate the protein content in supernatants. The protein samples (60 μ g in each sample) were subjected to SDS-PAGE and transferred to polyvinylidene fluoride membranes using a transfer apparatus (Bio-Rad, Hercules, CA, United States). The membranes were blocked for 2 h, then the primary antibody anti-HIF-1 α was added and incubated at 4 °C overnight, and the corresponding HRP-conjugated secondary antibody (Cell Signaling Technology) was added and incubated for 1 h. Protein-antibody complexes were detected by Clarity Western ECL Substrate (Bio-Rad), and results were authenticated with the ImageJ software (Gene Co. Ltd., China).

Statistical analysis

Data were presented as the mean \pm SD. One-way analysis of variance or general linear model with repeated measures was used to analyze the data sets with three or more groups, and least significant difference *post hoc* test for multiple comparisons. Student's *t*-test was used to analyze data sets with two groups. *P* < 0.05 was considered significant.

RESULTS

Efficacy of PN on experimental colitis was dependent upon initial time of administration

After the colitis model was established, PN administration (1.0 g/kg) was initiated at days 3 and 7 for seven consecutive days (Figure 1A). Compared

with the day 7 group, DAI scores, injuries of colonic mucosa and microvessels, serum concentrations of pro-inflammatory cytokines (IL-6 and TNF- α), and expression of HIF-1 α and MPO were significantly lower (Figure 1B, C, E-G and I; Figure 2A, C and E; Figure 3A), and serum concentrations of anti-inflammatory cytokines (IL-4 and IL-10), MVD and SOD were significantly higher (Figure 1D, H and J; Figure 3C) in the day 3 group. The earlier PN was administered, the more effective it was in treating acute colitis.

PN repaired colonic mucosal injuries and microvessels

The rats in the IA- and DSS-induced experimental colitis groups had obvious injuries of the colonic mucosa and microvessels, as well as lower MVD. After being treated with PN (0.5, 1.0 and 2.0 g/kg) for seven consecutive days, colonic mucosal injuries and microvessels significantly improved and MVD increased compared with the model groups. The efficacy of PN in attenuating mucosal and microvascular injuries was dose dependent (Figure 4A-E). The effects of medium- and high-dose PN were superior to those of low-dose PN, but there were no significant differences between the medium- and high-dose groups (Figure 4A-E).

PN improved impaired VP

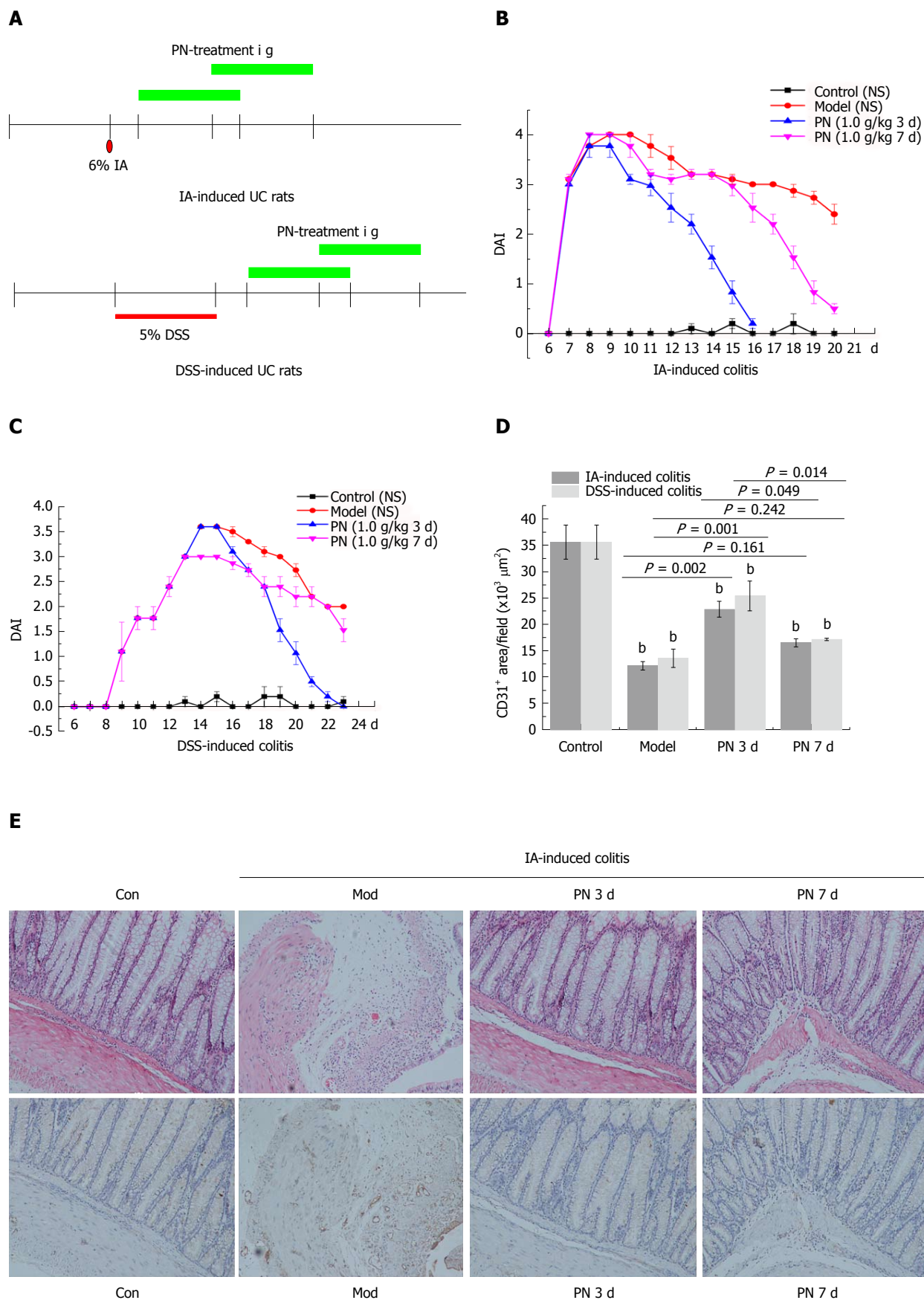
VP increased significantly in the groups with experimental colitis induced by DSS and IA compared with the normal control group. PN improved impaired VP in a dose-dependent manner. The effects of medium and high doses of PN were superior to those of low dose PN, but there were no significant differences between the medium- and high-dose groups (Figure 5).

PN reversed the disordered ratio of VEGFA165/VEGFA121

The serum concentrations of VEGFA165 and VEGFA121 and the ratio of VEGFA165/VEGFA121 increased significantly in the groups with experimental colitis induced by IA and DSS compared with the normal control group. PN significantly decreased elevated VEGFA165, VEGFA121 and VEGFA165/VEGFA121 ratio in a dose-dependent manner in rats with experimental colitis. The effects in the medium- and high-dose groups were superior to those of the low-dose group, but there were no significant differences between the medium- and high-dose groups (Figure 6A-C).

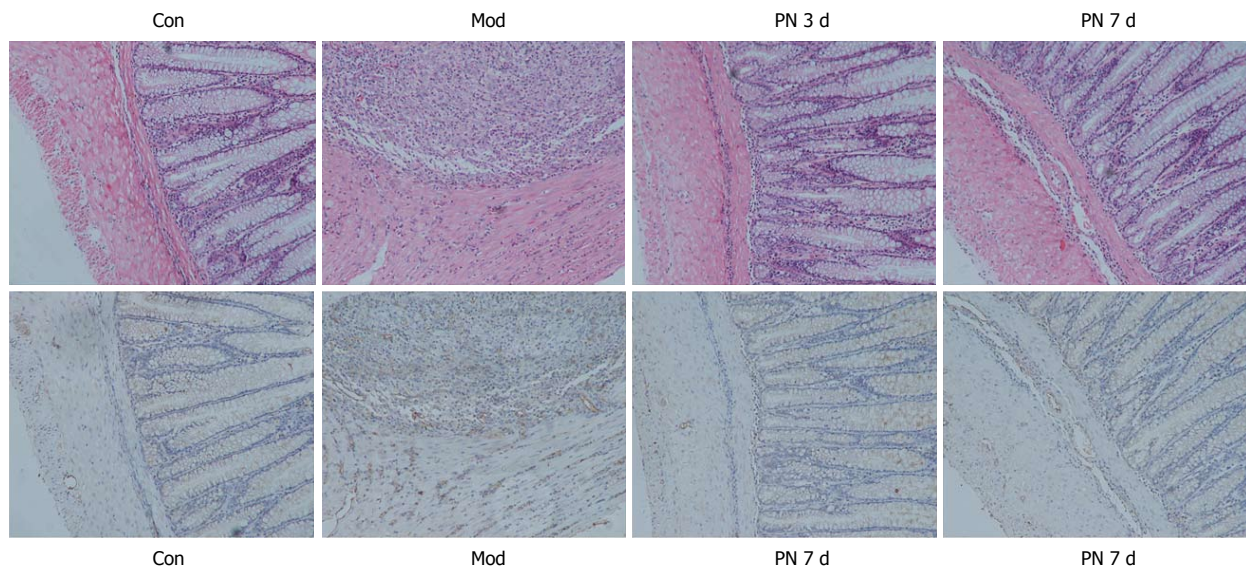
PN adjusted imbalance of pro-inflammatory and anti-inflammatory cytokines

The pro-inflammatory cytokines (IL-6 and TNF- α) increased significantly and the anti-inflammatory cytokines (IL-4 and IL-10) decreased significantly in the experimental colitis groups compared with the normal control group. PN down-regulated elevated IL-6 and TNF- α and up-regulated reduced IL-4 and IL-10 in a dose-dependent manner compared with the model control group (Figure 6D-G). The effects of medium

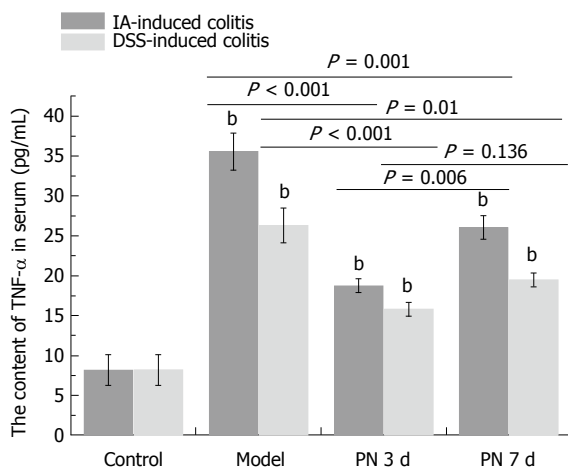


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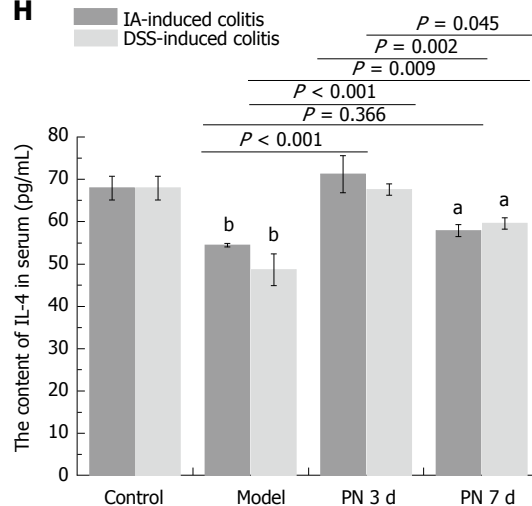
DSS-induced colitis



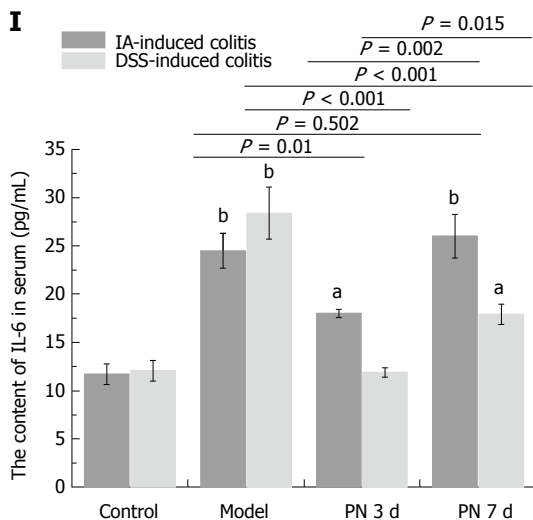
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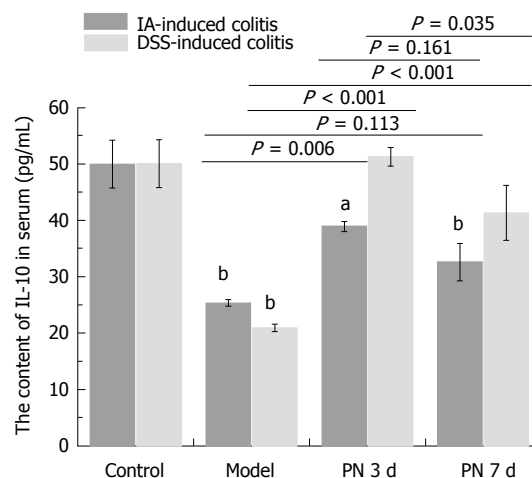


Figure 1 Efficacy of *Panax notoginseng* in experimental colitis was dependent on initial time of administration. A: PN administration (1.0 g/kg) was initiated at day 3 and 7 for seven consecutive days; B, C, G, J: DAI scores and serum concentrations of TNF- α and IL-6 in the day 3 group were significantly lower than those in the day 7 group; D, E: The pathological lesions of colonic mucosa and microvessels in the day 3 group were less than those in the day 7 group; F: MVD in the day 3 group was significantly higher than that in the day 7 group. Results are expressed as mean \pm SD of three independent experiments performed in triplicate. ^a $P < 0.05$, ^b $P < 0.01$ vs normal control. DAI: Disease activity index; IL: Interleukin; MVD: Microvessel density; PN: *Panax notoginseng*; TNF: Tumor necrosis factor.

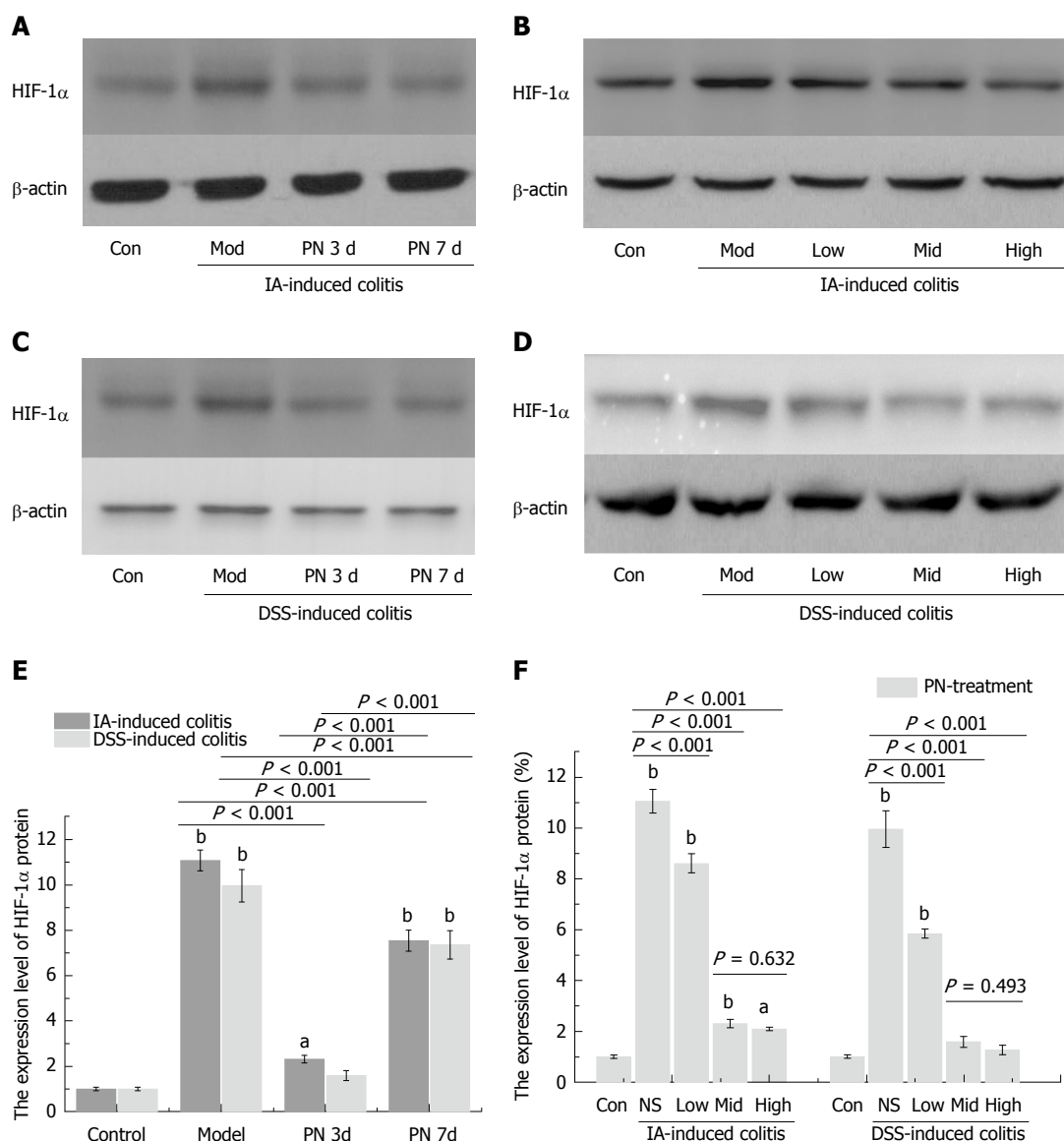


Figure 2 *Panax notoginseng* improved hypoxia in colonic mucosa. A-F: Increased expression of hypoxia-inducible factor-1 α in colonic mucosa of the experimental colitis group was down-regulated by PN in a time- and dose-dependent manner. Results are expressed as mean \pm SD of three independent experiments performed in triplicate. ^a $P < 0.05$, ^b $P < 0.01$ vs normal control. PN: *Panax notoginseng*.

and high dose PN were superior to those of low dose PN, but there were no significant differences between the medium- and high-dose groups.

PN improved hypoxia in colonic mucosa

Expression of HIF-1 α in colonic mucosa was significantly up-regulated in the experimental colitis groups compared with the normal control group. PN down-regulated increased expression of HIF-1 α in a dose-dependent manner compared with the model control group (Figure 2B, D and F). The effects of medium and high dose PN were superior to those of low dose PN, but there were no significant differences between the medium- and high-dose groups (Figure 2B, D and F).

PN blocked oxidative stress in colonic mucosa

The activity of MPO and SOD in colonic tissue was used

to evaluate the anti-oxidative effect of PN. The activity of MPO increased and the activity of SOD decreased in the colon in the experimental colitis groups compared with the normal control group. PN down-regulated elevated MPO and up-regulated decreased SOD in a dose-dependent manner compared with the control group. The effects of medium and high dose PN were superior to those of low dose PN, but there were no significant differences between the medium- and high-dose groups (Figure 3B and D).

DISCUSSION

PN, also known as Sanqi or Tianqi, is a common Chinese herbal medicine with various activities and is used to treat cardiovascular diseases, pain, inflammation and hemorrhagic injury^[16]. In recent

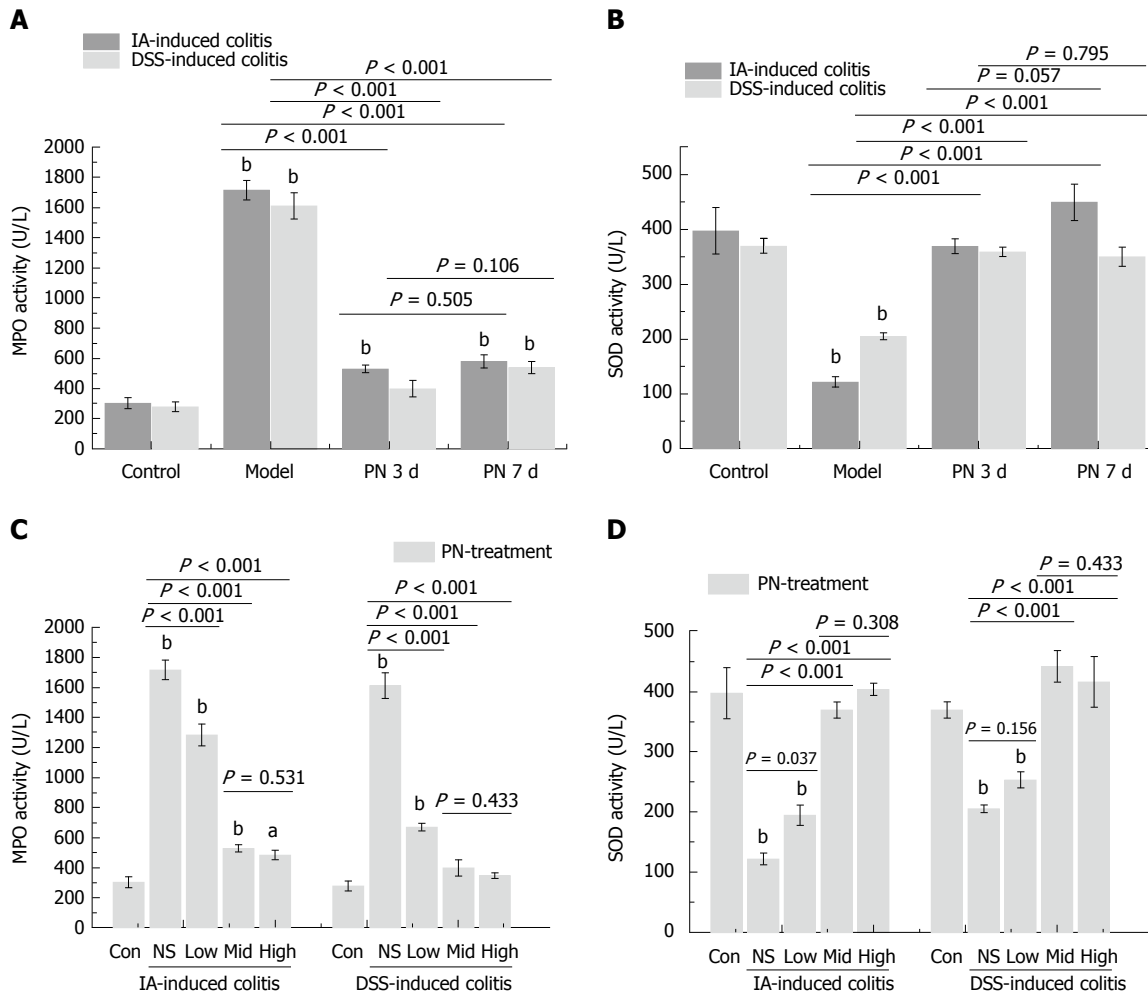


Figure 3 *Panax notoginseng* blocked oxidative stress in colonic mucosa. Activities of MPO and SOD in colonic tissue were used to evaluate the anti-oxidative effect of PN. A-D: The increased activity of MPO and decreased activity of SOD in the experimental colitis groups were reversed by PN in a time- and dose-dependent manner. Results are expressed as mean \pm SD of three independent experiments performed in triplicate. ^a $P < 0.05$, ^b $P < 0.01$ vs normal control. MPO: Myeloperoxidase; PN: *Panax notoginseng*; SOD: Superoxide dismutase.

years, PN has been used to treat UC, with the effects of promoting repair of mucosal injury and attenuating inflammatory responses^[17]. However, its mechanism of action is unclear.

It is well known that the efficacy of a drug is closely related to the initial administration time and its dose^[18]. In previous studies, the initial time of PN administration was usually based on personal experience rather than experimental evidence, which affected the standardization and efficacy of PN treatment. We found that initiating PN at day 3 after establishing the colitis models was more effective than initiating at day 7, demonstrating improved mucosal injury, microvascular impairment, inflammatory response and hypoxia. In our other study that has not been published, we found mild mucosal injury and increased vessel permeability and concentrations of TNF- α , IL-6 and MPO on day 3 in a colitis model. This suggested that the changes of vessel permeability and inflammatory cytokines occurred in the early stage of colitis and preceded mucosal injury. That may be why early initiation of PN treatment (day 3) improved colitis

more than initiating treatment on day 7. In addition, the efficacy of PN was dose dependent. The efficacy of medium and high doses was superior to that of the low dose, but there were no significant differences between the medium and high doses. This provided empirical evidence for using PN early and choosing the optimal dose in UC treatment.

Maintaining oxygen supply and metabolic clearance are the two crucial roles of colonic vessels. The severity of active UC is associated with mucosal hypoxia, which may result from increased oxygen consumption of inflammatory cells and decreased oxygen supply caused by vascular dysfunction^[19,20]. The imbalance between oxygen consumption and supply, as well as excessive serum cytokines, leads to increased epithelial cell apoptosis and consequent impairment of mucosal barrier function^[2,19]. Therefore, the levels of serum cytokines and expression of hypoxia- and oxidative stress-related proteins in colonic mucosa could reflect the status of hypoxia.

The serum concentrations of cytokines, including anti-inflammatory (IL-4 and IL-10) and pro-inflammatory (L-6

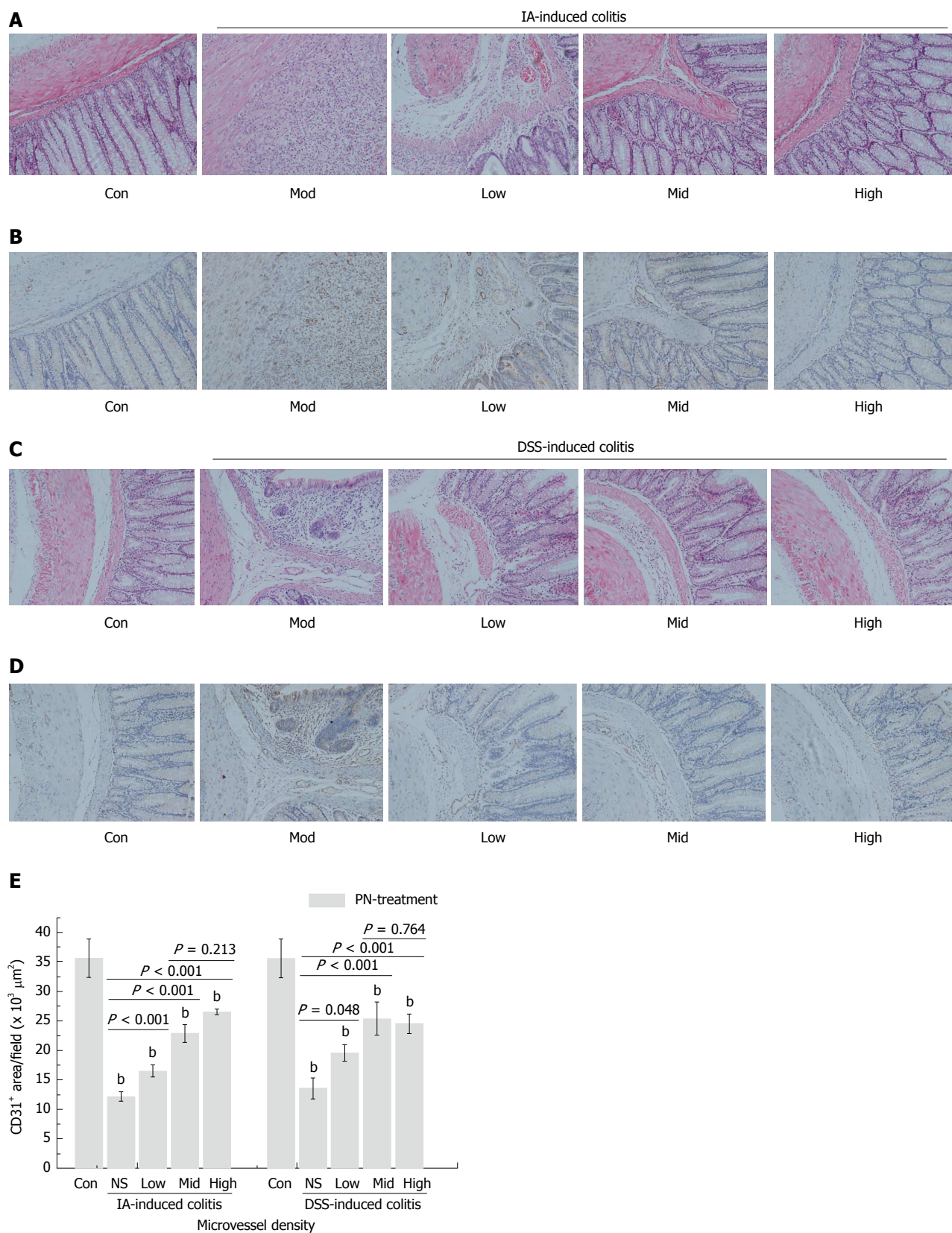


Figure 4 *Panax notoginseng* repaired colonic mucosal injuries and microvessels. IA- and DSS-induced experimental colitis groups were treated with PN (0.5, 1.0 and 2.0 g/kg) for seven consecutive days. A, C: Colonic mucosal injuries and microvessels significantly improved; B, D, E: Microvessel density increased compared with the control groups in a dose-dependent manner. Results are expressed as mean \pm SD of three independent experiments performed in triplicate. ^b $P < 0.01$ vs normal control. DSS: Dextran sodium sulfate; IA: Iodoacetamide; PN: *Panax notoginseng*.

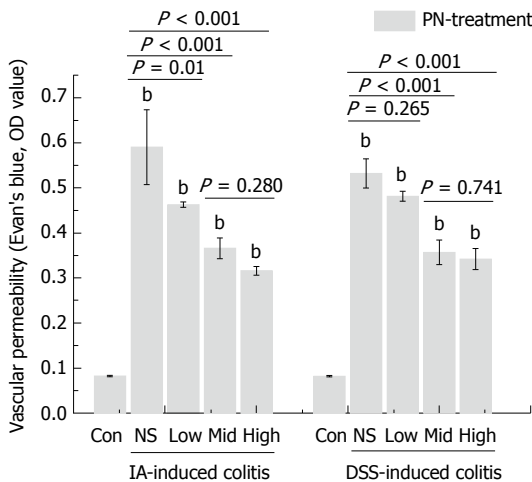


Figure 5 *Panax notoginseng* improved impaired vascular permeability. A and B: Increased VP in the IA- and DSS-induced experimental colitis groups was decreased by medium and high doses of PN. Results are expressed as mean \pm SD of three independent experiments performed in triplicate. ^a $P < 0.01$ vs normal control. DSS: Dextran sodium sulfate; IA: Iodoacetamide; PN: *Panax notoginseng*; VP: Vascular permeability.

and TNF- α) cytokines are indicators of inflammatory status^[21,22]. In addition, HIF-1 α is a crucial marker protein expressed under hypoxic conditions^[23,24]. Its expression increases when hypoxia occurs in tissues and is usually used to assess the severity of hypoxia^[25,26]. MPO and SOD, two kinds of oxidative enzymes, are crucial markers used to assess oxidative stress^[27,28]. They could reflect the severity of oxidative stress and hypoxia in colonic mucosa^[19,29]. VEGF, especially VEGFA, is an important cytokine implicated in angiogenesis^[8]. VEGFA121 and VEGFA165, two isoforms of VEGF, are closely correlated to the angiogenesis of colonic microvessels^[8,30].

Our previous study demonstrated that increased ratio of VEGFA165/VEGFA121 was in proportion to impairment of microvessels. In the present study, 7-d PN treatment reduced serum concentrations of VEGFA165 and VEGFA121 and the ratio of VEGFA165/VEGFA121, and attenuated impairment of microvessels compared with the colitis groups. PN down-regulated the expression of MPO and HIF-1 α while up-regulating the expression of SOD in colonic mucosa. These findings demonstrate that PN attenuates hypoxia and oxidative stress in colonic mucosa. The changes in VEGFA isoforms, HIF-1 α , MPO and SOD were dose dependent. The effects of medium and high doses were superior to those of low dose, but there were no significant differences between the medium and high doses. This suggested that the effects of PN reached a plateau when the dose was increased to a certain value. The optimal dose of PN is 1.0 g/kg for treating experimental colitis in rats.

In summary, PN is promising in UC treatment. It could improve hypoxia and relieve oxidative stress in the colon, attenuate vessel impairment and/or

promote angiogenesis, and finally promote repair of colonic mucosa. Early use of PN at an optimal dose might yield better efficacy. However, there are still some unresolved problems in the present study that need further study. For example, what is the real effective component in PN for UC treatment? What are the mechanisms of PN attenuating oxidative stress and regulating angiogenesis? These are important questions for using PN for treatment of UC and warrant exploration in future studies.

ARTICLE HIGHLIGHTS

Research background

Panax notoginseng (PN) is a Chinese herbal medicine commonly used to treat ulcerative colitis (UC) and vascular diseases. Microvascular injury plays an important role in the pathogenesis of UC, but PN's effects on microvascular injury in UC are unclear. To clarify the effects of PN on microvascular injury is important for treating UC.

Research motivation

The effects of PN on microvascular injury in colitis, its initial administration time, its dosage and its related mechanisms were investigated. These are important questions for using PN for treatment of UC.

Research objectives

To clarify the effects of PN on microvascular injury and related affecting factors, as well as its mechanisms.

Research methods

Dextran sodium sulfate (DSS)- or iodoacetamide (IA)-induced rat colitis models were used. PN administration was initiated at 3 d and 7 d after the model was established at doses of 0.5, 1.0 and 2.0 g/kg for seven consecutive days. The severity of colitis was evaluated by disease activity index (DAI). The pathological lesions were observed under microscope. Microvessel density (MVD) was evaluated by immunohistochemistry. Vascular permeability was evaluated using the Evans blue method. The serum concentrations of vascular endothelial growth factor (VEGF)A121, VEGFA165, interleukin (IL)-4, IL-6, IL-10 and tumor necrosis factor (TNF)- α were detected by enzyme-linked immunosorbent assay. Myeloperoxidase (MPO) and superoxide dismutase (SOD) were measured to evaluate the level of oxidative stress. Expression of hypoxia-inducible factor (HIF)-1 α protein was detected by western blotting. One-way ANOVA or general linear model with repeated measures was used to analyze the data sets with three or more groups, and least significant difference post hoc test for multiple comparisons. Student's *t*-test was used to analyze data sets with two groups. $P < 0.05$ was considered significant.

Research results

Obvious colonic inflammation and injuries of colonic mucosa and microvessels were observed in DSS- and IA-induced colitis in rats. DAI scores, the serum concentrations of VEGFA121, VEGFA165, VEGFA165/VEGFA121, IL-6 and TNF- α , and the concentrations of MPO and HIF-1 α in the colon were significantly higher while the serum concentrations of IL-4 and IL-10 and MVD in colon were significantly lower in the colitis model groups than in the normal control group. PN promoted repair of the injuries of colonic mucosa and microvessels, attenuated inflammation and decreased DAI scores in rats with colitis. PN decreased the serum concentrations of VEGFA121, VEGFA165, VEGFA165/VEGFA121, IL-6 and TNF- α and the concentrations of MPO and HIF-1 α in the colon. It also increased the serum concentrations of IL-4 and IL-10 as well as the concentration of SOD in the colon. The efficacy of PN was dosage dependent. In addition, DAI scores in the group initiating PN administration on day 3 were significantly lower than in the group initiating PN administration on day 7.

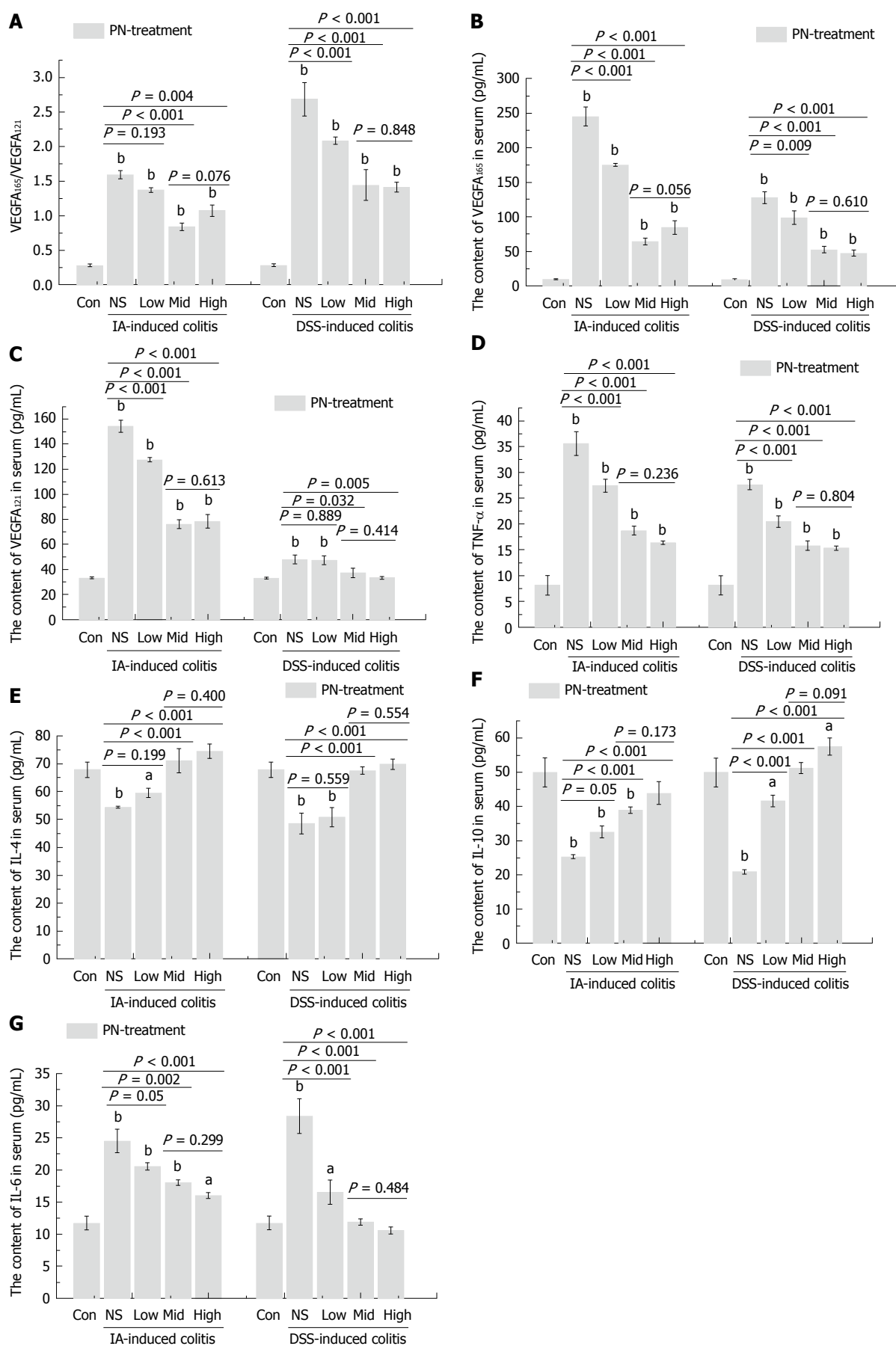


Figure 6 *Panax notoginseng* reversed the disordered ratio of VEGF₁₆₅/VEGF₁₂₁ and adjusted the imbalance of pro-inflammatory and anti-inflammatory cytokines. A-C: Increased serum concentrations of VEGF₁₆₅ and VEGF₁₂₁, as well as the increased ratio of VEGF₁₆₅/VEGF₁₂₁ in the experimental colitis groups were down-regulated by medium and high doses of PN; D-F: The increased serum concentrations of IL-6 and TNF-α and decreased serum concentrations of IL-4 and IL-10 in the experimental colitis groups were reversed by PN in a dose-dependent manner. Results are expressed as mean ± SD of three independent experiments performed in triplicate. ^a*P* < 0.05, ^b*P* < 0.01 vs normal control. IL: Interleukin; PN: *Panax notoginseng*; TNF: Tumor necrosis factor; VEGF: Vascular endothelial growth factor.

Research conclusions

PN repaired microvessel injury in experimental colitis via attenuating inflammation and oxidative stress in the colonic mucosa. The efficacy of PN was related to the initial administration time and the dose.

Research perspectives

Finding the real effective component in PN and clarifying the mechanisms of PN attenuating oxidative stress and regulating angiogenesis will be conducted in the future studies.

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Basic Study

Fructo-oligosaccharide intensifies visceral hypersensitivity and intestinal inflammation in a stress-induced irritable bowel syndrome mouse model

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Abstract

AIM

To determine whether fructo-oligosaccharide (FOS) affects visceral sensitivity, inflammation, and production of intestinal short-chain fatty acids (SCFA) in an irritable bowel syndrome (IBS) mouse model.

METHODS

Mice were randomly assigned to daily oral gavage of saline solution with or without FOS (8 g/kg body weight) for 14 d. Mice were further assigned to receive either daily one-hour water avoidance stress (WAS) or sham-

WAS for the first 10 d. After 2 wk, visceral sensitivity was measured by abdominal withdrawal reflex in response to colorectal distension and mucosal inflammation was evaluated. Gas chromatography, real-time reverse transcription PCR, and immunohistochemistry assays were used to quantify cecal concentrations of SCFA, intestinal cytokine expression, and number of intestinal mast cells per high-power field (HPF), respectively.

RESULTS

Mice subjected to WAS exhibited visceral hypersensitivity and low-grade inflammation. Among mice subjected to WAS, FOS increased visceral hypersensitivity and led to higher cecal concentrations of acetic acid (2.49 ± 0.63 mmol/L *vs* 1.49 ± 0.72 mmol/L, $P < 0.05$), propionic acid (0.48 ± 0.09 mmol/L *vs* 0.36 ± 0.05 mmol/L, $P < 0.01$), butyric acid (0.28 ± 0.09 mmol/L *vs* 0.19 ± 0.003 mmol/L, $P < 0.05$), as well as total SCFA (3.62 ± 0.87 mmol/L *vs* 2.27 ± 0.75 mmol/L, $P < 0.01$) compared to saline administration. FOS also increased ileal interleukin (IL)-23 mRNA (4.71 ± 4.16 *vs* 1.00 ± 0.99 , $P < 0.05$) and colonic IL-1 β mRNA (2.15 ± 1.68 *vs* 0.88 ± 0.53 , $P < 0.05$) expressions as well as increased mean mast cell counts in the ileum (12.3 ± 2.6 per HPF *vs* 8.3 ± 3.6 per HPF, $P < 0.05$) and colon (6.3 ± 3.2 per HPF *vs* 3.4 ± 1.2 per HPF, $P < 0.05$) compared to saline administration in mice subjected to WAS. No difference in visceral sensitivity, intestinal inflammation, or cecal SCFA levels was detected with or without FOS administration in mice subjected to sham-WAS.

CONCLUSION

FOS administration intensifies visceral hypersensitivity and gut inflammation in stress-induced IBS mice, but not in the control mice, and is also associated with increased intestinal SCFA production.

Key words: Fructo-oligosaccharide; Stress; Irritable bowel syndrome; Visceral hypersensitivity; Intestinal inflammation; Short chain fatty acids; FODMAP

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Core tip: Fructo-oligosaccharide is a component of Fermentable Oligosaccharides, Disaccharides, Monosaccharides, and Polyols (FODMAP), which has been associated with triggering symptoms in patients with irritable bowel syndrome (IBS). In a stress-induced IBS mouse model, daily fructo-oligosaccharide (FOS) administration further intensified visceral hypersensitivity and low-grade intestinal inflammation compared to saline. FOS administration also led to increased intestinal production of individual and total short-chain fatty acids (SCFA) in mice subjected to stress. However, no difference in visceral sensitivity, intestinal inflammation, or cecal concentrations of SCFA was observed among sham-stressed mice receiving FOS or saline. Our findings suggest a mechanism of FODMAP-

induced gastrointestinal symptoms associated with increased production of SCFA specific to IBS.

Chen BR, Du LJ, He HQ, Kim JJ, Zhao Y, Zhang YW, Luo L, Dai N. Fructo-oligosaccharide intensifies visceral hypersensitivity and intestinal inflammation in a stress-induced irritable bowel syndrome mouse model. *World J Gastroenterol* 2017; 23(47): 8321-8333 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v23/i47/8321.htm> DOI: <http://dx.doi.org/10.3748/wjg.v23.i47.8321>

INTRODUCTION

Irritable bowel syndrome (IBS) is a functional gastrointestinal disorder characterized by chronic abdominal pain associated with changes in bowel habit and frequency affecting more than a tenth of the general population^[1,2]. Many factors contribute to the development of IBS, including altered visceral pain perception, low-grade intestinal inflammation, change in microbiota, and psychosocial factors^[3]. The complex pathophysiology of IBS has posed challenges to developing effective interventions, and therapeutic gains with conventional pharmacologic therapies have been marginal at less than 15%^[4].

Importance of dietary factors in triggering symptoms is increasingly being recognized in patients with IBS. Specifically, poorly absorbed, fermentable carbohydrates categorized as Fermentable Oligosaccharides, Disaccharides, Monosaccharides, and Polyols (FODMAP) have been studied closely^[5]. Consumption of food high in FODMAP content triggers abdominal pain, bloating, and flatulence in patients with IBS. Furthermore, several randomized trials have demonstrated that low FODMAP diet reduces gastrointestinal symptoms in patients with IBS^[6-9]. Although largely unexplored, the accumulation of intestinal fluid from osmotic load of poorly digested carbohydrates and excessive colonic gas production associated with ingestion of FODMAP have been proposed as a mechanism for development of gastrointestinal symptoms^[10,11]. Intestinal dysmotility, visceral hypersensitivity, altered microbiota, and change in metabolic output also likely contribute to the pathophysiology of gastrointestinal symptoms associated with ingestion of FODMAP in IBS patients^[11-13]. In addition, the production of short-chain fatty acids (SCFA), such as acetic, propionic, and butyric acids, may also be important in the development of symptoms in IBS^[14].

Fructo-oligosaccharide (FOS) is one of the most frequently consumed FODMAP components in the general diet. The aim of our study was to investigate the effects of FOS on visceral sensitivity, intestinal SCFA production, and intestinal inflammation in a stress-induced IBS mouse model. Water avoidance

stress (WAS) was utilized to simulate psychological stress in IBS, and a WAS mouse model was used to evaluate the effects of FOS administration on visceral hypersensitivity and intestinal immune activation^[15]. Individual (acetic, propionic, and butyric acids) as well as total SCFA concentrations were also quantified in the cecum to determine the effects of FOS administration in a stress-induced IBS mouse model.

MATERIALS AND METHODS

Animals

Three-week-old female C57BL/6 mice (Slac Laboratory Animal Co. Ltd. Shanghai, China) were used as described in a previous study using WAS to develop a stress-induced IBS mouse model^[16]. Mice were housed in pathogen-free conditions with temperature ($21 \pm 1^\circ\text{C}$) and light/dark cycle (12/12 h) regulation. A purified rodent diet (AIN-76A) without any FODMAP content and demineralized water were supplied freely on demand.

Animal care and use statement

All animal experiment protocols were reviewed and approved by the Animal Care and Use Committee of Zhejiang University prior to initiating this study. All animals received humane care in compliance with the criteria described in "The Guide for the Care and Use of Laboratory Animals."

Experimental design

To evaluate the effects of FOS on WAS-induced visceral hypersensitivity and intestinal inflammation, 32 mice were randomly divided into four groups of eight mice (sham-WAS + saline administration, sham-WAS + FOS administration, WAS + saline administration, and WAS + FOS administration). Mice were administered daily with oral gavage of saline solution with or without FOS (8 g/kg body weight) for 14 d. FOS dose was derived according to the Meeh-Rubner formula^[8]. Mice were subjected to either WAS or sham-WAS during the first 10 d. For WAS, mice were placed on a glass platform (3 cm length \times 3 cm width \times 9 cm height) surrounded by water (25°C) in the middle of a plastic container (45 cm \times 30 cm \times 25 cm) as previously described^[15]. Control mice assigned to sham-WAS were placed in the same container without water. Food consumption quantity, body weight, and indexes were recorded daily prior to subjecting mice to daily WAS or sham-WAS.

Assessment of visceral sensitivity

Abdominal withdrawal reflex in response to colorectal distension was evaluated to assess visceral sensitivity as described previously^[17]. Semi-quantitative abdominal withdrawal reflex score (0-4) was utilized to evaluate pain responses at different magnitudes of

colorectal distention (20, 40, 60, and 80 mmHg)^[18]. With gradual colorectal distention to 100 mmHg, the pressure eliciting abdomen lifting was recorded as pain threshold and that eliciting body arching was recorded as volume threshold. To achieve accuracy, all pressure and threshold measurements were repeated three times and recorded by two independent operators blinded to WAS or FOS assignment.

Histological evaluation of inflammation

Mice were sacrificed by cervical dislocation, and intestinal tissues were harvested for histological evaluation. Intestinal tissue was fixed in formalin and processed with hematoxylin and eosin stains. The absence or presence of neutrophil infiltration in the lamina propria and the degree of interstitial edema in the intestinal tissues were graded based on previous description^[18]. Stained slides were examined by two independent observers blinded to WAS or FOS assignment.

Quantification of SCFA production

SCFA production was quantified using gas chromatography as previously described^[19]. Cecal contents (50 mg) were homogenized in 0.5 mL of distilled water and 0.1 mL of 25% (w/v) metaphosphoric acid was added to the suspension. The samples were subsequently centrifuged at $14000 \times g$ for 20 min, and the supernatant was filtered through a membrane filter (pore size $0.22 \mu\text{m}$). SCFA in the samples were then separated with InertCap FFAP columns (0.25 mm \times 30 mm \times 0.25 mm), and the peaks were integrated with GC Solution software (Shimadzu, GC-2010 Plus, Japan). Single-point internal standard method was used to quantify SCFA.

Intestinal cytokine mRNA detection

Intestinal expression of cytokines such as tumor necrosis factor (TNF)- α , interleukin (IL)-6, IL-23, IL-10, and IL-1 β was evaluated. Total RNA was isolated from 100 mg of ileal and colonic tissues by using a RNA Extraction Kit (Takara, Japan) and processed with a PrimeScript RT reagent Kit (Takara, Japan) to synthesize cDNA. Primers used are listed in Table 1. Quantitative real-time PCR was performed in triplicate for each sample with Lightcycler 480 instrument (Roche Applied Science, Penzberg, Germany). Reaction conditions for amplification of DNA were as follows: 95°C for 30 s and 40 cycles of 95°C for 5 s and 60°C for 30 s. Cytokine transcript levels were normalized with β -actin, and relative gene expression was expressed as the fold change ($2^{-\Delta\Delta\text{Ct}}$) relative to the expression in the control samples.

Immunohistochemistry

Intestinal mucosal mast cells were estimated by

Table 1 Primer sequences

Gene	Forward primer (5'-3')	Reverse primer (5'-3')
TNF- α	GGCTTCCGAATTCACCTGGAG	CCCCGCCCTTCCAAATAAA
β -actin	GCAGGAGTACGATGAGTCCG	ACGCAGCTCAGTAACAGTCC
IL-6	GTATGAACAACGATGATGCACTTG	ATGGTACTCCAGAAGACCAGAGGA
IL-23	AATAATGCTATGGCTGTTC	CCTTGAGTCCTGTGGGT
IL-10	ACTGCACCCACTTCCCAGT	ATGTTGTCCAGCTGGTCCTT
IL-1 β	TTGACGGACCCAAAAGATG	AGAAGGTGCTCATGTCCTCA

IL: Interleukin; TNF: Tumor necrosis factor.

immunohistochemistry. After incubating in xylene to dewax and in ethanol to rehydrate, tissue section was incubated with 0.3% hydrogen peroxide in methanol to block endogenous peroxidase activity, followed by visualizing antigen with heat-mediation. After blocking slides with 3% goat serum at room temperature for 20 min to prevent nonspecific staining, the section was treated with mouse anti-mast cell tryptase antibody (1:20000, Abcam, Cambridge, United Kingdom) for 1 hour at room temperature. After washing, the section was treated with HRP-labeled goat anti-mouse IgG (Zhongshan Gold Bridge, Beijing, China) for 30 min. Diaminobenzidine (DAB kit, Zhongshan Gold Bridge, Beijing, China) and hematoxylin were used to visualize the reaction. Four to five non-overlapping fields were randomly selected. The number of mucosal mast cells was counted under a light microscope (400 \times magnification, Leica Company, Wetzlar, Germany) by two independent observers and is expressed as cells per high power field (HPF).

Statistical analysis

Data are presented as mean \pm SD or median with 5th and 95th percentiles. Differences between two groups were determined by Student's *t*-test for normally distributed data or Wilcoxon two-sample otherwise. Comparisons among three or more groups were performed by one-way analysis of variance (ANOVA) for normally distributed data or Kruskal-Wallis one-way ANOVA for non-normally distributed data. Rate of weight gain was analyzed by repeated measures ANOVA using the factors of WAS administration and time. Statistical analyses were conducted using SPSS (IBM, Armonk, NY, United States; version 22) and Graphpad Prism (GraphPad Software, San Diego, CA, United States; version 6.0). A two-tailed *P*-value < 0.05 was considered statistically significant. The statistical methods of this study were reviewed by Professor Yunxian Yu from Department of Epidemiology and Health Statistics of Zhejiang University.

RESULTS

WAS-induced visceral hypersensitivity in the IBS mouse model

Of the 32 randomized mice, five (one in the sham-

WAS + saline group, one in the sham-WAS + FOS group, two in the WAS + saline group, and one in the WAS + FOS group) died due to gavage trauma and were excluded from the outcome analysis.

During the first 10 d, mice receiving WAS had lower rate of weight gain compared to mice receiving sham-WAS (Figure 1A). No difference in quantity of consumed feed were observed between mice receiving WAS or sham-WAS. Mice subjected to WAS had higher mean abdominal withdrawal reflex scores at colorectal distention pressures of 20, 40, 60, and 80 mmHg compared to mice subjected to sham-WAS (Figure 1B). Furthermore, mice subjected to WAS had lower pain and volume thresholds compared to mice subjected to sham-WAS (Figure 1C and D).

FOS intensifies WAS-induced visceral hypersensitivity

Among mice subjected to WAS, mice that received FOS administration for 14 d had higher mean abdominal withdrawal reflex scores at a colorectal distention pressure of 20 mmHg compared to those receiving saline administration (Figure 2A). Furthermore, mice that received FOS administration had lower pain and volume thresholds compared with those receiving saline administration following WAS (Figure 2B and C). However, no difference in mean abdominal withdrawal reflex scores, pain thresholds, or volume thresholds was observed between mice administered with FOS or saline following sham-WAS.

FOS has no effect on intestinal histological score

No difference in neutrophil counts or degree of interstitial edema in the lamina propria was observed between the WAS and sham-WAS groups (Figure 3A and C). Furthermore, no difference in histologic score was observed among all four groups (sham-WAS + saline, sham-WAS + FOS, WAS + saline, WAS + FOS) at 14 d of the experiment (Figure 3B and D).

FOS increases cecal SCFA concentrations following WAS

No difference in levels of SCFA in the cecum was found between mice subjected to WAS and sham-WAS that received saline administration. Among mice subjected to WAS, mice administered with FOS had higher mean

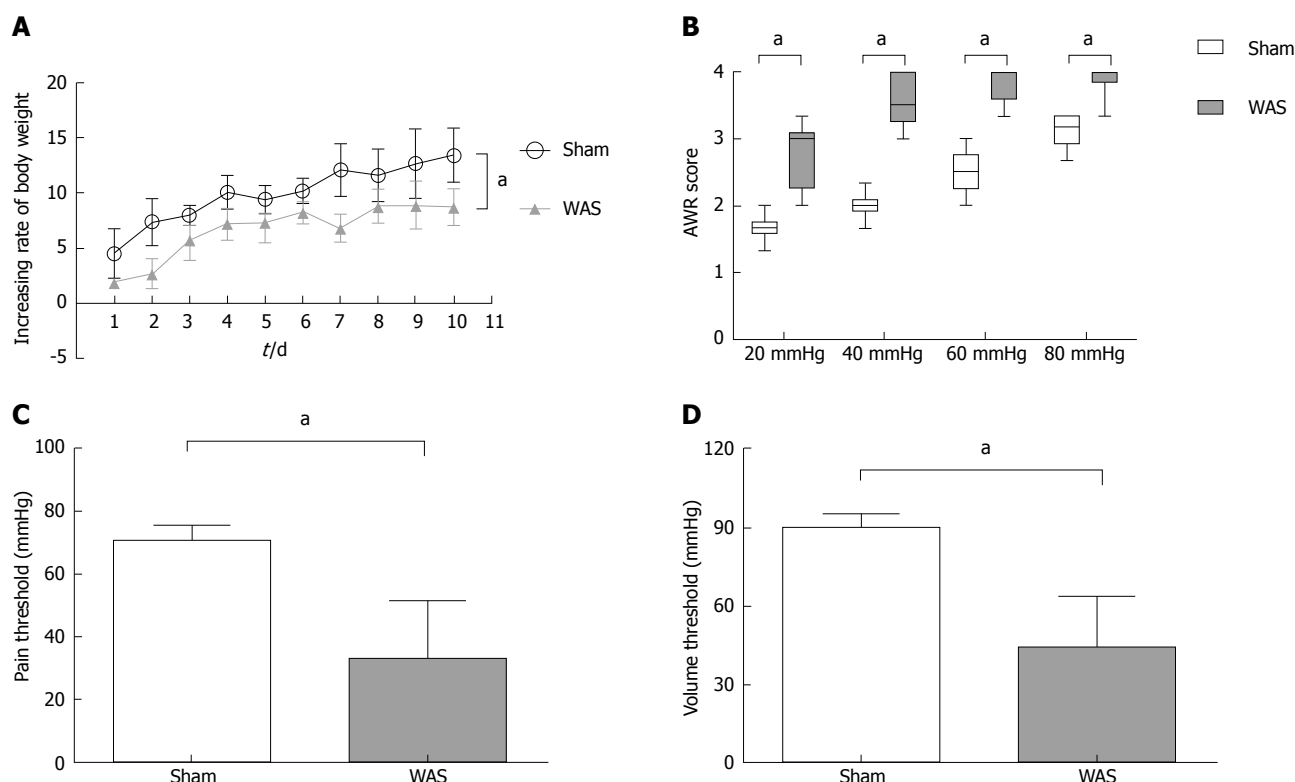


Figure 1 Effect of water avoidance stress on rate of weight gain and visceral sensitivity. **A:** Rate of weight gain (g) was lower in the water avoidance stress (WAS) group ($n = 13$) compared to the sham-WAS ($n = 14$) group. Values represent mean weight gain \pm SD, repeated analysis of variance (ANOVA); **B:** Abdominal withdrawal reflex (AWR) scores in response to colorectal distension were increased in the WAS group compared to the sham-WAS group. Lines within the box represent the median value, ends of the box represent 25th and 75th percentiles, and the error bars represent 5th and 95th percentiles. Wilcoxon two-sample test; **C:** Pain threshold was decreased in the WAS group compared to the sham-WAS group. Values represent mean \pm SD, Student's *t*-test; **D:** Volume threshold was decreased in the WAS group compared to the sham-WAS group. Values represent means values \pm SD, Student's *t*-test. ^a $P < 0.05$, Sham vs WAS.

concentrations of acetic acid (2.49 ± 0.63 mmol/L vs 1.49 ± 0.72 mmol/L, $P < 0.01$, one-way ANOVA), propionic acid (0.48 ± 0.09 mmol/L vs 0.36 ± 0.05 mmol/L, $P < 0.01$, one-way ANOVA), butyric acid (0.28 ± 0.09 mmol/L vs 0.19 ± 0.003 mmol/L, $P < 0.05$, one-way ANOVA), and total SCFA (3.62 ± 0.87 mmol/L vs 2.27 ± 0.75 mmol/L, $P < 0.01$, one-way ANOVA) measured in the cecum compared to the mice administered with saline for 14 d (Figure 4). However, among mice subjected to sham-WAS, no difference in concentrations of individual or total SCFA was found between those administered with FOS or saline for 14 d.

FOS-mediated intestinal cytokine expression following WAS

Mice subjected to WAS had higher expression of IL-6 (8.25 ± 3.95 vs 1.86 ± 1.66 , $P < 0.01$, one-way ANOVA) and TNF- α (2.05 ± 1.73 vs 0.56 ± 0.28 , $P < 0.05$, one-way ANOVA) mRNA in the ileal specimen, as well as higher IL-6 (1.60 ± 1.10 vs 0.46 ± 0.29 , $P < 0.05$, one-way ANOVA) and IL-1 β (0.88 ± 0.53 vs 0.34 ± 0.35 , $P < 0.05$, one-way ANOVA) mRNA expression in the colonic specimen compared to those receiving sham-WAS (Figure 5). Among mice subjected to WAS, mice administered with FOS for 14 d

had higher expression of IL-23 mRNA (4.71 ± 4.16 vs 1.00 ± 0.99 , $P < 0.05$, one-way ANOVA) in the ileum and IL-1 β mRNA (2.15 ± 1.68 vs 0.88 ± 0.53 , $P < 0.05$, one-way ANOVA) in the colon compared to the mice administered with saline. However, among mice subjected to sham-WAS, no difference in IL-6, IL-23, TNF- α , IL-10, or IL-1 β mRNA expression in the ileum or colon was found between mice administered with FOS or saline for 14 d.

FOS increases the mucosal mast cell counts following WAS

Mice subjected to WAS had higher mean mast cell counts in the ileum (8.3 ± 3.6 per HPF vs 4.9 ± 1.4 per HPF, $P < 0.05$, one-way ANOVA) and colon (3.4 ± 1.2 per HPF vs 1.8 ± 1.5 per HPF, $P < 0.05$, one-way ANOVA) compared to those subjected to sham-WAS (Figure 6). Among mice subjected to WAS, mice administered with FOS for 14 d had greater mast cell infiltration in the ileum (12.3 ± 2.6 per HPF vs 8.3 ± 3.6 per HPF, $P < 0.05$, one-way ANOVA) and colon (6.3 ± 3.2 per HPF vs 3.4 ± 1.2 per HPF, $P < 0.05$, one-way ANOVA) compared to the mice administered with saline. However, among mice subjected to sham-WAS, no difference in mast cell infiltration in the ileum or

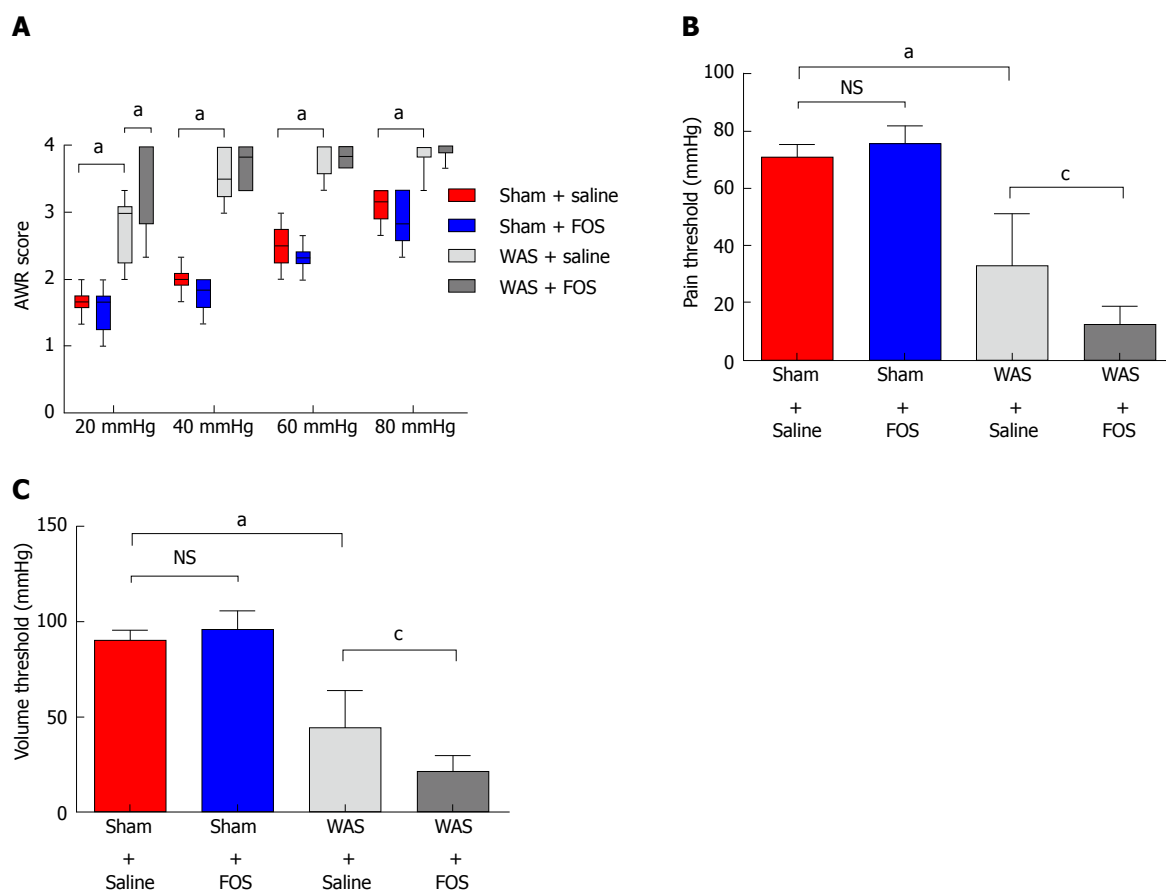


Figure 2 Effect of oral gavage of fructo-oligosaccharide on visceral sensitivity. A: Fructo-oligosaccharide (FOS) increased abdominal withdrawal reflex (AWR) scores in response to colorectal distension compared to saline administration following water avoidance stress (WAS). Values represent median, 25th, 75th, 5th, and 95th percentiles. Sham + saline ($n = 7$), sham + FOS ($n = 7$), WAS + saline ($n = 6$), WAS + FOS ($n = 7$). Kruskal-Wallis one-way ANOVA; B: Pain threshold decreased in FOS-administered compared to saline-administered mice following WAS. Values represent mean \pm SD, one-way ANOVA; C: Volume threshold decreased in FOS compared to saline-administered mice following WAS. Values represent mean \pm SD, one-way ANOVA. ^a $P < 0.05$, sham + saline vs WAS + saline; ^c $P < 0.05$, WAS + saline vs WAS + FOS.

colon was observed between mice administered with FOS or saline for 14 d.

DISCUSSION

We evaluated the effects of administration of high-dose FOS, a component of FODMAP, on visceral sensitivity and gut inflammation using a stress-induced IBS mouse model. Mice subjected to WAS exhibited visceral hypersensitivity and low-grade inflammation demonstrated by higher mucosal expression of pro-inflammatory cytokines and increased number of intestinal mast cells. Among mice subjected to WAS, FOS administration further intensified visceral hypersensitivity and also led to higher intestinal expression of IL-23 and IL-1 β with increasing mucosal mast cell counts. Furthermore, FOS administration in mice subjected to WAS led to higher intestinal production of individual (acetic, propionic, and butyric acids) as well as total SCFA. However, FOS administration did not affect visceral sensitivity, intestinal inflammation, or intestinal SCFA production in control mice.

The effect of psychological stress as an inciting and/or exacerbating factor on altered brain-gut axis is central to the pathophysiology of IBS. In our study, mice subjected to WAS demonstrated visceral hypersensitivity and low-grade immune activation, characterized by increased expression of pro-inflammatory cytokines and mucosal mast cell infiltration yet without overt difference in intestinal histological scores compared to the control mice. These findings are consistent with prior studies that demonstrated the effects of stress on visceral hypersensitivity and intestinal immune activation in rodents^[15,20]. Therefore, a WAS-induced IBS mouse model was used to study the effects of FOS administration on visceral sensitivity and mucosal inflammation typical in IBS.

Although the role of food intolerance-induced IBS symptoms has been long recognized, correlations with a specific food group have been difficult to demonstrate^[21,22]. A key observation in our study is that FOS consumption further intensified visceral hypersensitivity already present in mice subjected to WAS. This result is consistent with the clinical studies that demonstrated adverse effects of high

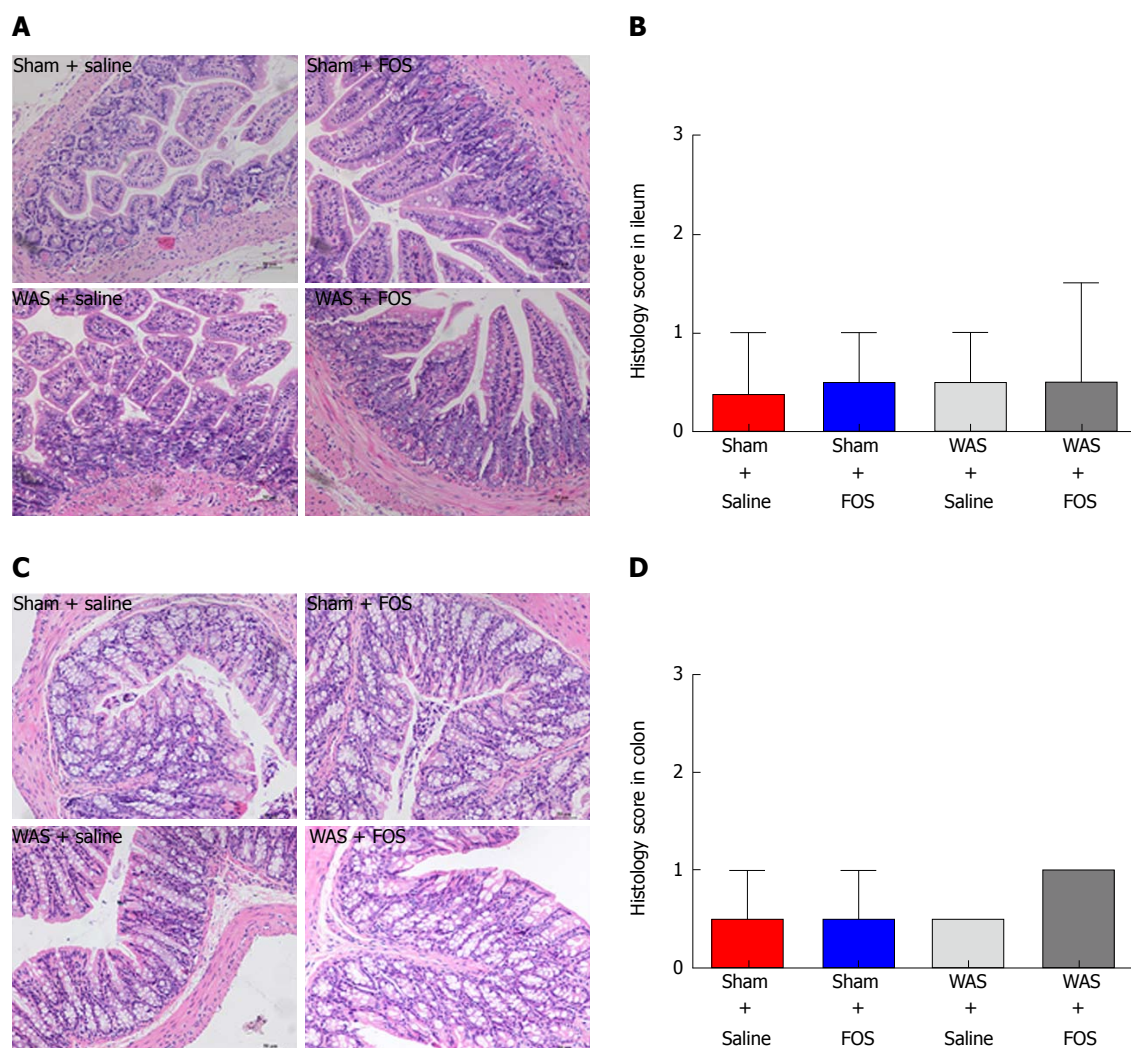


Figure 3 Effect of oral gavage of fructo-oligosaccharide on intestinal histological scores. A: Ileal tissues stained with hematoxylin-eosin (HE) for evaluation of inflammation score (magnification, 200 \times); B: No difference in structural histology among the four groups; C: Colonic tissues stained with H&E for evaluation of inflammation score (magnification, 200 \times); D: No difference in structural histology among the four groups. Values represent median with 5th and 95th percentiles; sham + saline ($n = 7$), sham + FOS ($n = 7$), WAS + saline ($n = 6$), WAS + FOS ($n = 7$); Kruskal-Wallis one-way ANOVA. FOS: Fructo-oligosaccharide; WAS: Water avoidance stress.

FODMAP diet as an individual component or as an aggregate in exacerbating gastrointestinal symptoms in IBS^[6,8,23,24]. Along the same vein, our findings are concordant with studies that demonstrated the efficacy of dietary restriction of FODMAP in improving gastrointestinal symptoms, such as abdominal pain, diarrhea, bloating, flatulence, and quality of life in IBS patients^[7,9,25]. Interestingly, FOS had no effect on visceral sensitivity in mice exposed to sham-WAS. Prior studies also demonstrated that high FODMAP diet-induced gastrointestinal symptoms in IBS patients but not in healthy volunteers except increased flatus^[6,8]. Our findings highlight the direct effects of FODMAP on visceral hypersensitivity as a mechanism of FODMAP-induced IBS symptoms other than proposed mechanisms such as osmotic effects of poorly absorbed carbohydrates and increased colonic gas production from intestinal fermentation. A recent study indicated that hypersensitivity to colorectal distension, rather

than excessive gas fermented by FODMAP, was the primary factor contributing to IBS symptoms^[26]. Our finding that FOS consumption increased visceral hypersensitivity in the IBS mouse model, but not in control mice, suggests that stress-induced visceral hyperalgesia is a prerequisite for FODMAP-induced visceral hypersensitivity. Similarly, anxiety was demonstrated to be a robust predictor of inducing abdominal symptoms after ingestion of lactose, another FODMAP component, in a previous study among patients with IBS^[27].

SCFA are byproducts of FODMAP fermentation. For example, IBS patients on a low FODMAP diet have altered fecal fermentation producing lower levels of stool SCFA including acetic acid and butyric acid^[28-30]. Our study showed that high-dose FOS administration increased production of individual (acetic, propionic, and butyric acids) and total SCFA, which was also associated with increased visceral hypersensitivity

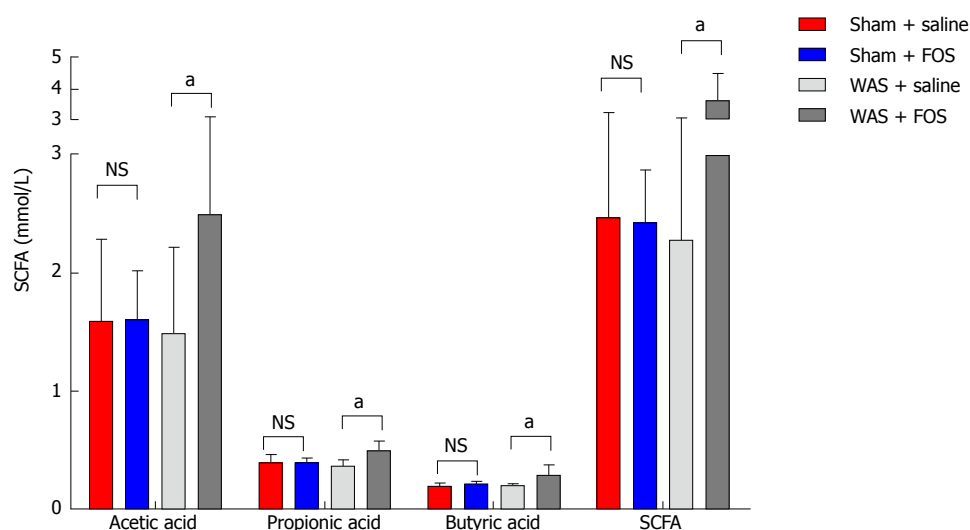


Figure 4 Effect of oral gavage of fructo-oligosaccharide on short chain fatty acid concentrations. The average concentrations of total SCFA, acetic, propionic, and butyric acids increased in FOS-administered mice compared to saline-administered mice following WAS intervention. No difference was observed in total SCFA, acetic, propionic, and butyric acid levels with FOS or saline administration in mice following sham-WAS. Values represent mean \pm SD; sham + saline ($n = 7$), sham + FOS ($n = 7$), WAS + saline ($n = 6$), WAS + FOS ($n = 7$); one-way ANOVA. * $P < 0.05$, WAS + saline vs WAS + FOS. SCFA: Short chain fatty acids; FOS: Fructo-oligosaccharide; WAS: Water avoidance stress.

and intestinal inflammation already present in the IBS mouse model. Although inconsistent effects, SCFA clearly play a role in the regulation of visceral pain and intestinal immune activation. For example, butyric acid reduced visceral pain in humans, but induced visceral hypersensitivity in rats^[31]. Intracolonic infusion of 0.5% acetic acid led to visceral hypersensitivity in rats^[32]. In addition, SCFA may also act as pro-inflammatory substrates to induce immune responses^[33], but in others cases, exert anti-inflammatory properties^[34]. SCFA inhibited regulatory T cell differentiation and suppressed IL-10 expression in IBS^[35]. However, butyric acid exacerbated dextran sodium sulfate-induced colitis in a murine model and increased IL-23 production by stimulating dendritic cells^[36].

Interestingly, administration of FOS in control mice did not increase the levels of individual or total SCFA production, highlighting the difference in fermentation of FOS between stressed and sham-stressed conditions. Stress-induced alteration in microbiota may lead to the change of fermentation products^[16]. Alternatively, stress-induced release of corticotropin-releasing hormone may accelerate intestinal transit, reducing absorption of SCFA^[37]. However, SCFA production was comparable between mice subjected to WAS or sham-WAS in the absence of FOS administration. Although studies have generally reported higher stool concentrations of SCFA in IBS patients, some have demonstrated similar SCFA levels in IBS and non-IBS patients, likely explained by a lack of rigorous control of dietary factors^[14,38,39]. In our study, feed void of FODMAP content as an essential substrate for SCFA may account for the lack of difference in SCFA production between WAS and sham-WAS group despite possible difference in fermentation capacity of the two

groups.

In our study, FOS administration in mice subjected to WAS was associated with low-grade inflammation, which is consistent with prior studies on IBS. FOS administration increased the expression of pro-inflammatory cytokines, such as IL-23 in the ileum and IL-1 β in the colon, following WAS. Specifically, IL-23 is important in regulating intestinal inflammation by activating lymphocytes, as well as inducing and promoting release of other inflammatory mediators. Although FOS administration exerted anti-inflammatory effects in some studies^[40], others have also demonstrated that FOS administration induced pro-inflammatory cytokine profile, including elevated IL-10 and a reduction in IL-6, typically observed in active Crohn's disease^[41]. Given the pivotal role of low-grade mucosal inflammation as a trigger of IBS symptoms^[42-44], the increased pro-inflammatory cytokines may have played a role in worsening visceral hypersensitivity in FOS-administered mice following WAS. In addition to increased production of pro-inflammatory cytokines, mice subjected to WAS had further increased mucosal mast infiltration with FOS. Our findings are in line with a study that demonstrated an eight-fold reduction of urinary histamine, a measure of mast cell activation, among IBS patients receiving a low compared to high FODMAP diet^[45]. Mast cells play an important role in mucosal immune activation in IBS by releasing a variety of pro-inflammatory mediators^[46]. For example, tryptase released by mast cells can activate protease-activated receptor-2, which is important in inducing visceral hypersensitivity^[47]. In addition to mucosal mast cell activation by WAS, FOS-induced SCFA production may also contribute to further recruitment of mucosal mast cells and secretion

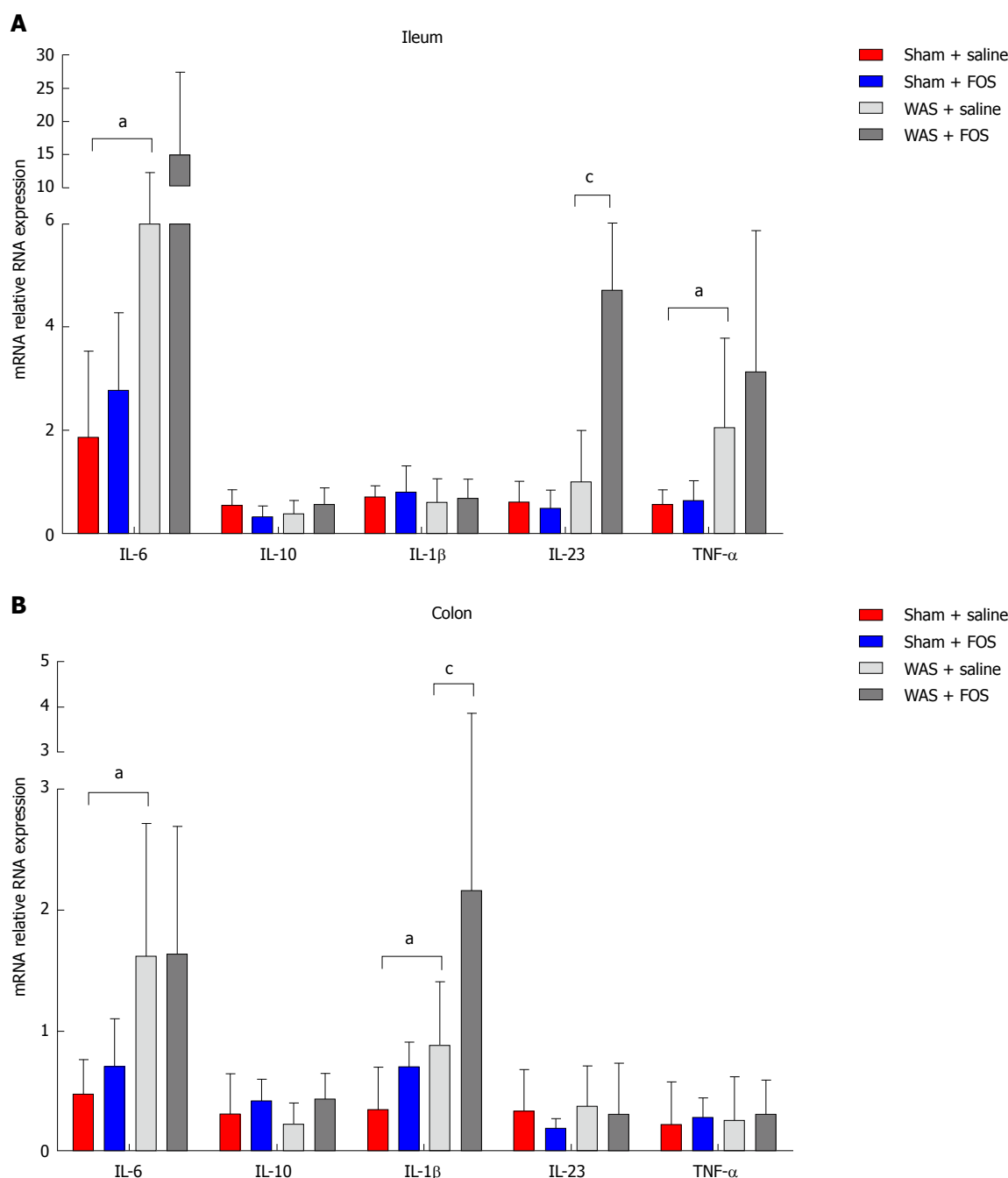


Figure 5 Effect of oral gavage of fructo-oligosaccharide on intestinal cytokine expression. A: Among saline-administered mice, IL-6 and TNF- α expressions increased in the ileum in the WAS group compared to the sham-WAS group. IL-23 expression increased in FOS compared to saline-administered mice following WAS; B: In saline-administered mice, colonic IL-6 and IL-1 β expression increased in the WAS group compared to the sham-WAS group. IL-1 β expression increased in FOS compared to saline-administered mice in the WAS group. Values represent mean \pm SD; sham + saline ($n = 7$), sham + FOS ($n = 7$), WAS + saline ($n = 6$), WAS + FOS ($n = 7$); one-way ANOVA. ^a $P < 0.05$, sham + saline vs WAS + saline; ^c $P < 0.05$, WAS + saline vs WAS + FOS. FOS: Fructo-oligosaccharide; WAS: Water avoidance stress; IL: Interleukin; TNF: Tumor necrosis factor.

of histamine^[48,49].

Our study has several limitations. First, FOS is only one component of FODMAP that was studied, and effects of the other FODMAP components on visceral hypersensitivity and immune activation are unknown. Second, although our study demonstrated the effects of FOS on stress-induced visceral hypersensitivity and intestinal inflammation, detailed mechanism was beyond the scope of the study and will be invaluable

in future studies. Finally, although the WAS-induced mouse model exhibited visceral hypersensitivity and low-grade inflammation, experimental models are not able to fully encompass the complex biopsychosocial components of IBS, and our findings should be interpreted with caution.

In conclusion, administration of FOS, a component of FODMAP, intensified visceral hypersensitivity and gut inflammation in the stressed-induced IBS mice, but

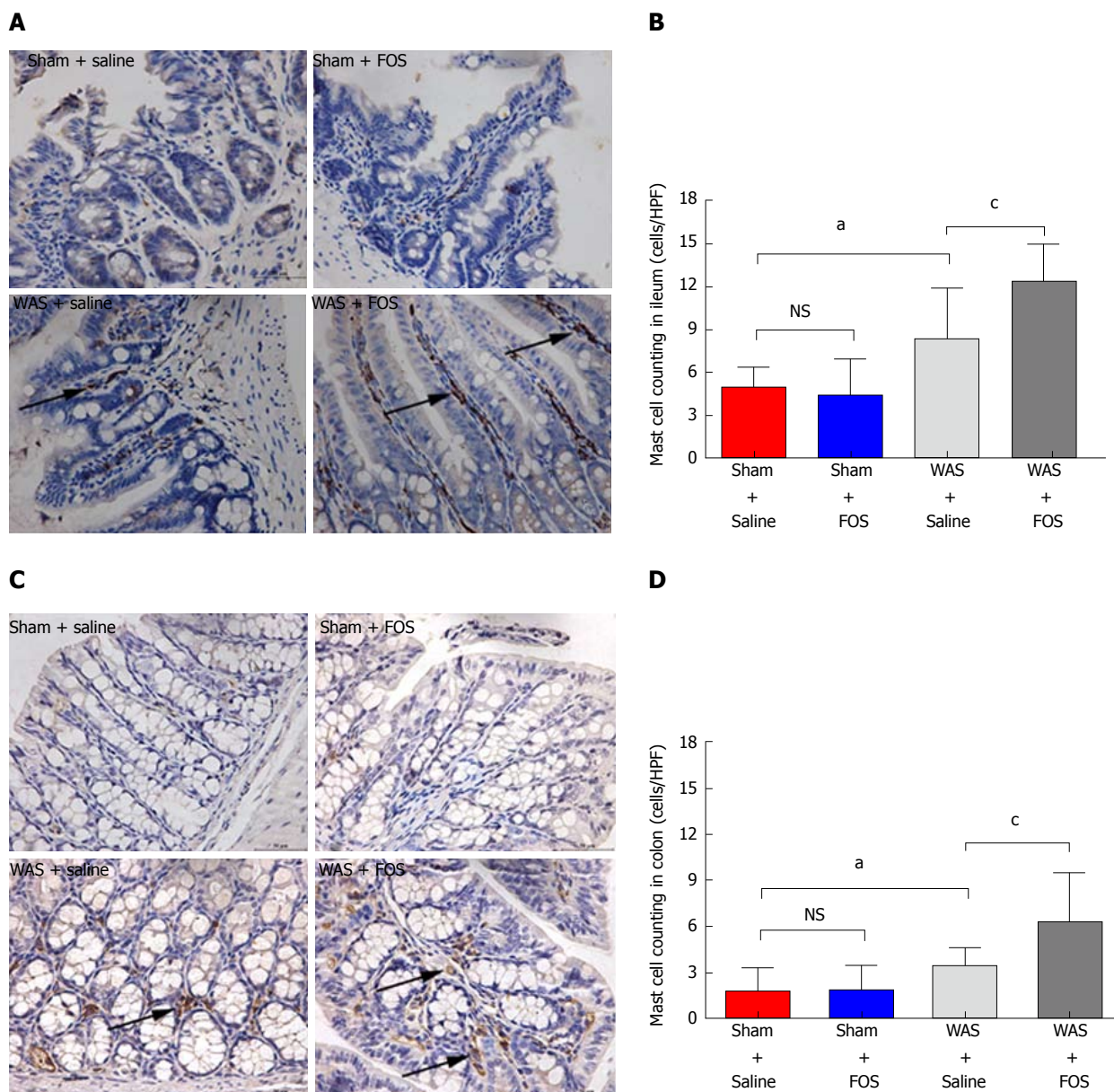


Figure 6 Effect of oral gavage of fructo-oligosaccharide on the number of mucosal mast cells (arrows). A: Ileal tissues stained with mast cell tryptase (magnification, 400 ×); B: In saline administered mice, mast cell counts increased in WAS compared to the sham-WAS group. Mast cell counts increased in FOS compared to saline administered mice following WAS; C: Colon stained with mast cell tryptase (magnification, 400 ×); D: In saline-administered mice, mast cell count increased in the WAS group compared to the sham-WAS group. Mast cell count increased in FOS compared to saline-administered mice following WAS. Values represent mean ± SD; sham + saline ($n = 7$), sham + FOS ($n = 7$), WAS + saline ($n = 6$), WAS + FOS ($n = 7$); one-way ANOVA. ^a $P < 0.05$, sham + saline vs WAS + saline; ^c $P < 0.05$, WAS + saline vs WAS + FOS. FOS: Fructo-oligosaccharide; WAS: Water avoidance stress.

not in the control mice. A parallel increased production of intestinal SCFA was also observed with FOS administration in the IBS mice but not in the control mice. Our findings suggest a mechanism of FODMAP-induced gastrointestinal symptoms specific to IBS.

ARTICLE HIGHLIGHTS

Research background

The impact of dietary factors in exacerbating symptoms of irritable bowel syndrome (IBS) is being increasingly recognized. Specifically, abdominal pain following the consumption of Fermentable Oligosaccharides, Disaccharides, Monosaccharides, and Polyols (FODMAP) is common, and dietary restriction of

FODMAP improves symptoms of IBS.

Research motivation

Although osmotic effects of poorly absorbed carbohydrates and increased colonic gas production from intestinal fermentation are proposed, evidence providing specific mechanism of FODMAP-induced IBS symptoms is sparse. With wide acceptance of low-FODMAP diet as a treatment for IBS, clarifying the specific mechanism is important for optimal application in clinical practice.

Research objectives

The aim of the study was to explore the effects of high-dose fructo-oligosaccharides (FOS), a component of FODMAP, on visceral sensitivity, inflammation, and production of intestinal short-chain fatty acids (SCFA) using an IBS mouse model. FOS administration intensified visceral hypersensitivity

and gut inflammation already present in the stress-induced IBS mice, but not in the control mice, and was also associated with increased cecal SCFA production. The results provide a biologic framework for FODMAP-induced IBS symptoms that supports the application of low FODMAP therapy in clinical practice.

Research methods

The effects of FOS on visceral sensitivity, SCFA production, and intestinal inflammation were examined by using a water avoidance stress (WAS)-induced IBS mouse model. Mice were randomly assigned to receive daily WAS or sham-WAS for 10 d while receiving daily oral gavage of saline solution with or without high-dose FOS. After 2 wk, visceral sensitivity was measured by abdominal withdrawal reflex in response to colorectal distension and mucosal inflammation was measured by histologic analyses. Furthermore, intestinal SCFA production, cytokine expression, and mast cell counts were evaluated.

Research results

FOS administration intensified visceral hypersensitivity, increased mucosal mast cell counts, and mediated intestinal cytokine expression in the stressed-induced IBS mice, but not in the control mice. A parallel increase in cecal SCFA levels was also observed with FOS administration in the IBS mice but not in the control mice. These findings suggest that visceral hypersensitivity and gut inflammation intensified by FODMAP diet may lead to worsening IBS symptoms. Examining the effects of other FODMAP components other than FOS on visceral hypersensitivity and immune activation, as well as, detailed molecular mechanism may be invaluable in future studies.

Research conclusions

Administration of high-dose FOS, a component of FODMAP, intensified visceral sensitivity and intestinal inflammation in a stress-induced IBS mouse model, and was also associated with increased production of SCFA. These findings suggest a mechanism of FODMAP-induced gastrointestinal symptoms specific to IBS and are consistent with clinical studies that demonstrate the efficacy of low-FODMAP diet in treatment of individuals with IBS.

Research perspectives

The importance of dietary factors in triggering symptoms is increasingly being recognized in patients with IBS. FOS administration intensifies visceral hypersensitivity and gut inflammation in stress-induced IBS mice, and is also associated with increased intestinal SCFA production.

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Basic Study

Morin enhances hepatic Nrf2 expression in a liver fibrosis rat model

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Abstract

AIM

To investigate whether morin can reduce hepatic fibrosis by activating the NF-E2-related factor 2 (Nrf2) signaling pathway.

METHODS

Twenty male Sprague-Dawley rats were randomly divided into four groups: control group, morin group, carbon tetrachloride (CCl₄) group, and morin + CCl₄ group. Rats in both the CCl₄ and morin + CCl₄ groups were injected intraperitoneally with CCl₄ at a dose of 2 mL/kg twice a week. Rats in both the morin and morin + CCl₄ groups were treated orally with morin at a dose of 50 mg/kg twice a week. Control rats were treated with vehicle only twice a week. At the end-point of the 8 wk of the experimental period, serum AST, ALT, and ALP were measured, and the liver specimens

were obtained for pathological assessment. Real-time PCR and Western blot methods were used to analyze the expression of α -smooth muscle actin (α -SMA), collagen I, collagen III, Nrf2, heme oxygenase (HO-1), and quinone oxidoreductase 1 (NQO1) using frozen liver specimens.

RESULTS

Morin-treated rats in the morin + CCl₄ group had less hyperplasia of fiber tissue, minimal inflammatory cells, and less body weight loss with favorable liver enzyme measurements compared to rats treated with CCl₄ only. Additionally, morin-treated rats had significantly lower mRNA and protein expression of α -SMA, collagen I, and collagen III, but significantly higher mRNA and protein expression of Nrf2, HO-1, and NQO1 compared to rats treated with CCl₄ only ($P < 0.05$).

CONCLUSION

Morin could play a protective role by inducing the expression of Nrf2 and its downstream antioxidant factors (HO-1 and NQO1) and reducing the expression of α -SMA, collagen I, and collagen III in CCl₄-induced liver fibrosis rats.

Key words: Liver fibrosis; Rat; Morin; Nrf2

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Core tip: We constructed a liver fibrosis rat model with carbon tetrachloride (CCl₄). The Sprague-Dawley rats were randomly divided into four groups: control group, morin group, CCl₄ group, and morin + CCl₄ group. α -SMA, collagen I, collagen III, NF-E2-related factor 2 (Nrf2), heme oxygenase (HO-1), and quinone oxidoreductase 1 (NQO1) were analyzed by real-time PCR and Western blot methods using frozen liver specimens. We found that morin could reduce hepatic fibrosis by inducing the expression of Nrf2 and its downstream antioxidant factors in the CCl₄-induced rat liver fibrosis model.

Sang L, Wang XM, Xu DY, Sang LX, Han Y, Jiang LY. Morin enhances hepatic Nrf2 expression in a liver fibrosis rat model. *World J Gastroenterol* 2017; 23(47): 8334-8344 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v23/i47/8334.htm> DOI: <http://dx.doi.org/10.3748/wjg.v23.i47.8334>

INTRODUCTION

Hepatic fibrosis refers to a series of pathogenic factors and pathological changes in the pathogenesis of a variety of liver diseases with liver extracellular matrix (ECM) metabolic abnormalities^[1]. Previous studies have found that the development and progression of liver fibrosis are significantly related to oxidative

stress in which a large number of free radicals lead to cell metabolic disorders and subsequent destruction of normal liver cells^[2-5]. Although there is currently no effective therapy for curing liver fibrosis, previous studies showed that the pathological changes in liver fibrosis could be reversed^[6,7].

Oxidative stress is closely related to the occurrence of liver disease^[8]. A large number of studies have shown that oxidative stress may promote the activation of hepatic satellite cells (HSCs) and increase collagen production^[9]. In the past decade, numerous studies proved that NF-E2-related factor 2 (Nrf2) plays a role as an important transcription factor in normal liver cells, and its activation could increase the expression of the downstream specific genes, such as the quinone oxidoreductase 1 (NQO1), heme oxygenase (HO-1), and glutathione, which play a role against oxidative stress^[10,11]. Studies have shown that Nrf2 activation could resist oxidative stress caused by hepatic ischemia and injury, liver fibrosis, and drug-induced liver damage^[12-15].

Flavonoids are rich in a variety of fruits, vegetables, and components of herbal-containing dietary agents and play an important role in preventing many kinds of diseases. Morin (3, 5, 7, 2', 4'-pentahydroxyflavone) is a kind of flavonoid that consists of a yellowish pigment found in onion and apple^[16], almond (P. guajava L.)^[17], fig (*Chlorophora tinctoria*)^[18], and other moraceae, including in food and herbal medicines^[19] (Figure 1). It has been shown that morin possesses biological properties, including antioxidant^[20,21], anti-inflammatory^[22], anti-apoptosis^[23,24], and anticancer^[19] activities. Morin also protects various human cells, such as myoblasts^[25], hepatocytes^[26], and erythrocytes, against oxidative damages^[27].

Carbon tetrachloride (CCl₄) intraperitoneal injection is a classical method for establishing an animal model of hepatic fibrosis, and the toxicity of CCl₄ leads to liver cell necrosis and mitochondrial damage along with aggravating oxidative stress. In addition, the abundant release of inflammatory and fibrogenic cytokines induced by CCl₄ could further augment the degree of hepatic fibrosis^[28]. A previous study demonstrated that morin protected against acute liver damage^[29] and ameliorated liver fibrosis^[20] induced by CCl₄, where morin inhibited proliferation and induced apoptosis of activated HSCs by suppressing the Wnt/ β -catenin and NF- κ B signaling pathways. However, there is no molecular evidence of the effects of morin on the Nrf2 signaling pathway. To our knowledge, *in vivo* investigation of the effect of morin on the Nrf2 signaling pathway and Nrf2 expression in the CCl₄-induced liver fibrosis model has not been reported. The purpose of this study was to investigate whether morin could reduce hepatic fibrosis by inducing the expression of Nrf2 and its downstream antioxidant enzymes using pathology as a gold standard in a rat

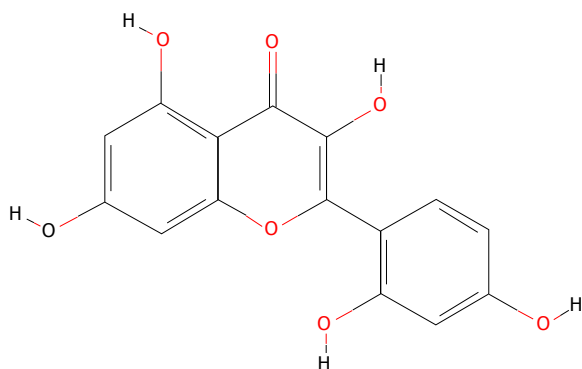


Figure 1 Chemical structure of morin. (<https://pubchem.ncbi.nlm.nih.gov/compound/morin>).

model of CCl₄-induced hepatic fibrosis.

MATERIALS AND METHODS

Chemicals and reagents

The chemical agents used in this study included CCl₄ and olive oil (Sinopharm Chemical Reagent Co., Ltd, Shanghai, China) as well as morin (Sigma Chemical Co., St Louis, MO, United States). Serum aspartate transaminase (AST), alanine transaminase (ALT), and alkaline phosphatase (ALP) assay kits were purchased from Nanjing Jiancheng Bioengineering Institute (Nanjing, China). The antibodies against Nrf-2, HO-1, NQO1, collagen I, collagen III, and α -SMA were obtained from Proteintech Group Inc. (Chicago, IL, United States). All other reagents used were in the purest form available commercially.

Animals and experimental design

This study was performed in accordance with the Guide for Care and Use of Laboratory Animals published by the National Institutes of Health of China (Guide for the Care and Use of Laboratory Animals, 1996) and was approved by the Animal Care and Use Committee of China Medical University. Twenty male Sprague-Dawley rats with an average body weight of 200–220 g (Changsheng Biotechnology Co., Ltd, Liaoning, China) were used in this study. All rats were fed a standard laboratory diet for a week at room temperature (20–22 °C) with a light/dark cycle of 12 h. Then, the rats were randomly divided into four groups of five rats each, *i.e.*, control group, morin group, CCl₄ group, and morin + CCl₄ group. The control rats were treated with vehicle only (olive oil) equivalent to the treatment group. The rats in the morin group were treated with morin at a dose of 50 mg/kg (suspended in water as previously described^[30]) by oral administration and 2 mL/kg of olive oil by intraperitoneal injection twice a week. The rats in the CCl₄ group were injected intraperitoneally with CCl₄ at a dose of 2 mL/kg [mixed with olive oil (40%, V/V)] twice a week. The rats in the morin + CCl₄ group were treated with the same doses

of morin and CCl₄ *via* the same routes as the morin group and the CCl₄ group. Body weights of animals were recorded twice per week. After 8 wk of treatment, animals were kept fasting for 24 h. Under 10% chloral hydrate anesthesia, the following procedures were performed, including obtaining blood samples from the heart for biochemical tests and resecting the liver and spleen for histopathological analysis. Liver tissues were weighted and cut in 10 mm × 10 mm × 3 mm pieces. Half of the specimen was fixed in 10% formaldehyde for histopathology and the other half was immediately frozen in -80 °C for PCR and Western blot tests.

Biochemical analysis

The blood samples were centrifuged at 3000 *g* for 10 min at 20 °C, and the serum was collected from the supernatant. The values of AST, ALT, and ALP were measured using commercial assay kits according to the manufacturer's protocols.

Histopathological assessment

Specimens of the liver were embedded in paraffin and cut into 5- μ m-thick sections after 24 h of fixation. Then, the samples were stained with hematoxylin and eosin (HE). The degree of liver fibrosis was analyzed and determined by an experienced pathologist. The liver fibrosis was categorized into five degrees, *i.e.*, F0 = no fibrosis, F1 = portal fibrosis without septa, F2 = portal fibrosis with rare septa, F3 = numerous septa without cirrhosis, and F4 = cirrhosis according to reference criteria^[31].

Quantitative real-time PCR

Total cellular RNA was extracted from tissues using TRIzol (Invitrogen). Reverse transcription of 1 μ g of RNA was done using RT reagents (TAKARA) following the manufacturer's instructions. Quantitative real-time PCR was done using SYBR Green PCR master mix (Applied Biosystems) in a total volume of 20 μ L on the 7900HT fast Real-time PCR system (Applied Biosystems) using the following cycling parameters: 50 °C for 2 min, 95 °C for 10 min, and 40 cycles of 95 °C for 15 s and 60 °C for 60 s. A dissociation procedure was performed to generate a melting curve for confirmation of amplification specificity. GAPDH was used as the reference gene. The relative levels of gene expression were represented as $\Delta C_t = C_{t\text{gene}} - C_{t\text{reference}}$, and the fold change of gene expression was calculated by the $2^{-\Delta\Delta C_t}$ method. Experiments were repeated in triplicate. The primer sequences are listed in Table 1.

Western blot analysis

Total proteins from tissues were extracted in lysis buffer (Pierce, United States) and quantified using the Bradford method. A total of 40 μ g of protein were separated using 10% SDS-PAGE (80 V–120 V) and then electrophoretically transferred to a PVDF membrane

Table 1 Primer sequences

Name	Primer sequence
Rat Collagen I for	5'-ACTGGTACATCAGCCCAAAACCC-3'
Rat Collagen I rev	5'-GGAATCCATCGGTCATGCTCT-3'
Rat Collagen III for	5'-GAGACTCCCATCATAGATATCGC-3'
Rat Collagen III rev	5'-AGCAAACAGGGCCAATGTCC-3'
Rat α -SMA for	5'-GCTATGCTCTGCCTCATGCC-3'
Rat α -SMA rev	5'-CACGCTCAGCAGTAGTCACGAA-3'
Rat Nrf2 for	5'-ACACAGCATAGCCCATCTCGT-3'
Rat Nrf2 rev	5'-ACCAACCTGGATGAGCGACAC-3'
Rat NQO1 for	5'-CCACGCAGAGAGGACATCATT-3'
Rat NQO1 rev	5'-TTCGACCACCTCCCATCCTT-3'
Rat HO-1 for	5'-CTTCCCAGCATCGACAAC-3'
Rat HO-1 rev	5'-CTGTACCCCTGTGCTTGACC-3'
Rat Gapdh for	5'-GCTGGTCATCAACGGGAAA-3'
Rat Gapdh rev	5'-CGCCAGTAGACTCCACGACAT-3'

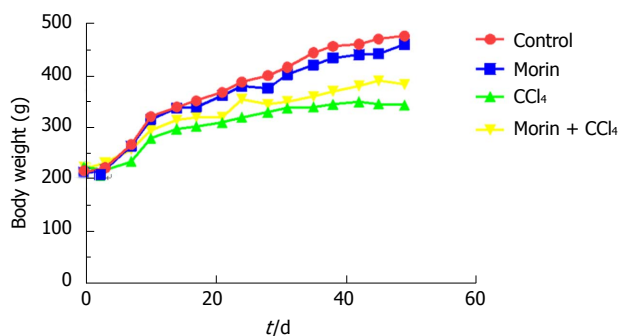


Figure 2 Changes in body weight among different groups. Body weight increased observably in the control and morin groups. The CCl₄ group had slow weight growth, but morin treatment was associated with increased body weight.

(80 V 100 min) (Millipore, Bedford, MA, United States). The membrane was blocked with 5% dry milk and incubated overnight at 4 °C with antibodies against HO-1 (1:800; Proteintech), NQO-1 (1:1000; Proteintech), Nrf2 (1:800; Proteintech), collagen I (1:800, Proteintech), collagen III (1:1000, Proteintech), α -SMA (1:1000, Proteintech), and GAPDH (1:4000, Proteintech). After washing, the membrane was incubated with a horseradish peroxidase-conjugated secondary antibody (Santa Cruz Biotechnology) at 37 °C for 2 h. Protein bands were visualized by enhanced chemiluminescence (Pierce) and detected using BioImaging Systems (UVP, Upland, CA, United States). The relative protein levels were calculated based on GAPDH protein as a loading control. Western blot images were measured with ImageJ software, and the relative gray values of protein expression were analyzed semi-quantitatively.

Statistical analysis

The experimental data are expressed as the mean \pm SD. Statistical analyses were performed using one-way analysis of variance (ANOVA) between groups, and unpaired comparisons were analyzed using the least significant difference method LSD *t*-test. A *P*-value of 0.05 or less was considered statistically significant.

All analyses were conducted using SPSS version 17.0 (SPSS, Inc., Chicago, IL, United States) and Prism GraphPad software Version 6.01 (GraphPad Software Inc., San Diego, CA, United States).

RESULTS

General observation

A total of four rats died before the end-point of the study, including two in the CCl₄ group, one in the morin + CCl₄ group, and one in the morin group. All animals in the control group survived. Normal diet and daily activities were recorded in the control and morin groups, with body weight increasing rapidly. The CCl₄ group presented poor feeding and daily activities with slow weight growth. The morin + CCl₄ group presented milder symptoms compared with the CCl₄ group, with increased body weight, which was, however, lower than that in the control and morin groups (Figure 2).

Histological changes in the liver

The results of HE staining showed that the liver cells appeared with a normal morphology and regular lobular structure in the control and morin groups. The liver tissue of CCl₄ group rats showed inflammatory cell infiltration, with portal and central veins surrounded by fibrous tissue accompanied by fibrous septa. The lobular structure was fuzzy with clearly visible false lobules. In the morin + CCl₄ group, the liver tissue demonstrated less hyperplasia of fiber tissue and minimal inflammatory cells compared to the CCl₄ group (Figure 3A-D).

Liver-spleen ratio and liver weight index

Both the CCl₄ and morin + CCl₄ groups had increased liver-spleen ratio (LSR) and liver weight index (LWI) compared with the control and morin groups (*P* < 0.05). The LWI between the CCl₄ and morin + CCl₄ groups showed a significant difference (*P* < 0.05), while no statistically significant difference was found for LSR (*P* > 0.05) (Table 2).

Biochemical findings

The CCl₄ and morin + CCl₄ groups had increased ALT, AST, and ALP levels compared to the control and morin groups (*P* < 0.05), and CCl₄ without morin treatment dramatically increased ALT, AST, and ALP values (Table 3).

mRNA expression of α -SMA, collagen I, collagen III, Nrf2, HO-1, and NQO1

Compared with the control and morin groups, significantly higher mRNA expression of α -SMA, collagen I, and collagen III was observed in liver tissues in the CCl₄ and morin + CCl₄ groups (*P* < 0.05). However, the mRNA expression of these molecules in the morin + CCl₄ group was significantly less than that in the CCl₄ group (*P* < 0.05) (Figure 4).

In the CCl₄ and morin + CCl₄ groups, mRNA expression

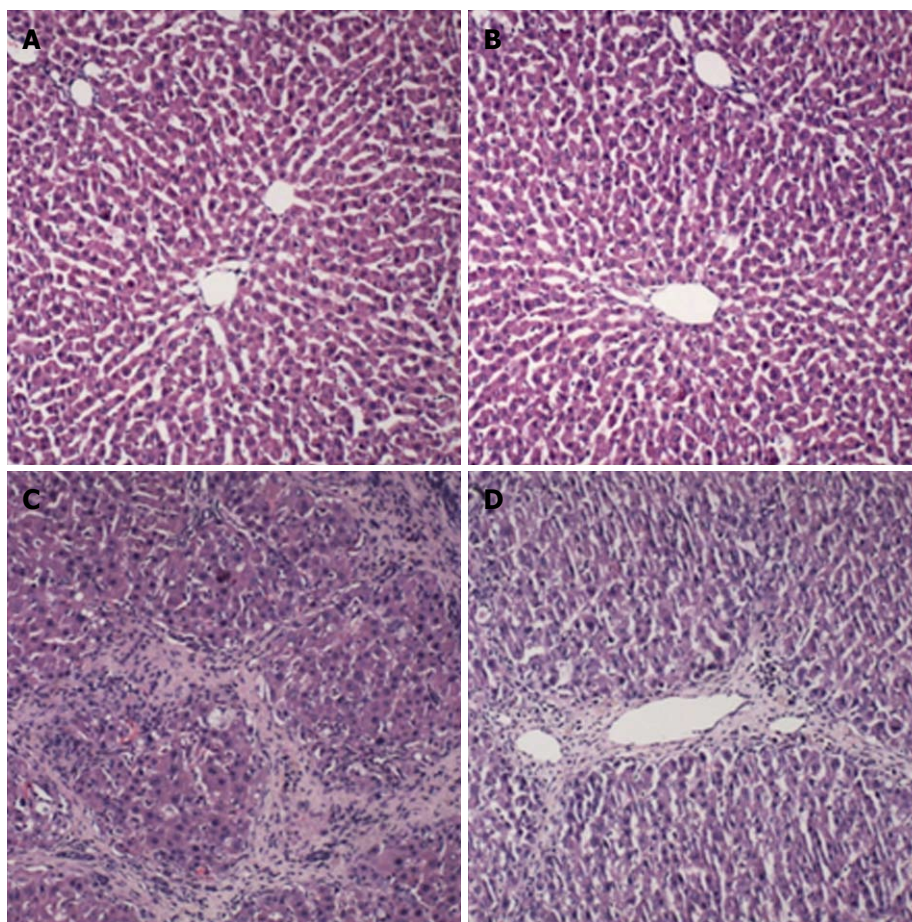


Figure 3 Histological changes of liver samples. A: Control group: treated with vehicle only; B: Morin group: treated with morin at a dose of 50 mg/kg twice a week; C: CCl₄ group: injected with CCl₄ at a dose of 2 mL/kg twice a week; D: Morin + CCl₄ group: treated with the same volume of morin and CCl₄ as the morin and CCl₄ groups. Liver tissues were stained with H&E ($\times 100$).

Table 2 Comparison of liver-spleen ratio and liver weight index among different groups

	Control (<i>n</i> = 5)	Morin (<i>n</i> = 4)	CCl ₄ (<i>n</i> = 3)	Morin + CCl ₄ (<i>n</i> = 4)	<i>F</i>	<i>P</i> value
LSR	12.27 \pm 1.92	12.67 \pm 1.60	16.43 \pm 1.37 ^{ac}	15.11 \pm 1.99 ^{ac}	4.668	0.022
LWI%	2.78 \pm 0.25	2.80 \pm 0.27	4.77 \pm 0.47 ^{ac}	4.17 \pm 0.39 ^{ace}	32.345	< 0.001

^a*P* < 0.05 *vs* control group, ^c*P* < 0.05 *vs* morin group, ^e*P* < 0.05 *vs* CCl₄ group. Liver-spleen ratio (LSR): Liver wet weight/spleen wet weight; liver weight index (LWI): (Liver wet weight/body weight) \times 100%.

Table 3 Serum parameters among different groups

	Control (<i>n</i> = 5)	Morin (<i>n</i> = 4)	CCl ₄ (<i>n</i> = 3)	Morin + CCl ₄ (<i>n</i> = 4)	<i>F</i>	<i>P</i> value
ALT (IU/L)	101.75 \pm 15.46	108.00 \pm 48.72	493.33 \pm 199.38 ^{ac}	291.50 \pm 111.92 ^{ace}	11.403	0.001
AST (IU/L)	339.25 \pm 72.59	257.80 \pm 98.22	1027.67 \pm 206.60 ^{ac}	585.50 \pm 131.85 ^{ace}	26.280	< 0.001
ALP (IU/L)	137.75 \pm 29.75	160.80 \pm 40.90	377.67 \pm 41.07 ^{ac}	266.50 \pm 58.90 ^{ace}	22.093	< 0.001

^a*P* < 0.05 *vs* control group, ^c*P* < 0.05 *vs* morin group, ^e*P* < 0.05 *vs* CCl₄ group.

values of *NQO1*, *HO-1*, and *Nrf2* were significantly higher than those in the control and morin groups (*P* < 0.05), while these mRNA values of the morin + CCl₄ rats were significantly different compared to those of the CCl₄ group (*P* < 0.05) (Figure 5).

Protein expression of α -SMA, collagen I, collagen III, Nrf2, HO-1, and NQO1

Compared with the control and morin groups, high expression of protein of α -SMA, collagen I, and collagen III in liver tissues in the CCl₄ and morin +

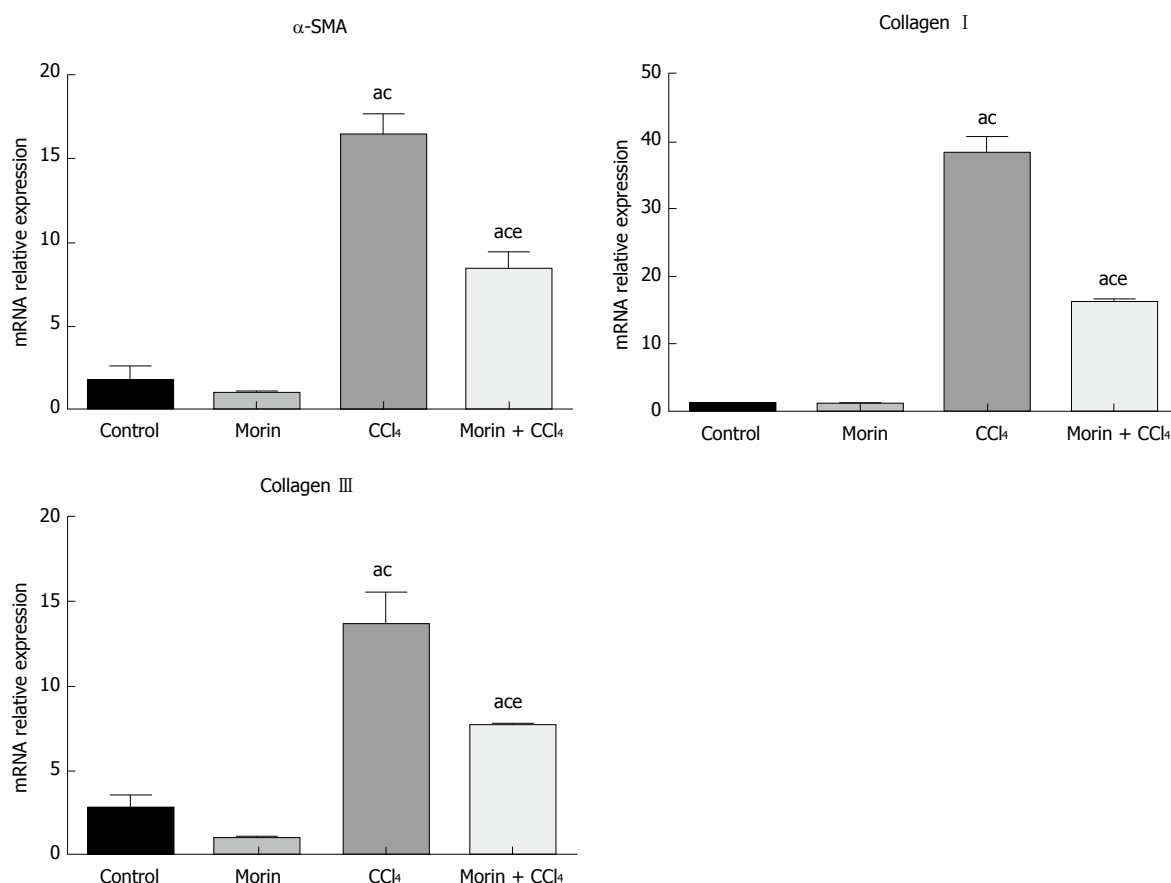


Figure 4 The mRNA expression of α -SMA, collagen I, and collagen III. ^a $P < 0.05$ vs control group, ^b $P < 0.05$ vs morin group, ^c $P < 0.05$ vs CCl₄ group. In the control and morin groups, there was only minimal expression. The CCl₄ and morin + CCl₄ groups showed significantly increased expression ($P < 0.05$), while the expression levels in the morin + CCl₄ group were lower than those of the CCl₄ group ($P < 0.05$).

CCl₄ groups had a statistically significant difference ($P < 0.05$). However, the morin + CCl₄ group had less expression of these protein factors compared to the CCl₄ group ($P < 0.05$) (Figure 6).

In the CCl₄ and morin + CCl₄ groups, the protein expression of Nrf2, HO-1, and NQO1 was statistically higher than that in the control and morin groups ($P < 0.05$), while these protein factors of the morin + CCl₄ rats had more expression compared to the CCl₄ group ($P < 0.05$) (Figure 7).

DISCUSSION

Liver fibrosis is a process of continuous damage to the liver blood vessels and hepatic cells with nodule formation, which may develop into cirrhosis and cancerous lesions. Research of fibrosis at the cellular and molecular levels suggested that the progression of liver injury was closely related to oxidative stress and lipid peroxidation^[32,33], leading to cell destruction and inducing hepatic fibrosis. HSCs can be activated by lipid peroxides acting as products of cell damage. After HSC activation, lipid droplets and vitamin A in the cytoplasm could be reduced or exhausted with α -SMA expression, accompanied by liver structural and functional changes resulting from redundant secretion

of ECM^[34]. However, it is possible to reverse liver fibrosis and early cirrhosis with effective interventions. Previous studies have shown that antioxidants have a protective effect by inhibiting the expression of α -SMA in HSC^[35], thus, inhibition of oxidative stress in the liver may reduce and even reverse liver fibrosis^[36].

Pathological features of liver fibrosis are reflected by fibrous tissue hyperplasia around the portal area and central vein and forming an interval of destruction of the lobular structure, accompanied by regenerative nodules and even early cirrhosis^[37]. The pathological findings in this study showed that liver tissue in the CCl₄ group had liver cell necrosis, fibrous tissue hyperplasia, interval widening, and pseudolobuli replacing normal lobular architecture. In the morin + CCl₄ group, the liver tissue showed minimal cell necrosis with less interstitial collagen fibers and lobular structure damage compared with the CCl₄ group. Thus, morin could effectively protect the liver tissue by reducing inflammation and inhibiting collagen deposition and fiber hyperplasia.

There are various enzymes that take part in liver metabolism. The damaged liver cells by pathogenic factors will produce free enzymes that are released into the bloodstream^[20]. Liver function and status could be assessed by assaying the contents of serum enzymes. Aminotransferases play an important role in

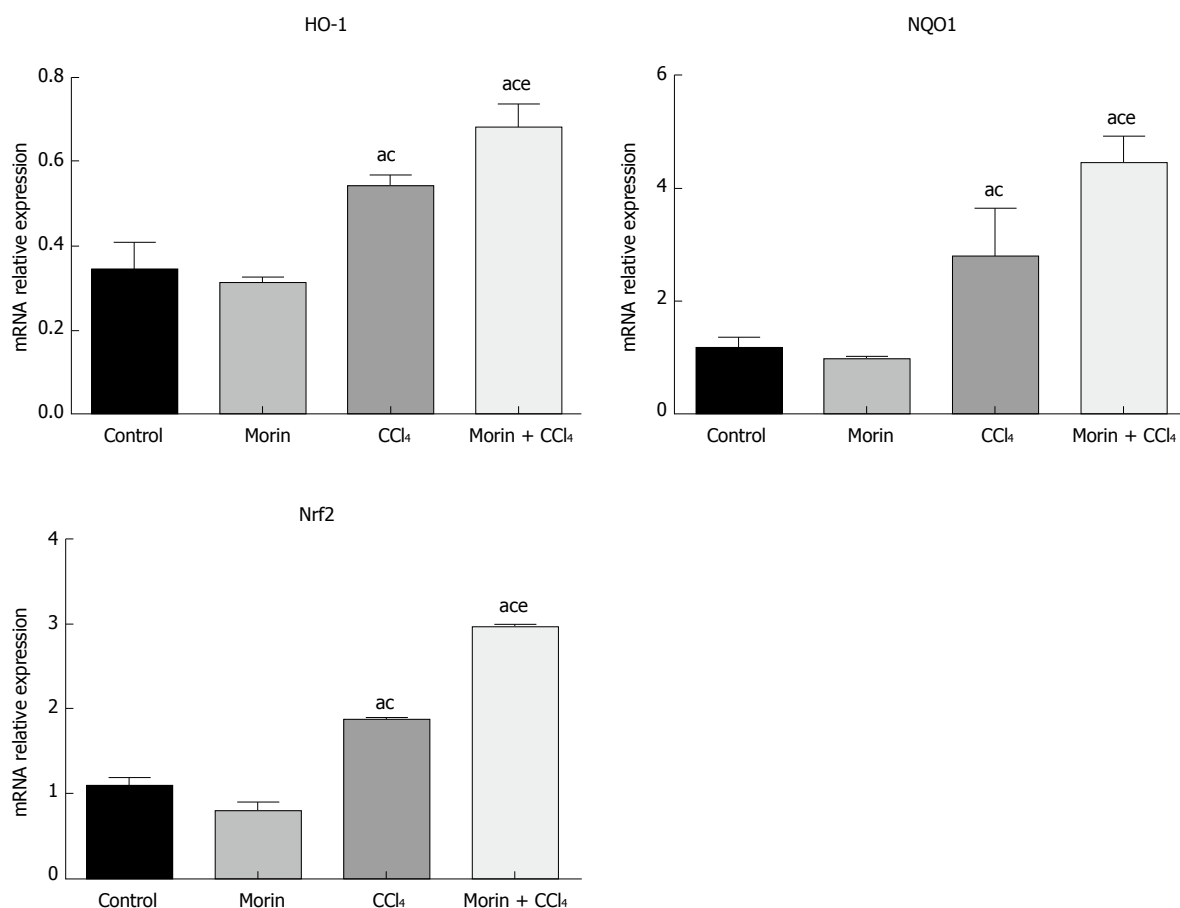


Figure 5 The mRNA expression of *HO-1*, *NQO1*, and *Nrf2*. ^a $P < 0.05$ vs control group, ^c $P < 0.05$ vs morin group, ^e $P < 0.05$ vs CCl₄ group. The expression was increased obviously in the CCl₄ and morin + CCl₄ groups compared to the control and morin groups ($P < 0.05$). The expression levels in the morin + CCl₄ group were significantly higher than those of the CCl₄ group ($P < 0.05$).

hepatic metabolism. When the liver cells are damaged, the serum ALT and AST levels as well as ALP level will be increased^[38]. In this study, in the CCl₄-induced liver fibrosis rat model, the values of serum ALT, AST, and ALP were reduced with morin administration, which implied that morin can reduce liver cell injury and thus prevent liver fibrosis. This also gives support for morin being able to condition the hepatocytes, protect against membrane frailty, and decrease the outflow of enzymes into circulation. These results are in accordance with previous studies that showed the ability of morin to inhibit hepatotoxicity^[39,40].

The amount of collagen accounts for 5%-10% of the total protein in human liver tissue. If the liver injury leads to fibrosis, the collagen content in the liver protein will be significantly increased up to approximately 50%, becoming an important component of ECM^[41] and ultimately leading to irreversible cirrhosis changes^[42]. Liver fibrosis is a common histological change in liver disease, which is mainly manifested by excessive deposition of ECM, such as type I and type III collagen, and the expression of α -SMA^[43]. At present, it is believed that the ECM actively participates in the occurrence and development of fibrosis, which has a great influence on HSC activation^[44-46]. Both *in vitro*

and *in vivo* experiments found that ECM synthesis was increased when liver tissue was damaged and further caused the activation of HSCs, which was based on the secretion of type I and III collagen^[47-49], ultimately promoting the occurrence of liver fibrosis. In our study, using both real-time PCR and Western blot methods, it was found that the control and morin groups had only minimal expression of collagen I, collagen III, and α -SMA, which may represent normal physiological function of the liver, while their expression in the CCl₄ group was significantly increased and had great relevance to the severity of liver fibrosis. With morin intervention reducing the expression of collagen I, collagen III, and α -SMA, the degree of liver fibrosis was relieved, which was evidenced by liver histopathology and serum measurements. All these results suggested that the anti-fibrotic effect of morin may be related to the down-regulation of the expression of collagen I, collagen III, and α -SMA.

Nrf2 is a key nuclear transcription factor in the oxidative stress of various cells^[50]. Under normal circumstances, Nrf2 and Keap1 are in a binding state in the cytoplasm^[51]; they will appear dissociated when oxidative stress is occurring^[52] and combine with antioxidant components as dimers, which are

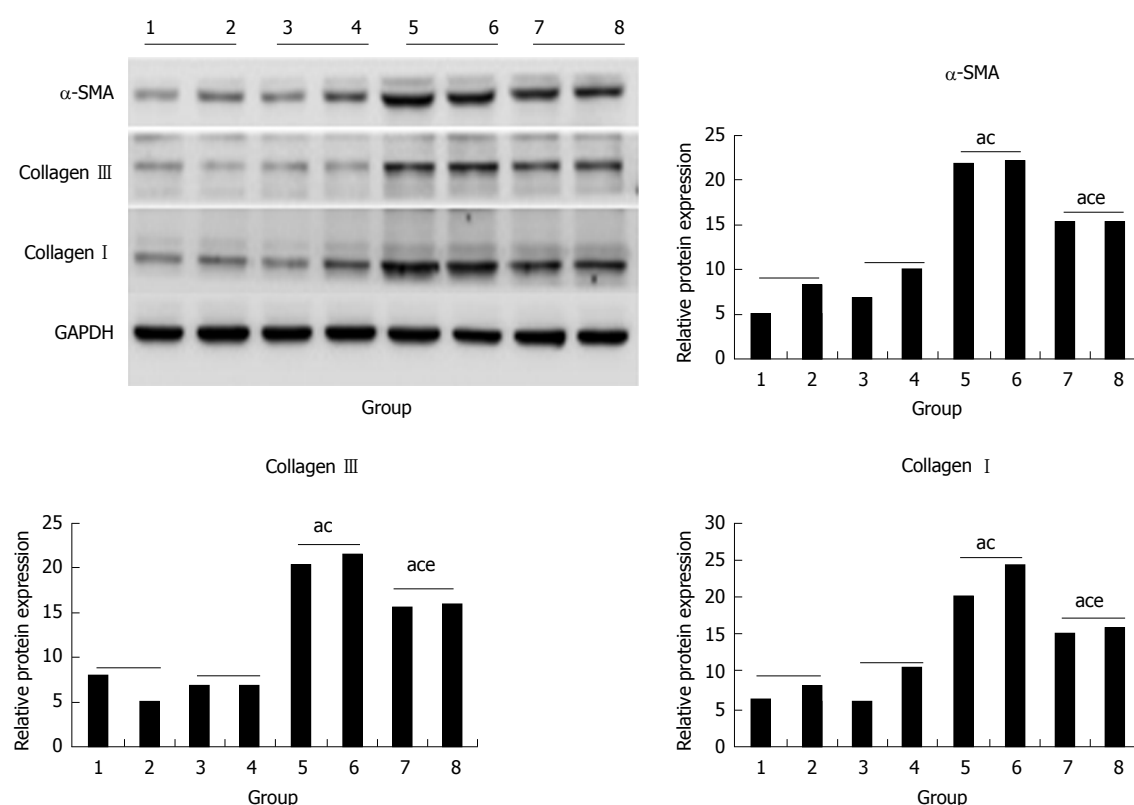


Figure 6 The protein expression of α -SMA, collagen III, and collagen I. (1, 2) control group, (3, 4) morin group, (5, 6) CCl₄ group, (7, 8) morin + CCl₄ group. ^a $P < 0.05$ vs control group, ^b $P < 0.05$ vs morin group, ^c $P < 0.05$ vs CCl₄ group. The CCl₄ and morin + CCl₄ groups showed significantly increased expression compared to the control and morin groups ($P < 0.05$), and the expression levels in the morin + CCl₄ group were lower than those in the CCl₄ group ($P < 0.05$).

involved in the synthesis of antioxidase and phase II detoxification enzymes and prevent the occurrence of liver fibrosis by improving the antioxidant capacity of the liver^[53]. HO-1 and NQO-1 are well characterized Nrf2-dependent antioxidant defense genes. Studies have suggested that Nrf2 and its downstream antioxidant factors HO-1 and NQO1 may contribute to improvement of liver fibrosis^[54]. It has been reported that morin could promote the nuclear translocation of Nrf2 in order to play its biological role and be used as an exogenous agonist of Nrf2^[55]. In this study, a CCl₄ induced liver fibrosis model, along with morin as an intervention, was used to observe the expression of Nrf2 and its downstream products NQO1 and HO-1 in different groups. The results showed that the expression of Nrf2, NQO1, and HO-1 was slightly increased in the CCl₄ group compared with the control and morin groups ($P < 0.05$). This might be due to Nrf2 activation acting as a cellular adaptive response against CCl₄-induced toxicity. Nrf2 activation was initiated as soon as the subjects were challenged by CCl₄-induced oxidative stress. However, it was unable to completely overcome the toxicity, while the adaptively stimulated Nrf2 might alleviate or delay the deleterious effects of CCl₄. The expression of Nrf2 and its downstream products NQO1 and HO-1 was evidently increased in the morin-treated group, indicating that morin administration could enhance this effect. Additionally, this supports morin playing an

important role in the prevention and treatment of liver fibrosis via the Nrf2 pathway.

This study has several limitations. First, the sample size was small, which easily led to individual differences and statistical error between the groups. Second, the anti-fibrotic mechanism of morin may be related to activation of the Nrf2 antioxidant pathway and expression of its downstream antioxidant enzymes. Further experiments are needed to confirm the specific mechanism of the morin intervention.

In summary, our current study showed that morin could play a protective role by inducing the expression of Nrf2 and its downstream antioxidant factors (HO-1 and NQO1) and reducing the expression of α -SMA, collagen I, and collagen III in a rat model of CCl₄-induced hepatic fibrosis. Although further studies are required, our study demonstrated that morin could effectively alleviate chronic liver damage by activation of the Nrf2 pathway.

ARTICLE HIGHLIGHTS

Research background

Previous studies have shown that the pathological changes of liver fibrosis, which refer to a series of pathogenic factors and pathological changes in the pathogenesis of a variety of liver diseases, could be reversed. In the past decade, numerous studies demonstrated that NF-E2-related factor 2 (Nrf2) as a transcription factor plays an important role against oxidative stress in normal liver cells. Morin possesses biological properties, including antioxidant, anti-inflammatory, anti-apoptosis, and anticancer activities. To our knowledge,

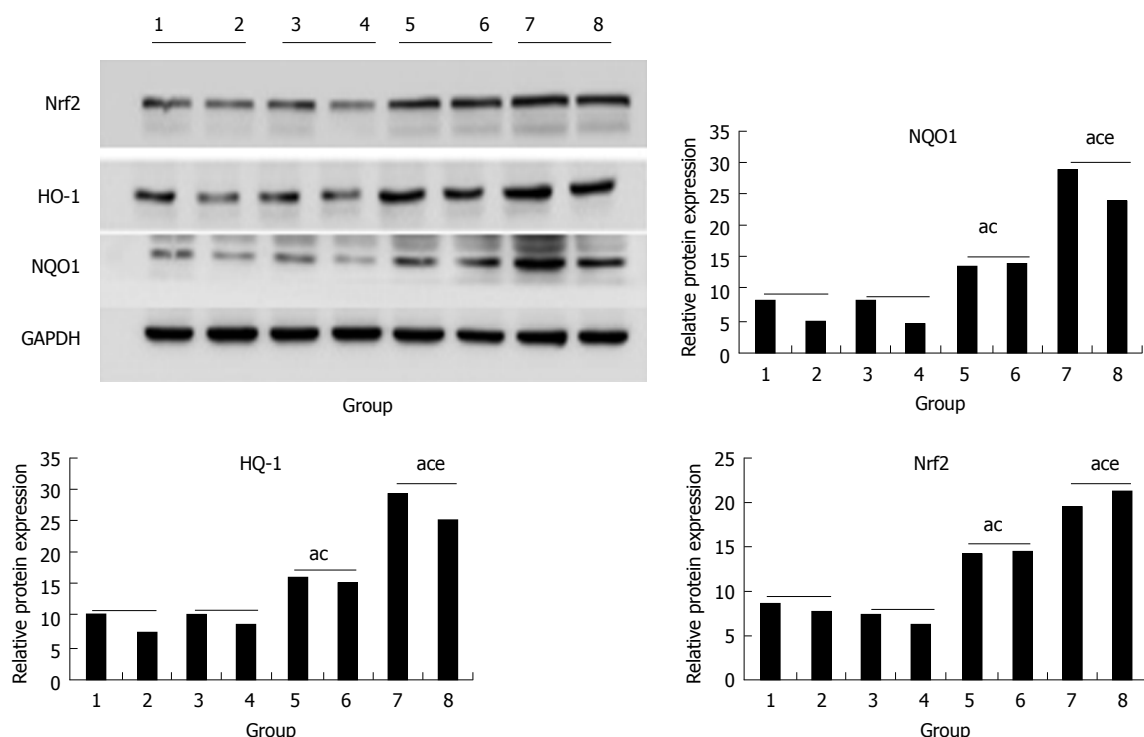


Figure 7 The protein expression of Nrf2, HO-1, and NQO1. (1, 2) control group, (3, 4) morin group, (5, 6) CCl₄ group, (7, 8) morin + CCl₄ group. ^a*P* < 0.05 vs control group, ^c*P* < 0.05 vs morin group, ^e*P* < 0.05 vs CCl₄ group. In the CCl₄ and morin + CCl₄ groups, the protein expression was increased compared to the control and morin groups (*P* < 0.05); the morin + CCl₄ group had a more significant change compared to the CCl₄ group (*P* < 0.05).

in vivo investigation of the effect of morin on the Nrf2 signaling pathway and Nrf2 expression in a CCl₄-induced liver fibrosis model has not been reported previously.

Research motivation

Previous studies demonstrated that morin protected acute liver damage and ameliorated liver fibrosis induced by CCl₄, and morin inhibited proliferation and induced apoptosis of activated hepatic satellite cells by suppressing the Wnt/β-catenin and the NF-κB signaling pathways. However, there is no molecular evidence about the effects of morin on the Nrf2 signaling pathway.

Research objectives

The purpose of this study was to investigate whether morin can reduce hepatic fibrosis by inducing the expression of Nrf2 and its downstream antioxidant enzymes in a rat model of CCl₄-induced hepatic fibrosis.

Research methods

Twenty male Sprague-Dawley rats were randomly divided into four groups: control group, morin group, carbon tetrachloride (CCl₄) group, and morin + CCl₄ group. At the end-point of the experimental period, serum AST, ALT, and ALP were measured, and the liver specimens were obtained for pathological assessment. α-SMA, collagen I, collagen III, NF-E2-related factor 2 (Nrf2), heme oxygenase (HO-1), and quinone oxidoreductase 1 (NQO1) were analyzed by real-time PCR and Western blot methods using frozen liver specimens.

Research results

Rats in the morin + CCl₄ group had less hyperplasia of fiber tissues, minimal inflammatory cells, and less body weight loss with favorable liver enzyme measurements compared to rats treated with CCl₄ only. Additionally, morin-treated rats had significantly lower mRNA and protein expression of α-SMA, collagen I, and collagen III, but significantly higher mRNA and protein expression of Nrf2, HO-1, and NQO1 compared to rats treated with CCl₄ only (*P* < 0.05).

Research conclusions

Our study showed that morin could play a protective role by inducing the expression of Nrf2 and its downstream antioxidant factors (HO-1 and NQO1) and reducing the expression of α-SMA, collagen I, and collagen III in a rat model of CCl₄-induced hepatic fibrosis.

Research perspectives

Although further studies are required, our study demonstrated that morin could effectively alleviate chronic liver damage by activation of the Nrf2 pathway.

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Basic Study

Circular RNA circ-LDLRAD3 as a biomarker in diagnosis of pancreatic cancer

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Informed consent statement: All cancer specimen and blood samples from the patients and healthy volunteers were taken after informed written consent and ethical permission were obtained prior to study enrollment.

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Abstract

AIM

To analyze the diagnostic value of a circular RNA (circRNA), circ-LDLRAD3, in pancreatic cancer.

METHODS

Expression levels of circ-LDLRAD3 were tested in both cells and clinical samples; the latter included 30 paired pancreatic cancer tissues and adjacent non-tumorous tissues, 31 plasma samples from patients with pancreatic cancer, and 31 plasma samples from healthy volunteers. Real-time quantitative reverse transcription

polymerase chain reaction (qRT-PCR) was performed to measure expression levels of circ-LDLRAD3 in cells and clinical samples; then, the relationship between clinicopathological factors of patient samples and expression of circ-LDLRAD3 in pancreatic cancer was analyzed. The diagnostic value of circ-LDLRAD3 was verified by receiver operating characteristic (ROC) curve analysis.

RESULTS

Circ-LDLRAD3 was up-regulated in pancreatic cancer cell lines ($P < 0.01$), pancreatic cancer tissues ($P < 0.01$), and plasma samples from patients with pancreatic cancer ($P < 0.01$). High expression of circ-LDLRAD3 was significantly associated with venous invasion, lymphatic invasion, and metastasis. The area under the ROC curve of circ-LDLRAD3 alone or combination with CA19-9 was 0.67 and 0.87, respectively, with a sensitivity and specificity of 0.5738 (alone) and 0.7049 (alone), and 0.8033 (combination) and 0.9355 (combination), respectively.

CONCLUSION

These data suggest that circ-LDLRAD3 may be a biomarker in the diagnosis of pancreatic cancer.

Key words: Circular RNA; Pancreatic cancer; Biomarker

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Core tip: Circular RNAs (circRNAs), a novel class of stable endogenous RNAs, play important roles in the occurrence and progression of cancer; however, little is known about their diagnostic value in pancreatic cancer. Our study focused on a novel circRNA, circ-LDLRAD3. Expression levels of circ-LDLRAD3 were tested in both cells and clinical samples, including tissue samples and plasma samples. Then, the relationship between clinicopathological factors of patient samples and expression of circ-LDLRAD3 in pancreatic cancer was analyzed. The diagnostic value of circ-LDLRAD3 was verified by ROC curve analysis. Our study suggests that circ-LDLRAD3 may be a new biomarker in the diagnosis of pancreatic cancer.

Yang F, Liu DY, Guo JT, Ge N, Zhu P, Liu X, Wang S, Wang GX, Sun SY. Circular RNA circ-LDLRAD3 as a biomarker in diagnosis of pancreatic cancer. *World J Gastroenterol* 2017; 23(47): 8345-8354 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v23/i47/8345.htm> DOI: <http://dx.doi.org/10.3748/wjg.v23.i47.8345>

INTRODUCTION

Pancreatic cancer is a malignancy of the digestive system with insidious onset and rapid development,

resulting in delayed and difficult early diagnoses and poor prognosis^[1,2]. The incidence and mortality of pancreatic cancer are rising every year worldwide, and it is the 7th and 4th leading cause of mortality from all malignant tumors in China^[3] and the United States^[4], respectively. Surgical resection remains the major means of treatment for pancreatic cancer; however, the 5-year survival rate for patients undergoing a complete resection remains as low as 6%^[5]. The key to improving the prognosis of pancreatic cancer mostly lies in early diagnosis and early treatment, which can be achieved by detection of relevant molecular markers among patients with high risk, followed by early and timely interventions^[6-8].

Circular RNAs (circRNAs) are a class of noncoding RNAs with continuous, covalently closed circular structures, which have been further found to exhibit species conservation and tissue specificity^[9]. With the emergence of next-generation sequencing, especially RNA sequencing technology, circRNAs have been found to be extensively expressed in the cytoplasm. In addition, they have been garnering attention because of their specificity of expression, complexity of regulation, and important role in pathogenesis of many diseases, especially cancer^[10]. Unlike their linear counterparts, circRNAs are characterized by stable ring structure formed by a covalently closed continuous loop. Without free 3' and 5' ends, these molecules are not easily degraded by nucleases, which makes them ideal biomarkers for detection of disease^[11]. Investigators have identified disease-specific patterns of circRNA expression, which can serve as biomarkers for diseases^[12], especially cancer^[10,13]. However, there has been little investigation into the association of circRNAs with pancreatic cancer.

In this study, we focused our investigation on circRNA-hsa_circ_0006988, whose gene is located at chr11:36248634-36248980. Its gene symbol is LDLRAD3 (low density lipoprotein receptor class A domain containing 3), therefore we will refer to circRNA-hsa_circ_0006988 as circ-LDLRAD3 instead of its original name in circBase^[14] (<http://www.circbase.org>). We chose circ-LDLRAD3 as a target for further study because we previously identified that it may be up-regulated in a previous microarray screening (<http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE69362>)^[15] and associated with pancreatic cancer in circBase^[14] and circ2Traits^[16]. By expanding the sample size, we found that the expression levels of circ-LDLRAD3 were higher in both pancreatic cancer tissues and plasma from patients with pancreatic cancer as compared to control samples. Moreover, up-regulated expression of circ-LDLRAD3 was significantly related to major clinicopathological factors of patients with pancreatic cancer. Our results make clear that circ-LDLRAD3 may serve as a biomarker in the diagnosis of pancreatic cancer.

MATERIALS AND METHODS

Clinical samples

Thirty samples of pancreatic cancer and their paired adjacent pancreatic tissues were obtained from patients with pancreatic cancer treated at Shengjing Hospital of China Medical University (Shenyang, China) from September 2016 to June 2017. Paired normal tissue samples were obtained 5 cm from the pancreatic cancer tissue and were confirmed to contain no tumor cells after evaluation by two experienced pathologists. All specimens were immediately stored in liquid nitrogen until use.

Peripheral blood samples (4 mL) were collected from another 31 patients with pancreatic cancer and 31 healthy volunteers prior to any medical interventions at Shengjing Hospital of China Medical University (Shenyang, China) from October 2016 to July 2017. Plasma samples were isolated as previously described. The anti-coagulant for peripheral blood samples was ethylenediaminetetraacetic acid (EDTA). Clinical information was collected for all patients and healthy volunteers.

Tumors were staged according to the 8th tumor-node-metastasis (TNM) staging system drafted by the International Union Against Cancer. No patients received radiotherapy, chemotherapy, or targeted therapy before surgery. All patients and healthy volunteers provided written informed consent before the procedure. The Institutional Review Board of China Medical University approved this study based on the Helsinki Declaration.

Cell culture

The normal pancreatic cell lines, HPC-Y5 and HPDE6-C7, were obtained from the Cell Bank of the Chinese Academy of Sciences (Shanghai, China). The pancreatic cancer cell lines, Capan-2, Panc-1, SW1990, and AsPC-1, were obtained from ATCC (Manassas, United States). HPC-Y5, HPDE6-C7, and Panc-1 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) (Gibco, Gaithersburg, MD, United States); Capan-2 and AsPC-1 cells were cultured in RPMI-1640 medium (Gibco, Gaithersburg, MD, United States); and SW1990 cells were cultured in Leibovitz's L-15 medium (Gibco, Gaithersburg, MD, United States). All media contained 10% fetal bovine serum (FBS) (Gibco, Gaithersburg, MD, United States) and all cells were cultured in a humidified atmosphere consisting of 5% CO₂ and 95% air at 37 °C.

Total RNA extraction

Total RNA from all cell lines, pancreatic cancer tissues, and paired adjacent tissues was extracted using TRIzol reagent (Invitrogen, Carlsbad, CA, United States). Total RNA in plasma samples was extracted using a mirVana PARIS Kit (Ambion, Carlsbad, CA, United States) following the manufacturer's instructions. Quantity

and quality of RNA were determined spectrophotometrically at 260 nm and 280 nm. The integrity and contamination were confirmed using denaturing agarose gel electrophoresis.

Reverse transcription

Total RNA was reverse transcribed using a PrimeScript reagent kit with gRNA Eraser (Random primers) (TaKaRa, Dalian, China) according to the manufacturer's instructions.

Sanger sequencing

To precisely examine the primer sequences of circ-LDLRAD3, Sanger sequencing was utilized. In brief, a T vector carrying the target fragment was utilized for Sanger sequencing in order to determine the back-spliced junction of circ-LDLRAD3. The following divergent primers were synthesized by Geneseed Biotech (Guangzhou, China): 5'-CTTGCTGGACCAGAGAAC-3' (forward) and 5'-CATGAGGTTGTTCCGCTTC-3' (reverse). Sanger sequencing was performed by the same company.

circ-LDLRAD3 detection using qRT-PCR

Real-time quantitative reverse transcription polymerase chain reactions (qRT-PCR) was performed using a Roche 480II system (Roche, Basel, Switzerland) utilizing SYBR Premix Ex Taq II (Tli RNaseH Plus) (Takara, Dalian, China), following the manufacturer-provided instructions. Primers for GAPDH were synthesized by Sangon Biotech (Shanghai, China) as follows: 5'-GCACCGTCAAGGCTGAGAAC-3' (forward) and 5'-TGGTGAAGACGCCAGTGA-3' (reverse). The data were analyzed using the comparative cycle threshold (Δ CT) method after three independent experiments. All results are expressed as the mean \pm SD.

Serological tumor-associated marker analysis

Serum carbohydrate antigen 19-9 (CA19-9) and carcinoembryonic antigen (CEA) were measured using a Roche E601 machine (Roche, Basel, Switzerland) with a cutoff value of 40 U/mL and 5 ng/mL, respectively.

Statistical analysis

All statistical data were analyzed using SPSS 23.0 (SPSS, Chicago, IL, United States), GraphPad 7.0 (GraphPad Software, La Jolla, CA, United States), and SigmaPlot 12.5 (SigmaPlot Software, La Jolla, CA, United States). Differences in expression levels of circ-LDLRAD3 between pancreatic cancer tissues and paired adjacent non-tumorous tissues were compared by using paired *t*-tests, and differences in expression levels of circ-LDLRAD3 between plasma samples from patients with pancreatic cancer and those from healthy volunteers were compared by Student's *t*-tests. A Fisher's exact test was used to analyze the association between circ-LDLRAD3 expression and patients' clinicopathological factors. A Spearman's

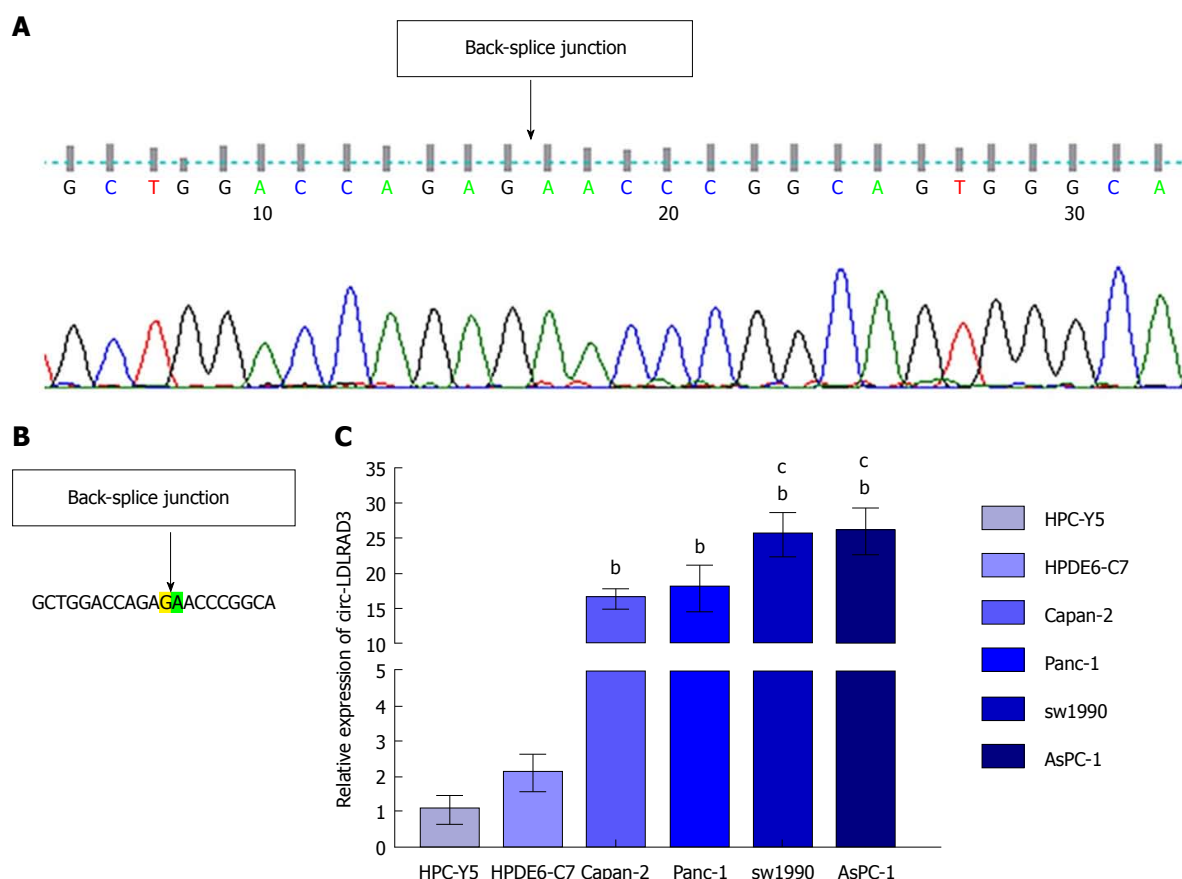


Figure 1 Circ-LDLRAD3 expression levels in pancreatic cell lines and pancreatic cancer cell lines. A: Sanger sequencing of circ-LDLRAD3 showed the back-splice junction. B: The back-splice junction of circ-LDLRAD3 in CircBase. C: Relative expression of circ-LDLRAD3 in human pancreatic cell lines and pancreatic cancer cell lines. ^a $P < 0.01$ vs pancreatic cell lines (HPC-Y5 and HPDE6-C7); ^c $P < 0.05$ vs primary pancreatic cancer cell lines (Capan-2 and Panc-1).

rank correlation coefficient was introduced to further calculate bivariate correlations. The receiver operating characteristics (ROC) curve was established to evaluate the diagnostic value of circ-LDLRAD3; the cutoff value of circ-LDLRAD3 was calculated using Youden index (specificity + sensitivity - 1). The comparison of the area under the ROC curve (AUC) was analyzed by Z-test. P values < 0.05 were considered statistically significant.

RESULTS

Circ-LDLRAD3 expression is up-regulated in pancreatic cancer lines

Sanger sequencing of circ-LDLRAD3 qRT-PCR product was first conducted to determine the back-junction of circ-LDLRAD3. The results of the back-splice junction of circ-LDLRAD3 indicated there was no difference between our product and that found in CircBase (Figure 1A and B). Next, expression levels of circ-LDLRAD3 were tested in normal pancreatic cell lines (HPC-Y5 and HPDE6-C7) and pancreatic cancer cell lines (Capan-2, Panc-1, SW1990, and AsPC-1). These results indicate that the relative expression levels of circ-LDLRAD3 were higher in pancreatic cancer cell lines than in normal pancreatic cell lines ($P < 0.01$). In

addition, the relative expression levels of circ-LDLRAD3 in metastatic pancreatic cancer cell lines (SW1990 and AsPC-1) were higher than those in primary pancreatic cancer cell lines (Capan-2 and Panc-1) ($P < 0.05$) (Figure 1C).

Circ-LDLRAD3 expression is up-regulated in pancreatic cancer tissues and plasma of patients with pancreatic cancer

Expression of circ-LDLRAD3 was measured via qRT-PCR in 30 pancreatic cancer tissues compared with paired adjacent non-tumorous tissues and in plasma samples of patients with pancreatic cancer compared with healthy volunteers. Lower ΔCT values indicate higher expression of circ-LDLRAD3. As shown in Figure 2A, expression of circ-LDLRAD3 was up-regulated in pancreatic cancer tissues ($P < 0.01$), while expression of circ-LDLRAD3 in plasma samples with pancreatic cancer were higher than those in healthy volunteers ($P < 0.01$, Figure 2B).

Upregulation of circ-LDLRAD3 is associated with clinicopathological factors in patients with pancreatic cancer

The above data demonstrated that circ-LDLRAD3 expression was significantly up-regulated in pancreatic

Table 1 Clinicopathological factors of patients' tissue samples and expression of circ-LDLRAD3 in pancreatic cancer

Characteristic	<i>n</i> (%)
Age (yr)	
≥ 60	19 (63.3)
< 60	11 (36.7)
Gender	
Male	9 (30)
Female	21 (70)
Tumor diameter (cm)	
≤ 4	19 (63.3)
> 4	11 (36.7)
CA19-9	
Positive	19 (63.3)
Negative	11 (36.7)
CEA	
Positive	16 (56.7)
Negative	13 (43.3)
Clinical stage	
I A	3 (10)
I B	10 (33.3)
II A	7 (23.3)
II B	9 (30)
III	1 (3.3)
IV	0 (0)
T classification	
T1	3 (10)
T2	15 (50)
T3	11 (36.7)
T4	1 (3.3)
N classification	
N0	20 (66.7)
N1	10 (33.3)
N2	0 (0)
Metastasis	
M0	30 (100)
M1	0 (0)
Venous invasion	
No	24 (80)
Yes	6 (20)
Lymphatic invasion	
No	23 (76.7)
Yes	7 (23.3)
Expression of circ-LDLRAD3	
Low expression	12 (40)
High expression	18 (60)

cancer tissues and plasma samples of patients with pancreatic cancer; hence, we analyzed the association between circ-LDLRAD3 and clinicopathological factors of patients with pancreatic cancer.

As shown in Tables 1-3, in pancreatic cancer tissues, a strong association was observed between circ-LDLRAD3 expression and venous invasion ($P = 0.025$) and lymphatic invasion ($P = 0.014$). However, no association was found between circ-LDLRAD3 expression and other clinicopathological factors including age ($P = 0.279$), gender ($P = 0.255$), tumor diameter ($P = 0.279$), CA19-9 ($P = 0.643$), CEA ($P = 0.88$), clinical stage ($P = 0.256$), T classification ($P = 0.274$), N classification ($P = 0.429$), and metastasis (none). A Spearman analysis of correlation between circ-LDLRAD3 and various clinicopathological factors indicated that expression of circ-LDLRAD3

Table 2 Correlation between circ-LDLRAD3 expression and clinicopathological factors of pancreatic cancer patients (tissue samples)

Characteristic	Circ-LDLRAD3		<i>P</i> value
	Low or none, <i>n</i>	High, <i>n</i>	
Age (yr)			
≥ 60	9	10	0.279
< 60	3	8	
Gender			
Male	7	14	0.255
Female	5	4	
Tumor diameter (cm)			
≤ 4	9	10	0.279
> 4	3	8	
CA19-9			
Positive	5	6	0.643
Negative	7	12	
CEA			
Positive	5	8	0.88
Negative	7	10	
Clinical stage			
I A	2	1	0.256
I B	6	4	
II A	1	6	
II B	3	6	
III	0	1	
IV	0	0	
T classification			
T1	2	1	0.274
T2	8	8	
T3	2	8	
T4	0	1	
N classification			
N0	9	11	0.429
N1	3	7	
N2	0	0	
Metastasis			
M0	12	18	None
M1	0	0	
Venous invasion			
No	12	12	0.025
Yes	0	6	
Lymphatic invasion			
No	12	11	0.014
Yes	0	7	

was correlated with clinical stage ($P = 0.022$), T classification ($P = 0.003$), venous invasion ($P = 0.025$), and lymphatic invasion ($P = 0.008$).

In the plasma of patients with pancreatic cancer (Tables 4-6), circ-LDLRAD3 levels were significantly associated with CA19-9 ($P = 0.03$), N classification ($P = 0.049$), venous invasion ($P = 0.005$), and lymphatic invasion (0.014). No association was found between circ-LDLRAD3 and age, gender, tumor diameter, CEA, clinical stage, T classification, or metastasis. In Spearman analysis, circ-LDLRAD3 expression was correlated with clinical stage ($P < 0.001$), metastasis ($P = 0.004$), venous invasion ($P = 0.029$), and lymphatic invasion ($P < 0.001$).

Potential diagnostic value of circ-LDLRAD3 as a biomarker in pancreatic cancer

To identify whether circ-LDLRAD3 can serve as a

Table 3 Spearman analysis of correlation between circ-LDLRAD3 and clinicopathological factors of pancreatic cancer patients (Δ CT values in tissues)

Variable	Circ-LDLRAD3 expression level	
	Spearman correlation	P value
Age (yr)	-0.22	0.243
Gender	-0.122	0.521
Tumor diameter (cm)	-0.303	0.104
CA19-9	0.028	0.883
CEA	0.019	0.919
Clinical stage	-0.415	0.022
T classification	-0.519	0.003
N classification	-0.196	0.299
Metastasis	None	None
Venous invasion	-0.607	< 0.001
Lymphatic invasion	-0.478	0.008

biomarker in pancreatic cancer, Δ CT values were further evaluated. The area under the ROC curve (AUC) was 0.67; the cutoff value, sensitivity, and specificity were 9.315, 0.5738, and 0.7049, respectively. When combined with CA19-9, the AUC was increased to 0.87 and the sensitivity and specificity were 0.8033 and 0.9355, respectively (Figure 3).

DISCUSSION

There have been few recent therapeutic advances in the treatment of pancreatic cancer. For more than 10 years, surgery and chemotherapy with gemcitabine have been the standard treatment methods^[17-19]; yet, only 13%-15% of patients with pancreatic cancer are likely to undergo pancreaticoduodenectomy^[20]. Furthermore, patients with pancreatic cancer are prone to experience multidrug chemotherapy resistance^[21]. There are several challenges in the diagnosis and treatment of pancreatic cancer. First, there is difficulty in making an early diagnosis. The pathological and biological characteristics of pancreatic cancer result in early symptoms which lack specificity^[22]. Distant metastases have already occurred in roughly 50% of patients with pancreatic cancer at the time of treatment while the resection rate was only 15%^[23]. Second, the heterogeneity of pancreatic cancer makes it difficult to treat. Whole genome analysis of pancreatic cancer shows that 12 core signaling pathways have genetic changes. Alterations in multiple genes and multiple pathways increase the difficulty of achieving effective treatment, resulting in poor prognoses^[20,24]. Therefore, the key to the diagnosis and treatment of pancreatic cancer lies in early detection and diagnosis. Risk assessment of pancreatic cancer-relevant molecular markers in patients and early and timely intervention to prevent deterioration will have a positive effect on the diagnosis and treatment of pancreatic cancer^[25,26].

CircRNAs are a novel class of RNAs with O-shaped closed structure that exist in the living cells. Unlike

Table 4 Clinicopathological factors of plasma samples of patients with pancreatic cancer and expression of circ-LDLRAD3

Characteristic	n (%)
Age (yr)	
≥ 60	15 (48.4)
< 60	16 (51.6)
Gender	
Male	19 (61.3)
Female	12 (38.7)
Tumor diameter (cm)	
≤ 4	21 (67.7)
> 4	10 (32.3)
CA19-9	
Positive	25 (87.1)
Negative	4 (12.9)
CEA	
Positive	10 (32.3)
Negative	21 (67.7)
Clinical stage	
I A	5 (16.1)
I B	6 (19.4)
II A	6 (19.4)
II B	5 (16.1)
III	6 (19.4)
IV	3 (9.7)
T classification	
T1	5 (16.1)
T2	15 (48.4)
T3	9 (29.0)
T4	2 (6.5)
N classification	
N0	21 (67.7)
N1	6 (19.4)
N2	4 (12.9)
Metastasis	
M0	28 (90.3)
M1	3 (9.7)
Venous invasion	
No	19 (61.3)
Yes	12 (38.7)
Lymphatic invasion	
No	21 (67.7)
Yes	10 (32.3)
Expression of circ-LDLRAD3	
Low expression	9 (29)
High expression	22 (71)

traditional linear RNA molecules, circRNAs are resistant to degradation by exonuclease and RNases because there are no 5'-end, 3'-end, or even poly(A) tail^[27]. Hence, circRNA can stably exist in cells for a long period of time. Furthermore, circRNA molecules in human cells are ten-fold more numerous than the number of homogenetic linear isomer RNA molecules^[28]. CircRNA molecules have highly conserved sequences, a stable existence, and tissue-specific expression; circRNAs have been demonstrated to regulate gene expression in post-transcriptional ways^[29]. For example, circRNAs can act as microRNA (miRNA) sponges. Li *et al.*^[30] reported that circ-ITCH competitively sponged miRNA-7, miRNA-17, and miRNA-214, leading to higher expression of the ITCH gene. The ITCH gene product has been shown to inhibit Dvl2 phosphor-

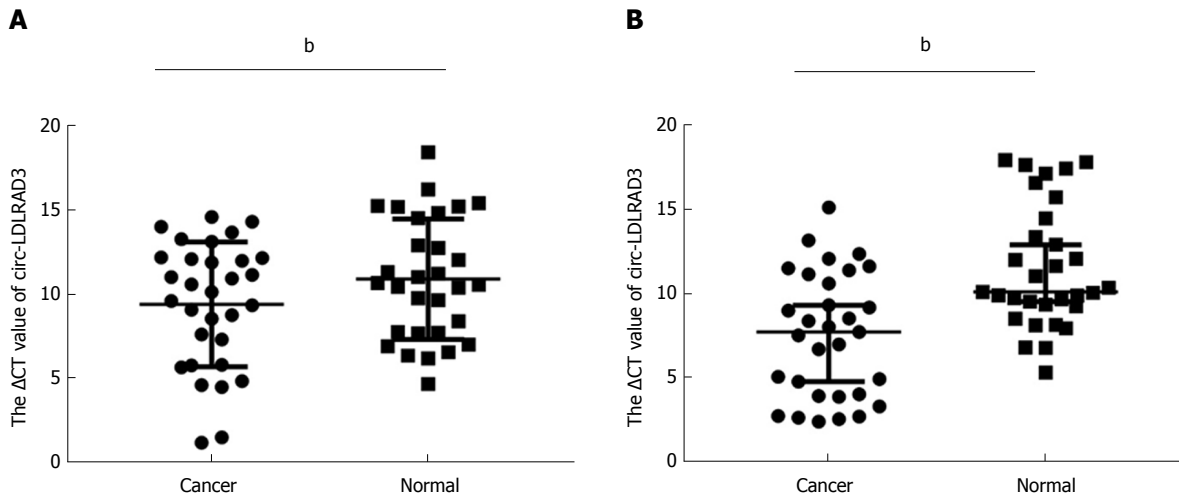


Figure 2 The expression levels of circ-LDLRAD3 in pancreatic cancer samples. A: The expression levels of circ-LDLRAD3 in pancreatic cancer tissues and paired non-tumorous tissues ($n = 30$ each). Lower Δ CT value indicates higher expression of circ-LDLRAD3. B: The expression levels of circ-LDLRAD3 in plasma samples of patients with pancreatic cancer and healthy controls ($n = 31$ each). $^bP < 0.01$.

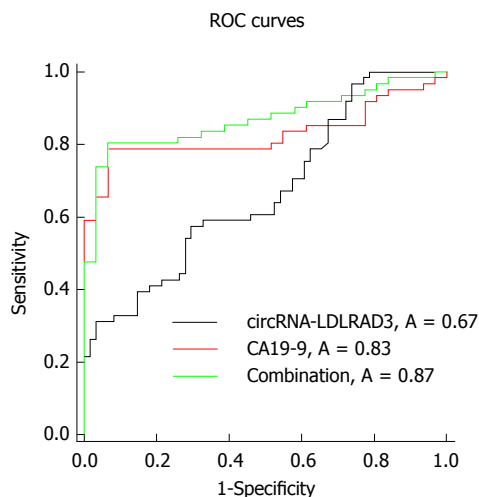


Figure 3 Receiver operating characteristic curves of circ-LDLRAD3 alone or in combination with CA19-9.

ylation and, furthermore, to inhibit the Wnt signaling pathway to prevent tumorigenesis in the esophagus^[30]. In addition, many differentially expressed circRNAs have been investigated in tissue, blood^[31], saliva^[32], and other bodily fluid^[33] samples, suggesting that circRNA molecules can serve as biomarkers in many diseases including diabetes mellitus^[34], coronary artery disease^[35], and cancer^[10]. CircRNAs, together with other known biomarkers, may be able to improve the accuracy of specificity of diagnosis in certain diseases. However, little work has been published thus far regarding the role of circRNAs in pancreatic cancer.

This is the first study to report the expression pattern of circ-LDLRAD3 and its diagnostic value in pancreatic cancer. The expression of circ-LDLRAD3 was higher in pancreatic cancer cell lines, pancreatic cancer tissues, and plasma samples of patients with

pancreatic cancer when compared to matched control samples. Moreover, the expression of circ-LDLRAD3 in metastatic pancreatic cell lines was higher than that in primary cell lines and there was a strong correlation between circ-LDLRAD3 expression and venous and lymphatic invasion in both tissues and plasma samples. Interestingly, in plasma samples, circ-LDLRAD3 was found to be associated with metastasis. Considering that there were no pancreatic cancer tissue samples with metastasis in the 30 patients tested, we strongly believe that the expression of circ-LDLRAD3 correlates with venous invasion, lymphatic invasion, and metastasis. These data indicate that circ-LDLRAD3 has potential to be a novel biomarker of metastatic pancreatic cancer with invasion potential.

This study provides a new avenue for the early diagnosis of pancreatic cancer, which has traditionally been clinically difficult^[32]. The sensitivity and specificity of tumor marker CA19-9 in the diagnosis of pancreatic cancer are 79%-81% and 82%-90%, respectively. However, about 3%-7% of pancreatic cancer patients are Lewis antigen negative and also do not express CA19-9; abnormal CA19-9 levels are not detected in this type of patients^[20,36,37]. In this study, serum levels of circ-LDLRAD3 were found to be closely related to blood CA19-9 levels. Compared with the diagnostic value of circ-LDLRAD3 alone in pancreatic cancer, whose AUC, sensitivity, and specificity were 0.67, 0.5738, and 0.7049, respectively, the combination of circ-LDLRAD3 and CA19-9 increased the diagnostic value, with corresponding values for AUC, sensitivity, and specificity were 0.87, 0.8033, and 0.9355, respectively. These results suggest that circ-LDLRAD3 has potential as a novel biomarker in the diagnosis of pancreatic cancer.

However, due to the limited number of available

Table 5 Correlation between circ-LDLRAD3 expression and clinicopathological factors of pancreatic cancer patients (plasma samples)

Characteristic	Circ-LDLRAD3		P value
	Low or one, <i>n</i>	High, <i>n</i>	
Age (yr)			
≥ 60	5	10	0.609
< 60	4	12	
Gender			
Male	5	14	0.675
Female	4	8	
Tumor diameter (cm)			
≤ 4	6	15	0.935
> 4	3	7	
CA19-9			
Positive	6	21	0.030
Negative	3	1	
CEA			
Positive	5	5	0.076
Negative	4	17	
Clinical stage			
I A	3	2	0.060
I B	3	3	
II A	3	3	
II B	0	5	
III	0	6	
IV	0	3	
T classification			
T1	3	2	
T2	3	12	0.282
T3	3	6	
T4	0	2	
N classification			
N0	9	12	0.049
N1	0	6	
N2	0	4	
Metastasis			
M0	9	19	0.244
M1	0	3	
Venous invasion			
No	9	10	0.005
Yes	0	12	
Lymphatic invasion			
No	9	12	0.014
Yes	0	10	

tissue and plasma samples from patients with pancreatic cancer, only 30 paired pancreatic cancer tissues and 31 matched plasma samples were analyzed. Studies utilizing a large number of samples in multiple centers should be implemented in future. The study of circ-LDLRAD3 function in pancreatic cancer is also likely to improve the understanding of the occurrence and progression mechanisms of pancreatic cancer.

In conclusion, our data indicate that circ-LDLRAD3 expression was significantly up-regulated in pancreatic cancer cell lines, pancreatic cancer tissues, and pancreatic cancer plasma samples. Furthermore, circ-LDLRAD3 expression was correlated with lymphatic invasion, venous invasion, and metastasis. Therefore, circ-LDLRAD3 has potential as a novel biomarker indicative of tumor invasion capacity in the diagnosis

Table 6 Correlation between circ-LDLRAD3 expression and clinicopathological factors of pancreatic cancer patients (Δ CT values in plasma samples)

Variable	Circ-LDLRAD3 expression level	
	Spearman correlation	P value
Age (yr)	-0.108	0.562
Gender	0.059	0.752
Tumor diameter (cm)	-0.102	0.584
CA19-9	-0.398	0.027
CEA	-0.085	0.650
Clinical stage	-0.603	< 0.001
T classification	-0.129	0.491
N classification	-0.271	0.140
Metastasis	-0.5	0.004
Venous invasion	-0.392	0.029
Lymphatic invasion	-0.611	< 0.001

of pancreatic cancer.

ARTICLE HIGHLIGHTS

Research background

Pancreatic cancer is a malignancy with a very poor prognosis. There have been few recent therapeutic advances in the treatment of pancreatic cancer for more than 10 years. The key to improving the prognosis of pancreatic cancer mostly lies in early diagnosis and early treatment. Circular RNAs (circRNAs) are a class of noncoding RNAs characterized by stable ring structure formed by a covalently closed continuous loop, which makes them stable in cells, tissues, and body fluid. Therefore, they can serve as ideal biomarkers for detection of diseases, especially cancer. This study indicates that circ-LDLRAD3 has potential as a novel biomarker indicative of tumor invasion capacity in the diagnosis of pancreatic cancer.

Research motivation

This study aimed to analyze and evaluate the diagnostic value of a new circular RNA, circ-LDLRAD3, in pancreatic cancer. And research data suggest that circ-LDLRAD3 may be used as a biomarker in pancreatic cancer diagnosis.

Research objectives

The main objectives in this study were pancreatic cancer and a new circular RNA, circ-LDLRAD3. The results showed that the expression level of circ-LDLRAD3 was up-regulated in pancreatic cancer and it can serve as a biomarker in pancreatic cancer.

Research methods

The expression levels of circ-LDLRAD3 were detected using real-time quantitative reverse transcription polymerase chain reaction (qRT-PCR) in pancreatic cancer cell lines, normal pancreatic cell lines, paired pancreatic cancer tissues and adjacent non-tumorous tissues, and plasma samples from patients with pancreatic cancer and healthy volunteers. The relationship between circ-LDLRAD3 expression and patients' clinicopathological factors was analyzed, the diagnostic value of circ-LDLRAD3 was further calculated alone and combined with CA19-9.

Research results

Our study found that expression levels of circ-LDLRAD3 were up-regulated in pancreatic cell lines, pancreatic cancer tissues, and plasma samples from pancreatic cancer patients. It may serve as a new biomarker in the diagnosis of pancreatic cancer. Studies utilizing a large number of samples in multiple centers should be implemented in future. The study of circ-LDLRAD3 function in pancreatic cancer is also likely to improve the understanding of the occurrence

and progression mechanisms of pancreatic cancer.

Research conclusions

This study indicated that the expression of a new circular RNA, circ-LDLRAD3, was significantly up-regulated in pancreatic cancer cell lines, pancreatic cancer tissues, and pancreatic cancer plasma samples. Furthermore, circ-LDLRAD3 expression was correlated with lymphatic invasion, venous invasion, and metastasis. Therefore, circ-LDLRAD3 has potential as a novel biomarker indicative of tumor invasion capacity in the diagnosis of pancreatic cancer. It is highly believed that the key to improving the prognosis of pancreatic cancer mostly lies in early diagnosis and early treatment. Therefore, searching for ideal biomarkers is essential. Circular RNAs are a class of non-coding RNAs which are stable because of their unique circular structure. Previous studies have confirmed that some circRNAs can serve as biomarkers in certain diseases. In this study, we focused a new circular RNA, circ-LDLRAD3, and hypothesized that expression levels of circ-LDLRAD3 were up-regulated in pancreatic cancer. Moreover, this study verified the hypothesis and found that expression levels of circ-LDLRAD3 were significantly up-regulated in pancreatic cancer cell lines, pancreatic cancer tissues, and pancreatic cancer plasma samples, whose expression levels were correlated with lymphatic invasion, venous invasion, and metastasis. Therefore, circ-LDLRAD3 may be a new biomarker in the diagnosis of pancreatic cancer.

Research perspectives

This is the first study to report the expression pattern of circ-LDLRAD3 and its diagnostic value in pancreatic cancer and provides a new avenue for the early diagnosis of pancreatic cancer. However, due to the limited number of available tissue and plasma samples from patients with pancreatic cancer, studies utilizing a large number of samples in multiple centers should be implemented in future. The study of circ-LDLRAD3 function in pancreatic cancer is also likely to improve the understanding of the occurrence and progression mechanisms of pancreatic cancer. And more types of circular RNAs and their relationship with pancreatic cancer should be verified in the future research.

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Case Control Study

Rifaximin ameliorates hepatic encephalopathy and endotoxemia without affecting the gut microbiome diversity

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Abstract

AIM

To determine the efficacy of rifaximin for hepatic encephalopathy (HE) with the linkage of gut microbiome in decompensated cirrhotic patients.

METHODS

Twenty patients (12 men and 8 women; median age, 66.8 years; range, 46-81 years) with decompensated cirrhosis (Child-pugh score > 7) underwent cognitive neuropsychological testing, endotoxin analysis, and fecal microbiome assessment at baseline and after 4 wk of treatment with rifaximin 400 mg thrice a day. HE was determined by serum ammonia level and number connection test (NCT)-A. Changes in whole blood endotoxin activity (EA) was analyzed by endotoxin

activity assay. Fecal microbiome was assessed by 16S ribosome RNA (rRNA) gene sequencing.

RESULTS

Treatment with rifaximin for 4 wk improved hyperammonemia (from 90.6 ± 23.9 $\mu\text{g/dL}$ to 73.1 ± 33.1 $\mu\text{g/dL}$; $P < 0.05$) and time required for NCT (from 68.2 ± 17.4 s to 54.9 ± 20.3 s; $P < 0.05$) in patients who had higher levels at baseline. Endotoxin activity was reduced (from 0.43 ± 0.03 to 0.32 ± 0.09 ; $P < 0.05$) in direct correlation with decrease in serum ammonia levels ($r = 0.5886$, $P < 0.05$). No statistically significant differences were observed in the diversity estimator (Shannon diversity index) and major components of the gut microbiome between the baseline and after treatment groups (3.948 ± 0.548 at baseline *vs* 3.980 ± 0.968 after treatment; $P = 0.544$), but the relative abundances of genus *Veillonella* and *Streptococcus* were lowered.

CONCLUSION

Rifaximin significantly improved cognition and reduced endotoxin activity without significantly affecting the composition of the gut microbiome in patients with decompensated cirrhosis.

Key words: Gut microbiome; Hepatic encephalopathy; Liver cirrhosis; Endotoxin; Rifaximin

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Core tip: Hepatic encephalopathy (HE) is characterized by deficits in cognitive, psychiatric, and motor function and ranges in severity from minimal to overt HE and coma. Rifaximin is used for standard treatment of HE, targeting reduction of ammonia and gut bacterial translocation. This study demonstrates that rifaximin improves hyperammonemia and cognitive impairment with the linkage of decreased endotoxin activity in patients with decompensated cirrhosis. The diversity and major components of gut microbiome analyzed by 16S rRNA gene sequencing are not altered by treatment with rifaximin. This is the first report of systemic and local effects of rifaximin in Japanese patients.

Kaji K, Takaya H, Saikawa S, Furukawa M, Sato S, Kawaratani H, Kitade M, Moriya K, Namisaki T, Akahane T, Mitoro A, Yoshiji H. Rifaximin ameliorates hepatic encephalopathy and endotoxemia without affecting the gut microbiome diversity. *World J Gastroenterol* 2017; 23(47): 8355-8366 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v23/i47/8355.htm> DOI: <http://dx.doi.org/10.3748/wjg.v23.i47.8355>

INTRODUCTION

Hepatic encephalopathy (HE) is a spectrum of neuropsychiatric syndromes that form major complications

in patients with acute or chronic liver disease^[1]. It is characterized by a wide range of changes in the mental state from minimal signs of altered brain function to deep coma. Minimal HE is the earliest stage; it occurs in up to 80% of patients with cirrhosis and manifests as abnormalities in the central nervous system function^[2,3]. Recent pathophysiological evidence suggests that alterations in the gut microbiome could be critical to bacterial translocation, hyperammonemia, and systemic inflammation, leading to the development of HE^[4-7]. Bacterial overgrowth in the gut microbiome is closely associated with the severity of liver disease, and patients with overt HE reportedly reveal significant changes in the enteric microbiota compared with those with minimal HE^[4-7]. Recent randomized clinical trial has reported that fecal microbiota transplantation, a newly developed microbiome-targeted therapy, has the potential to improve HE^[8].

Considering the pathogenesis of microbe-based HE, endotoxemia is known to play a potentially cardinal role in the development of systemic inflammation and neuroinflammation. In patients with advanced cirrhosis, endotoxin increases the permeability of the blood-brain barrier and enhances astrocyte swelling via nitric oxide and prostanoid production in the brain microglia^[9-11]. Clinical evidence suggests that endotoxemia is correlated with the severity of HE and increased incidence of overt HE^[10-12]. Furthermore, accumulating evidences have revealed that microbiota-targeted therapies, such as probiotics, prebiotics, synbiotics, and antibiotics, may cause at least partial improvement of endotoxemia^[13-16].

Rifaximin, an oral antibiotic with broad-spectrum activity against aerobic and anaerobic Gram-positive and Gram-negative bacteria, is widely used for the prevention of HE and is proposed to have beneficial effects on overt HE and survival. It could exert antimicrobial activity against ammonia-producing enteric bacteria including (1) Gram-positive aerobic bacteria such as *Streptococcus* and *Bacillus*; (2) Gram-negative facultative anaerobic bacteria such as *E. coli*, *Klebsiella*, *Citrobacter*, *Enterobacter*, and *Proteus*; (3) Gram-positive obligatory anaerobic bacteria such as *Clostridium*; and (4) Gram-negative obligatory anaerobic bacteria such as *Bacteroides*^[17]. However, in Japan, it has only been recently available for patients with HE^[18]. Several studies of cirrhotic patients in the Western countries have reported the possible mechanisms of action of rifaximin, suggesting that it could decrease endotoxin levels without altering the relative abundance of pathogenic bacteria^[19-21]. To the best of our knowledge, the effects of rifaximin on the gut microbiome in patients from the Eastern countries have not been assessed. Moreover, the relationship between the endotoxin activity and microbial alteration at the gene level has not been elucidated.

The present study aimed to evaluate the impact of rifaximin on the endotoxin activity and gut microb-

iota identified by 16S ribosome RNA (rRNA) gene sequencing in patients with decompensated cirrhosis.

MATERIALS AND METHODS

Patients and study design

The study was conducted from January to May 2017 at the Third Department of Internal Medicine of Nara Medical University. The subjects were patients with decompensated cirrhosis (Child-Pugh score > 7) due to several causes, aged 18 years or older, who had been diagnosed by clinical, biochemical, and ultrasound findings ($n = 45$). The exclusion criteria were cardiac and/or respiratory failure or invasive cancer within the past 5 years; renal failure with serum creatinine > 200 $\mu\text{mol/L}$; clinical or biochemical signs of infection 28 d prior to inclusion; concomitant inflammatory bowel diseases and/or irritable bowel syndrome; previous history of gastrectomy, enterectomy, and/or liver transplantation; and developed portosystemic shunt. Patients who consumed nonabsorbable disaccharides, probiotics, prebiotics, synbiotics, or other antibiotics 28 d prior to inclusion were also excluded. Finally, 20 patients except for the patients to meet the exclusion criteria ($n = 17$) and decline to participate ($n = 8$) were finally analyzed.

All subjects were treated with rifaximin 400 mg thrice a day for 4 wk, and the complete investigational program was performed at baseline and after 4 wk of treatment. The program comprised general laboratory tests, measurement of serum ammonia levels, neuropsychological testing, measurement of whole blood endotoxin activity, and analysis of fecal microbiota (Figure 1).

The study protocols conformed to the principles outlined in the 1964 Declaration of Helsinki and its later amendments and were approved by the Ethics Committee of Nara Medical University (approval number 994) and were registered at UMIN000029127. All subjects provided written informed consent prior to their inclusion in the study.

Neuropsychological test system

To objectively evaluate cognitive performance, we used the number connection test (NCT)-A distributed by the Japan Society of Hepatology, as previously described^[22,23]. The hardware consisted of a touch screen tablet such as iPad (Apple, Cupertino, CA, United States).

Measurement of endotoxin activity

Whole blood endotoxin activity was assessed with the commercially available Endotoxin Activity Assay (EAA) kit (Spectral Diagnostics, Toronto, Canada), which uses a luminol chemiluminescence method. In brief, the EAA is based on the principle that endotoxin binds to antiendotoxin antibodies and is delivered to neutrophils

by complement receptors. In the presence of β -glucan and luminol, the neutrophils undergo a respiratory burst accompanied by light emission. The light produced is quantified by a chemiluminometer, and its intensity is proportional to the amount of endotoxin present in the sample^[24].

Analysis of fecal microbiota composition

Fecal samples were collected before and 4 wk after rifaximin administration and placed in 1.5-mL tubes, snap-frozen on dry ice, and stored at -80°C . 16S rRNA analysis of fecal samples was performed at Takara Bio (Shiga, Japan). DNA was extracted with the MoBio Powerlyzer Powersoil DNA Isolation Kit (MoBio Laboratories, Carlsbad, CA, United States). The V4 hypervariable region of the bacterial 16S rRNA gene was amplified from the fecal DNA extracts using the modified universal bacterial primer pairs 341F (5'-TCGTCGGCAGCGTCAGATGTGTATAAGA GACAGCCTACGGGNGGCWGCAG-3') and 806R (5'-GTCTCGTGGGCTCGGAGATGTGTATAAGAGA CAGGGACTACHVGGGTWTCTAAT-3') with Illumina adaptor overhang sequences. Amplicons were generated, cleaned, indexed, and sequenced according to the Illumina MiSeq 16S Metagenomic Sequencing Library Preparation protocol (http://support.illumina.com/downloads/16s_metagenomic_sequencing_library_preparation.html), with brief modifications.

Sequencing data were combined and sample identification was assigned to multiplexed reads using the MOTHUR software environment^[25]. The data were denoised; low-quality sequences, pyrosequencing errors, and chimeras were removed; and then the sequences were clustered into operational taxonomic units (OTUs) at 97% identity using the CD-HITOTU pipeline (available from <http://eeizhong-lab.ucsd.edu/cd-hit-otu>)^[26]. OTUs containing fewer than four reads per individual diet/animal combination were excluded due to the likelihood of a sequencing artifact. The samples were normalized by random resampling sequences used to the lowest number of sequences per sample (each diet/animal combination) using Daisychopper (<http://www.festinalente.me/bioinf/>). Taxonomic classification of OTUs was done with the Ribosomal Database Project Classifier^[27].

Statistical analysis

Differences between the paired groups were analyzed by the Mann-Whitney U test. Correlations were calculated with the Spearman rank test. The data are expressed as means \pm SD. A two-tailed p -value less than 0.05 was considered to indicate statistical significance. Analyses were performed with EZR (Saitama Medical Center, Jichi Medical University), which is a graphical user interface for R (The R Foundation for Statistical Computing, version 2.13.0). Specifically, EZR is a modified version of R comman-

Table 1 Characteristic features of patients at baseline (*n* = 20)

	Baseline	Treatment	<i>P</i> value
Age	66.8 (46-81)		
Sex (male/female)	12/8		
Etiology			
Alcohol	4		
HBV	4		
HCV	8		
NASH	2		
Alcohol + HBV	1		
Alcohol + HCV	1		
Child class (A/B/C)	0/18/2		
MELD score	8.3 (2.6-15.0)	7.5 (1.2-15.0)	0.474
AST (U/L)	50 ± 22	53 ± 29	0.791
ALT (U/L)	32 ± 14	31 ± 14	0.755
Albumin (g/dL)	3.3 ± 0.6	3.3 ± 0.5	0.980
Total bilirubin (mg/dL)	1.8 ± 0.9	1.6 ± 0.8	0.545
Prothrombin time (INR)	1.28 ± 0.11	1.26 ± 0.11	0.630
CRP (mg/dL)	0.3 ± 0.6	0.2 ± 0.2	0.533
WBC (10 ³ /μL)	3.4 ± 1.1	3.5 ± 1.0	0.847
Platelet (10 ⁴ /μL)	8.1 ± 4.1	7.7 ± 3.5	0.710
BTR	3.7 ± 1.5	4.2 ± 3.7	0.601

Data of age and MELD score are given in median and total range. The other data are given in mean ± SD. BTR: Branched chain amino acid and tyrosine ratio; CRP: C-reactive protein; HBV: Hepatitis B virus; HCV: Hepatitis C virus; NASH: Nonalcoholic steatohepatitis.

der (version 1.6-3) that includes statistical functions frequently used in biostatistics^[28].

RESULTS

Characteristic features of patients

The demographic and baseline clinical characteristics of the patients are presented in Table 1. Twenty patients with decompensated cirrhosis (12 men and 8 women; median age, 66.8 years; range, 46-81 years) were included in the study. The etiology of cirrhosis was identified as alcohol (40%), hepatitis B virus (HBV) (20%), hepatitis C virus (HCV) (20%), nonalcoholic steatohepatitis (NASH) (10%), alcohol and HBV (5%), and alcohol and HCV (5%). The majority of the patients (90%) were classified as Child-Pugh B, and the others (10%) were classified as Child-Pugh C; the median Model of End-stage Liver Disease (MELD) score was 8.6 (range, 2.6-15.0). Administration of rifaximin did not have any adverse effects, including hepatotoxicity and nephrotoxicity (Supplementary Figure 1), on any patient during the research period. Also, no significant changes were observed in MELD score, serum albumin, total bilirubin, prothrombin time, C-reactive protein (CRP), white blood cells (WBC), platelet and branched chain amino acid & tyrosine ratio (BTR) after 4 wk treatment of rifaximin (Table 1). The numbers of patient with the high endotoxin activity/delayed NCT/high ammonia are 11/10/16. All of the patients with high endotoxin activity were included in the high ammonia group. Seven patients with delayed NCT were included in the high ammonia group.

Effect of rifaximin on hepatic encephalopathy

The mean levels of serum ammonia among all patients remain unaltered after 4 wk of treatment with rifaximin as compared with baseline (66.7 ± 29.8 μg/dL at baseline vs 62.7 ± 27.6 μg/dL after treatment, *P* = 0.440; Figure 2A), although the mean levels among the patients who revealed high levels of serum ammonia (> 70 μg/dL) at baseline were significantly decreased after treatment (90.6 ± 23.9 μg/dL at baseline vs 73.1 ± 33.1 μg/dL after treatment, *P* < 0.05; Figure 2B). In coincidence with serum ammonia levels, the mean time required for NCT among all patients did not differ from baseline after treatment (51.7 ± 18.7 s at baseline vs 45.0 ± 18.4 s after treatment, *P* = 0.267; Figure 3A), whereas the mean time required for NCT among patients who revealed prolongation of NCT (> 50 s) at baseline was significantly shortened after treatment (68.2 ± 17.4 s at baseline vs 54.9 ± 20.3 s after treatment, *P* < 0.05; Figure 3B).

Endotoxin activity

The mean endotoxin activity among all patients remained unaltered after 4 weeks of treatment compared with baseline (0.27 ± 0.14 at baseline vs 0.29 ± 0.15 after treatment, *P* = 0.641; Figure 4A). The mean endotoxin activity among patients who reported high levels of endotoxin activity (> 0.4) at baseline was significantly decreased after treatment (0.43 ± 0.03 at baseline vs 0.32 ± 0.09 after treatment, *P* < 0.05; Figure 4B). Univariate correlation analysis demonstrated that the decrease in the endotoxin activity level after 4 weeks of treatment (Δ EA) correlated directly with the decrease in the serum ammonia level (Δ NH₃; *r* = 0.5886, *P* < 0.05; Figure 4C).

Microbiota composition of the feces

For gut microbiome analysis using 16S rRNA gene sequencing, fecal samples were collected before and after 4 wk of treatment with rifaximin from the 20 patients. In total, 33222538 raw reads were obtained from all 40 fecal samples. After filtering, 17958952 high-quality sequences were produced, with an average of 448473 ± 64690 reads per sample. No statistically significant differences were observed in the diversity estimator (Shannon diversity index) between the baseline and treatment groups (3.948 ± 0.548 at baseline vs 3.980 ± 0.968 after treatment, *P* = 0.544; Figure 5A). UniFrac principal coordinate analysis (PCoA) also revealed no significant clustering between the microbiota composition before and after rifaximin treatment (Figure 5B). The overall microbiota composition and average relative abundances for each group at the phylum, class, and order levels are shown in Figure 4C-E. There were 7 phyla, 12 classes, and 18 orders in the fecal samples. The dominant phyla of both groups were *Firmicutes*, *Actinobacteria*, *Bacteroides*, and *Proteobacteria*; the dominant classes of both

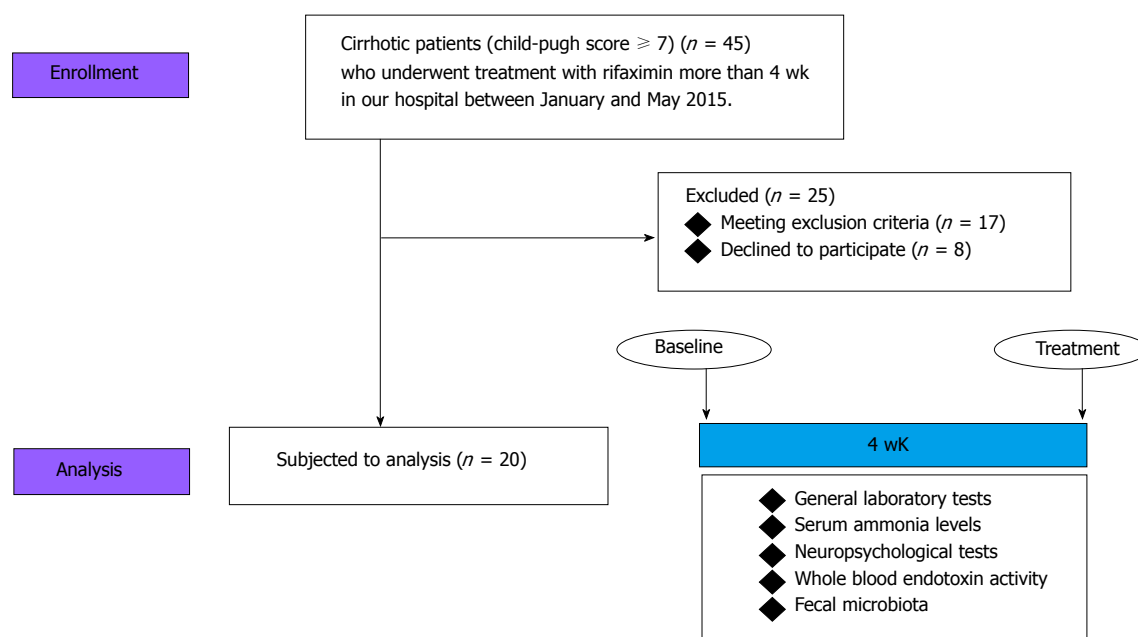


Figure 1 The selection of the study population and experimental design. 20 patients except for 25 patients to meet the exclusion criteria and decline to participate were finally analyzed.

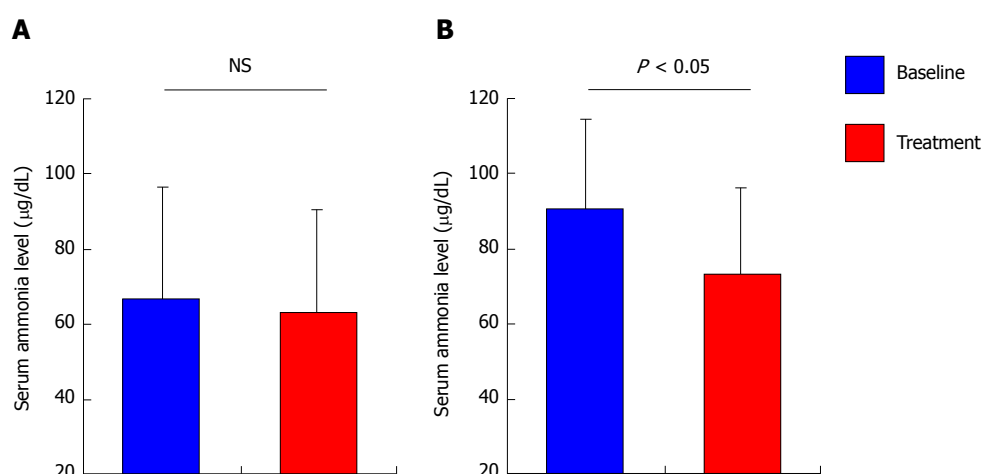


Figure 2 Effect of rifaximin on serum ammonia level. Comparison of the mean levels of serum ammonia between baseline and 4 wk post-rifaximin among (A) total patients ($n = 20$) and (B) the patients who showed high levels of serum ammonia ($> 70 \mu\text{g/dL}$) at baseline ($n = 16$). Data are means \pm SD.

groups were *Clostridia*, *Actinobacteria*, *Bacteroidia*, *Bacilli*, and *Coriobacteria*; and the dominant orders of both groups were *Bacillales*, *Actinomycetales*, *Bacteroidales*, *Coriobacteriales*, and *Bifidobacteriales*. At the phylum level, no differences were observed in the average abundance in the patients from baseline to after treatment with rifaximin (Figure 5C). Likewise, we did not observe any changes in the average abundance at the class and order levels (Figure 5D and E).

Furthermore, we evaluated the changes in abundance of selected genera after treatment with rifaximin. We selected seven genera revealing elevation in the feces of cirrhotic patients compared with healthy controls, as previously described^[29]. The relative abundances of *Veillonella* and *Streptococcus* were lower in the treatment group than in the baseline

group (Figure 6A and B), whereas no significant differences were observed in the relative abundances of *Lactobacillus*, *Prevotella*, *Haemophilus*, *Megasphaera*, and *Fusobacterium* (Figure 6C-G).

DISCUSSION

The results of this study demonstrate that rifaximin improves cognitive performance with reduced serum ammonia levels and endotoxin activity in patients with decompensated cirrhosis. The 16S rRNA gene analysis found no significant differences in the predominant organisms from before treatment to after treatment, indicating that rifaximin exerts its pharmacological actions independently of modification of the gut microbiota. Evidence reveals the effects of rifaximin on

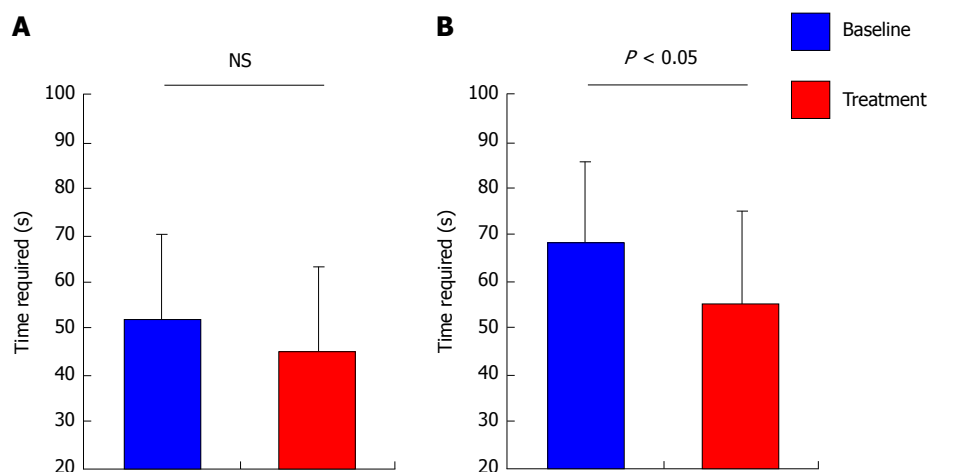


Figure 3 Effect of rifaximin on cognitive disturbance. Comparison of the mean time required for NCT between baseline and 4 wk post-rifaximin among (A) total patients ($n = 20$) and (B) the patients who showed prolongation for NCT (> 50 s) at baseline ($n = 10$). Data are means \pm SD.

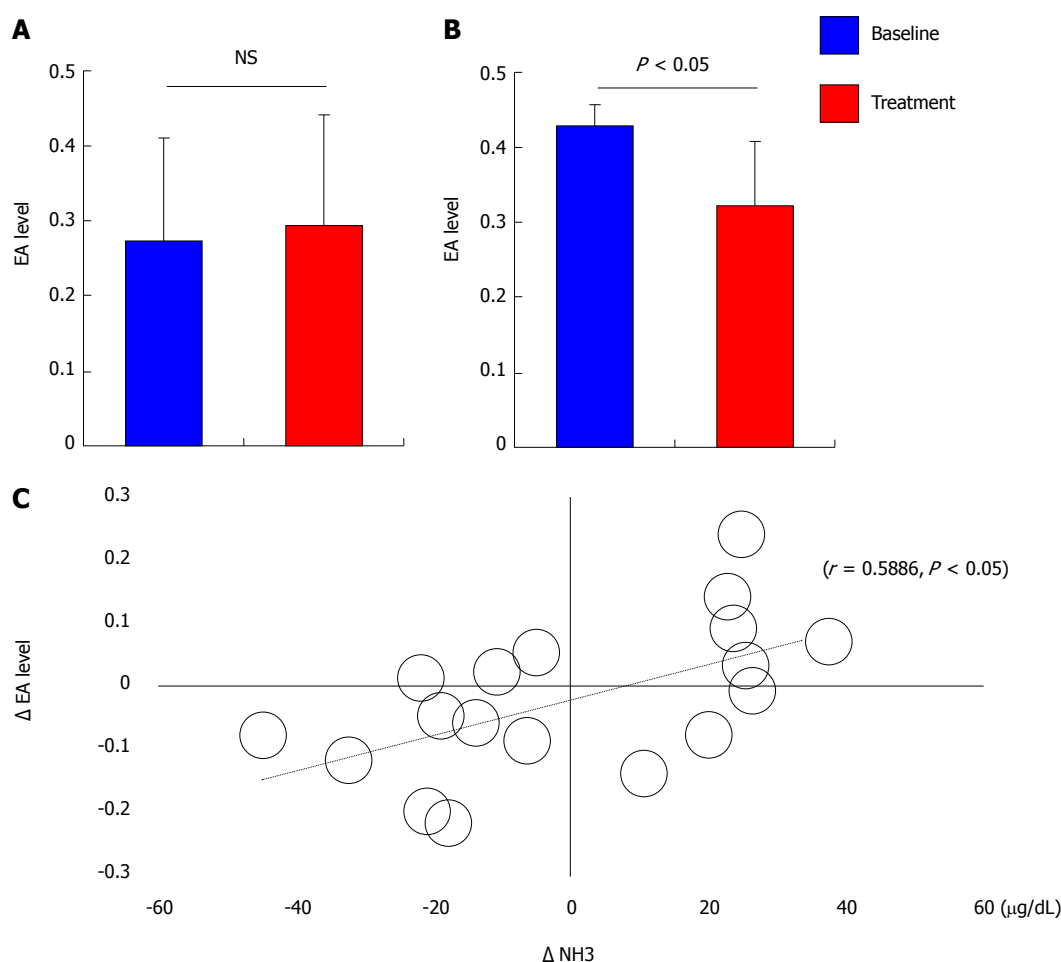
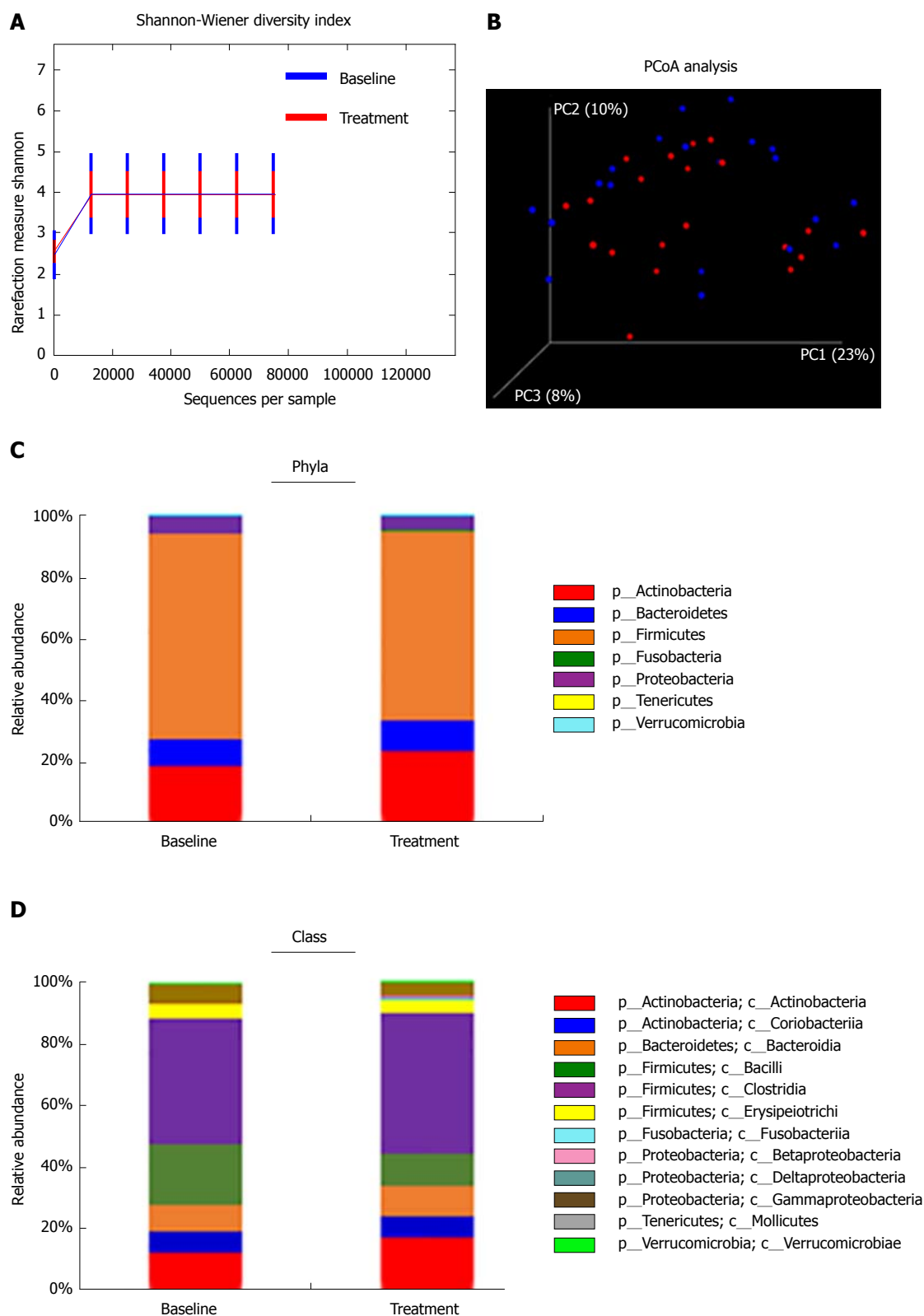


Figure 4 Effect of rifaximin on endotoxin activity. Comparison of the endotoxin activities between baseline and 4 wk post-rifaximin among (A) total patients ($n = 20$) and (B) the patients who showed high levels of EA (> 0.4) at baseline ($n = 11$). C: Univariate correlation analysis between the decrease in EA level (Δ EA) and that in serum ammonia level (Δ NH₃) by treatment with rifaximin ($r = 0.5886$, $P < 0.05$). Data are means \pm SD.

HE with the link between endotoxin and gut microbiota in patients from Western countries^[19-21]. Because the microbial taxa involved are slightly different in cirrhotic patients from Eastern and Western countries, we undertook this study to assess the effects of rifaximin

on HE in cirrhotic patients from Eastern countries^[30,31].

Rifaximin exerts its antibiotic actions via inhibition of bacterial RNA synthesis by binding to the β -subunit of bacterial DNA-dependent RNA polymerase^[32]. The activity of rifaximin is targeted to the gastrointestinal



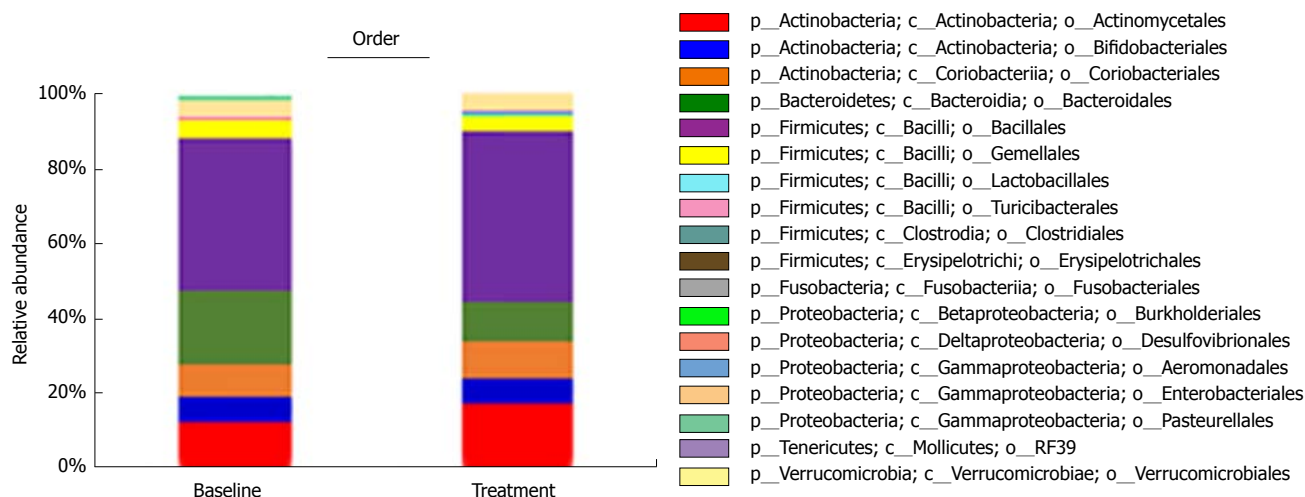


Figure 5 Effect of rifaximin on the diversity and major compositions of gut microbiome. A: Shannon diversity between baseline and treatment groups (mean index \pm SD 3.948 \pm 0.548 at baseline vs 3.980 \pm 0.968 at treatment, $P = 0.544$). B: Pco analysis (PcoA) of gut microbiota. Baseline samples (blue) were clustered together compared to 4 wk post-rifaximin (red). C-E: Effects of rifaximin on alterations in the composition of gut microbiome in phylum (C), class (D) and order (E).

tract due to its nonsystemic absorption; thus the use of rifaximin is a viable option as treatment for HE. A meta-analysis including 19 randomized, controlled trials of rifaximin for HE demonstrated that it lowered serum ammonia (mean difference, $-7.10 \mu\text{g/dL}$; 95%CI: -12.29 to -1.91) and improved NCT (mean difference, -5.29 s; 95%CI: -10.05 to -0.53)^[33]. Consistent with this evidence, the present results indicate that 4 wk of administration of rifaximin significantly reduced the serum ammonia levels and shortened the time required for NCT in cirrhotic patients who had reported higher levels at baseline. Recent clinical trials suggest that these beneficial effects of rifaximin on HE are strongly associated with improved endotoxemia^[19,21,34]. Basic *in vivo* studies revealed the mechanistic insight for the ability of rifaximin to lower plasma endotoxin levels. Kang *et al.*^[20] reported that rifaximin improved endotoxemia induced by humanization with stools from patients with minimal HE in germ-free mice^[20]. Zhu *et al.*^[35] demonstrated that rifaximin attenuates liver fibrosis and portal hypertension by inhibiting the lipopolysaccharides (LPS)/toll-like receptor (TLR) 4 pathway in bile duct-ligation induced fibrotic mice. Unlike other studies, we assessed the plasma endotoxin activity by an EAA. Most quantitative limulus amoebocyte lysate (LAL) tests, which are widely used to measure the endotoxin levels, are not endotoxin-specific, as these tests detect both endotoxin from Gram-negative bacteria and (1-3)- β -D-glucan from fungus, which are microbial products translocated from the intestine. Therefore, these tests are unable to detect spillover endotoxemia in liver diseases due to the complexity of the measurement, difficulty in standardization, and low sensitivity. The EAA is a novel and simple method to assess blood levels of endotoxin with higher sensitivity as compared with these tests^[24,36]. In fact, our results demonstrated

that rifaximin significantly decreased the plasma EA levels, but not endotoxin concentration as detected by LAL tests, in patients with cirrhosis (data not shown). Additionally, our results reveal a direct correlation between ΔNH_3 and ΔEA , indicating that a decrease in endotoxin activity is crucial in the effect of rifaximin on hyperammonemia. It has been reported that ammonia and LPS synergistically facilitate cytotoxic edema and precoma in cirrhotic rats^[37]. Another report suggested that bacterial LPS inhibit the hepatic ammonia removal via glutamine synthesis^[38]; however, a large-scale prospective study would be required to elucidate the exact mechanism of interaction between the generation of ammonia and endotoxemia.

As described in the previous reports, we focused on the modulation of gut microbiota as the major cause of the ability of rifaximin to lower plasma endotoxin activity levels in cirrhotic patients^[19-21,33,39]. Remarkably, although rifaximin exerts its effects on a wide spectrum of Gram-positive and Gram-negative organisms, recent evidence suggests that its pharmacological action may involve the alteration of bacterial function and virulence rather than reduction of the bacterial population^[39]. In a previous cohort study, Bajaj *et al.*^[40] assessed the modulation of the gut microbiome in cirrhotic patients with minimal HE and demonstrated that rifaximin affected neither the overall abundance of bacteria nor the bacterial load. They revealed that rifaximin's clinical activity might be attributed to effects on metabolic function of the gut microbiota, rather than a change in the relative bacterial abundance^[19,40]. Our results also reveal no significant changes in the relative abundances at the phylum, class, and order levels as well as in the overall diversity of fecal microbiota between baseline and follow-up samples. Furthermore, a previous quantitative metagenomic study reported that several genera of bacteria, including *Veillonella*, *Streptococcus*,

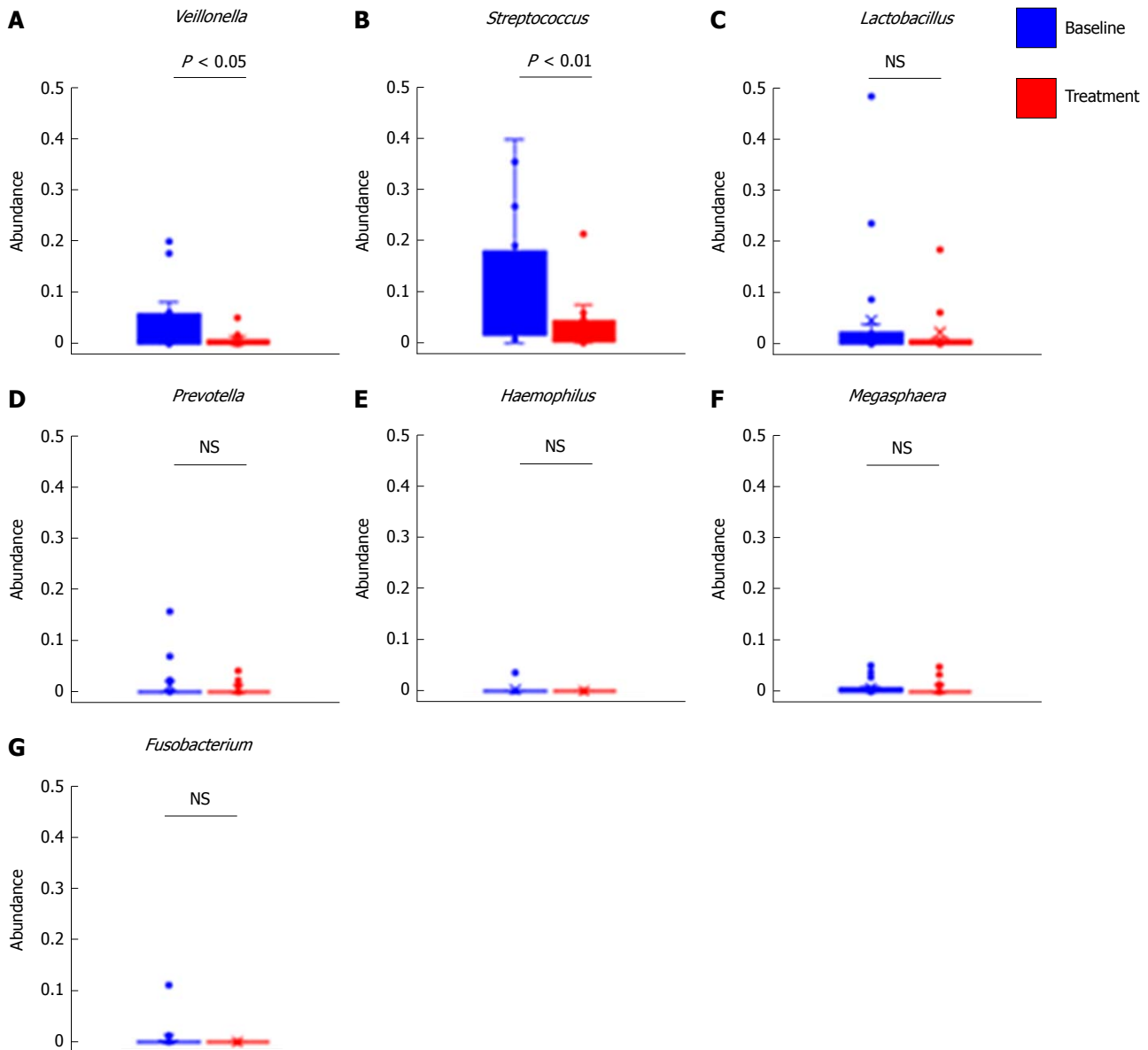


Figure 6 Alterations in abundances of selected genera by treatment with rifaximin. Relative abundances of (A) *Veillonella*, (B) *Streptococcus*, (C) *Lactobacillus*, (D) *Prevotella*, (E) *Haemophilus*, (F) *Megaspiera* and (G) *Fusobacterium*. Data are means \pm SD.

Lactobacillus, *Prevotella*, *Haemophilus*, *Megaspiera*, and *Fusobacterium*, were more abundant in cirrhotic patients^[29]; therefore, we next evaluated the effects of rifaximin on the levels of these organisms. Notably, present metagenomics revealed a marked decrease in *Veillonella* after treatment with rifaximin. *Veillonella* is an anaerobic Gram-negative coccus, and previous studies reported that its abundance increased in the colonic mucosa of cirrhotic patients with HE as compared with those without HE, and decreased less in patients treated with rifaximin and nonabsorbable disaccharide than in those receiving nonabsorbable disaccharide monotherapy^[34]. These findings indicate that *Veillonella* may be a candidate fecal marker for the presence of HE. *Veillonella* primarily requires lactic acid for fermentation, and hence it has a symbiotic relationship with *Streptococcus*, which produces

lactate metabolically^[41]. Based on their symbiosis, we also observed a significant decrease in *Streptococcus* in parallel with *Veillonella* in the fecal samples from patients treated with rifaximin.

In the present study, endotoxin-generating Gram-negative bacteria were unchanged by treatment with rifaximin. The role of rifaximin in lowering plasma endotoxin levels without modifying the overall composition of the gut microbiome remains unclear. We suggest two possible mechanisms: first, the impact of metabolic modifications in the gut microbiota^[19]; second, the possibility that rifaximin may contribute in improvement of the intestinal barrier function. A recent *in vitro* study using human intestinal epithelial cells reported that *Clostridium difficile* toxin A-induced cell apoptosis and deprivation of tight junction proteins (TJPs) were suppressed by treatment with rifaximin

through the pregnane X receptor-dependent inhibition of the TLR4/MyD88/NF- κ B pathway^[42]. Our previous report also demonstrated that antibiotics improved the intestinal permeability and enhanced TJP expression in the rat nonalcoholic steatohepatitis model^[43]. A further basic analysis is needed to elucidate the association of these mechanisms.

A limitation of this study was the small sample size. Further studies are essential to evaluate the effects of rifaximin in a larger population and in other Asian countries, as well as the effects of its long-term administration. Additionally, assessment of proinflammatory cytokines such as tumor necrosis factor- α and interleukin-6 is currently in progress for deeper analysis of the interaction between endotoxemia and the development of HE.

In conclusion, rifaximin significantly improved cognition and reduced endotoxin activity with minor modification of the gut microbiome in patients with decompensated cirrhosis. This is the first report of systemic and local effects of rifaximin in Japanese patients.

ARTICLE HIGHLIGHTS

Research background

Hepatic encephalopathy (HE) is characterized by deficits in cognitive, psychiatric, and motor function and ranges in severity from minimal to overt HE and coma. Rifaximin is used for standard treatment of HE, targeting reduction of ammonia and gut bacterial translocation. Currently, rifaximin has been suggested to partially affect gut microbiome in the patients with HE.

Research motivation

The effects of rifaximin on the gut microbiome in patients from the Eastern countries have not been assessed. Moreover, the relationship between the endotoxin activity and microbial alteration at the gene level has not been elucidated. Recently, 16S rRNA gene sequencing has been established as a novel method to directly access the genetic content of entire communities of organisms. Some evidence reveals the effects of rifaximin on HE with the linkage of gut microbiota in patients from Western countries. Because the microbial taxa involved are slightly different in cirrhotic patients from Eastern and Western countries, we undertook this study to assess the effects of rifaximin on HE in cirrhotic patients from Eastern countries. Moreover, the relationship between the endotoxin activity and microbial alteration at the gene level.

Research objectives

To determine the efficacy of rifaximin for hepatic encephalopathy (HE), evaluated with serum ammonia level, NCT and endotoxin activity, with the linkage of gut microbiome in decompensated cirrhotic patients.

Research methods

Twenty patients with decompensated cirrhosis were enrolled for this study. They were treated with rifaximin 400 mg three times a day for 4 wk. The measurement of serum ammonia level and number connection test (NCT)-A were performed to evaluate their status of hepatic encephalopathy before and after treatment of rifaximin. Endotoxemia was assessed by blood endotoxin activity assay (EAA). The 16S ribosome RNA gene sequencing was performed for analysis of fecal microbiome, and the diversity and compositions of gut microbiome were compared between before and after treatment of rifaximin.

Research results

This study demonstrates that rifaximin improves hyperammonemia and cognitive impairment with the linkage of decreased endotoxin activity in

patients with decompensated cirrhosis. The diversity and major components of gut microbiome analyzed by 16S rRNA gene sequencing are not altered by treatment with rifaximin, although the relative abundances of genus *Veillonella* and *Streptococcus* were lowered.

Research conclusions

This study demonstrates that rifaximin significantly improves hepatic encephalopathy with minor modification of the gut microbiome in Japanese patients with decompensated cirrhosis. Rifaximin markedly improved cognition and reduced endotoxin activity without significantly affecting the composition of the gut microbiome indicating that the effect of rifaximin is independent of modification of gut microbial diversity. This effect of rifaximin on gut microbiome in Japanese cirrhotic patients is similar to the patients in the West. On the other hands, rifaximin modified minor compositions of gut microbiome such as decreased relative abundances of genus *Veillonella* and *Streptococcus* in current subjects. So far, the mechanism of decreased endotoxin activity by rifaximin is still obscure, but we speculate that it is possibly related to the pharmacological action of rifaximin to improve intestinal barrier function. In conclusion, rifaximin is an effective medical agent for the patients with hepatic encephalopathy.

Research perspectives

This study demonstrates that rifaximin improves hyperammonemia and cognitive impairment with the linkage of decreased endotoxin activity in patients with decompensated cirrhosis. These effects of rifaximin are independent of alteration of gut microbial diversity, indicating that rifaximin has a potential capacity to decrease ammonia and endotoxin level other than the effect on gut microbiome such as the improvement of intestinal barrier function. Therefore, we will examine the effect of rifaximin on intestinal tight junction protein in the clinical practice to elucidate above hypothesis in near future after the approval of ethical committee. We consider that the best method is to analyze the alteration of intestinal tight junction protein before and after treatment with rifaximin in the biopsy tissues from the patients with decompensated cirrhosis.

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Retrospective Study

Association between white opaque substance under magnifying colonoscopy and lipid droplets in colorectal epithelial neoplasms

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Informed consent statement: Patients were not required to give informed consent as this is a retrospective study.

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Abstract

AIM

To examine the association between white opaque substance (WOS) and histologically verified lipid

droplets in colorectal epithelial neoplasms.

METHODS

We reviewed colonoscopy records at our institution from 2014 to 2016 and identified cases of endoscopically or surgically resected colorectal epithelial neoplasms observed by magnifying narrow-band imaging (M-NBI) colonoscopy. Immunohistochemistry was used to stain tumors with a monoclonal antibody specific to adipophilin as a marker of lipids. The expression and distribution of adipophilin were compared between WOS-positive and WOS-negative lesions and among tumors classified by histologic type and depth of invasion.

RESULTS

Under M-NBI colonoscopy, 81 lesions were positive for WOS and 48 lesions were negative for WOS. The rate of adipophilin expression was significantly higher in WOS-positive lesions (95.1%) than in WOS-negative lesions (68.7%) ($P = 0.0001$). The incidence of deep adipophilin expression was higher in WOS-positive lesions (24.7%) than in WOS-negative lesions (4.2%) ($P = 0.001$). The incidence of deep expression was predominant among cancers with massive submucosal invasion (62.5%) compared to adenoma (7.2%) and high-grade dysplasia or cancers with slight submucosal invasion (12.7%) ($P = 0.0001$).

CONCLUSION

The distribution of lipid droplets may be closely associated with the visibility of WOS under M-NBI colonoscopy, and with histologic grade and depth of tumor invasion.

Key words: White opaque substance; Adipophilin; Magnifying narrow-band imaging; Colorectal neoplasm; Magnifying endoscopy

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Core tip: We investigated the association between the distribution of the lipid droplets and endoscopically-verified white opaque substance (WOS) in colorectal neoplasms. The incidence of deep adipophilin expression was higher in WOS-positive lesions than in WOS-negative lesions. The incidence of deep expression was predominant among cancers with massive submucosal invasion compared to adenoma and high-grade dysplasia or cancers with slight submucosal invasion. We thus concluded that the distribution of lipid droplets may be closely associated with the visibility of WOS, and also with histologic grade and depth of tumor invasion.

Kawasaki K, Eizuka M, Nakamura S, Endo M, Yanai S, Akasaka R, Toya Y, Fujita Y, Uesugi N, Ishida K, Sugai T, Matsumoto T. Association between white opaque substance under magnifying colonoscopy and lipid droplets in colorectal epithelial neoplasms.

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INTRODUCTION

White opaque substance (WOS) under magnifying narrow-band imaging (M-NBI) endoscopy has been reported to be a novel endoscopic finding in gastric neoplasms^[1]. Yao *et al*^[1] reported that WOS is valuable for distinguishing between gastric adenocarcinoma and gastric adenoma. WOS has also been observed in other gastrointestinal tract neoplasms, such as esophageal adenocarcinoma originating from esophageal glands, duodenal neoplasms, and colorectal neoplasms^[2-4]. We recently reported that irregular distribution of WOS in colorectal neoplasms may be a sign of massively invading submucosal colorectal cancer^[5].

In gastric and duodenal neoplasms, WOS has been shown to represent an accumulation of lipid droplets^[3,6]. Lipid staining for gastric and duodenal epithelial neoplasms has demonstrated that most gastric neoplasms with WOS are positive for oil red O staining^[6], and the distribution of Sudan IV-positive lipid droplets corresponds approximately with that of WOS in duodenal neoplasms^[3]. To date, however, only a single study investigated the histopathological features of WOS in colorectal epithelial neoplasms^[7]. In that study, Imamura *et al*^[7] reported that WOS in colorectal epithelial neoplasms was composed of lipid droplets. However, the association between the distribution of the lipid droplets and endoscopically-verified WOS in colorectal neoplasms remains unclear.

We conducted a single-center, retrospective analysis to examine the association between lipid droplets and WOS in colorectal epithelial neoplasms. We also compared the distribution of lipid droplets among tumors classified by histologic type and depth of invasion.

MATERIALS AND METHODS

Study population and data collection

The present investigation was based on retrospective data collection. We reviewed the endoscopy database at our institution from 2014 to 2016, and identified all patients with a diagnosis of colorectal epithelial neoplasm removed by endoscopic submucosal dissection (ESD) or laparoscopic surgery. Among those tumors, we excluded colorectal epithelial neoplasms, which cannot be observed by M-NBI colonoscopy. We also excluded cancers invading the proper muscular layer. The protocol of this retrospective study was approved by the Institutional Review Board at Iwate Medical University.

Demographics of study subjects were extracted

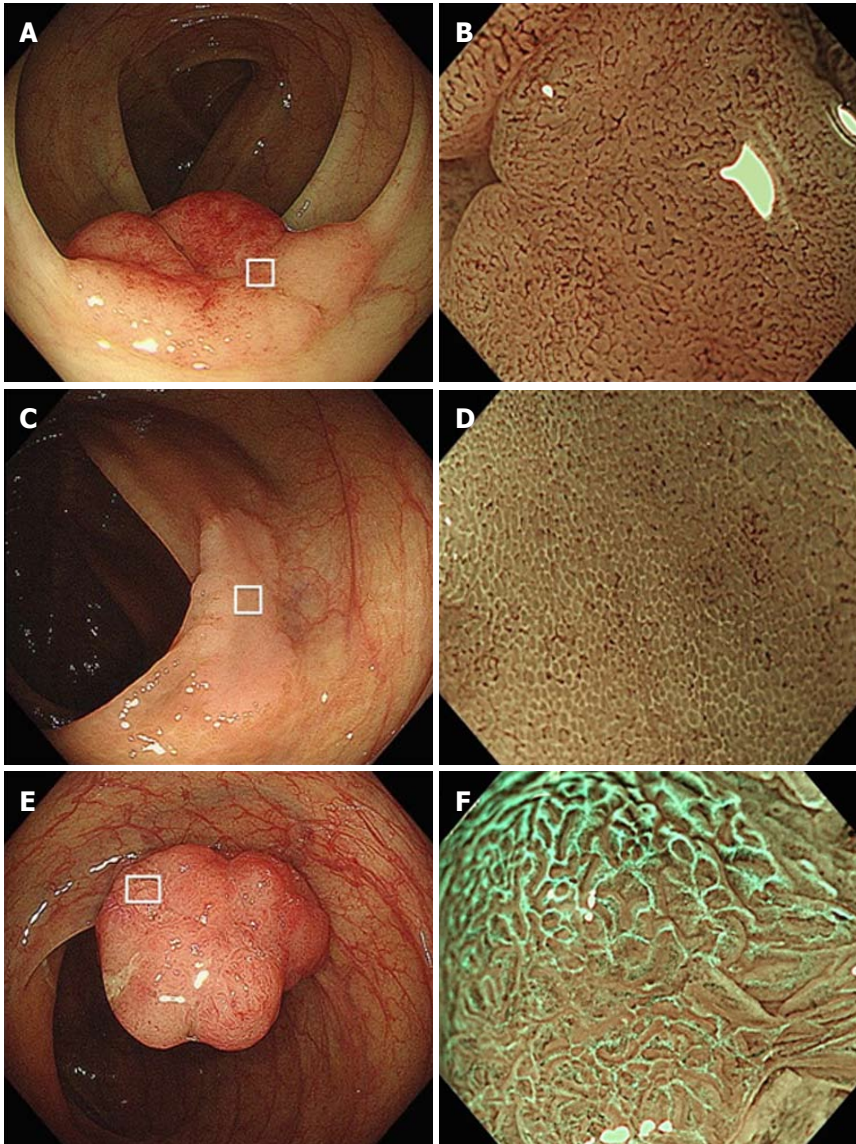


Figure 1 Magnifying endoscopic features with narrow-band imaging. A: Conventional view. A protruding lesion is detected in the transverse colon. B: M-NBI of the box in Figure 1a shows a clear microvascular pattern without WOS; C: Conventional view. A flat-elevated lesion is detected in the ascending colon. D: M-NBI of box in Figure 1C shows WOS obscuring the microvascular pattern. This WOS is regarded as regular WOS, since it is well-organized and distributed symmetrically with a regular reticular pattern. E: Conventional view. A protruding lesion is detected in the ascending colon. F: M-NBI of the box in Figure 1e shows WOS obscuring the microvascular pattern. This WOS is regarded as irregular WOS, since it is disorganized and distributed asymmetrically with an irregular speckled pattern. M-NBI: Narrow-band imaging; WOS: White opaque substance.

via chart review. Evaluated characteristics included age, sex, colonoscopic findings (size, location, and morphology), and M-NBI colonoscopic findings. The location of each lesion was classified as right side (cecum to transverse colon) or left side (descending colon to rectum). The gross morphology was defined as protruding type or flat-elevated type, based on the Paris classification^[8].

Definition of white opaque substance

As has been reported previously^[5], we defined WOS as a whitish area on M-NBI colonoscopy that obscures the microvascular pattern within the colorectal epithelial neoplasm (Figure 1). When M-NBI colonoscopy revealed an area of WOS, the lesion was considered

WOS-positive. WOS was further classified into regular and irregular WOS based on the classifications proposed by Yao *et al.*^[1] and by our group^[5]. Regular WOS was defined as WOS observed in a well-organized and symmetrical distribution with a regular reticular, maze-like, or speckled pattern. In contrast, irregular WOS was defined as WOS that appeared in a disorganized and asymmetrical distribution with an irregular reticular or speckled pattern (Figure 1).

Histopathological evaluation

Histological diagnosis was based on the WHO classification proposed in 2000^[9]. The grade of dysplastic change was classified as adenoma, high-grade dysplasia (HGD), or carcinoma. Carcinoma was defined

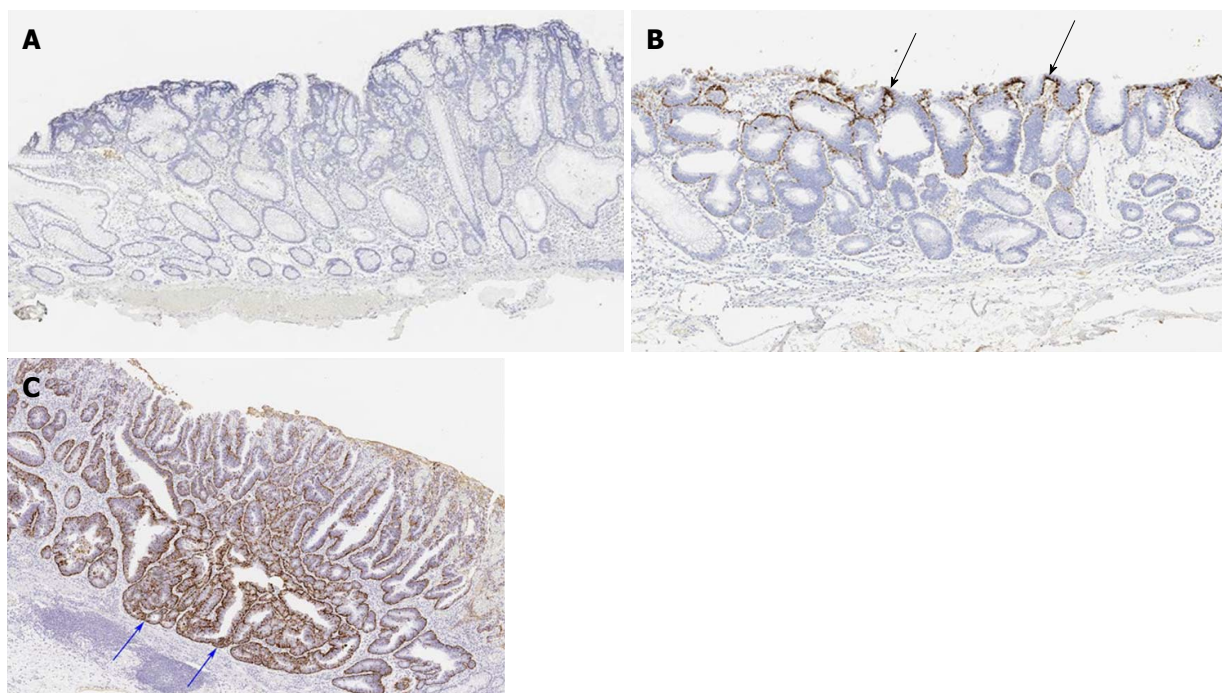


Figure 2 Histopathological findings: Adipophilin immunostaining. A: Score 0, adipophilin is not detected within the neoplastic epithelium; B: Score 1, adipophilin is detected within the neoplastic epithelium. The depth of adipophilin expression is superficial (black arrows); C: Score 2, adipophilin is detected within the neoplastic epithelium. Adipophilin expression is deep (blue arrows).

as neoplastic glands that had invaded the submucosal layer. Carcinomas were further classified as those with massive submucosal invasion (mSM) and those with slight submucosal invasion (sSM). mSM carcinoma was defined as having a vertical invasion depth $> 1000 \mu\text{m}$, while sSM carcinoma was defined as having an invasion depth $< 1000 \mu\text{m}$ ^[8,10].

Immunohistochemical staining

All sections were deparaffinized in xylene and rehydrated in a graded ethanol series. Slides were autoclaved in citrate buffer (pH 9.0) at 97°C for 20 min by PT Link (Dako EnVision System, Denmark). To clarify lipid accumulation, a primary antibody against adipophilin (pre-dilution, AP125; Fitzgerald Industries International, Concord, MA, United States) was used. Immunostaining for adipophilin was performed using an autoimmunostaining system (Dako EnVision System, Denmark).

The intensity of adipophilin expression was tabulated as negative, weak, moderate and strong. When the intensity of adipophilin-positive neoplastic cells was moderate or strong, the lesion was regarded as being adipophilin-positive. Depth of adipophilin expression was scored as 0 (negative), 1 (superficial expression), and 2 (deep expression) (Figure 2). When the depth of proper mucosal layer was divided into five equal layers, score 1 (superficial expression) was defined as having a vertical adipophilin expression no more than one-fifth, while score 2 (deep expression) was defined as having a vertical adipophilin expression more than one-fifth.

Scores were determined by two pathologists (ME and TS). When any difference in scoring occurred, the pathologists discussed the case until a consensus was obtained.

Statistical analysis

Parametric data are expressed as mean \pm SD. Nonparametric data are expressed as numbers and percentages. Comparisons between any two or among any three groups were performed by chi-squared test, the Tukey honestly significant difference test, or Student's *t* test where appropriate. $P < 0.05$ were considered significant. All statistical computations were performed with JMP version 11 (Statistical Discovery Program, Cary, NC, United States).

RESULTS

Demographic data

A total of 129 lesions in 120 patients were included in this study. Overall, 81 lesions in 74 patients were regarded as WOS-positive and 48 lesions in 46 patients as WOS-negative (Figure 1). Neither age at the time of diagnosis of colorectal epithelial neoplasms (70.6 ± 8.6 years in patients with WOS-positive lesions and 68.6 ± 9.1 years in the remaining patients) nor gender (55.4% male in patients with WOS-positive lesions and 45.8% in the remaining patients) differed between patient groups.

Comparisons of endoscopic and histological characteristics between WOS-positive and WOS-negative lesions are shown in Table 1. No differences in tumor

Table 1 Endoscopic and histologic characteristics of white opaque substance-positive and white opaque substance-negative lesions *n* (%)

		WOS-positive (81 lesions)	WOS-negative (48 lesions)	<i>P</i> value
Size (mm), mean ± SD		32.8 ± 16.4	34.7 ± 18.3	0.51
Location	Right side of the colon	51 (63.0)	22 (45.8)	0.07
	Left side of the colon	30 (37.0)	26 (54.2)	
Morphology	Protruded type	20 (24.7)	14 (29.2)	0.68
	Flat-elevated type	61 (75.3)	34 (70.8)	
Histologic type	Adenoma	22 (27.2)	20 (41.7)	0.12
	HGD or carcinoma	59 (72.8)	28 (58.3)	

WOS: White opaque substance; HGD: High-grade dysplasia.

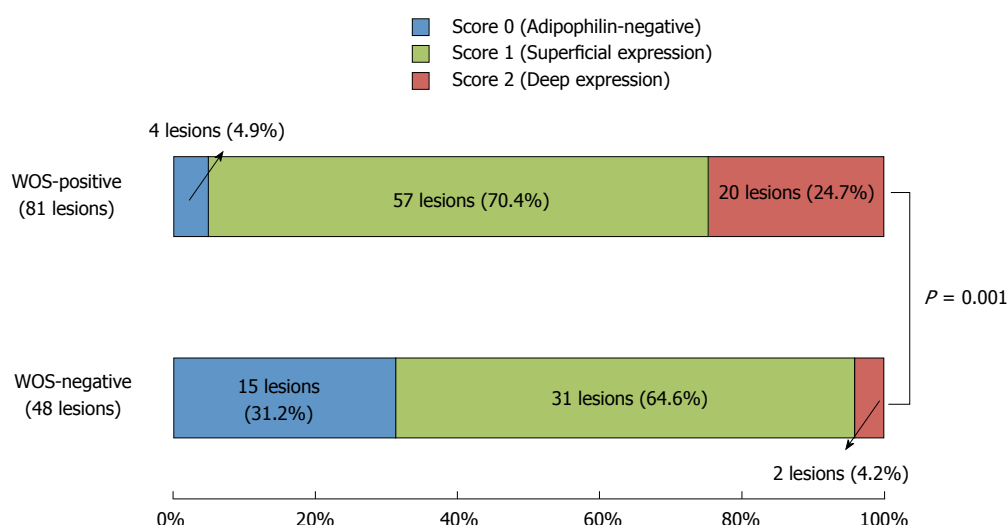


Figure 3 Comparison of the distribution of adipophilin between white opaque substance-positive and -negative lesions. The scores for adipophilin expression depth are significantly different between the two groups of lesions ($P = 0.001$), with a predominance of score 2 (deep expression) in WOS-positive lesions (24.7%) compared to WOS-negative lesions (4.2%).

Table 2 Immunohistochemical analysis of adipophilin expression in white opaque substance-positive and white opaque substance-negative lesions *n* (%)

		WOS-positive (81 lesions)	WOS-negative (48 lesions)	<i>P</i> value
Adipophilin	Positive	77 (95.1)	33 (68.7)	0.0001
	Negative	4 (4.9)	15 (31.3)	

WOS: White opaque substance.

size, tumor location, gross morphology, or histologic type were observed between the two groups.

Association between adipophilin expression and white opaque substance under magnifying narrow-band imaging colonoscopy

Table 2 compares adipophilin positivity rates between WOS-positive and WOS-negative lesions. Overall, 77 of the 81 WOS-positive lesions (95.1%) were adipophilin-positive, while 33 of the 48 WOS-negative lesions (68.7%) were adipophilin-positive (Figures 1 and 2). The adipophilin positivity rate was significantly higher in WOS-positive lesions than in WOS-negative lesions

($P = 0.0001$).

Figure 3 compares the distribution of adipophilin expression between WOS-positive and WOS-negative lesions. The score for depth of adipophilin expression was significantly different between the two groups ($P = 0.001$), with a predominance of score 2 (deep expression) (Figure 2) in WOS-positive lesions (24.7%) compared to 4.2% in WOS-negative lesions.

WOS-positive lesions were further classified as having regular WOS (65 lesions) or irregular WOS (16 lesions) (Figure 1). Figure 4 compares the distribution of adipophilin between lesions with regular vs. irregular WOS. Deep adipophilin expression was more frequent in lesions with irregular WOS (56.3%) than in those with regular WOS (16.9%) ($P = 0.0006$) (Figure 2).

Association between histologic type/depth of invasion and adipophilin expression

Histologically, the 129 lesions included 42 adenomas, 63 HGDs, 8 sSM carcinomas, and 16 mSM carcinomas. Figure 5 compares adipophilin expression between adenomas, HGD/sSM carcinomas, and mSM carcinomas. A score of 2 (deep expression) was more frequent in mSM carcinomas (62.5%) than in ade-

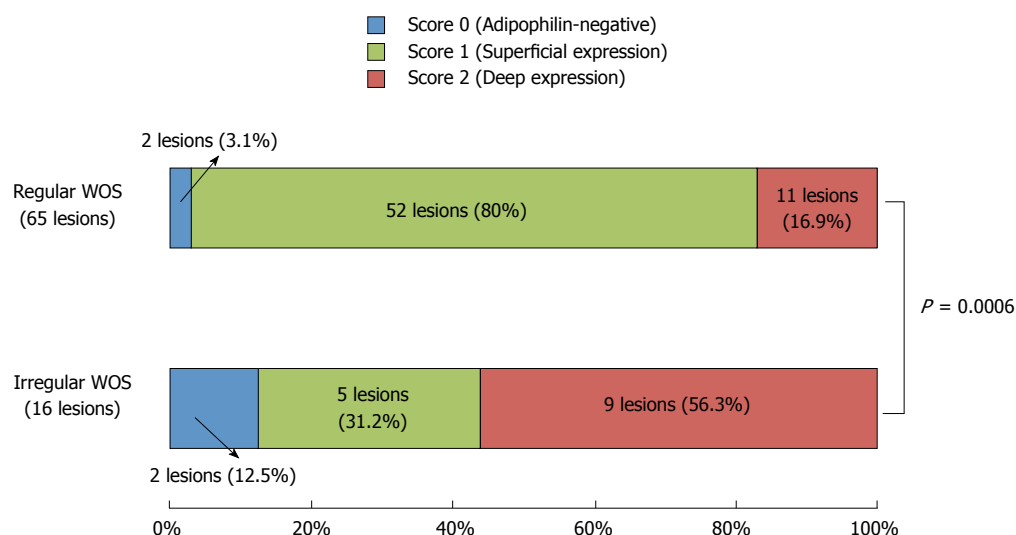


Figure 4 Comparison of the distribution of adipophilin between regular white opaque substance and irregular white opaque substance lesions. Score 2 (deep expression) is more frequent in lesions with irregular WOS (56.3%) than in lesions with regular WOS (16.9%) ($P = 0.0006$).

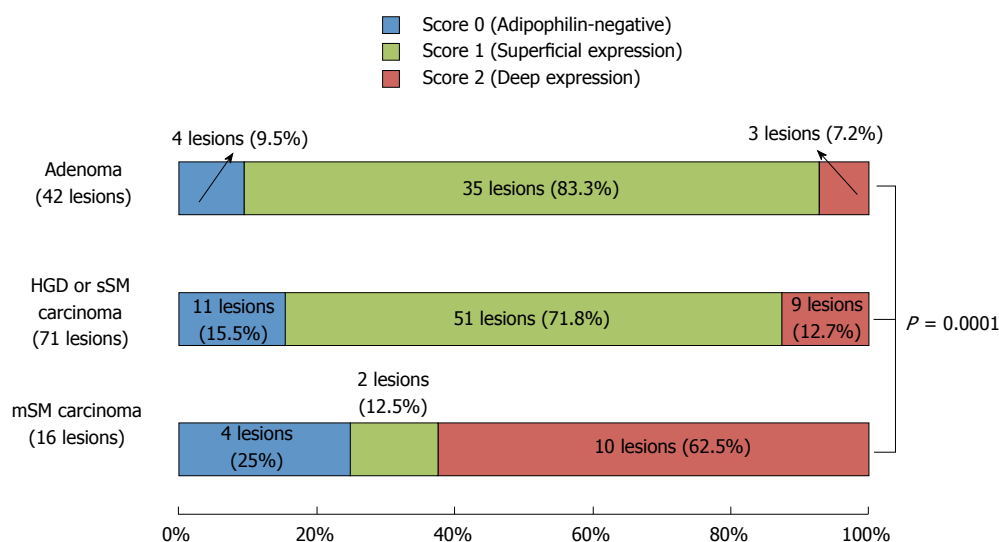


Figure 5 Immunohistochemical findings of colorectal lesions by histologic type. Score 2 (deep expression) is more frequent in mSM carcinomas (62.5%) than in adenomas (7.2%) or in HGDs/sSM carcinomas (12.7%) ($P = 0.0001$). HGD: High-grade dysplasia; sSM: Slight submucosal invasion; mSM: Massive submucosal invasion.

nomas (7.2%) and HGDs/sSM carcinomas (12.7%) ($P = 0.0001$). Figure 6 shows a case of mSM carcinoma with irregular WOS.

DISCUSSION

In this study, we confirmed that colorectal neoplasms with positive WOS under M-NBI colonoscopy had more profound accumulation of adipophilin compared to those without WOS. We also confirmed that the depth of adipophilin accumulation within tumors was associated with the visibility and morphology of WOS. Furthermore, we showed that the depth of adipophilin accumulation was associated with histologic grade of

dysplasia and depth of invasion in colorectal tumors.

WOS under M-NBI endoscopy appears as a white deposit that obscures the subepithelial microvascular pattern in gastric neoplasms, duodenal neoplasms, esophageal adenocarcinoma originating from esophageal glands, and colorectal neoplasms, as well as in gastric and colorectal hyperplastic polyps^[1-5,11,12]. WOS has been histopathologically and immunohistochemically verified to be a consequence of intramucosal accumulation of lipid droplets. Gastric neoplasms with WOS have been shown to be positive for oil red O staining and adipophilin^[6,13-15], and duodenal neoplasms with WOS have been reported to be positive for Sudan IV^[13].

Only a single clinicopathologic study that evaluated

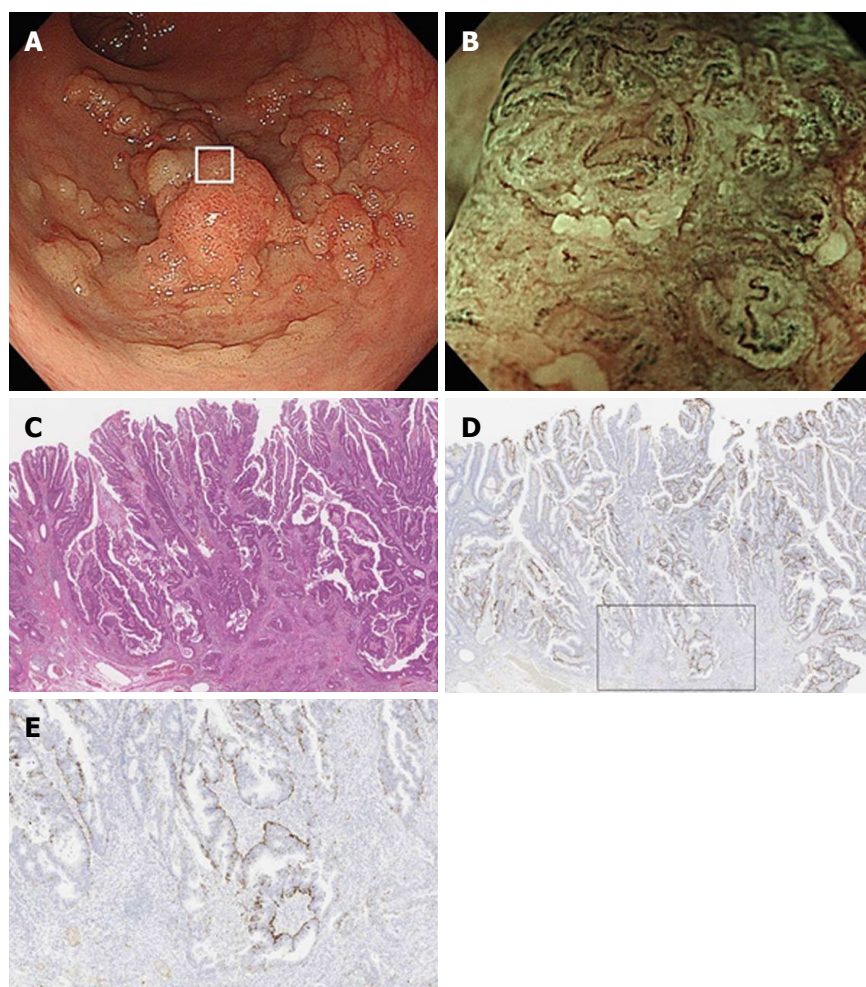


Figure 6 Endoscopic and histologic features of a lesion with irregular white opaque substance. A: Colonoscopy shows a protruding lesion in the rectum; B: Magnifying endoscopic findings with narrow-band imaging of box in Figure 6a show irregular WOS. WOS is disorganized and asymmetrical; C: Histological examination of the resected specimen shows well to moderately differentiated adenocarcinoma invading the deep submucosal layer (invasion depth; 4,320 μ m); D: A low-power view with adipophilin staining shows that the depth of adipophilin expression is deep; E: A high-power view of the box in Figure 6d shows that adipophilin is detected within the neoplastic epithelium. WOS: White opaque substance.

WOS in colorectal neoplasms has been published^[7]. In that study, Imamura *et al*^[7] reported that 19 (47.5%) of 40 colorectal lesions with WOS were positive for oil red O staining, while only 5 (5%) of 40 colorectal lesions without WOS were positive. Moreover, the investigators reported that 40 (100%) of 40 colorectal lesions with WOS were positive for adipophilin, while 25 (62.5%) of 40 colorectal lesions without WOS were positive. In the present study, we observed similar trends in the association between WOS and adipophilin expression. Thus, it seems possible that as in gastric and duodenal neoplasms, WOS in colorectal tumors represents accumulation of lipid droplets in the neoplastic epithelium.

The incidence of lipid droplets in gastric neoplasms without WOS has been reported in the literature to be extremely low. Yao *et al*^[6] reported that only 1 (4.3%) of 23 WOS-negative lesions was positive for oil red O. Ueo *et al*^[13] also reported a similar trend: only 2 (7.4%) of 27 WOS-negative lesions were positive for

adipophilin. With regards to colorectal neoplasms, only 2 (5%) of 40 WOS-negative lesions were positive for oil red O^[7]. As observed in our present investigation, however, Imamura *et al*^[7] reported a much higher rate (62.5%) of adipophilin-positive expression in biopsy specimens obtained from WOS-negative colorectal tumors. The study investigators^[7] also observed a significantly lower density of adipophilin in WOS-negative lesions compare to WOS-positive lesions. These observations strongly suggest that WOS under M-NBI is less sensitive for the detection of lipid droplets in colorectal tumors than in gastric tumors.

In the present study, we also investigated the histological distribution of lipid droplets in colorectal lesions that were completely resected by endoscopy or surgery. Thus, we were able to assess the depth of adipophilin expression in the samples. We found that deep expression of adipophilin was more frequent in WOS-positive lesions than in WOS-negative lesions. Thus, it seems possible that WOS under M-NBI may

be representative of the total volume of lipid droplets in colorectal tumors.

The present results also showed that deep adipophilin expression was more frequent in mSM carcinomas than in adenomas or HGDs/sSM carcinomas. Yao *et al*^[6] reported that 10 (36.5%) of 26 gastric neoplasms were positive for oil O red staining within surface epithelial cells, while 16 (61.5%) of 26 gastric neoplasms were positive for oil O red both in surface epithelial cells and in the subepithelial region. Ueo *et al*^[13] reported that surface plus cryptal accumulation of adipophilin was frequent in gastric cancers, while surface accumulation was observed in most gastric adenomas. It thus seems possible that the intraepithelial localization of lipid droplets in gastric tumors differs according to the histological grade of dysplasia. However, the association between lipid droplet accumulation and tumor invasion depth has not been examined in gastric epithelial tumors to date. In contrast, our results suggested that the depth of adipophilin expression may be correlated with the depth of invasion, rather than the histologic grade, in colorectal tumors.

In our previous analysis, the incidence of mSM carcinoma was significantly and conspicuously higher among lesions with irregular WOS (82.4%) than among those with regular WOS (1.4%)^[5]. In the present study, lesions with irregular WOS had deeper adipophilin expression than those with regular WOS. It thus seems possible that irregular WOS may be a consequence of massive lipid droplet accumulation in colorectal tumors. This speculation appears to partially explain the high rate of submucosal invasion in lesions with irregular WOS. Lipid droplets may be directly or indirectly associated with the malignant potential of colorectal neoplasms.

The present study has several limitations. First, since we included only lesions removed by ESD or surgery and did not include small lesions, it remains unclear whether the present observations are applicable to smaller colorectal lesions. Second, the present results were based on a retrospective analysis in a single center. A need exists for prospective analysis of a greater number of colorectal epithelial neoplasms to determine the association between lipid droplets and WOS under NBI, and also the clinical significance of WOS for the diagnosis and treatment of colorectal tumors.

In conclusion, the present immunohistochemical study showed that WOS observed in colorectal tumors under M-NBI colonoscopy represents lipid droplets. In addition, the distribution of lipid droplets may be closely associated with the visibility of WOS under M-NBI colonoscopy, and also with histologic grade and depth of tumor invasion. Further prospective studies are warranted to establish the clinical significance of WOS for the diagnosis and treatment of colorectal epithelial tumors and the association between lipid

droplets and WOS under NBI.

ARTICLE HIGHLIGHTS

Research background

White opaque substance (WOS) under magnifying narrow-band imaging (M-NBI) endoscopy is a novel endoscopic finding for the diagnosis of gastrointestinal tract neoplasms. In previous studies, WOS has been shown to contain lipid droplets. However, the association between the distribution of the lipid droplets and endoscopically verified WOS in colorectal neoplasms remains unclear.

Research motivation

The elucidation of WOS or lipid droplets in colorectal epithelial tumors will help the diagnosis and treatment of colorectal epithelial tumors.

Research objectives

To examine the association between WOS and histologically verified lipid droplets in colorectal epithelial neoplasms.

Research methods

We conducted this retrospective study involving 129 lesions of endoscopically or surgically resected colorectal epithelial neoplasms observed by M-NBI colonoscopy. Immunohistochemistry was used to stain tumors with a monoclonal antibody specific to adipophilin as a marker of lipids. The expression and distribution of adipophilin were compared between WOS-positive and WOS-negative lesions and among tumors classified by histologic type and depth of invasion.

Research results

81 lesions were positive for WOS and 48 lesions were negative for WOS. The rate of adipophilin expression was significantly higher in WOS-positive lesions (95.1%) than in WOS-negative lesions (68.7%) ($P = 0.0001$). The incidence of deep adipophilin expression was higher in WOS-positive lesions (24.7%) than in WOS-negative lesions (4.2%) ($P = 0.001$). The incidence of deep expression was predominant among cancers with massive submucosal invasion (62.5%) compared to adenoma (7.2%) and high-grade dysplasia or cancers with slight submucosal invasion (12.7%) ($P = 0.0001$).

Research conclusions

The distribution of lipid droplets may be closely associated with the visibility of WOS under M-NBI colonoscopy, and with histologic grade and depth of tumor invasion.

Research perspectives

Our study showed that WOS under M-NBI colonoscopy appears to represent lipid droplets and the distribution of lipid droplets may be closely associated with the visibility of WOS with histologic grade and depth of tumor invasion. The accumulation of lipid droplets may directly or indirectly represent the malignant potential of colon cancer cells. Further prospective studies are warranted to establish the clinical significance of WOS for the diagnosis and treatment of colorectal epithelial tumors and the association between lipid droplets and WOS under NBI.

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Retrospective Study

Nomogram based on tumor-associated neutrophil-to-lymphocyte ratio to predict survival of patients with gastric neuroendocrine neoplasms

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Abstract

AIM

To assess the predictive value of the tumor-associated neutrophil-to-lymphocyte ratio in terms of the clinical outcomes of patients with gastric neuroendocrine neoplasms after radical surgery.

METHODS

Data were retrospectively collected from 142 patients who were diagnosed with gastric neuroendocrine neoplasms and who underwent radical gastrectomy at our department from March 2006 to March 2015. These data were retrospectively analyzed, and a receiver operating characteristic curve analysis was used to identify the optimal value of the tumor-associated neutrophil-to-lymphocyte ratio. Univariate and multivariate survival analyses were used to identify prognostic factors. A nomogram was then applied to predict clinical outcomes after surgery.

RESULTS

The tumor-associated neutrophil-to-lymphocyte ratio was significantly associated with tumor recurrence, especially with liver metastasis and lymph node metastasis ($P < 0.05$ for both), but not with clinical characteristics ($P > 0.05$ for all). A multivariate Cox regression analysis identified the tumor-associated

neutrophil-to-lymphocyte ratio as an independent prognostic factor for recurrence-free survival and overall survival ($P < 0.05$ for both). The concordance index of the nomograms, which included the tumor-associated neutrophil-to-lymphocyte ratio, Ki-67 index, and lymph node ratio, was 0.788 (0.759) for recurrence-free survival (overall survival) and was higher than the concordance index of the traditional TNM staging system [0.672 (0.663)].

CONCLUSION

The tumor-associated neutrophil-to-lymphocyte ratio is an independent prognostic factor in patients with gastric neuroendocrine neoplasms. Nomograms that include the tumor-associated neutrophil-to-lymphocyte ratio, Ki-67 index, and lymph node ratio have a superior ability to predict clinical outcomes of postoperative patients.

Key words: Gastric neuroendocrine neoplasms; Tumor-associated neutrophil-to-lymphocyte ratio; Tumor recurrence; Prognosis

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Core tip: The study aimed to assess the predictive value of the tumor-associated neutrophil-to-lymphocyte ratio in terms of the clinical outcomes of 142 patients diagnosed with gastric neuroendocrine neoplasms. We demonstrated that the tumor-associated neutrophil-to-lymphocyte ratio was significantly correlated with tumor recurrence, especially with liver and lymph node metastasis. Moreover, the tumor-associated neutrophil-to-lymphocyte ratio was found to be an independent predictor of recurrence-free survival and overall survival, and combining it with the Ki-67 index and lymph node ratio could improve prognosis prediction in patients with gastric neuroendocrine neoplasms, as could applying the traditional TNM staging system.

Cao LL, Lu J, Lin JX, Zheng CH, Li P, Xie JW, Wang JB, Chen QY, Lin M, Tu RH, Huang CM. Nomogram based on tumor-associated neutrophil-to-lymphocyte ratio to predict survival of patients with gastric neuroendocrine neoplasms. *World J Gastroenterol* 2017; 23(47): 8376-8386 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v23/i47/8376.htm> DOI: <http://dx.doi.org/10.3748/wjg.v23.i47.8376>

INTRODUCTION

Gastric neuroendocrine neoplasms (g-NENs) are a highly heterogeneous and poorly understood group of relatively rare tumors that are derived primarily from enterochromaffin-like cells (ECL-cells) localized in the gastric mucosa^[1]. Due to an increased understanding of g-NENs and improved diagnostic techniques, the incidence of g-NENs, which account for 6% of all

neuroendocrine neoplasms, is increasing every year^[2]. However, due to significant differences in the clinical pathology and biological characteristics, our knowledge regarding g-NENs is still very limited^[3,4]. The World Health Organization (WHO, 2010) classifies g-NENs into the following subclasses: neuroendocrine tumors (g-NETs), neuroendocrine carcinoma (g-NEC), and mixed adenoneuroendocrine carcinoma (g-MANEC)^[5]. In addition to an early diagnosis, an important and effective component of proper management is the identification of the prognostic factors in patients with g-NENs. According to the American Joint Committee on Cancer/Union for International Cancer Control (AJCC/UICC), the TNM staging system, which accounts for invasion depth, lymph node status, and metastases, is one of the most important prognostic factors in patients with g-NENs^[6,7]. However, the prognostic factors of these tumors are complex and multifaceted and have not been clearly defined thus far^[8,9]. In the past decade, increasing evidence has suggested that both tumor-associated neutrophils (TANs) and tumor-associated lymphocytes (TALs) are significantly associated with patient prognosis. Elevated TANs and reduced TALs correlate with advanced stage and poor prognosis in a variety of human tumors, including cervical cancer^[10], hepatocellular carcinoma^[11], and pancreatic cancer^[12]. However, few studies have focused on the relationship between the tumor-associated neutrophil-to-lymphocyte ratio (TA-NLR) and the prognosis of patients with neuroendocrine neoplasms, particularly g-NENs.

This study investigated the utility of the TA-NLR as a prognostic indicator and evaluated its clinical value for the diagnosis and postoperative surveillance of patients undergoing radical surgery for g-NENs.

MATERIALS AND METHODS

General conditions

Overall, 173 patients who were diagnosed with g-NENs at Fujian Medical University Union Hospital between March 2006 and March 2015 were identified from a prospective database. The exclusion criteria for this study were as follows: metastatic disease confirmed preoperatively or during surgery ($n = 11$), perioperative death ($n = 1$), and incomplete/inaccurate medical records ($n = 19$). In all, 142 patients who underwent radical surgery were included in this study. The pathological data of these patients were reconfirmed by two pathologists according to the North American Neuroendocrine Tumor Society (NANETS) guidelines (2010)^[13]. In total, 27 (19.0%) patients were diagnosed with g-NETs, 45 (31.7%) with g-NEC, and 70 (49.3%) with g-MANEC. The ethics committee of Fujian Union Hospital approved this retrospective study. Written consent was obtained from the patients, and their information was stored in the hospital database and used for research.

Table 1 Characteristics of the 142 patients with gastric neuroendocrine neoplasms with different tumor-associated neutrophil-to-lymphocyte ratios

Clinicopathological feature	TA-NLR		Univariate analysis	Multivariate analysis
	≤ 0.21 (n = 71)	> 0.21 (n = 71)	P value	P value
Age (yr)			0.322	
≤ 70	57	52		
> 70	14	19		
Gender			0.851	
Male	52	51		
Female	19	20		
Tumor site			0.099	
Upper	39	26		
Middle	10	15		
Lower	17	18		
Mixed	5	12		
Tumor size (cm)			0.593	
≤ 3.5	25	22		
> 3.5	46	49		
Ki-67 index (%)			0.081	
≤ 2	13	6		
≥ 3, ≤ 20	8	16		
> 20	50	49		
Depth of invasion			0.044	0.406
T1	14	8		
T2	7	3		
T3	34	29		
T4	16	31		
Lymph node ratio			0.043	0.355
0	25	13		
> 0, ≤ 0.2	25	24		
> 0.2, ≤ 0.4	15	18		
> 0.4	6	16		
Lymphovascular invasion			0.610	
No	43	40		
Yes	28	31		
ASA status			0.805	
1 + 2	61	62		
3 + 4	10	9		
Postoperative complication			0.041	0.071
No	57	46		
Yes	14	25		
Surgical approach			0.855	
Endo/laparoscopic	49	50		
Open	22	21		

TA-NLR: Tumor-associated neutrophil-to-lymphocyte ratio.

Immunohistochemistry analysis

Immunohistochemical staining for CD8 or CD15 was performed using formalin-fixed, paraffin-embedded tumor tissue sections (4-μm-thick) from 142 g-NENs (Figure 2A). Briefly, the slides were baked at 65 °C for 2 h, deparaffinized with xylene, and rehydrated in graded alcohol. The slides were subjected to antigen retrieval *via* the high-pressure method in antigen retrieval solution. Endogenous peroxidase was inactivated using 3% H₂O₂ in methanol. Non-specific binding was blocked *via* incubation in 1% bovine serum albumin (BSA; Sigma-Aldrich; St. Louis, MO, United States) in phosphate buffered saline (PBS). Subsequently, the slides were incubated overnight at 4 °C with a primary monoclonal mouse antibody against CD8 or CD15 (1:100 dilution; Zhongshan Golden Bridge Biotech, Beijing, China). Normal goat

serum was used as a negative control. After being washed with PBS, tissue sections were incubated with the secondary antibody (Zhongshan Golden Bridge Biotech, Beijing, China) for 20 min at room temperature and then stained with diaminobenzidine (DAB). Finally, the slides were counterstained in hematoxylin, dehydrated, and mounted with a coverslip.

Two pathologists who were blinded to the clinical data reviewed the immunoreactivity under a light microscope. Inflammatory cells that had infiltrated the tumor nest and tumor stroma were analyzed, and inflammatory cells that were confined to lymph vascular spaces or within the vicinity of tumor necrosis or secretions were excluded from the analysis. Cases with tumor-infiltrating inflammatory cells present in 10 non-overlapping high- power fields (× 40) were

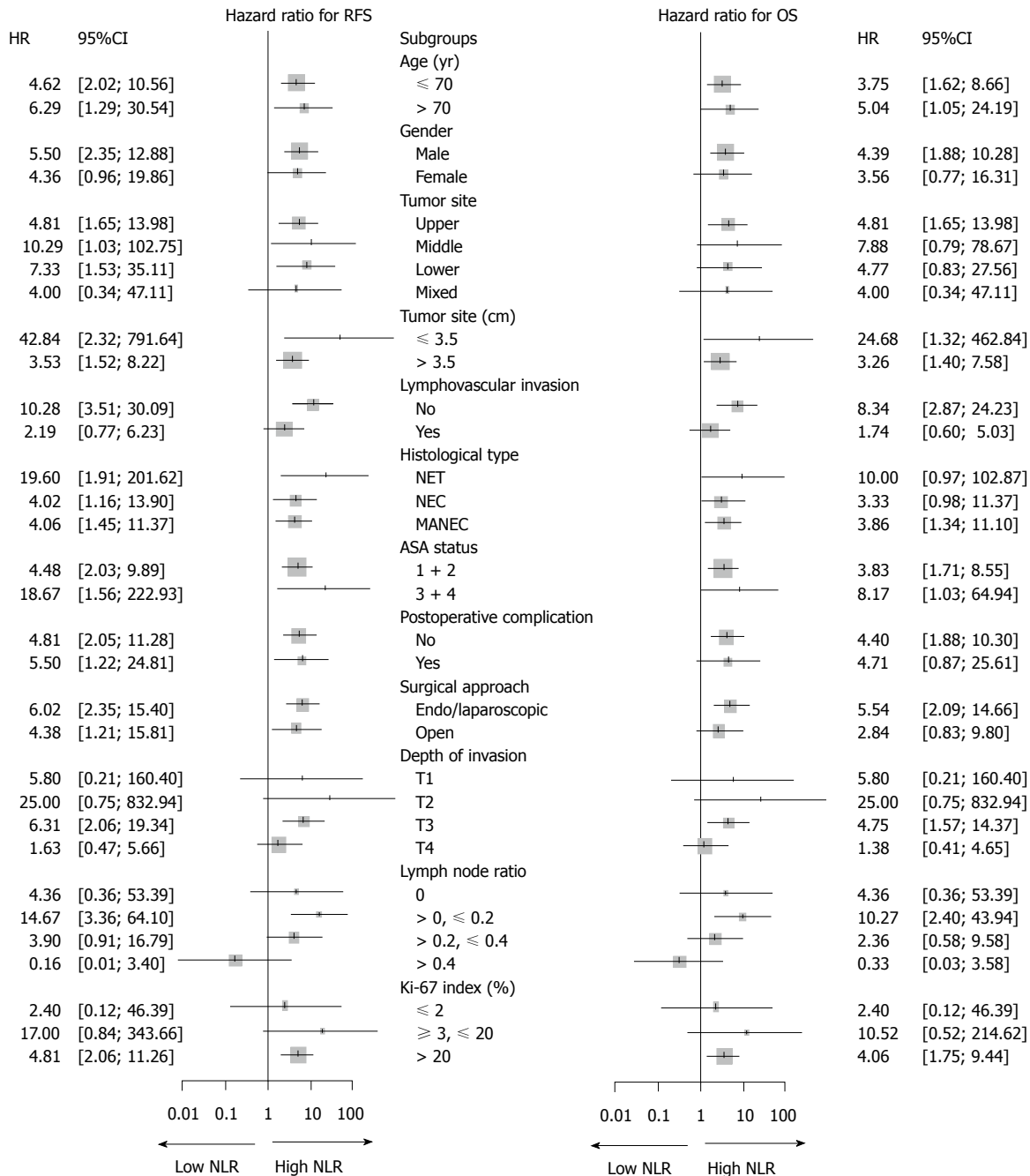


Figure 1 Forest plot showing the hazard ratios (oblongs) and 95% CIs (bars) for RFS (left) and OS (right) (according to subgroups) among 142 patients with gastric neuroendocrine neoplasms undergoing radical surgery. Long-term survival, including RFS and OS, was better among patients with a low TA-NLR than in patients with a high TA-NLR. g-NENs: Gastric neuroendocrine neoplasms. RFS: Recurrence-free survival; OS: Overall survival; TA-NLR: Tumor-associated neutrophil-to-lymphocyte ratio.

examined in representative areas on two slides of a given tumor (*i.e.*, a total of 20 fields per neoplasm). The number of tumor-related inflammatory cells was assessed in a semiquantitative manner using the mean value of high-power fields based on a $\times 40$ objective (magnification $\times 400$)^[14,15]. The TA-NLR was calculated as the average number of neutrophils (CD15-positive cells) divided by the average number of T lymphocytes

(CD8-positive cells). A receiver operating characteristic (ROC) curve analysis was performed in relation to the occurrence of recurrence and death from any cause. For all 142 patients, a TA-NLR of 0.21 had the highest sensitivity and specificity for both outcomes. Therefore, patients were categorized into the following two groups: low TA-NLR group (≤ 0.21 , 71 patients) and high TA-NLR group (> 0.21 , 71 patients).

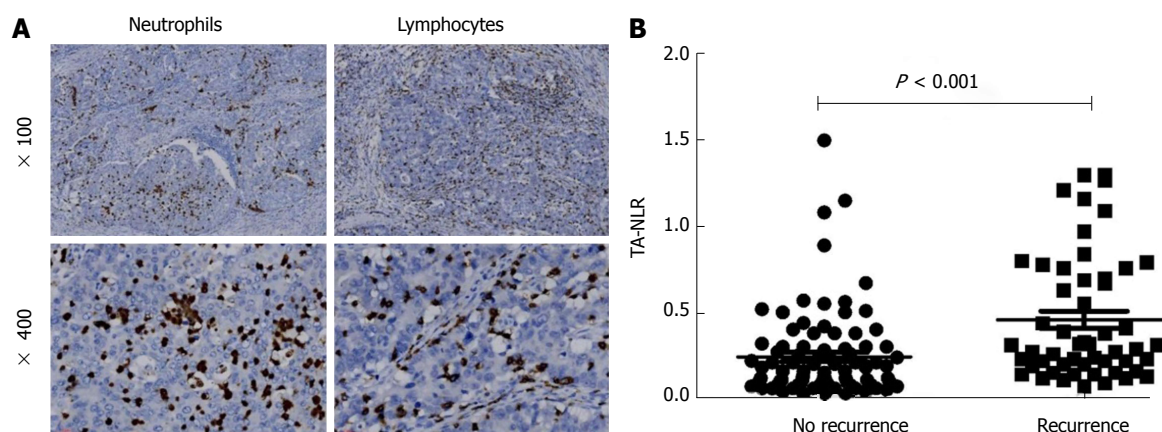


Figure 2 Relationship between the tumor-associated neutrophil-to-lymphocyte ratio and tumor recurrence. A: Representative immunohistochemical staining for CD15 (left) and CD8 (right); B: Significant differences in the TA-NLR were observed between the recurrence group ($0.46\% \pm 0.05\%$, mean \pm SE) and the non-recurrence group ($0.24\% \pm 0.03\%$, $P < 0.001$). TA-NLR: Tumor-associated neutrophil-to-lymphocyte ratio.

Table 2 Characteristics of 142 patients with gastric neuroendocrine neoplasms with different levels of tumor-associated neutrophil-to-lymphocyte ratios

Patient feature	TA-NLR		Univariate analysis <i>P</i> value
	≤ 0.21 (<i>n</i> = 71)	> 0.21 (<i>n</i> = 71)	
Symptom			
Abdominal pain	46	41	0.390
Dysphagia	14	12	0.665
Nausea	12	9	0.480
Vomiting	5	5	1.000
Acid-reflux	9	4	0.156
Anemia	10	13	0.495
Abdominal distention	6	8	0.575
Gastrointestinal blood loss	12	14	0.665
Weight loss	24	29	0.386
No symptoms	2	7	0.105
Medical history			
Hypertension	19	15	0.432
Diabetes	7	3	0.202
Coronary heart disease	4	4	1.000
Chronic gastritis	44	38	0.796
Family history	5	6	0.754
Smoking	26	27	0.862
Drinking	6	4	0.515

TA-NLR: Tumor-associated neutrophil-to-lymphocyte ratio.

Postoperative follow-up

The patients were monitored after surgery *via* telephone interviews, outpatient visits, and letters. Our department follows a standardized surveillance protocol and follows patients at three-month intervals for the first two years, six-month intervals for years two to five, and at least once per year five years after surgery. The postoperative follow-up data included clinical symptoms and signs, laboratory tests, imaging examinations, and endoscopy and biopsy results. In this study, the median follow-up time was 40 mo (range, 2-106 mo). The overall survival (OS) time was calculated as the number of months from the date of surgery to the date of last contact, date of death from any cause, or the date the end point was realized. The

recurrence-free survival (RFS) time was calculated as the number of months from the date of surgery to the date of identification of disease recurrence (either radiological or histological), the date of death or last contact, or the date the end point was realized.

Statistical analysis

All enumeration and measurement data were analyzed using SPSS 17.0 for Windows (SPSS, Chicago, IL, United States). χ^2 test, Fisher's exact test, or unpaired Student's *t* test was utilized to compare the differences between the TA-NLR groups and the clinicopathological factors, as appropriate. A univariate survival analysis was performed using the Kaplan-Meier method. A multivariate survival analysis was performed using a Cox proportional hazards model, and the significant variables from the univariate analysis were included in the model. R software (version 3.2.0) was utilized to develop the nomograms and the forest plot. $P < 0.05$ was considered significant.

RESULTS

TA-NLR is not associated with clinicopathological factors

The univariate analysis revealed that the TA-NLR was associated with the invasion depth, LNR (lymph node ratio), and postoperative complications ($P < 0.05$ for all; Table 1). However, the multivariate analysis revealed no significant differences in the clinicopathological factors between the two groups ($P > 0.05$ for all; Table 1). In addition, no significant differences were observed in the clinical symptoms, medical history, family history, active and past smoking histories, or history of heavy alcohol use between the two groups ($P > 0.05$ for all; Table 2).

Elevated TA-NLR is associated with a poor prognosis

As shown in Figure 1, the RFS and OS were analyzed according to age, gender, tumor site and size,

Table 3 Variables associated with recurrence-free survival according to the Cox proportional hazards regression model

Variable	Univariate analysis			Multivariate analysis		
	Hazard ratio	95%CI	P value	Hazard ratio	95%CI	P value
Age (yr)			0.790			
≤ 70	Reference					
> 70	1.083	0.602-1.950				
Gender			0.126			
Male	Reference					
Female	0.608	0.322-1.149				
Tumor site			0.770			
Upper	Reference					
Middle	0.825	0.389-1.751				
Lower	0.885	0.465-1.682				
Mixed	1.348	0.588-3.091				
Tumor size (cm)			0.004			0.671
≤ 3.5	Reference			Reference		
> 3.5	2.740	1.385-5.421		NA	NA	
Lymphovascular invasion			0.144			
No	Reference					
Yes	1.471	0.876-2.468				
ASA status			0.190			
1 + 2	Reference					
3 + 4	1.551	0.804-2.993				
Postoperative complication			0.029			
No	Reference			Reference		0.305
Yes	1.869	1.065-3.278		NA	NA	
Surgical approach			0.249			
Endo/laparoscopic	Reference					
Open	0.733	0.432-1.243				
Depth of invasion			0.005			0.557
T1	Reference			Reference		
T2	5.328	0.483-58.789		NA	NA	
T3	11.722	1.587-86.603		NA	NA	
T4	19.301	2.629-141.682		NA	NA	
Lymph node ratio			< 0.001			< 0.001
0	Reference			Reference		
> 0, ≤ 0.2	5.490	1.623-18.568		3.338	0.962-11.581	
> 0.2, ≤ 0.4	8.091	2.393-27.351		4.6	1.317-16.066	
> 0.4	17.946	5.239-61.480		10.266	2.906-36.266	
Ki-67 index (%)			0.004			< 0.001
≤ 2	Reference			Reference		
≥ 3, ≤ 20	3.013	0.639-14.203		1.501	0.305-7.393	
> 20	7.047	1.709-29.053		4.999	1.140-21.927	
TA-NLR			< 0.001			< 0.001
≤ 0.21	Reference			Reference		
> 0.21	3.366	1.890-5.992		2.974	1.630-5.426	

TA-NLR: Tumor-associated neutrophil-to-lymphocyte ratio.

lymphovascular invasion, ASA status, postoperative complications, surgical approach, invasion depth, LNR, and Ki-67 index. The hazard ratios and 95% confidence interval (CI) for the RFS and OS were compared between the subgroups. The long-term survival time, including RFS and OS, was shorter in the high TA-NLR group compared with the low TA-NLR group.

TA-NLR is an independent prognostic factor for RFS and OS

The univariate analysis found that larger tumor size, occurrence of postoperative complications, greater invasion depth, higher LNR, higher Ki-67 index, and higher TA-NLR were prognostic indicators of poorer RFS ($P < 0.05$ for all; Table 3). The tumor size,

invasion depth, LNR, Ki-67 index, and TA-NLR were identified as prognostic indicators of OS ($P < 0.05$ for all; Table 4). According to the multivariate analysis, the Ki-67 index, LNR, and TA-NLR were independent prognostic factors of RFS and OS ($P < 0.05$ for all; Tables 3 and 4).

TA-NLR is significantly correlated with recurrence site

The TA-NLR was significantly higher in the recurrence group than in the non-recurrence group ($P < 0.05$ for both; Figure 2B). Details regarding the recurrence site following surgery are listed in Table 5. The recurrence rate was significantly higher in the high TA-NLR group compared with the low TA-NLR group ($P < 0.001$). Additionally, an elevated TA-NLR was significantly associated with both liver metastasis and lymph node

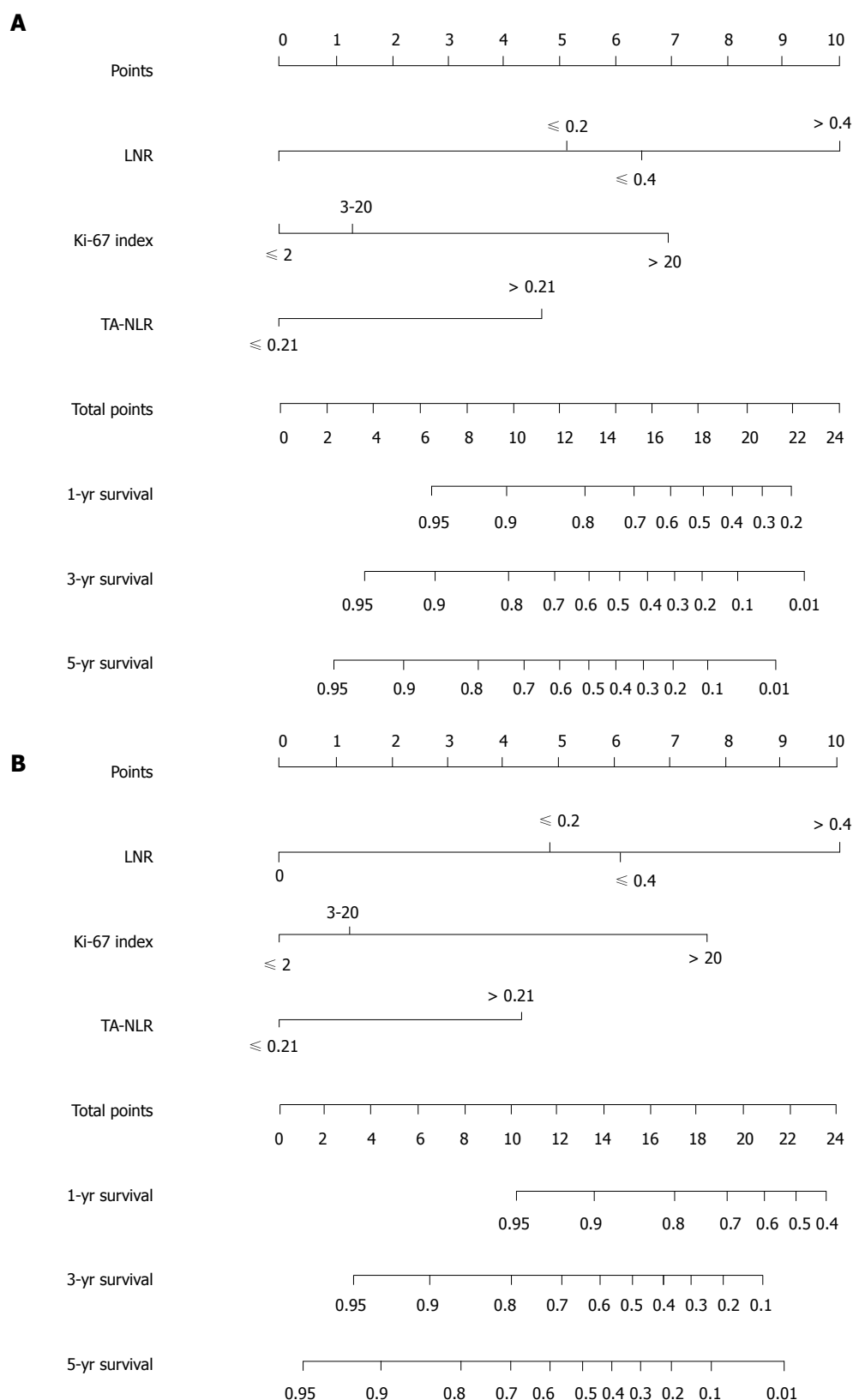


Figure 3 Nomograms for the prediction of recurrence-free survival (A) and overall survival (B) in patients following gastric neuroendocrine neoplasm resection; the C-index was 0.788 and 0.759 for RFS and OS, respectively. LNR: Lymph node ratio; TA-NLR: Tumor-associated neutrophil-to-lymphocyte ratio.

metastasis ($P < 0.05$ for both).

TA-NLR, combined with the Ki-67 index and LNR, is a superior prognostic prediction system

Prognostic nomograms were established using R

software (Figure 3). The C-index of the nomograms for RFS (OS), which included the TA-NLR, LNR, and Ki-67 index, was 0.788 (0.759). However, the C-index of the TNM staging system for RFS (OS) was 0.673 (0.662) (Figure 4). Thus, both the TNM staging system and

Table 4 Variables associated with overall survival according to the Cox proportional hazards regression model

Variable	Univariate analysis			Multivariate analysis		
	Hazard ratio	95%CI	P value	Hazard ratio	95%CI	P value
Age (yr)			0.566			
≤ 70	Reference					
> 70	1.197	0.648-2.211				
Gender			0.190			
Male	Reference					
Female	0.640	0.329-1.247				
Tumor site			0.190			
Upper	Reference					
Middle	0.687	0.313-1.509				
Lower	0.540	0.255-1.145				
Mixed	1.441	0.628-3.307				
Tumor size (cm)			0.002			0.214
≤ 3.5	Reference			Reference		
> 3.5	3.591	1.618-7.969		NA	NA	
Lymphovascular invasion			0.214			
No	Reference					
Yes	1.417	0.818-2.455				
ASA status			0.118			
1 + 2	Reference					
3 + 4	1.736	0.870-3.465				
Postoperative complication			0.320			
No	Reference					
Yes	1.380	0.732-2.603				
Surgical approach			0.276			
Endo/laparoscopic	Reference					
Open	0.736	0.425-1.276				
Depth of invasion			0.024			0.646
T1	Reference			Reference		
T2	5.524	0.501-60.954		NA	NA	
T3	10.793	1.455-80.038		NA	NA	
T4	15.632	2.116-115.464		NA	NA	
Lymph node ratio			< 0.001			0.002
0	Reference			Reference		
> 0, ≤ 0.2	4.791	1.402-16.370		2.854	0.813-10.027	
> 0.2, ≤ 0.4	6.676	1.956-22.790		3.724	1.054-13.162	
> 0.4	14.677	4.218-51.074		9.152	2.528-33.129	
Ki-67 index (%)			0.002			< 0.001
≤ 2%	Reference			Reference		
≥ 3%, ≤ 20%	2.168	0.437-10.751		1.584	0.313-8.008	
> 20%	6.582	1.589-27.269		5.535	1.238-24.752	
TA-NLR			< 0.001			0.003
≤ 0.21	Reference			Reference		
> 0.21	2.938	1.610-5.360		2.617	1.389-4.928	

TA-NLR: Tumor-associated neutrophil-to-lymphocyte ratio.

the nomograms had superior abilities to predict clinical outcomes for patients with g-NENs.

DISCUSSION

Neuroendocrine neoplasms, particularly g-NENs in the digestive system, are a unique subgroup of tumors with great clinical heterogeneity and varied biology. In recent years, with the growing popularity of upper gastrointestinal endoscopy and increasing improvements in diagnostic techniques, the reported incidence of g-NENs has increased each year, and currently, the incidence is approximately 0.3 per 100 thousand^[16,17]. According to previous studies, a patient's prognosis is significantly associated with the clinical and pathological parameters as well as the

biological characteristics of g-NENs^[18-20]. However, the independent prognostic factors for g-NEN patients are still controversial. To our knowledge, studies have reported individual prediction models for the prognosis of g-NENs. We evaluated the prognostic value of TA-NLR in patients with g-NENs and further established a tumor prognosis prediction model to provide a basis for individual clinical therapy.

In most cases, the clinical symptoms of g-NENs are not typical because they depend on the location and invasiveness of the primary tumor or metastases. The symptoms mainly include abdominal pain, abdominal distension, difficulty swallowing, nausea, and vomiting. In this study, abdominal pain was the most common symptom, followed by weight loss, difficulty swallowing, and gastrointestinal bleeding; this finding

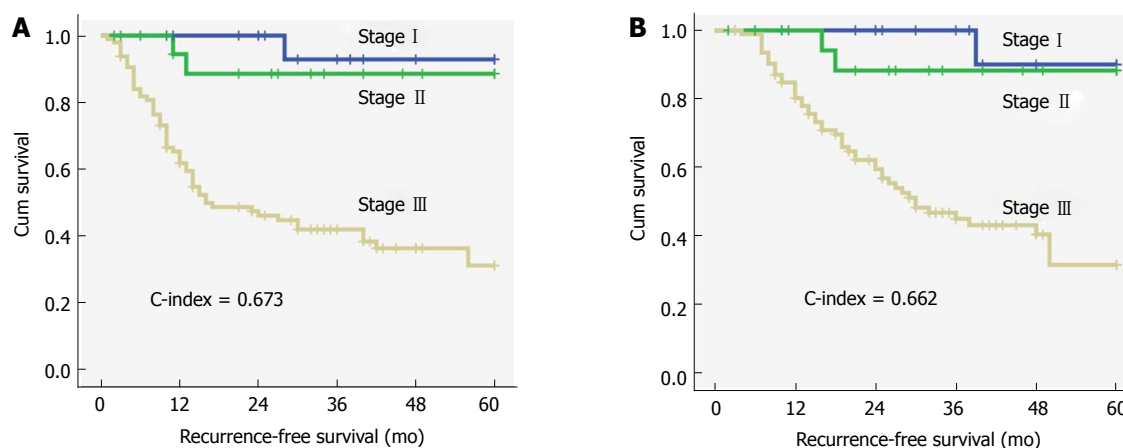


Figure 4 Survival curves for recurrence-free survival (A) and overall survival (B) according to the traditional TNM staging system (NCCN 2015); the C-index was 0.673 and 0.662 for RFS and OS, respectively.

Table 5 Site of recurrence after surgery

Site of recurrence	TA-NLR		P value
	≤ 0.21 (n = 71)	> 0.21 (n = 71)	
Liver	10	28	0.001
Peritoneal cavity	6	9	0.413
Lymph node	2	11	0.009
Lung	3	4	0.721
Bone	0	5	0.058
Adrenal gland	1	4	0.366
Pancreas	2	2	1.000
Locoregional recurrence	2	3	0.683
Spleen	0	2	0.496
Kidney	1	1	1.000
Brain	0	1	1.000
Number of patients with recurrence	15	38	< 0.001

TA-NLR: Tumor-associated neutrophil-to-lymphocyte ratio.

is consistent with previous reports^[21]. In addition, approximately 6% of the patients without any clinical symptoms were diagnosed *via* physical examinations. Among asymptomatic patients, approximately 40% were diagnosed with g-NEC or g-MANEC, although most of them were diagnosed with g-NETs. Therefore, postoperative follow-up is still essential for patients who have no clinical symptoms.

In recent years, substantial evidence has revealed that pathological stage is closely related to the prognosis of patients with g-NENs. Deep tumor invasion, lymph node metastasis, and distant metastasis were associated with decreased long-term survival^[8,9,22]. The Ki-67 index, as a marker of cell proliferation, is widely used to evaluate the malignant potential of neuroendocrine tumors. The European Neuroendocrine Tumor Society (ENETS) and the WHO adopted a three-tier classification system based on the Ki-67 index for gastrointestinal pancreatic neuroendocrine tumors (G1: ≤ 2%; G2: 3%-20%; G3: > 20%). The Ki-67 index combined with a pathological staging system improves the diagnosis and prognosis prediction of patients with

neuroendocrine tumors, and it is thus widely used in clinical practice. In this study, the rate of lymph node metastasis and the Ki-67 index were independent risk factors for OS and RFS in patients with g-NENs. In addition, increasing evidence has confirmed that the tumor-associated inflammatory response is closely related to the prognosis of patients with malignant tumors^[12,23,24]. However, the relationship between the tumor-associated inflammatory response and g-NENs is unclear. Our study is the first to confirm that the TA-NLR is significantly associated with the prognosis of patients with g-NENs. We observed, through a univariate analysis, that the RFS and OS rates in patients with a TA-NLR > 0.21 were significantly lower than the rates in patients with a TA-NLR < 0.21. The multivariate analysis further revealed that the TA-NLR was an independent risk factor for patients with g-NENs.

Postoperative local recurrence and distant metastasis are the leading causes of death for patients with malignant tumors. Liver metastasis, peritoneal metastasis, and lymph node metastasis were the main types of tumor recurrence. The proportions of patients with these types of recurrence were 72%, 28%, and 25%, respectively. The spleen, kidney, and brain were relatively rare sites of recurrence. Our results are similar to those of previous reports^[9]. In the present study, the TA-NLR was closely related to tumor recurrence, and a high incidence of liver metastasis and lymph node metastasis was observed in patients with a high TA-NLR. Thus, during the postoperative follow-up period, clinicians should utilize the prognostic value of the TA-NLR, as well as clinical characteristics, to discover potential hepatic or lymph node metastases at an earlier time point.

Nomograms, as a new type of statistical prediction model, are currently widely used in clinical practice for the majority of cancer types^[25,26]. Prognostic nomograms enable the use of a combination of multiple relevant clinical predictors and can be utilized

to predict RFS and OS for individual patients. For many cancers, nomograms compare favorably to the traditional TNM staging system and have been proposed as an important tool in clinical practice^[13,27]. In this study, we established prognostic nomograms for g-NENs by combining the TA-NLR, Ki-67 index, and LNR. This combination had a high predictive ability, as did the traditional TNM staging system. Therefore, the combination of the TA-NLR, Ki-67 index, and LNR, as a novel prognostic system, may provide simple, more accurate prognostic predictions.

This study had some limitations. The study was uncontrolled and performed in a single institution. The results should be confirmed by subsequent prospective studies. Some heterogeneity was also present in this study, as it included multiple histological types (including NET, NEC, and MANEC), which do not represent a specific progression of a unique pathologic process. Due to the low incidence of g-NENs and the limited number of samples in the study, a statistical analysis could not be conducted for any one histological type. We will focus on each of the three histological types in the future, after more cases have been accumulated. However, to our knowledge, our study enrolled more patients with g-NENs than similar reports in the literature, and for the first time, we demonstrated that the TA-NLR was able to predict long-term survival relatively accurately in patients. Our study could be the basis for a subsequent prospective clinical study.

As a simple and inexpensive inflammatory biomarker, the TA-NLR is significantly correlated with tumor recurrence, especially with liver and lymph node metastasis. The TA-NLR is an independent predictor of RFS and OS, and its combination with the Ki-67 index and LNR could improve prognosis prediction in g-NEN patients undergoing radical surgery, as could the traditional TNM staging system.

COMMENTS

Background

The incidence of gastric neuroendocrine neoplasms (g-NENs), which account for 6% of all neuroendocrine neoplasms, is increasing every year. In addition to an early diagnosis, an important and effective component of proper management is the identification of the prognostic factors in patients with g-NENs. However, the prognostic factors for these tumors are complex and multifaceted and have not been clearly defined thus far. Few studies to date have focused on the relationship between the tumor-associated neutrophil-to-lymphocyte ratio (TA-NLR) and the prognosis of patients with g-NENs.

Research frontiers

In the past decade, increasing evidence has suggested that both tumor-associated neutrophils (TANs) and tumor-associated lymphocytes (TALs) are significantly associated with patient prognosis. Elevated TANs and reduced TALs correlate with advanced stage and poor prognosis in a variety of human tumors, including cervical cancer, hepatocellular carcinoma, and pancreatic cancer.

Innovations and breakthroughs

This study enrolled more patients with g-NENs than similar reports in the literature and, for the first time, demonstrated that the TA-NLR was able to predict long-term survival relatively accurately in patients.

Applications

This study established a novel prognostic system that included the TA-NLR, Ki-67 index, and lymph node ratio, which may provide simple, more accurate prognostic predictions. Moreover, as a simple and inexpensive inflammatory biomarker, the TA-NLR is significantly correlated with tumor recurrence, especially with liver and lymph node metastasis. Thus, during the postoperative follow-up period, clinicians should utilize the prognostic value of the TA-NLR, as well as clinical characteristics, to discover potential hepatic or lymph node metastases at an earlier time point.

Terminology

Gastric neuroendocrine neoplasms (g-NENs), a highly heterogeneous and poorly understood group of relatively rare tumors, are derived primarily from enterochromaffin-like cells (ECL-cells) localized in the gastric mucosa. The World Health Organization (WHO, 2010) classifies g-NENs into the following subclasses: neuroendocrine tumors (g-NETs), neuroendocrine carcinoma (g-NEC), and mixed adenoneuroendocrine carcinoma (g-MANEC).

Peer-review

Previous studies have established that elevated TANs and reduced TALs correlate with advanced stage and poor prognosis in a variety of human tumors, including cervical cancer, hepatocellular carcinoma, and pancreatic cancer. In this study, the authors demonstrated that the TA-NLR is an independent predictor of RFS and OS and that it is also significantly correlated with tumor recurrence, especially with liver and lymph node metastasis. However, as the authors indicate, this study was uncontrolled and was performed within a single institution. The results should therefore be confirmed in subsequent prospective studies.

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Retrospective Study

Impact of cigarette smoking on recurrence of hyperlipidemic acute pancreatitis

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Abstract

AIM

To investigate the impact of cigarette smoking on the recurrence rate and recurrence-free survival in patients with hyperlipidemic acute pancreatitis (HLAP).

METHODS

A total of 863 patients were admitted to our hospital for acute pancreatitis (AP) from January 2013 to March

2016, of whom 88 diagnosed with HLAP were enrolled in this retrospective study. Demographic data, medical history, previous episodes of pancreatitis, consumption of alcohol and cigarettes, as well as biochemical and hematological data were carefully recorded for univariate and multivariate analyses. During follow-up, the information on current smoking status and recurrent AP was gathered. Recurrence-free survival (RFS) was calculated using the Kaplan-Meier method, and the differences between groups were compared using the log-rank test.

RESULTS

No significant differences were observed between the three groups in age or medical history of hyperlipidemia, fatty liver, diabetes mellitus, hypertension, or AP. The current smokers had a remarkably higher recurrence rate and a greater incidence of repeated episodes of AP (50.0% and 77.8%, respectively) than non-smokers (9.8% and 39.0%), and these two percentages were reduced to 9.1% and 36.4% for patients who gave up smoking. The median follow-up time was 13.5 mo and HLAP recurred after hospital discharge in 23 (26.1%) patients. Multivariate analysis identified current smoking (HR = 6.3, $P = 0.020$) as an independent risk factor contributing to HLAP recurrence. Current smokers had significantly worse RFS than non-smokers (23 mo *vs* 42 mo), but no significant difference was documented between ex-smokers (34 mo) and non-smokers. The RFS was not significantly different between light and heavy smokers.

CONCLUSION

Smoking is associated with worse RFS and an increased rate of HLAP recurrence. Continued smoking correlates with a compromised survival and smoking cessation should be recommended.

Key words: Acute pancreatitis; Hyperlipemia; Smoking; Recurrence; Epidemiology

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Core tip: This present study retrospectively enrolled hyperlipidemic acute pancreatitis (HLAP) patients in a large regional central hospital and revealed that cigarette smoking was associated with worse recurrence-free survival and an increased rate of HLAP recurrence. For smokers, continued smoking might be strongly correlated with HLAP recurrence and compromised survival. Therefore, smoking cessation should be strongly recommended.

Xiang JX, Hu LS, Liu P, Tian BY, Su Q, Ji YC, Zhang XF, Liu XM, Wu Z, Lv Y. Impact of cigarette smoking on recurrence of hyperlipidemic acute pancreatitis. *World J Gastroenterol* 2017; 23(47): 8387-8394 Available from: URL: <http://www.wjgnet.com>

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INTRODUCTION

Acute pancreatitis (AP) is a potentially life-threatening acute inflammatory condition of the pancreas with high morbidity and mortality^[1]. It is widely believed that the most common etiologies of AP are gallstone disease and alcohol abuse. Currently, 12%-20% of patients with AP have previous hypertriglyceridemia, which has become a well-recognized etiology^[2-4]. Hyperlipidemic acute pancreatitis (HLAP) is a form of AP occurring in the presence of severe hypertriglyceridemia and in the absence of other causes^[5]. Some researchers even report hypertriglyceridemia as the underlying cause in more than half of all gestational pancreatitis cases^[6]. Numerous studies have suggested that compared with other types of pancreatitis, HLAP is associated with more complications, a longer course of disease, and a higher recurrence rate^[7].

The exact pathophysiology of HLAP is not entirely certain. At present, it is believed that HLAP is related to injury in the pancreatic tissue (including acinar cells and pancreatic ducts) and microcirculation disturbance caused by free fatty acids (FFAs) that are produced by the pancreatic lipase, which catalyzes decomposition of triglycerides^[5]. The resulting ischemia creates an acidic environment, which further triggers FFAs toxicity. In this way, inflammation can be initiated and amplified within the pancreas. Earlier studies found that cigarette smoking was independently associated with pancreatic cancer and chronic pancreatitis (CP) by leading to pancreatic calcification and abnormal secretion of the pancreatic ducts^[8-10]. Notably, some recent studies identified that smoking was significantly associated with non-biliary AP instead of biliary AP^[11,12], indicating different pathophysiological mechanisms for these subtypes. However, whether cigarette smoking has any long-term impact on HLAP recurrence has not yet been investigated. Because the nicotine in tobacco can cause lipid metabolism disturbance and oxidative stress and further increase blood viscosity and microcirculation dysfunction in the pancreas^[13], it is reasonable to hypothesize that smoking may be associated with the high recurrence rate of HLAP.

This present study retrospectively enrolled HLAP patients treated at a large regional central hospital. The impact of cigarette smoking on recurrence rate and recurrence-free survival (RFS) in HLAP patients was investigated.

MATERIALS AND METHODS

Patients and diagnosis

A total of 863 patients were admitted to our hospital

for AP from January 2013 to March 2016, of whom 90 were hospitalized at least twice. Among these patients, those with biliary pancreatitis, alcoholic pancreatitis, and other causes were excluded from the study. The remaining 88 patients identified with HLAP were enrolled. The diagnosis of HLAP was made if patients had AP in the presence of serum total triglyceride (TG) > 11.3 mmol/L (1000 mg/dL), or had a serum TG level of 5.65 to 11.3 mmol/L accompanied by chylous serum after excluding other known risk factors for AP. The diagnosis of AP requires at least two of the following three features: acute upper abdominal pain often radiating through to the back, serum amylase and/or lipase levels \geq three times the upper limit of normal, and evidence of pancreatitis upon abdominal imaging^[14]. The work described was carried out in accordance with The Code of Ethics of the World Medical Association. This research was approved by the Ethics Committees of the First Affiliated Hospital of Medical College, Xi'an Jiaotong University.

Tobacco and alcohol exposure assessment

Data on tobacco exposure were obtained from the baseline questionnaire and telephone follow-up. All patients were asked if they smoke regularly and, if so, for how many years they had smoked and the average number of cigarettes they smoked per day. Smoking data included smoking pack-years (PY, packages of cigarettes smoked per day multiplied by the number of years for which the individual has smoked). Smoking status was defined as non-smoker (<100 cigarettes during lifetime), ex-smoker, and current smoker. An ex-smoker was defined as one who had quit smoking for more than 6 mo before the end of follow-up after being discharged from the hospital. A current smoker was defined as one who smoked at least 1 cigarette per day for over 1 year and continued to smoke within 1 year prior to follow-up. Tobacco exposure was characterized as none, light smoker ($0 < \text{PY} < 10$), and heavy smoker ($\text{PY} \geq 10$)^[15,16]. Since alcohol and smoking are often linked behaviors^[17], there are questions about the independent influence of smoking. Therefore, exposure of alcohol was also investigated and taken into consideration. In accordance with previous guidelines, a high drinker was defined as one who has drunk at least 40 g/d (20 g/d for female) for over 5 years. Smokers and alcohol abusers were routinely encouraged to give up these bad habits.

Treatment and follow-up

All the patients were treated with comprehensive routine therapy, including restriction of oral intake, fluid expansion, parenteral nutrition, analgesia, proton pump inhibitor administration, inhibition of pancreatic enzyme secretion, antibiotics, early enteral nutrition, and plasma exchange, if needed^[18].

The information on demographic data, history of

hypertriglyceridemia, fatty liver, diabetes, hypertension, previous episodes of pancreatitis, consumption of alcohol and cigarettes, as well as biochemical and hematological data were carefully recorded at admission. During follow-up, we gathered information on current smoking status and recurrent AP. Repeated episodes were defined as patients who were diagnosed with AP more than twice during their lifetime. To avoid recurrence, all patients were counseled to continue dietary fat restriction after treatment. Lipid lowering agents were used for patients when necessary.

Statistical analysis

Statistical analyses were performed with the SPSS 21.0 software package (SPSS Inc., Chicago, IL, United States). Continuous variables are expressed as the mean \pm SE, and comparisons between groups were performed using non-parametric tests, the *t*-test, or ANOVA, as appropriate. Categorical variables were compared between groups using the χ^2 test or Fisher's exact test. RFS was calculated using the Kaplan-Meier method, and the differences between groups were compared using the log-rank test. Risk factors for the recurrence of HLAP were analyzed by univariate analysis first, and those with $P < 0.20$ and possible clinical effect were included in multivariate analysis using a Cox proportional hazards model. A P value < 0.05 was considered statistically significant. The statistical methods and results of this study were reviewed by a biostatistician (Qian Li, PhD) at Xi'an Jiaotong University.

RESULTS

Of the 88 patients with HLAP who were enrolled and analyzed (66 men and 22 women with a mean age of 40.9 ± 1.1 year), 36 and 11 patients were documented as current smokers and ex-smokers, respectively, while 41 patients had no cigarette smoking history. The characteristics of the patients in the three groups are shown in Table 1. No significant differences were observed between the three groups in age or medical history of hyperlipemia, fatty liver, diabetes mellitus, hypertension, or AP. Of the current smokers, 55.6% had a history of AP, which was significantly higher than that of non-smokers (31.7%). Cigarette smoking patients were more likely to have concomitant alcohol abuse than non-smokers ($P = 0.037$), and more male patients than females tended to smoke. Biochemical tests of serum TG, cholesterol (CHOL), amylase, lipase, and calcium levels were not significantly different between these groups, and nor were the leukocyte and platelet counts. Patients who currently or previously smoked had higher systemic inflammatory response syndrome (SIRS) proportions and bedside index for severity in acute pancreatitis (BISAP) scores than non-smokers, but the differences were not significant.

Table 1 Clinical characteristics of the hyperlipidemic acute pancreatitis patients

Clinical parameter	Non-smokers No. or mean	Current-smokers No. or mean	Ex-smokers No. or mean	P value
N	41	36	11	
Age (yr)	42.7 ± 2.0	39.1 ± 1.2	40.0 ± 2.2	0.446
Gender (M/F)	20/21	36/0	10/1	< 0.001 ^a
Heavy alcohol drinking	4	12	2	0.037 ^a
Smoking pack-years	0	13.5 ± 2.2	7.8 ± 2.9	< 0.001 ^a
History of hyperlipemia	39	30	8	0.084
History of fatty liver	16	15	4	0.943
History of diabetes mellitus	10	14	5	0.258
History of hypertension	8	3	2	0.364
History of AP	13	20	3	0.065 ^a
Leukocyte (× 10 ⁹ /L)	12.5 ± 0.5	13.1 ± 0.7	12.3 ± 1.1	0.506
Platelet count (× 10 ⁹ /L)	185.2 ± 11.8	171.7 ± 8.2	175.6 ± 20.1	0.680
Serum TG (mmol/L)	18.3 ± 1.7	15.1 ± 1.5	17.1 ± 2.9	0.358
Serum CHOL (mmol/L)	9.0 ± 0.6	8.4 ± 0.6	8.0 ± 0.8	0.316
Serum amylase (U/L)	569.3 ± 104.5	507.7 ± 111.4	629.8 ± 121.9	0.270
Serum lipase (U/L)	1297.1 ± 228.1	874.1 ± 170.2	1214.4 ± 582.0	0.406
Serum calcium (mmol/L)	2.0 ± 0.04	2.1 ± 0.04	2.0 ± 0.08	0.197
SIRS	17	17	8	0.182
BISAP score	0.8 ± 0.1	0.8 ± 0.2	1.4 ± 0.3	0.224
Hospitalization (d) ¹	10.9 ± 0.8	8.6 ± 0.7	11.9 ± 1.6	0.015 ^a
Recurrence of AP	4	18	1	< 0.001 ^a
Repeated episodes of AP	16	28	4	0.001 ^a

^a*P* < 0.05, current *vs* non-smokers; ¹Exclude those discharged without medical advice or transferred to a different hospital. AP: Acute pancreatitis; TG: Total triglyceride; CHOL: Cholesterol; SIRS: Systemic inflammatory response syndrome; BISAP: Bedside index for severity in acute pancreatitis.

In addition, current smokers had a remarkably higher recurrence rate and a greater incidence of repeated episodes of AP (50.0% and 77.8%, respectively) than non-smokers (9.8% and 39.0%, respectively). It is worth noting that these two percentages were reduced to 9.1% and 36.4% for patients who gave up smoking after being discharged from the hospital.

The median follow-up time was 13.5 mo (2-42 mo) by September 2016. HLAP recurred after discharge from the hospital in 23 (26.1%) patients. To investigate the risk factors contributing HLAP recurrence, we examined 19 potential variables and analyzed them by univariate analysis, as shown in Table 2. Univariate analysis identified that smoking history and smoking pack-years were risk factors associated with higher HLAP recurrence. Specifically, current smoking was a risk factor relative to non-smoking (HR = 5.1, 95%CI: 1.7-15.2, *P* = 0.003). Smoking PY < 10 was a protective factor relative to PY ≥ 10 (HR = 0.4, 95%CI: 0.2-0.9, *P* = 0.035). Additionally, the following three variables had a *P* value < 0.20: gender (*P* = 0.168), history of hyperlipemia (*P* = 0.141), and history of AP (*P* = 0.117). When introducing the five variables with *P* value < 0.20 in univariate analysis into multivariate analysis using a Cox proportional hazards model, we identified current smoking (HR = 6.3, 95%CI: 1.3-29.8, *P* = 0.020) as an independent risk factor contributing to HLAP recurrence (Table 3).

During follow-up, four non-smoker patients, one ex-smoker patient, and 18 current smoker patients experienced AP recurrence with different RFS time. Figure 1 shows the Kaplan-Meier curves for RFS of

non-, ex-, and current smokers. The median RFS time in non-smokers, ex-smokers, and current smokers was 42 mo, 34 mo, and 23 mo, respectively (*P* = 0.002). Current smokers had significantly worse RFS than non-smokers (*P* = 0.001), but no significant difference in RFS was documented between ex-smokers and non-smokers (*P* = 0.962). Kaplan-Meier curves for RFS in non-smokers (PY = 0), light smokers (0 < PY < 10), and heavy smokers (PY ≥ 10) are shown in Figure 2. The median RFS of non-smokers, light smokers, and heavy smokers was 42 mo, 23 mo, and 30 mo, respectively, indicating that the RFS was significantly worse in smokers than in non-smokers (*P* = 0.014). However, the RFS was not significantly different between light and heavy smokers (*P* = 0.749).

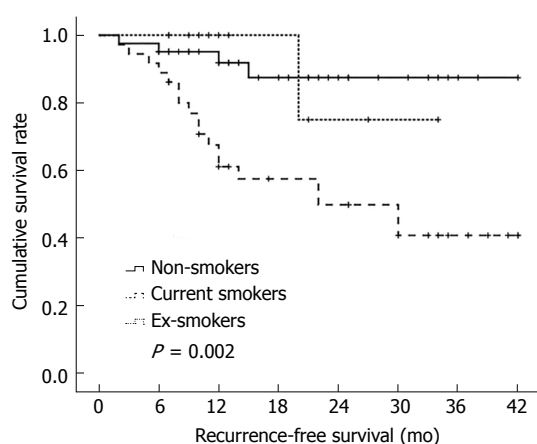
DISCUSSION

In the present study, univariate and multivariate analyses identified that cigarette smoking status is an independent risk factor contributing to the recurrence of HLAP^[19]. To the best of our knowledge, this is the first study evaluating the influence of cigarette smoking on HLAP recurrence. A recent comprehensive study analyzed 2810 patients (1065 with gallstone-related AP, 1222 with non-gallstone related AP, and 523 with recurrent AP/CP) and demonstrated that cigarette smoking was associated with non-gallstone-related AP and recurrent AP/CP^[12]. Unfortunately, this study did not evaluate any correlation between cigarette smoking and HLAP recurrence. As non-gallstone-related AP was a broad category including various

Table 2 Univariate analysis of risk factors for recurrence of hyperlipidemic acute pancreatitis

Variable	n	Number of recurrence	HR (95%CI)	P value
Age (≤ 40 / > 40 , yr)	46/42	14/9	0.7 (0.3-1.6)	0.417
Gender (M/F)	66/22	20/3	2.4 (0.7-7.9)	0.168
Heavy alcohol drinking (Y/N)	18/70	6/17	1.4 (0.5-3.5)	0.507
Smoking history (Y/N)	47/41	19/4	4.3 (1.4-12.5)	0.009
Non-smokers	41	4	1 (ref.)	1 (ref.)
Current-smokers	36	18	5.1 (1.7-15.2)	0.003
Ex-smokers	11	1	1.1 (0.1-9.5)	0.959
Smoking pack-years (> 0 but < 10 / ≥ 10)	64/24	11/12	0.4 (0.2-0.9)	0.035
0	41	4	1 (ref.)	1 (ref.)
0 < PY < 10	23	7	3.8 (1.1-13.1)	0.032
≥ 10	24	12	4.6 (1.5-14.2)	0.009
History of hyperlipemia (Y/N)	77/11	18/5	0.5 (0.2-1.3)	0.141
History of fatty liver (Y/N)	35/53	9/14	1.0 (0.4-2.2)	0.917
History of diabetes mellitus (Y/N)	29/59	10/13	1.7 (0.7-3.9)	0.208
History of hypertension (Y/N)	13/75	2/21	0.7 (0.2-2.9)	0.585
History of AP (Y/N)	36/52	13/10	1.9 (0.8-4.4)	0.117
Leukocyte (< 10 / ≥ 10 , $\times 10^9$ /L)	23/65	5/18	0.6 (0.2-1.7)	0.379
Platelet count (< 100 / ≥ 100 , $\times 10^9$ /L)	4/84	1/22	0.8 (0.1-5.7)	0.790
Serum TG (≤ 11.3 / > 11.3 , mmol/L)	32/56	7/16	0.9 (0.4-2.2)	0.813
Serum CHOL (≤ 5.5 / > 5.5 , mmol/L)	16/72	4/19	1.0 (0.3-2.8)	0.934
Serum amylase (< 540 / ≥ 540 , U/L)	62/26	18/5	1.6 (0.6-4.4)	0.335
Serum lipase (< 600 / ≥ 600 , U/L) ¹	31/34	8/9	0.6 (0.2-1.5)	0.273
Serum calcium (< 2.0 / ≥ 2.0 , mmol/L)	28/60	8/15	1.0 (0.4-2.3)	0.918
SIRS (Y/N)	42/46	11/12	1.1 (0.5-2.4)	0.858
BISAP score (< 2 / ≥ 2)	69/19	19/4	1.3 (0.5-3.9)	0.610

¹Data of the parameter in 65 of patients were available. AP: Acute pancreatitis; TG: Total triglyceride; CHOL: Cholesterol; SIRS: Systemic inflammatory response syndrome; BISAP: Bedside index for severity in acute pancreatitis; HR: Hazard ratio; CI: Confidence interval.



No. of patients at risk								
Non-smokers	41	39	28	19	12	7	3	1
Current smokers	36	33	21	15	13	11	5	1
Ex-smokers	11	11	6	4	2	1	-	-

Figure 1 Kaplan-Meier curves for the recurrence-free survival of non-, ex-, and current smokers.

causes and pathogeneses, it was difficult to analyze the pathophysiologic mechanism of cigarette smoking leading to recurrent HLAP. The most significant difference between the study by Setiawan *et al.*^[12] and ours is that our study takes HLAP into consideration as a specific disease to evaluate the effects of cigarette smoking on HLAP recurrence.

Notably, current smokers appeared to be associated with a higher recurrence rate and a greater incidence

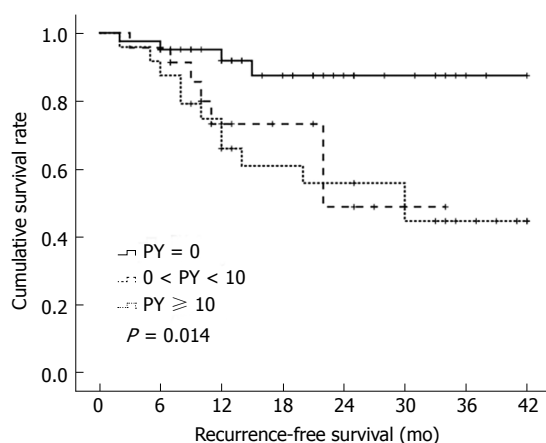
of repeated episodes of AP. In addition, the RFS was significantly worse in smokers than in non-smokers. However, the RFS was not significantly different between light and heavy smokers. This finding implied that cigarette smoking might contribute to HLAP recurrence but not in a dose- or duration-dependent manner; however, this could not be fully demonstrated in the present study due to short follow-up period and the small number of patients enrolled. Rebours *et al.*^[9] demonstrated in their study that alcoholic CP occurred earlier at a 20-pack-year threshold, and that patients had more calcifications. Similar results were observed in other research^[8,11,20] with regard to CP or AP. Therefore, the importance of smoking in the recurrence of different AP cases might differ. Whether the level of tobacco exposure influences HLAP recurrence and RFS needs to be further evaluated.

Interestingly, ex-smokers who had quit smoking for at least 6 mo after discharge from the hospital had a significant advantage with regard to the recurrence rate and RFS compared to current smokers in the present study. Univariate and multivariate analyses also demonstrated that current smoking was an independent risk factor contributing to HLAP recurrence. Therefore, lifestyle modifications are a key feature in the long-term management of HLAP^[21,22]. Due to a possible increase in recurrence rate related to smoking, doctors should always strongly suggest that the patient quit smoking once HLAP has been diagnosed. Moreover, current smokers had a higher

Table 3 Multivariate analysis of risk factors for recurrence of hyperlipidemic acute pancreatitis

Variable	Hazard ratio	95%CI	P value
Gender (Male)	0.5	0.1-3.0	0.449
Smoking history (Current)	6.3	1.3-29.8	0.020
Smoking pack-years (≥ 10)	1.0	0.4-2.7	0.969
History of hyperlipemia (Yes)	0.7	0.2-1.8	0.435
History of AP (Yes)	1.4	0.6-3.3	0.422

AP: Acute pancreatitis; CI: Confidence interval.



No. of patients at risk

PY = 0	41	39	28	19	12	7	3	1
0 < PY < 10	23	22	10	7	4	2	-	-
PY ≥ 10	24	22	17	12	11	10	5	1

Figure 2 Kaplan-Meier curves for the recurrence-free survival of non-, light, and heavy smokers.

proportion of concomitant alcohol abuse than non- & ex-smokers in our cohort. We also entered "high alcohol drinking" as a potential variable influencing HLAP recurrence^[23]. Although there were no data indicating a potential influence of high alcohol consumption on HLAP recurrence, it was recognized as a synergistic factor with smoking for AP occurrence and a negative prognostic factor for AP survival^[3,24,25]. However, only a small sample of alcohol drinkers were enrolled in our study since alcohol drinking is not as common among the Chinese population as in Western populations, which might contribute to bias in this data analysis.

Several studies have shown unique molecular characteristics and behavior patterns of pancreatic cancer related to tobacco use. However, the mechanism by which cigarette smoking promotes HLAP recurrence remains unknown. Some experimental and clinical studies indicated that cigarette smoke aggravates pancreatic acinar cell injury and pancreatic calcification by increasing oxidative stress and the production of pro-inflammatory cytokines^[8,20,26,27]. Nicotine, the main poisonous element of tobacco, might accumulate significantly in the pancreas and participate in regulating lipid peroxidation, resulting in

HLAP and HLAP recurrence.

There are several limitations in our present study. First, measurement error in self-reported tobacco use is possible and may have led to some degrees of nondifferential misclassification of exposure. Second, more-detailed classification is needed to clarify the potential dose- and duration-dependent correlation between cigarette smoking and HLAP. Finally, the number of cases within our exposure categories by pancreatitis type is small, and long-term follow-up is needed.

In the present study, we found that cigarette smoking was associated with worse RFS and an increased recurrence rate of HLAP. For smokers, continued smoking might be strongly correlated with HLAP recurrence and compromised survival. Therefore, smoking cessation should be strongly recommended. Future studies are needed to clarify possible changes in the metabolic and molecular characteristics of HLAP related to tobacco use and to determine whether these changes contribute to disease recurrence.

ARTICLE HIGHLIGHTS

Research background

Hyperlipidemic acute pancreatitis (HLAP) is a form of AP occurring in the presence of severe hypertriglyceridemia and in the absence of other causes. The exact pathophysiology of HLAP is not entirely certain. It is believed that HLAP is related to pancreatic tissue injury and microcirculation disturbance caused by free fatty acids. Some recent studies identified that smoking was significantly associated with non-biliary AP instead of biliary AP, but whether cigarette smoking has any long-term impact on HLAP recurrence has not yet been investigated. This is the first study evaluating the influence of cigarette smoking on HLAP recurrence.

Research motivation

Authors performed this study to better understand the relationship between cigarette smoking and HLAP recurrence, as well as the pathophysiologic mechanism of recurrent HLAP.

Research objectives

The main objective of this study was to investigate the impact of cigarette smoking on the recurrence rate and recurrence-free survival in HLAP. The authors found that cigarette smoking was associated with worse RFS and an increased recurrence rate of HLAP. These findings provide references for further clarifying the mechanism of HLAP.

Research methods

A total of 88 patients diagnosed with HLAP were enrolled in this retrospective study. Demographic data, medical history, previous episodes of pancreatitis, consumption of alcohol and cigarettes, as well as biochemical and hematological data were carefully recorded for univariate and multivariate analyses. During follow-up, the information on current smoking status and recurrent AP was gathered. Recurrence-free survival was calculated using the Kaplan-Meier method, and the differences between groups were compared using the log-rank test.

Research results

Current smokers had a remarkably higher recurrence rate and a greater incidence of repeated episodes of AP than non-smokers, and these two percentages were reduced to 9.1% and 36.4% for patients who gave up smoking. The median follow-up time was 13.5 mo. Multivariate analysis identified current smoking as an independent risk factor contributing to HLAP

recurrence. Current smokers had significantly worse RFS than non-smokers, but no significant difference was documented between ex-smokers and non-smokers.

Research conclusions

In the present study, the authors found that cigarette smoking was associated with worse RFS and an increased recurrence rate of HLAP. For smokers, continued smoking might be strongly correlated with HLAP recurrence and compromised survival. Smoking cessation for at least 6 mo would lead to a significant advantage in recurrence rate and RFS compared to current smokers.

Research perspectives

The study revealed that smoking is associated with worse RFS and higher recurrence rate of HLAP. Besides, smoking cessation for at least 6 mo would lead to a significant advantage in recurrence rate and RFS compared to current smokers. For the future research, more detailed classification is needed to clarify the potential dose- and duration-dependent correlation between cigarette smoking and HLAP. Besides, expanding the number of cases and long-term follow-up are needed.

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Clinical Trials Study

First-week clinical responses to dexlansoprazole 60 mg and esomeprazole 40 mg for the treatment of grades A and B gastroesophageal reflux disease

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and Chuah SK performed the research; Liang CM and Kuo MT analyzed the data and wrote the manuscript; all authors approved the final version of the manuscript.

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Abstract

AIM

To compare the one-week clinical effects of single doses of dexlansoprazole and esomeprazole on grades A and B erosive esophagitis.

METHODS

We enrolled 175 adult patients with gastroesophageal reflux disease (GERD). The patients were randomized in a 1:1 ratio into two sequence groups to define the order in which they received single doses of dexlansoprazole ($n = 88$) and esomeprazole ($n = 87$) for an intention-to-treat analysis. The primary end-points were the complete symptom resolution (CSR) rates at days 1, 3, and 7 after drug administration.

RESULTS

Thirteen patients were lost to follow-up, resulting in 81 patients in each group for the per-protocol analysis. The CSRs for both groups were similar at days 1, 3 and 7. In the subgroup analysis, the female patients achieved higher CSRs in the dexlansoprazole group than in the esomeprazole group at day 3 (38.3% *vs* 18.4%, $P = 0.046$). An increasing trend toward a higher CSR was observed in the dexlansoprazole group at day 7 (55.3% *vs* 36.8%, $P = 0.09$). In the esomeprazole group, female sex was a negative predictive factor for CSR on post-administration day 1 [OR = -1.249 ± 0.543 ; 95%CI: 0.287 (0.099-0.832), $P = 0.022$] and day 3 [OR = -1.254 ± 0.519 ; 95%CI: 0.285 (0.103-0.789), $P = 0.016$]. Patients with spicy food eating habits achieved lower CSRs on day 1 [37.3% *vs* 21.4%, OR = -0.969 ± 0.438 ; 95%CI: 0.380 (0.161-0.896), $P = 0.027$].

CONCLUSION

The overall CSR for GERD patients was similar at days 1-7 for both the dexlansoprazole and esomeprazole groups, although a higher incidence of CSR was observed on day 3 in female patients who received a single dose of dexlansoprazole.

Key words: Dexlansoprazole; Esomeprazole; One-week response; Complete symptom resolution rate; Gastroesophageal reflux disease

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Core tip: No existing report has investigated the short-term clinical effects of dexlansoprazole 60 mg *vs* esomeprazole 40 mg. This study compared the one-week clinical effects of a single dose of the two drugs for grades A and B erosive esophagitis. We enrolled 175 adult patients with gastroesophageal reflux disease (GERD) and randomized them in a 1:1 ratio into a dexlansoprazole ($n = 88$) or esomeprazole group ($n = 87$) for an intention-to-treat analysis (ITT). The primary end-points were the complete symptom resolution (CSR) rates at days 1, 3, and 7. The CSRs for both groups were similar at days 1, 3 and 7. In the subgroup analysis, female patients achieved higher CSRs in the dexlansoprazole group than in the esomeprazole group at day 3 (38.3% *vs* 18.4%, $P = 0.046$). In the esomeprazole group, female sex was a negative predictive factor for CSR at post-dose day 1 [OR = -1.249 ± 0.543 ; 95%CI: 0.287 (0.099-0.832), $P = 0.022$] and day 3 [OR = -1.254 ± 0.519 ; 95%CI: 0.285 (0.103-0.789), $P = 0.016$]. This pilot study suggested that the overall CSR rates for GERD patients were similar at days 1 through 7 for both the dexlansoprazole and esomeprazole groups, although a higher CSR was observed at day 3 in female patients who received a single dose of dexlansoprazole.

Liang CM, Kuo MT, Hsu PI, Kuo CH, Tai WC, Yang SC, Wu KL, Wang HM, Yao CC, Tsai CE, Wang YK, Wang JW, Huang CF, Wu DC, Chuah SK; Taiwan Acid-Related Disease Study Group. First-week clinical responses to dexlansoprazole 60 mg and esomeprazole 40 mg for the treatment of grades A and B gastroesophageal reflux disease. *World J Gastroenterol* 2017; 23(47): 8395-8404 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v23/i47/8395.htm> DOI: <http://dx.doi.org/10.3748/wjg.v23.i47.8395>

INTRODUCTION

Gastroesophageal reflux disease (GERD) is a common gastrointestinal disorder worldwide. GERD continues to increase in incidence with the aging population and the obesity epidemic^[1,2]. Based on the Montreal definition, GERD is diagnosed when the reflux of stomach contents causes troublesome symptoms^[3], such as heartburn and regurgitation, as well as other atypical or extraesophageal symptoms, such as chest pain, asthma, voice hoarseness, and sleep disturbance^[4]. Proton pump inhibitors (PPIs) are widely recognized as superior to other antisecretory therapies, including histamine-2 receptor antagonists (H₂RA), and thus play a critical role in pharmacological therapy for the treatment of GERD^[5]. Although PPIs represent the mainstay of treatment for healing erosive esophagitis,

symptom relief, and preventing complications, several studies have shown that up to 40% of GERD patients report either a partial or a complete lack of response of their symptoms after taking a standard once-daily PPI dose^[6-8].

A study comparing the pharmacokinetic effects of different PPIs 12-24 h post-dose showed that the mean percentage of time with a pH > 4 and the average of the pH mean were greater for dexlansoprazole than for esomeprazole (60% vs 42%, $P < 0.001$ and pH 4.5 vs 3.5, $P < 0.001$). However, this study did not report the clinical effects after the use of tablets^[9]. Rapid onset PPIs for fast symptom relief is an unmet need in GERD treatment. To date, no reports have investigated the differences in short-term clinical effects and timing to symptom relief of GERD between dexlansoprazole 60 mg and esomeprazole 40 mg. Therefore, we conducted a randomized, controlled, open-label study to compare the 7-d clinical effects of single doses of dexlansoprazole (60 mg) and esomeprazole (40 mg) in patients with Los Angeles (LA) grades A and B erosive esophagitis.

MATERIALS AND METHODS

Ethics statement

This study was funded by the Research Foundation of the Chang Gung Memorial Hospital, Taiwan (CMRPG8D1441). This open-labeled trial was conducted at Kaohsiung Chang Gung Memorial Hospital, Kaohsiung Medical University Hospital, and Kaohsiung Veterans General Hospital in Taiwan. The study protocol was approved by the Ethics Committees of the above three hospitals. All patients provided written informed consent prior to participation. This clinical trial has been registered in a publicly accessible registry (ClinicalTrials.gov number: NCT03128736).

Study population

We invited 243 eligible outpatients to join our study. The outpatients were at least 18 years old, presented with clinical symptoms of acid regurgitation, heart-burn, and a feeling of acidity in the stomach^[10], and had endoscopy-confirmed LA grade A or B erosive esophagitis^[11,12]. We enrolled a total of 175 patients using strict inclusion criteria. The exclusion criteria included (1) those who had been taking antisecretory agents, such as PPIs and H₂RA, within 2 wk prior to the endoscopy; (2) those who had coexistence of a peptic ulcer or gastrointestinal malignancies, and were pregnant; (3) those who had coexistence of a serious concomitant illness (*e.g.*, decompensated liver cirrhosis and uremia); (4) those who underwent previous gastric surgery; (5) those who were allergic to dexlansoprazole or esomeprazole; and (6) those who had a symptom score less than 12 on a validated

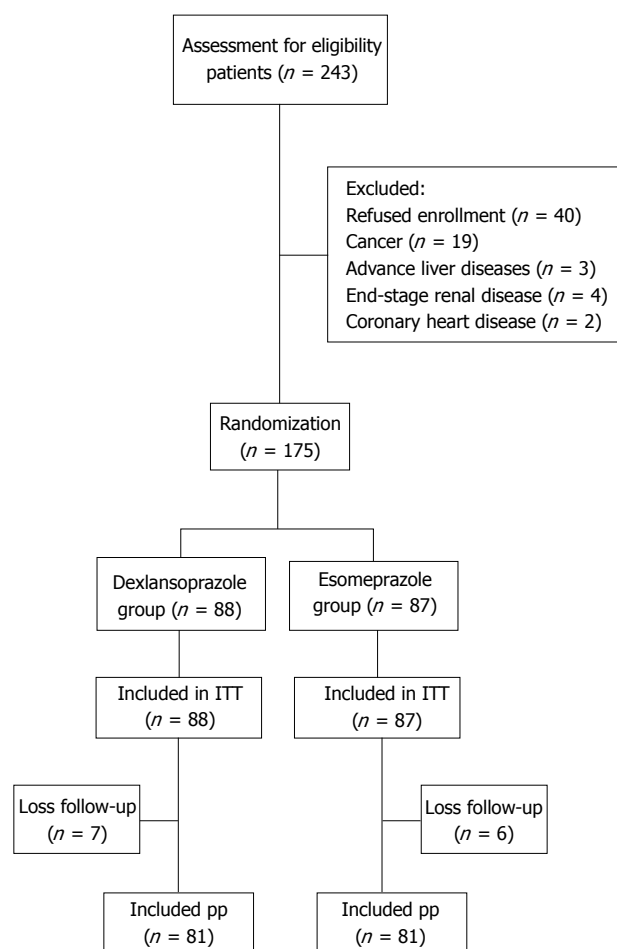


Figure 1 Schematic flowchart of the study design. ITT: Intention-to-treat; PP: Per protocol.

questionnaire (Chinese GERDQ)^[10].

Study protocol

Figure 1 shows the schematic flowchart of the study design. Eligible patients were randomly assigned to receive either dexlansoprazole 60 mg q.d. or esomeprazole 40 mg q.d. for 8 wk as an initial treatment. Randomization was conducted using a computer-generated list of random numbers in a 1:1 ratio into two sequence groups that defined the order in which the patients received a single dose of dexlansoprazole or esomeprazole for an intention-to-treat analysis. An independent staff member assigned the treatments according to consecutive numbers kept in sealed envelopes. Written informed consent was obtained from each patient.

Each patient completed diary cards during the study period. Complete symptom resolution (CSR) was defined as no reflux symptoms leading to troublesome feelings in the 7 d of initial treatment. The patients were asked to complete the Chinese GERDQ upon recruitment^[10]. The selected symptoms that best accounted for the differences between the

patients with GERD and the controls included acid regurgitation, heartburn, and a feeling of acidity in the stomach. The severity and frequency of symptoms in the questionnaire were graded on a five-point Likert scale as follows: (1) (none: no symptoms/none in the last month); (2) (mild: symptoms could be easily ignored/less than once per month); (3) (moderate: awareness of symptoms but easily tolerated/ \geq once per month); (4) (severe: symptoms sufficient to interfere with normal activities/ \geq once per week); and (5) (incapacitating: incapacitating symptoms with an inability to perform daily activities or requiring a day off work/ \geq once daily)^[10]. Blood samples were collected to measure the fasting blood sugar, serum cholesterol, and triglyceride levels. In addition, the body mass index (BMI) was calculated. Upon initial endoscopy, specimens taken from the greater curvature within 5 cm from the pylorus and from the greater curvature of the middle body were subjected to a microscopic examination for *Helicobacter pylori* (*H. pylori*) using a hematoxylin and eosin stain. No eradication therapy was administered during the study period.

Patient demographic data and follow-up

A complete medical history and demographic data were obtained from each patient. The collected variables included age (< 60 or \geq 60 years), sex, history of smoking, history of alcohol consumption (< 80 g/d or \geq 80 g/d), coffee ingestion (< 1 cup/d or \geq 1 cup/d), tea ingestion (< 1 cup/d or \geq 1 cup/d), coexistence of a systemic disease (yes or no), severity of erosive esophagitis, and BMI. A gastric biopsy for histology and an *H. pylori* examination were also performed. The patients returned to the clinics for drug refills and evaluation of reflux symptoms after one week. Adverse events were prospectively evaluated. The adverse events were assessed according to a 4-point scale system as follows: none; mild (discomfort, annoying but not interfering with daily work); moderate (discomfort sufficient to interfere with daily work); and severe (discomfort resulting in discontinuation of PPI therapy). Compliance was checked by counting the unused medication at the completion of 7 d of treatment.

End points

CSR was defined as no reflux symptoms sufficient to impair the quality of life before the end of the initial treatment phase. The main outcome measures were the CSR rates at days 1, 3 and 7 of the initial treatment period. All patients who started esomeprazole or dexlansoprazole as their initial treatment were included in the intent-to-treat (ITT) analysis. Patients with poor drug compliance were excluded from the per-protocol (PP) analysis. Poor compliance was defined as taking less than 80% of the total medication during the initial

treatment phase.

Statistical analysis

According to the observations in this study, the CSR rate after a once-daily PPI therapy was approximately 50% at day 7. Assuming that the two types of PPIs provided similar effects on the CSR rates with a standard deviation of less than 10%^[13], we estimated that we required at least 196 patients in each treatment group to demonstrate a 10% absolute difference in the CSR with a type I error of 0.05 and a statistical power of 80% and assuming a 10% loss to follow-up. As a consequence of not achieving the target number, our study was a pilot study.

In this pilot study, the χ^2 test with or without Yates correction for continuity and Fisher's exact test were used when appropriate to compare the rates of CSR, symptom relapse, and esophagitis relapse between the groups. The mean reflux symptom scores between groups were compared using the Wilcoxon rank sum test. All statistical analyses were performed using the SPSS program (version 10.1, Chicago, IL, United States). A *P* value less than 0.05 was considered significant.

RESULTS

From April 2014 to March 2016, two hundred and forty-three eligible symptomatic patients who had endoscopy-confirmed Los Angeles grade A or B erosive esophagitis were assessed. A total of 175 of these patients were recruited for randomization after excluding 68 patients who refused enrollment (*n* = 40), cancer patients (*n* = 19), and patients with advanced liver disease (*n* = 3), end-stage renal disease (*n* = 4), and coronary heart disease (*n* = 2). A total of 88 patients received the dexlansoprazole treatment, and 87 patients received the esomeprazole treatment. A total of 13 patients were lost during the follow-up period (seven in the dexlansoprazole group and 6 in the esomeprazole group) (Figure 1). The baseline characteristics of the two groups were similar in age, sex, diet habits, body mass index, and symptom scores (GERDQ) (Table 1). At days 1, 3, and 7 post-dose, the CSR rates for the dexlansoprazole vs esomeprazole groups were 25.9% vs 28.4% (*P* = 0.724), 33.3% vs 32.1% (*P* = 0.867), and 51.9% vs 48.1% (*P* = 0.637), respectively. The symptoms and frequencies of nighttime reflux were similar in both groups (Table 2). In the subgroup analysis based on sex, females had higher CSR rates in the dexlansoprazole group at day 3 (38.3% vs 18.4%, *P* = 0.046), and an increasing trend was observed at day 7 (55.3% vs 36.8%, *P* = 0.09) (Table 3). However, no significant differences were observed in the subgroup analyses based on age and body weight. After splitting

Table 1 Baseline characteristics of the patients [*n* = 81, *n* (%)]

Variables	Dexlansoprazole	Esomeprazole	<i>P</i> value
Age (mean ± SD, yr)	50.6 ± 13.3	49.9 ± 12.8	0.985
Male sex	34 (42.0)	43 (53.1)	0.137
Smoking	12 (14.8)	9 (11.1)	0.483
Alcohol use	22 (27.2)	22 (27.2)	1.000
Ingestion of coffee	44 (54.3)	36 (44.4)	0.209
Ingestion of tea	58 (71.6)	49 (60.5)	0.230
Betel nut	4 (4.9)	1 (1.2)	0.173
Spicy food	52 (64.2)	51 (63.0)	0.870
Sweet food	72 (88.9)	75 (92.6)	0.416
Body mass index	25.4 ± 4.8	24.9 ± 4.4	0.420
Waist girth	88.8 ± 12.2	88.7 ± 11.4	0.361
Metabolic syndrome	36 (44.4)	38 (46.9)	0.950
Atypical symptoms			
Chest pain	38 (46.9)	39 (48.1)	0.588
Dysphagia	20 (24.7)	22 (27.2)	0.557
Regurgitation of food	29 (35.8)	31 (38.3)	0.561
Nausea	26 (32.1)	23 (28.4)	0.544
Hiccup	37 (45.7)	44 (54.3)	0.300
Foreign body sensation (throat)	48 (59.3)	40 (49.4)	0.301
Foreign body sensation (chest)	16 (19.8)	16 (19.8)	0.604
Hoarseness	28 (34.6)	28 (34.6)	0.604
Throat cleaning	44 (54.3)	44 (54.3)	0.602
Cough	38 (46.9)	34 (42.0)	0.516
Sore throat	20 (24.7)	20 (24.7)	0.604
Dry mouth	54 (66.7)	52 (64.2)	0.590
Bad breath	29 (35.8)	30 (37.0)	0.590
Epigastric pain	36 (44.4)	45 (55.6)	0.197
Epigastric fullness	65 (80.2)	54 (66.7)	0.111
Insomnia	36 (44.4)	28 (34.6)	0.199
Sinusitis	7 (8.6)	14 (17.3)	0.102
Otitis media	5 (6.2)	5 (6.2)	1.000
Sugar	97.4 ± 12.5	97.0 ± 12.8	0.604
Cholesterol	205.3 ± 36.7	207.7 ± 35.4	0.971
Triglyceride	121.9 ± 57.2	113.7 ± 64.7	0.284
HDL	54.7 ± 18.2	55.3 ± 14.4	0.866
LDL	127.0 ± 32.7	127.5 ± 32.8	0.942
<i>H. pylori</i> infection			
Previous history - no	10 (12.3)	15 (18.5)	0.553
Current infection - no	10 (12.3)	12 (14.8)	0.703
Endoscopic findings			
Hiatal hernia	10 (12.3)	15 (18.5)	0.347
GEFV (grade 3 or 4)	7 (8.6)	8 (9.9)	0.521
Esophagitis grade B	15 (18.5)	13 (16.0)	0.678

HDL: High-density lipoprotein; LDL: low-density lipoprotein; *H. pylori*: *Helicobacter pylori*; GEFV: Gastroesophageal flap valve.

the data from the two PPI groups in the multivariate analysis, no dependent factor for CSR was found in the dexlansoprazole group (Table 4). In the esomeprazole group, female sex was a negative predictive factor for CSR at post-dose days 1 [OR = -1.249 ± 0.543; 95%CI: 0.287 (0.099-0.832), *P* = 0.022] and 3 [OR = -1.254 ± 0.519; 95%CI: 0.285 (0.103-0.789), *P* = 0.016]. In addition, patients with a habit of consuming spicy foods had lower CSR rates (37.3% vs 21.4%) on day 1 after the multivariate analysis [OR = -0.969 ± 0.438; 95%CI: 0.380 (0.161-0.896), *P* = 0.027] (Table 5). No dependent factor was found on days 3 and 7.

DISCUSSION

We conducted a randomized, controlled, open-label

study to compare the 7-d clinical effects of single doses of dexlansoprazole 60 mg and esomeprazole 40 mg for GERD patients. We observed that the overall CSR rates for GERD patients were similar at days 1 through 7 of treatment for both the dexlansoprazole and esomeprazole groups. However, in our subgroup analysis based on sex, we observed that females had higher CSR rates in the dexlansoprazole group at day 3 (38.3% vs 18.4%, *P* = 0.046), and an increasing trend was observed at day 7 (55.3% vs 36.8%, *P* = 0.09). The logistic regression analysis showed that female sex was a negative predictive factor for CSR on post-dose days 1 [OR = -1.249 ± 0.543; 95%CI: 0.287 (0.099-0.832), *P* = 0.022] and 3 [OR = -1.254 ± 0.519; 95%CI: 0.285 (0.103-0.789), *P* = 0.016] in the esomeprazole group. We also found

Table 2 Comparison of the complete symptom resolution rates and night-time breakthrough heartburn between dexlansoprazole and esomeprazole over one week [$n = 81$, n (%)]

Variables	Dexlansoprazole	Esomeprazole	<i>P</i> value
CSR Day 1	21 (25.9)	23 (28.4)	0.724
CSR Day 3	27 (33.3)	26 (32.1)	0.867
CSR Day 7	42 (51.9)	39 (48.1)	0.637
Night reflux	45 (76.3)	40 (74.1)	0.787
Night heart burn	20 (33.9)	18 (33.3)	0.949
Night acid reflux	20 (33.9)	19 (35.2)	0.886
Frequency of night symptoms	2.7 ± 2.0	2.7 ± 2.4	0.343

CSR: Complete symptom resolution.

Table 3 Comparison of the complete symptom resolution rates between dexlansoprazole and esomeprazole over one week (Subgroup analysis by gender) n (%)

Time	Gender	Dexlansoprazole	Esomeprazole	<i>P</i> value
CSR Day 1	Female	13 (27.7)	6 (15.8)	0.192
	Male	8 (23.5)	17 (39.5)	0.136
CSR Day 3	Female	18 (38.3)	7 (18.4)	0.046
	Male	9 (26.5)	19 (44.2)	0.109
CSR Day 7	Female	26 (55.3)	14 (36.8)	0.090
	Male	16 (47.1)	25 (58.1)	0.333

CSR: Complete symptom resolution.

that patients with the habit of eating spicy foods had lower CSR rates (37.3% vs 21.4%) on day 1 after the multivariate analysis [OR = -0.969 ± 0.438; 95%CI: 0.380 (0.161-0.896), $P = 0.027$].

Both dexlansoprazole and esomeprazole are potent PPIs for gastric acid suppression with excellent symptom relief for patients with GERD^[14-19]. The advantage of dexlansoprazole MR (Takeda Pharmaceuticals, Osaka, Japan) is that it employs a novel approach by which its dual delayed-release (DDR) formulation prolongs the plasma concentration and ultimately extends the duration of acid suppression^[14], thereby offering a twice-daily dosing effect in a one-time dose. Metz *et al*^[15] found that patients who received a 60-mg dose of dexlansoprazole MR satisfactorily controlled heartburn (median of 91%-96% for 24-h heartburn-free days and 96%-99% for heartburn-free nights). Moreover, Sharma *et al*^[16] reported that 92%-95% of patients were healed using dexlansoprazole MR for 8 wk. Conversely, esomeprazole (40 mg) is a delayed-release formulation with single-release characteristics that produces maximum plasma concentrations at approximately 1.6 h post-dose. Approximately 73%-75% heartburn-free days and 85%-91% heartburn-free nights were observed in patients who received 40 mg of esomeprazole for 4 wk^[17-19]. In addition, esomeprazole at 40 mg/d also achieved good healing rates (87%-94.1%) for erosive esophagitis after 8 wk of treatment^[18-20].

However, no direct head-to-head comparative

report has investigated the short-term clinical effects or timing to symptom relief of GERD between dexlansoprazole at 60 mg and esomeprazole at 40 mg. Wu *et al*^[21] reported an indirect comparative study that revealed that the dexlansoprazole 30 mg dose was more effective than esomeprazole at the 20 mg or 40 mg dose (RR = 2.01, 95%CI: 1.15-3.51; RR = 2.17, 95%CI: 1.39-3.38, respectively) for patients with non-erosive esophagitis at 4 wk. However, no significant differences were found in the healing rates of erosive esophagitis. A one-day comparative pH study showed that dexlansoprazole had a higher mean percentage of time with a pH > 4 than esomeprazole (58% and 48%, $P = 0.003$) at 0-24 h post-dose^[9]. Unfortunately, differences in the clinical effects between these two PPIs were not mentioned.

In this study, we found that the symptoms and frequencies of nighttime reflux were similar between the dexlansoprazole and esomeprazole groups ($P = 0.787$ and $P = 0.343$, respectively). At days 1, 3, and 7 post-dose, the CSR rates between the two groups were similar (25.9% vs 28.4%, $P = 0.724$, 33.3% vs 32.1%, $P = 0.867$, and 51.9% vs 48.1%, $P = 0.637$, respectively). Nevertheless, we also observed that female patients had higher CSR rates in the dexlansoprazole group ($P = 0.046$) and an increasing trend for the effect on day 7 ($P = 0.09$) when we performed the subgroup analysis based on sex. Remarkably, our logistic regression analysis showed that female sex was a negative predictive factor for CSR on post-dose days 1 [OR = -1.249 ± 0.543; 95%CI: 0.287 (0.099-0.832), $P = 0.022$] and 3 [OR = -1.254 ± 0.519; 95%CI: 0.285 (0.103-0.789), $P = 0.016$] in the esomeprazole group. These findings implied that esomeprazole at 40 mg required more time (3 d) than dexlansoprazole at 60 mg to attain CSR in females. Several possible mechanisms may underlie these observations. First, both esomeprazole and dexlansoprazole are extensively metabolized in the liver by oxidation, reduction, and subsequent conversion of sulfate, glucuronide and glutathione conjugates to inactive metabolites. Oxidative metabolites are formed by the cytochrome P450 (CYP)

Table 4 Multivariate analysis of the clinical factors predictive of complete symptom resolution within one week based on dexlansoprazole and esomeprazole administration

Time	PPI	Clinical factors	CSR	Coefficient of variation	Odds ratio (95%CI)	P value
Day 1	Dexlansoprazole	Null	15.80%	-1.249 ± 0.543	0.285 (0.103-0.789)	0.022
	Esomeprazole	Female				
Day 3	Dexlansoprazole	Null	18.40%	-1.254 ± 0.519	0.287 (0.099-0.832)	0.016
	Esomeprazole	Female				
Day 7	Dexlansoprazole	Null				
	Esomeprazole	Null				

CSR: Complete symptom resolution; PPI: Proton pump inhibitor.

Table 5 Multivariate analysis of the clinical factors predictive of complete symptom resolution within one week

Time	Clinical factor	CSR	Coefficient of variation	Odds ratio (95%CI)	P value
Day 1	Spicy food	No: 37.3% Yes: 21.4%	-0.969 ± 0.438	0.380 (0.161-0.896)	0.027
Day 3	Null				
Day 7	Null				

CSR: Complete symptom resolution.

enzyme system, mainly by CYP2C19 and CYP3A4^[22,23]. In the pharmacokinetics report of esomeprazole^[24], the mean exposure (AUC) to esomeprazole increases from 4.32 $\mu\text{mol}\cdot\text{h/L}$ on day 1 to 11.2 $\mu\text{mol}\cdot\text{h/L}$ on day 5 after a 40-mg once-daily dose, indicating that the pharmacokinetics of esomeprazole are time- and dose-dependent^[25]. For dexlansoprazole^[26,27], no accumulation of dexlansoprazole occurs after multiple once-daily doses of 60 mg, although the mean AUC and max concentration (C_{max}) values of dexlansoprazole are slightly higher (less than 10%) on day 5 than on day 1. We validated this finding by calculating the C_{max} of dexlansoprazole, which was 16 $\mu\text{mol}\cdot\text{h/L}$ on day 1 and 17.67 $\mu\text{mol}\cdot\text{h/L}$ on day 5. As a result, dexlansoprazole almost achieved the target concentration on day 1. Second, ample evidence has shown that estrogen and progestogen can enhance relaxation of the lower esophageal sphincters and induce GERD symptoms^[28-30], especially in postmenopausal women taking hormone replacement therapy (HRT)^[31-36]. These hypotheses might explain why female patients taking esomeprazole needed at least 3 more days to accumulate a sufficient plasma concentration to achieve plateau levels and desirable clinical effects.

Another observation in this study was the lower CSR rates in patients with the habit of eating spicy foods in the esomeprazole group at day 1 after the multivariate analysis. No reliable data are available in the existing literature regarding the role of diet or specific foods or drinks in GERD^[37]. Some foods are believed to induce or worsen GERD symptoms in daily clinical practice, and this belief has led to advising patients to avoid the suspect foods^[38]. Nebel *et al.*^[39] demonstrated that fried foods, spicy foods, and

alcohol were the most common precipitating factors of heartburn, but this study had no control group and did not quantify the intake of dietary items. In contrast, our study used a dietary questionnaire to estimate the frequency of the consumption of different types of food.

In addition to the above shortcoming, this study has other limitations. First, we enrolled only patients with Los Angeles grade A or B erosive esophagitis in this study and not those with Los Angeles grade C or D erosive esophagitis or Barrett's esophagus. As a result, the study may not represent the clinical effects of the entire GERD population. Second, this study used dietary questionnaires to estimate the frequency of consumption of different types of foods but did not quantify the fat or carbohydrate content. Nonetheless, this pilot study is the first important report to compare the clinical efficacy of a one-week dual delayed-release treatment with dexlansoprazole at 60 mg and esomeprazole at 40 mg for grades A and B GERD patients, since fast symptomatic relief is an important unmet need in the treatment of GERD.

In conclusion, the overall CSR rates for GERD were similar at days 1 through 7 for both the dexlansoprazole and esomeprazole groups, although a higher CSR was observed at day 3 in female patients who received a single dose of dexlansoprazole. Since rapid onset of proton-pump inhibitors for fast symptom relief is an unmet need for the treatment of GERD and no report have investigated the short-term clinical effects of dexlansoprazole 60 mg vs esomeprazole 40 mg, this finding of this pilot study is novel. Furthermore, these findings may have important implications for clinical practice when treating patients with grades A and B GERD. This issue was hampered by the small sample

size. Thus, we believe that large-scale comparative studies are necessary.

ARTICLE HIGHLIGHTS

Research background

Gastroesophageal reflux disease (GERD) is a common gastrointestinal disorder worldwide and continues to increase in incidence due to the aging population and obesity epidemic. Although proton pump inhibitors (PPIs) represent the mainstay of treatment for healing erosive esophagitis, symptom relief, and preventing complications, several studies have shown that up to 40% of GERD patients report either a partial or a complete lack of response of their symptoms after taking a standard once daily PPI dose. Rapid onset proton-pump inhibitors for fast symptom relief is an unmet need for GERD treatment. To date, no reports have investigated the short-term clinical effects and timing to symptom relief of gastroesophageal reflux disease (GERD) between dexlansoprazole (60 mg) and esomeprazole (40 mg). This report is the first randomized, controlled, open-label study to compare the 7-d clinical effects of single doses of dexlansoprazole at 60 mg and esomeprazole at 40 mg for LA grades A and B erosive esophagitis.

Research motivation

A study comparing the pharmacokinetic effects of different PPIs 12-24 h post-dose showed that the mean percentage of time with a pH > 4 and the average of the pH mean were greater for dexlansoprazole than for esomeprazole (60% vs 42%, $P < 0.001$ and pH 4.5 vs 3.5, $P < 0.001$). However, this study did not report the clinical effects after the use of tablets. Therefore, the significance of solving these problems for future research in this field should be based on large-scale, head-to-head comparisons of these PPIs on immediate symptom relief for GERD to fulfill the unmet need in real-world treatment.

Research objectives

The main objectives realized in this study motivated us to conduct this randomized, controlled, open-label study that compared the 7-d clinical effects of single doses of dexlansoprazole at 60 mg and esomeprazole at 40 mg for LA grades A and B erosive esophagitis.

Research methods

This study was funded by the Research Foundation of the Chang Gung Memorial Hospital, Taiwan (CMRPG8D1441), and has been registered in a publicly accessible registry (ClinicalTrials.gov number: NCT03128736). We enrolled 175 adult GERD subjects and randomized them in a 1:1 ratio into two sequence groups that defined the order in which they received single doses of dexlansoprazole ($n = 88$) and esomeprazole ($n = 87$) for an ITT. Written informed consent was obtained from each patient. The patients were asked to complete the Chinese GERDQ upon recruitment. Blood samples were collected to measure the fasting blood sugar, serum cholesterol, and triglyceride levels. In addition, the BMI was calculated. A complete medical history and demographic data were obtained from each patient. The primary end points were the complete symptom resolution (CSR) rates at days 1, 3, and 7. CSR was defined as no reflux symptoms sufficient to impair the quality of life before the end of the initial treatment phase. The main outcome measures were the CSR rates at days 1, 3 and 7 of the initial treatment period. All patients starting esomeprazole or dexlansoprazole as their initial treatment were included in the ITT analysis. Patients with poor drug compliance were excluded from the PP analysis.

Research results

Thirteen patients were lost during the follow up period, resulting in the inclusion of 81 patients in each group in the PP analysis. The CSRs for both groups were similar at days 1, 3 and 7. In the subgroup analysis, female patients achieved higher CSRs in the dexlansoprazole group than in the esomeprazole group at day 3 (38.3% vs 18.4%, $P = 0.046$). An increasing trend toward CSR was observed at day 7 (55.3% vs 36.8%, $P = 0.09$). In the esomeprazole group, female sex was a negative predictive factor for CSR at post-dose days 1 (OR = -1.249 \pm 0.543; 95%CI: 0.287 (0.099-0.832), $P = 0.022$) and 3 (OR = -1.254 \pm

0.519; 95%CI: 0.285 (0.103-0.789), $P = 0.016$). Patients with spicy food eating habits achieved lower CSRs on day 1 (37.3% vs 21.4%, OR = -0.969 \pm 0.438; 95%CI: 0.380 (0.161-0.896), $P = 0.027$).

Research conclusions

The conclusion of this study was that the overall CSR rates for GERD were similar on days 1 through 7 for both the dexlansoprazole and esomeprazole groups, although a higher incidence was observed on day 3 in female patients who received a single dose of dexlansoprazole. The findings of this study are novel, since no report has investigated the short-term clinical effects of dexlansoprazole 60 mg vs esomeprazole 40 mg. This comparison represents an unmet need for GERD treatment in real-world clinical practice. The findings in this study could have important implications for clinical practice in the future for the treatment of grade A and B GERD patients. Furthermore, this study observed that female sex was a negative predictive factor for CSR at post-dose days 1 and 3 in the esomeprazole group. These findings implied that esomeprazole at 40 mg required more time (3 d) than dexlansoprazole at 60 mg to attain CSR in females. The new theories proposed suggest that these observations could be due to differences in the pharmacokinetics of esomeprazole and dexlansoprazole. Esomeprazole is time- and dose-dependent, especially at days 1 and 5. No accumulation of dexlansoprazole occurs after multiple once-daily doses at 60 mg. The authors validated this possibility by calculating the C_{max} of dexlansoprazole, which was 16 $\mu\text{mol}\cdot\text{h/L}$ on day 1 and 17.67 $\mu\text{mol}\cdot\text{h/L}$ on day 5. As a result, dexlansoprazole almost achieved the target concentration on day 1. In addition, there is ample evidence that estrogen and progesterone enhance relaxation of the lower esophageal sphincters and induce GERD symptoms, especially in post-menopausal women taking hormone replacement therapy. These hypotheses could explain why female patients taking esomeprazole needed at least 3 more days to accumulate a sufficient plasma concentration to achieve plateau levels and desirable clinical effects.

Research perspectives

The important message of this study is that rapid onset PPIs for fast symptom relief remains an unmet need for GERD treatment. However, no report has investigated the short-term clinical effects of dexlansoprazole 60 mg vs esomeprazole 40 mg. Thus, the findings of this pilot study are novel and may have important implications for clinical practice in the future for the treatment of patients with grades A and B GERD. This pilot study was hampered by the small sample size. We believe that large-scale randomized controlled trials are necessary to further fulfill the future perspectives.

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Observational Study

Rate of adverse events of gastroduodenal snare polypectomy for non-flat polyp is low: A prospective and multicenter study

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Abstract

AIM

To evaluate the rate of adverse events (AEs) during consecutive gastric and duodenal polypectomies in several Spanish centers.

METHODS

Polypectomies of protruded gastric or duodenal polyps ≥ 5 mm using hot snare were prospectively included. Prophylactic measures of hemorrhage were allowed in predefined cases. AEs were defined and graded according to the lexicon recommended by the American Society for Gastrointestinal Endoscopy. Patients were followed for 48 h, one week and 1 mo after the

procedure.

RESULTS

308 patients were included and a single polypectomy was performed in 205. Only 36 (11.7%) were on prior anticoagulant therapy. Mean polyp size was 15 ± 8.9 mm (5-60) and in 294 cases (95.4%) were located in the stomach. Hemorrhage prophylaxis was performed in 219 (71.1%) patients. Nine patients presented AEs (2.9%), and 6 of them were bleeding ($n = 6$, 1.9%) (in 5 out of 6 AE, different types of endoscopic treatment were performed). Other 24 hemorrhagic episodes could be managed without any change in the outcome of the endoscopy and, consequently, were considered incidents. We did not find any independent risk factor of bleeding.

CONCLUSION

Gastroduodenal polypectomy using prophylactic measures has a rate of AEs small enough to consider this procedure a safe and effective method for polyp resection independently of the polyp size and location.

Key words: Polypectomy; Bleeding; Adverse events; Protruded polyps; Gastroduodenal; Foregut

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Core tip: The safety of polypectomy in the upper gastroduodenal tract is controversial because the reported rate in retrospective studies is higher than in colonic polypectomy but results come mainly from retrospective studies and they do not use the same standardized nomenclature and definitions for adverse events. To our knowledge, this is the first study using the ASGE lexicon for reporting adverse events of gastro-duodenal polypectomy and shows an acceptable low rate, confirming the safety of this procedure.

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INTRODUCTION

Gastric polyps are found in around 3%-6% of patients undergoing upper endoscopy^[1,2]. According to these sources, the most common gastric polyps are the hyperplastic and fundic gland types (70%-90%)

followed by adenomas, with a variable prevalence among countries depending on the use of proton pump inhibitor drugs (PPI) or the prevalence of *Helicobacter pylori* (*H. pylori*) infection. In western countries, where *H. pylori* infection is low and PPI use is very common, fundic gland polyps are seen more frequently^[2,3]. However, two retrospective Spanish series showed that in Spain the most frequent were hyperplastic polyps (50.9%), followed by fundic gland polyps (7.4%), adenomas (3%), and adenocarcinomas (1.9%)^[4,5].

Sporadic duodenal polyps are uncommon with a prevalence of 0.3% to 4.6%^[6,7]. Whereas multiple, small polyps in the duodenal bulb are benign a substantial number of them located in the descending duodenum are adenomas^[7]. Duodenal adenomas can occur sporadically or more commonly in patients with Familial Adenomatous Polyposis, occurring in 50% to 100%^[8]. Most of gastric and duodenal polyps are asymptomatic and are incidentally found at endoscopy performed for unrelated reasons.

Gastric and duodenal polyps have a risk of malignant transformation that depends on histologic type. The risk of gastric polyps undergoing malignant transformation is near 2% for hyperplastic polyps, 5% for tubular adenoma, and up to 30% for tubulovillous adenoma^[9-11]. However, polyp histology cannot be reliably distinguished by endoscopic appearance^[12,13] and biopsy is not always representative of the entire polyp^[14]. Therefore, polypectomy is warranted if feasible and clinically appropriate; this is supported by current guidelines^[15,16].

Endoscopic polypectomy has become standard in the management of most polyps in the gastrointestinal tract. Gastric and duodenal polyps can be safely removed with snare depending on size, location and presence of a stalk. However, bleeding is the most common adverse event (AE) of snare polypectomy, with an incidence of 6% to 7.2% in gastric polyps and up to 13.9% in duodenal polyps^[17,18]. Although these figures are higher than those reported in colonic polypectomies (0.3%-6%)^[19,20], the available evidence is limited by the fact that it is often based on retrospective studies performed at a single center or with a small number of patients and the nomenclature and definitions used for AEs are different. The need for standardized nomenclature and agreement on definitions for AEs was addressed by the American Society for Gastrointestinal Endoscopy (ASGE) in a workshop celebrated in 2008 and whose recommendations were published in 2010^[21]. Moreover, there are few studies that specifically evaluate risk factors and the efficacy of different hemostatic techniques in the prevention and control of post-polypectomy bleeding.

The aim of this study was to estimate the incidence and risk factors of several types of AEs associated with gastroduodenal polypectomy in several Spanish hospitals using a standardized lexicon specific for

endoscopic procedures.

MATERIALS AND METHODS

Patients

This is a prospective multicenter study performed at 15 Spanish hospitals. Patients with gastric and duodenal polyps that underwent endoscopic polypectomy were eligible for inclusion in the study. All patients included in the study had been previously diagnosed of gastric polyps and subsequently underwent a second endoscopy to perform the polypectomy. Therefore, when the physicians were aware that they had to perform the polypectomy, they previously asked the patient for consent to participate in the study. Inclusion criteria were: (1) protruded gastric or duodenal polyps ≥ 5 mm; and (2) polypectomy performed using an electrocautery snare. The exclusion criteria were: (1) age under 18 years; (2) prothrombin time $< 50\%$ or INR > 1.5 and platelet count < 50000 (blood test were only mandatory in patients with anticoagulation therapy or with conditions associated with coagulation disturbances); (3) aspirin intake during the previous 3 d; (4) clopidogrel intake during the previous 7 d; and (5) conditions associated with coagulation disturbances. The study protocol was approved by the Ethics Committee of each hospital and informed consent was obtained from all patients.

Three days before the procedure, oral anti-coagulants were replaced by subcutaneous low-molecular weight heparin. The patients were guided to reintroduce them 24-h after the procedure (the dose depended on the value of the previous INR value). Aspirin and clopidogrel were also reintroduced at usual doses.

Snare polypectomy was performed according to the conventional method encircling the polyp with a polypectomy snare and applying electrocautery current^[22]. Patients were placed in the left lateral decubitus position and sedation was administered according to the endoscopist or anaesthesiologist's preference.

Variables were recorded in database templates. The database included demographic characteristics, medical and drug history, indication of upper endoscopy, endoscopists' expertise (staff or fellow), morphological features and localization of polyps, technical information about the polypectomy procedure (bloc/peacemeal resection, cautery setting, hemorrhage prophylaxis technique), type of sedation, unexpected events and measures for correcting them, and patient outcome. Polyp size was determined endoscopically using an open biopsy forceps (7 mm in length, Boston Scientific Large Capacity with Needle Biopsy Forceps 2.8 mm). In cases with multiple polyps, the biggest one's characteristics were recorded.

Definition of AEs

AEs were defined, following the lexicon of ASGE

Workshop^[21], as an event that prevents completion of the polypectomy (planned procedure) and/or results in admission to hospital, prolongation of existing hospital stay, another procedure (needing sedation/anesthesia), or subsequent medical consultation. Unplanned events that did not interfere with completion of the planned procedure or changed the plan of care were classified as incidents.

Severity of AEs was graded as mild, moderate, severe and fatal according to ASGE classification. AEs were defined as mild or moderate if patients required less than 4 nights or between 4 to 10 nights of hospitalization respectively. They were classified as severe if unplanned or prolonged hospitalization was required for more than 10 nights or requiring intensive care unit admission or surgery. Finally they were graded as fatal if death occurred in relationship of the procedure.

Based on timing, AEs were defined as "intra-procedure" if they occurred during the exploration or in the recovery area, "early" if they occurred within 14 d and "late" from day 15th onward after polypectomy.

Assessment of AEs

AEs were assessed and recorded by a physician during and after the procedure while the patient was recovering from sedation or anesthesia and up to 24 h later in those admitted for observation. At 48 h, one week and day 30 after the procedure, a telephone call was made in order to ask the patient whether they had experienced any symptoms or required medical assistance. A standard questioner was used for the evaluation of late complications. Responses were recorded and entered into a database.

The completeness of data collection was monitored every 2 wk and missing data were proactively collected by contacting the patients and/or referring physicians, as far as this was possible.

Definition of hemorrhage

Bleeding was recorded as a potential AE when it required any form of intervention, either immediately after polypectomy during the index endoscopy, or in a repeat endoscopy, regardless of obtaining hemostasis, hospital admission, blood transfusion, or surgery. Depending on its activity, bleeding was classified as spurting or oozing; depending on its timing, it was classified as immediate-onset bleeding (evident during the examination) or late-onset bleeding (evident after the examination).

Immediate postpolypectomy bleeding was graded from G1 to G4 in severity based on objective endoscopic findings based on the time and continuity of bleeding as previously described (G1: Spontaneous hemostasis within 60 s, G2: Continuous but decreased oozing over 60 seconds, G3: Continuous oozing over 60 s that needs endoscopic treatment and G4: Active spurting)^[23].

After the procedure, bleeding was defined as a drop in Hb > 2 gr/dL or clinical evidence of bleeding (melena or hematemesis).

Bleeding prophylaxis

Prophylaxis of hemorrhage was allowed in the following situations:

Pedunculated polyps (Paris type 0-Ip): (1) Stalk \geq 5 mm and/or head \geq 20 mm: adrenaline injection or endoloop before or immediately after polypectomy; and (2) Visible vessel after polypectomy: adrenaline injection, endoloop or hemostatic clip.

Sessile polyps (Paris type 0-Is): oozing bleeding with spontaneous hemostasis in less than 30 s and polyp size > 20 mm: adrenaline injection, argon plasma coagulation (APC) or hemostatic clip^[23]. The technique was selected based on physician's preference.

Statistical analysis

Sample size calculation was performed assuming 10% of AE from the previous data published^[24,25]. With these numbers, we calculated that a total of 300 patients were required to achieve statistical significance (α error = 0.05, β error = 0.1).

Continuous variables were expressed as mean \pm SD. In cases with a multiple polypectomy, data provided correspond to the biggest one. Analysis was performed per patient and not per polyp. 95% confidence interval (CI) of AEs incidence was calculated by using standard formula. Comparisons were done using Fisher's test for categorical variables and *t* test for continuous variables. The chi-squared test and the Mann-Whitney *U* test, or Student's *t*-test were applied where appropriate for statistical analysis. In addition, a multivariate logistic regression analysis was carried out to assess the existence of predictive factors of AEs and the odds ratio (OR) was calculated to indicate the associated risk. *P* < 0.05 was considered statistically significant. All analyses were performed with SPSS for Windows, version 23.0 (SPSS Inc, Chicago, IL; United States).

RESULTS

From September 2012 to March 2015, a total of 326 patients with gastroduodenal polyps agreed to participate in the study. 18 patients were excluded because they did not meet inclusion criteria (polyp < 5 mm, *n* = 1; platelets < 50,000, *n* = 1; cold snare polypectomy, *n* = 9; Paris classification IIb or IIc polyp, *n* = 7). Then, 308 patients were finally included (Figure 1). Most of them were ASA I-II (*n* = 231, 75%) and only 36 (11.7%) were on anticoagulants. The most frequent indication was iron-deficiency anemia (*n* = 103, 33.4%). Characteristics of the patients are described in Table 1.

In 205 cases a single polypectomy was performed whereas in the other 103 it was multiple (mean 1.7 \pm 1.3, range 1-7). Polyp mean size was 15 \pm 8.9 mm

Table 1 Characteristics of patients *n* (%)

Characteristics	Value
Age (yr), mean \pm SD (range)	69.1 \pm 11.8 (22-92)
Gender: M/F	111/197 (36/64)
Smoker	21 (6.8)
Alcohol	35 (11.4)
Cirrhosis	20 (6.5)
Anticoagulation	36 (11.7)
Indication	
Iron-deficiency anemia	103 (33.4)
Polyp follow-up	68 (22.1)
Dyspepsia/ GERD	51 (16.6)
Upper hemorrhage	33 (10.7)
Pernicious anemia	7 (2.3)
Dysphagia	6 (1.9)
FAP	4 (1.3)
Others	36 (11.7)
ASA	
I	34 (10.4)
II	202 (62)
III	88 (27)
IV	2 (0.6)

GERD: Gastroesophageal reflux disease; FAP: Familial adenomatous polyposis; ASA: American Society of Anesthesiologist.

(5-60) and 179 of them (58.1%) were > 10 mm. The majority of them were located in the stomach ($n = 294$, 95.4%). The most frequent histological type was hyperplastic ($n = 224$, 72.7%). Characteristics of the resected polyps are described in Table 2.

Table 3 shows the technical details of the endoscopy and polypectomy. Polypectomies were performed by a staff endoscopist in 268 cases (87%) and at university hospitals in 251 cases (81.5%). Hemorrhage prophylaxis was performed in 219 (71.1%) patients; the most common technique was injection of adrenaline alone or in combination with clips, endoloops and APC.

All the patients were successfully contacted. A total of 41 patients (13.3%) presented 45 unexpected events: 30 bleeding, 10 abdominal pain, 2 respiratory desaturation, 1 spontaneous bacterial peritonitis, 1 esophageal laceration and 1 pneumothorax. However, following the ASGE lexicon, only 9 patients presented 9 (2.9%; 95%CI: 1-4.8) events that were considered AEs, and 6 of them were bleeding (5 in stomach and 1 in duodenum; 1.9%; 95%CI: 0.4-3.5). Severity and timing of these AEs are described in Table 4.

Bleeding was the most common unplanned event that occurred during the procedure ($n = 30$, 9.7%; 95%CI: 6.4-13.1). The majority of episodes could be managed without any change in the outcome of the endoscopy and, consequently, were considered incidents (24 out of 30, 80%). In 13 out of 24 incidents (54.1%) and in 5 out of 6 AE (83.3%), different types of endoscopic treatment were performed: injection alone in 3, clips alone in 3, injection plus clips in 10 and combination of injection, clips and APC in 2. In all the cases, bleeding was adequately controlled. Figure 2 shows the relationship between the use of prophylactic

Table 2 Characteristics of polyps (the biggest in case of multiple polyps) *n* (%)

Characteristics	Value
Paris classification of polyps	
0- Is	152 (49.4)
0- Ip	156 (50.6)
Size (mm), mean \pm SD (range)	15 \pm 8.9 mm (5-60)
Size	
5 mm	17 (5.5)
6-10 mm	109 (35.4)
11-20 mm	132 (42.9)
> 20 mm	47 (15.3)
Location	
Fundus	50 (16.2)
Body	112 (36.4)
Incisura	5 (1.6)
Antrum	119 (38.6)
Pylorus	8 (2.6)
Duodenum	14 (4.5)
Physician expertise	
Staff	268 (87)
Fellow	40 (13)
Polyp histology	
Hyperplastic	224 (72.7)
Adenoma	29 (9.4)
Fundic glands hyperplasia	25 (8.1)
Adenocarcinoma	8 (2.6)
Inflammatory fibroid	7 (2.3)
Neuroendocrine tumor	5 (1.6)
Others ¹	7 (2.3)
No retrieved	2 (0.6)

¹3 Brunner's gland hamartoma, 1 gastric inflammatory pseudopolyp, 1 spindle cell lipoma, 1gastric heterotopia polyp, 1 cystic gastritis.

measures, the presence and severity of bleeding and the use of endoscopic treatment.

There were no statistically significant differences in terms of age, gender, polyp histology and location in stomach or duodenum, technical details of polypectomy, hospital characteristics and use of prophylactic measures between patients who developed hemorrhagic episodes and those who did not. Only polyp size and endoscopist expertise were statistically significant in the univariate but not in the multivariate analysis (Table 5).

DISCUSSION

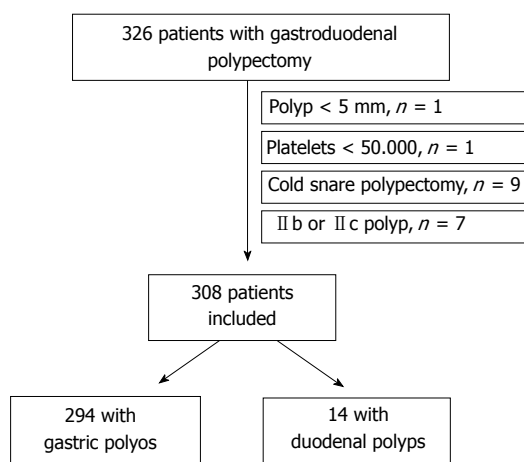
Bleeding is the most common adverse event of snare polypectomy in the upper gastrointestinal tract. In our study we found a 2.6% AEs rate (1.9% considering only bleeding) after resection of gastric and duodenal polyps which is lower than data reported in other series. To the best of our knowledge, this study is the first multicenter and prospective evaluation of AEs after gastroduodenal snare polypectomy using the lexicon recommended by the ASGE. Our results confirm the safety of gastric polypectomy when applying preventive measures and emphasize the need of using standardized systems to report AEs.

For years, polypectomy in the upper gastrointestinal

Table 3 Endoscopy and polypectomy technical details

Characteristics	n (%)
Sedation	302 (98.1)
University Hospital	251 (81.5)
Endoscopist Staff	268 (87)
Number of polyps resected	
1	205 (66.6)
2	55 (17.9)
3	20 (6.5)
4	8 (2.6)
5	7 (2.3)
> 5	13 (4.2)
Cautery settings	
Endocut	236 (76.6)
Hemorrhage prophylaxis	219 (71.1)
One technique	149 (68)
Two or more	70 (32)
Prophylactic technique	
Injection alone	119 (54.3)
Clips	16 (7.3)
Clips + injection	60 (27.4)
Endoloop	9 (4.1)
Endoloop + injection	5 (2.3)
APC	5 (2.3)
APC + injection	2 (0.9)
APC+ clips + injection	3 (1.4)

APC: Argon plasma coagulation.

**Figure 1** Study flow chart.

tract was considered less secure than that of colonic polyps. Two prospective studies with a lower number of patients evaluated the safety of gastric polypectomy. Muehldorfer *et al*^[14] studied the use of biopsy for the histological diagnosis of gastric polyps and the assessment of AEs was a secondary aim and the reported an incidence of hemorrhagic events was 7.2%. However, the definition of bleeding was broad including all the cases in which a therapeutic intervention was required regardless of the need of hospitalization or transfusion. Following the ASGE lexicon definition of AEs, the rate of AEs in this study would have been of three (1.3%): two bleeding episodes that required a blood transfusion and one perforation. The other

prospective study is a Taiwanese comparative study that assessed the efficacy of submucosal epinephrine injection before polypectomy of 151 sessile polyps (87 colonic and 64 upper GI) in the prevention of bleeding and perforation^[18]. This study showed a total of nine (5.96%) episodes of post-polypectomy hemorrhage, eight of them were immediate, and two perforations, with a total of 7.3% complications. However, most of the hemorrhagic episodes occurred in foregut polyps (10.9% vs 2.3%), were immediate and were controlled with additional endoscopic treatment. Only two patients required blood transfusion, cutting down the number of hemorrhagic AEs in foregut polypectomies to 3.1%.

Bardan *et al*^[26] performed a retrospective study (102 patients with gastric polyps) in which the primary outcome was the occurrence of immediate or delayed bleeding episodes. Although they reported seven episodes of bleeding (6.9%), six were detected immediately after polypectomy and were adequately treated by injection. Only one episode was considered severe because it required a blood transfusion 6 days after the polypectomy and fulfilled the definition of AEs by the ASGE lexicon, decreasing the rate of hemorrhagic AEs to 0.98%. The retrospective design of this study limits the conclusions and it could be argued that complication rate might be higher.

Kratzsch *et al*^[27] in the largest retrospective analysis (1416 foregut polyps) also found a low complication rate (3.1%) that is close to our findings. However, there is a lack of relevant information concerning the definition of AEs and use of prophylactic measures, and the retrospective design of this study limits the conclusions since it may underestimate complications.

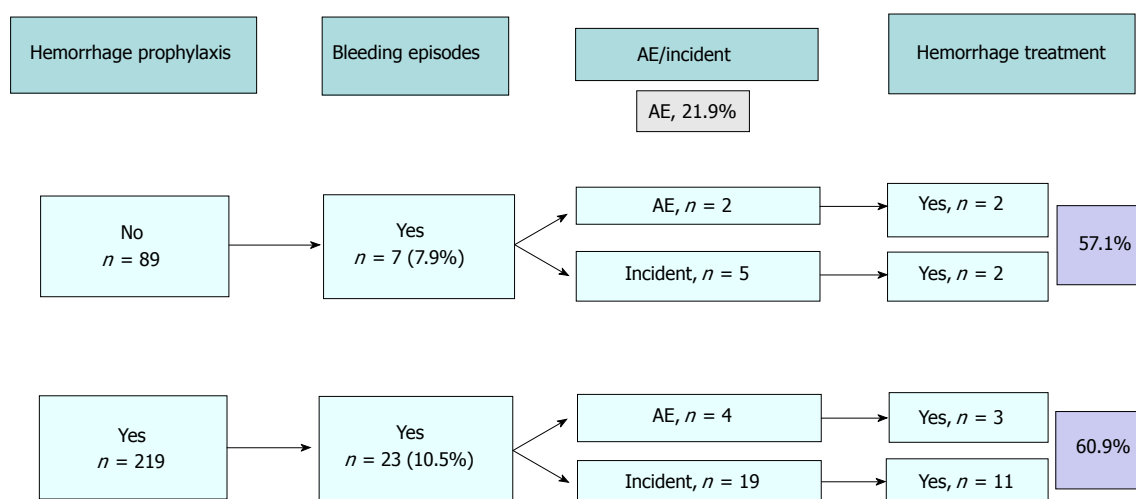
Information regarding the risks of duodenal resection is even scarcer. To date, the results of the two largest retrospective series treated with snare polypectomy showed a rate of hemorrhagic AEs of 7.8%-11%^[28,29] which is much higher than ours. Although duodenal polypectomy is usually technically more challenging than gastric polypectomy, location in the duodenum is not significantly associated with more hemorrhagic episodes, as showed in our study. We did not include flat polyps because these should be removed with mucosectomy which is technically challenging, more difficult than standard polypectomy, and associated with more AEs.

Polyp size has proved to be the main risk factor for significant unexpected events in colonic polypectomies. In fact, size is one factor that determines the complexity of polypectomy and as the complexity of polypectomy increases, a higher risk of complications is reported^[30,31]. The overall perforation and bleeding rates in these series were very low (0.05% and 0.65%, respectively). However, when the analysis was limited to bleeding requiring transfusion, unplanned hospital admission, interventional radiology or endoscopy, or surgery, the rate dropped to 0.13%. Again,

Table 4 Type and severity of adverse events according to ASGE lexicon

Unexpected events		Time of presentation	Severity (intraprocedural hemorrhage)	Admission or prolongation of hospitalization	Repeat endoscopy	AEs ASGE lexicon	
Type	n = 45					n = 9	Severity
Hemorrhage	30	Intraprocedural, n = 26	Grade 1, n = 11 Grade 3, n = 14 Grade 4, n = 1	Yes, n = 2	No	2	Mild
		3 d, n = 1		No	Yes	1	Moderate
		7 d, n = 2		Yes	Yes	2	Moderate (1) Severe (1)
		30 d, n = 1		Yes	Yes	1	Moderate
Abdominal pain	10	24 h		No	No		
Respiratory desaturation	2	Intraprocedural		No	No		
Pneumothorax	1	Intraprocedural		Yes	No	1	Moderate
SBP	1	7 d		Yes	No	1	Severe
Esophageal laceration	1	Intraprocedural		Yes	No	1	Mild

SBP: Spontaneous bacterial peritonitis.

**Figure 2** Relationship between the use of prophylactic measures, the presence and severity of bleeding episodes and the use of endoscopic treatment.

these results emphasize the importance of using standardized systems for reporting AEs. Because hemorrhage prophylaxis was allowed in polyps larger than 20 mm which have a high likelihood of bleeding, this fact could explain that size was not associated with a higher rate of hemorrhagic AEs in our series.

Although the rate of AEs in our study is low, the number of bleeding episodes is not negligible and many of them received prophylaxis (10.5%) or were treated endoscopically (60%) with injection, APC, hemostatic clips or a combination of methods which increases health care costs. Interestingly, the combination of two or more techniques did not improve the prophylactic effect of using one technique alone against bleeding. However, one could expect a higher number and more severe bleeding episodes if we had not systematically applied prophylactic measures, with an estimated high economical impact as well.

This study has several strengths. First, it is a multicenter study performed in many hospitals (tertiary and community) with a different volume of explorations that increase the generalizability of the results.

Second, preventive measures for post polypectomy bleeding were applied systematically. Third, all patients were systematically evaluated and reached three times (at 48 h, 7 and 30 d after the procedure), avoiding drop-outs that could bias the results. Fourth, we only included protruded polyps in order to avoid the use of other endoscopic resection techniques such as mucosectomy or endoscopic submucosal dissection which are more technically demanding and have a higher risk of complications. And finally, we used a standardized lexicon for endoscopic AEs.

One limitation of the study is that multiple polyps in the same patient were not considered separately and it is not possible to attribute the bleeding episode to the one that received prophylaxis or not. However, prophylactic measures were applied to the polyps with more risk of bleeding and the number of polyps was considered a variable in the analysis. The second limitation is that the number of AEs found is lower than the estimated figure used for the sample size calculation, which underpowers the results of the study. Unfortunately, this low rate prevented us from

Table 5 Univariate analysis of bleeding risk factors *n* (%)

	Bleeding, <i>n</i> = 30	No bleeding, <i>n</i> = 278	<i>P</i> value
Age (yr), mean ± SD	69.5 ± 10	69.1 ± 12	0.137
Gender			0.634
Male	12 (40)	99 (35.6)	
Female	18 (60)	179 (64.4)	
Anticoagulation ¹			0.217
Yes	14 (46.7)	98 (35.2)	
No	16 (53.3)	180 (64.7)	
ASA			0.515
I, II	20 (66.7)	201 (72.3)	
III, IV	10 (33.3)	77 (27.7)	
Paris classification of polyps ¹			
0-Ip	17 (56.7)	135 (48.6)	0.399
0-Is	13 (43.3)	143 (51.4)	
Polyp size ¹			< 0.036
≤ 10 mm	7 (23)	120 (43.2)	
> 10 mm	23 (77)	158 (56.8)	
Location			0.557
Stomach	28 (93.3)	266 (95.7)	
Duodenum	2 (6.7)	12 (4.3)	
Polyp histology ¹			0.092
Hyperplastic	18 (60)	206 (74.4)	
Others	12 (40)	72 (25.6)	
Polyp with dysplasia			0.053
Yes	8 (26.7)	36 (13.4)	
No	22 (73.3)	232 (86.6)	
Physician expertise ¹			< 0.026
Staff	30 (100)	238 (85.6)	
Fellow	0 (0)	40 (14.4)	
University hospital			0.207
Yes	27 (90)	224 (80.6)	
No	3 (10)	54 (19.4)	
Number of polyps resected			0.989
One	20 (66.7)	185 (66.5)	
More than one	10 (33.3)	93 (33.4)	
Use of endocut ¹			0.068
Yes	27 (90)	209 (75.2)	
No	3 (10)	69 (24.8)	
Hemorrhage prophylaxis ¹			0.479
Yes	23 (76.7)	196 (70.5)	
No	7 (23.3)	82 (29.5)	

¹Clinically relevant variables that have been included in the multivariate analysis.

studying risk factors for polypectomy-related AEs. Finally, we used definitions of hemorrhage and criteria for prophylaxis that apply to colonic polyps because we did not find any specific definition for gastric polyps. However, we assume that the mechanism of post-polypectomy hemorrhage must be similar regardless the localization of the polyp.

In conclusion, gastroduodenal polypectomy using prophylactic measures has a rate of AEs small enough to consider this procedure a safe and effective method for polyp resection independently of the polyp size and location.

ARTICLE HIGHLIGHTS

Research background

Gastric and duodenal polypectomy is commonly performed. Although there is a theoretical increased risk of bleeding, there is scarce information regarding the potential adverse events (AEs) of polypectomy in this setting. The aim of this

study was to evaluate the rate of AEs during consecutive gastric and duodenal polypectomies in several Spanish centers.

Research motivation

The safety of polypectomy in the upper GI tract is controversial because the reported rate in retrospective studies is higher than in colonic polypectomy but results come mainly from retrospective studies and they do not use the same standardized nomenclature and definitions for adverse events.

Research objectives

The aims of this study were to determine in a prospective study the rate of adverse events of gastroduodenal snare polypectomy for non-flat polyps; to evaluate the adverse events (early and late) that occur after a gastric and/or duodenal polypectomy as well as the predictive fractures for its development; to evaluate the different endoscopic techniques used in the prophylaxis of post-polypectomy hemorrhage.

Research methods

The research methods: (1) Multicenter, longitudinal and prospective study of all patients undergoing polypectomy of gastric or duodenal polyps ≥ 5 mm using an electrocautery polypectomy snare; (2) Patients with PT < 50% and

platelets < 50000 or clopidogrel in the 7 d prior to endoscopy were excluded; (3) Prophylactic measures of hemorrhage were allowed in certain predefined cases; (4) Intraprocedural hemorrhage was defined as bleeding that lasts more than 30 seconds and severity was graded from 1 to 4; (5) Late hemorrhage was defined as melena or hematochezia since discharge from endoscopy unit and up to 30 d. (6) Patients were followed during 30 d with serial phone calls; and (7) Predictive factors of complications were analyzed

Research results

308 patients were included and a single polypectomy was performed in 205. Hemorrhage prophylaxis was performed in 219 (71.1%) patients. Nine patients presented AEs (2.9%), and 6 of them were bleeding ($n = 6$, 1.9%) (In 5 out of 6 AEs, different types of endoscopic treatment were performed). Other 24 hemorrhagic episodes could be managed without any change in the outcome of the endoscopy and, consequently, were considered incidents. We did not find any independent risk factor of bleeding.

Research conclusions

The rate of adverse events of gastroduodenal snare polypectomy for non-flat polyp is low. However, the number of bleeding episodes is not negligible and many of them receive prophylaxis or are treated endoscopically with injection, APC, hemostatic clips or a combination of methods which increases health care costs. Prophylactic measures do not reduce the risk of hemorrhage. To our knowledge, this is the first study using the ASGE lexicon for reporting adverse events of gastro-duodenal polypectomy and shows an acceptable low rate, confirming the safety of this procedure. Because AEs of gastroduodenal polypectomies are low, there is no need of using more than one prophylactic endoscopic technique (clips, sclerosis, APC...) with the consequent reduction of costs.

Research perspectives

Gastroduodenal polypectomy using prophylactic measures has a rate of AEs small enough to consider this procedure a safe and effective method for polyp resection independently of the polyp size and location. The future research direction is to compare the use of prophylaxis or not before polypectomy in gastric polyps and the best method would be a prospective, comparative and randomized study.

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Chronic kidney disease severely deteriorates the outcome of gastrointestinal bleeding: A meta-analysis

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Abstract

AIM

To understand the influence of chronic kidney disease (CKD) on mortality, need for transfusion and rebleeding in gastrointestinal (GI) bleeding patients.

METHODS

A systematic search was conducted in three databases for studies on GI bleeding patients with CKD or end-stage renal disease (ESRD) with data on outcomes of mortality, transfusion requirement, rebleeding rate and length of hospitalization (LOH). Calculations were performed with Comprehensive Meta-Analysis software using the random effects model. Heterogeneity was tested by using Cochrane's Q and I^2 statistics. Mean difference (MD) and OR (odds ratio) were calculated.

RESULTS

1063 articles (EMBASE: 589; PubMed: 459; Cochrane: 15) were found in total. 5 retrospective articles and 1 prospective study were available for analysis. These 6 articles contained data on 406035 patients, of whom 51315 had impaired renal function. The analysis showed a higher mortality in the CKD group (OR = 1.786, 95%CI: 1.689-1.888, $P < 0.001$) and the ESRD group (OR = 2.530, 95%CI: 1.386-4.616, $P = 0.002$), and a rebleeding rate (OR = 2.510, 95%CI: 1.521-4.144, $P < 0.001$) in patients with impaired renal function. CKD patients required more unit red blood cell transfusion (MD = 1.863, 95%CI: 0.812-2.915, $P < 0.001$) and spent more time in hospital (MD = 13.245, 95%CI: 6.886-19.623, $P < 0.001$) than the controls.

CONCLUSION

ESRD increases mortality, need for transfusion, rebleeding rate and LOH among GI bleeding patients. Prospective patient registries and observational clinical trials are crucially needed.

Key words: Gastrointestinal bleeding; Chronic kidney disease; Mortality; Blood transfusion; Rebleeding

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Core tip: Acute gastrointestinal bleeding is a potentially life-threatening abdominal emergency that remains a common cause of hospitalization. Pre-existing chronic kidney disease (CKD) may worsen the prognosis. This is the first meta-analysis to compare CKD patients and normal renal function patients based on GI bleeding. We investigated these two groups in terms of mortality, transfusion amount, rebleeding rate and length of

hospitalization.

Hágendorn R, Farkas N, Vincze Á, Gyöngyi Z, Csupor D, Bajor J, Erőss B, Csécséi P, Vasas A, Szakács Z, Szapáry L, Hegyi P, Mikó A. Chronic kidney disease severely deteriorates the outcome of gastrointestinal bleeding: A meta-analysis. *World J Gastroenterol* 2017; 23(47): 8415-8425 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v23/i47/8415.htm> DOI: <http://dx.doi.org/10.3748/wjg.v23.i47.8415>

INTRODUCTION

Acute gastrointestinal bleeding (GI) is an abdominal emergency which remains a common cause of hospitalization^[1]. An accurate diagnosis of GI bleeding relies on prompt resuscitation, initial risk evaluation, and provisional clinical diagnosis followed by an appropriate definitive investigation which enables specific therapeutic interventions. GI bleeding involves any bleeding in the GI tract from the esophagus, stomach, small intestines or large intestines to the anus.

Upper GI bleeding has an annual incidence that ranges from 40 to 150 episodes per 100000 persons and a mortality rate of 6%-10%^[2], whereas lower GI bleeding has an annual incidence ranging from 20 to 27 episodes per 100000 persons and a mortality rate of 4%-10%^[3,4]. Since GI bleeding is a potentially life-threatening acute disorder, understanding the risk factors that worsen the disease is of great importance. Scoring systems have therefore been developed to predict the outcome of therapy. The Rockall score is one of these scoring systems. It includes pre-endoscopic (age, shock and comorbidity) and post-endoscopic (diagnosis and presence or absence of endoscopic stigmata of recent haemorrhage) factors^[5]. Several studies have demonstrated high mortality with higher Rockall scores^[6]. However, Laeeq *et al*^[7] have not found significantly higher mortality in patients with high pre-endoscopic Rockall score (> 5). The Rockall score only assesses the risk of mortality in patients with upper GI bleeding. The Glasgow Blatchford score is another scoring system which uses clinical and laboratory parameters. Neither scoring system makes distinction between pre-existing renal failure and acute renal failure due to haemorrhage. Both of these scoring systems have been designed for the risk assessment of upper GI bleeding. Previous studies have shown evidence of increased risk of GI bleeding in chronic kidney disease (CKD) patients and with end-stage renal disease (ESRD) requiring renal replacement therapy in comparison with the general population, but also an association with higher

mortality^[8-10]. Further studies have demonstrated that bleeding in CKD patients from the upper GI tract is more common than from the lower GI tract^[11]. The increased prevalence of small bowel erosions, ulcers and angioectasias is also well known in CKD patients and it may be as high as 33% and it often causes obscure gastrointestinal bleeding^[12-14]. However, no meta-analyses or systematic reviews have been conducted to assess the difference between CKD/ESRD patients and the normal renal function population with regard to GI bleeding.

The aim of this study was therefore to examine outcomes of GI bleeding, such as mortality, blood transfusion requirement, rebleeding rate and length of hospitalization (LOH) in CKD/ESRD patients compared to patients with normal renal functions.

MATERIALS AND METHODS

Search strategy

This study was conducted using the preferred reporting items for systematic review and meta-analysis protocols (PRISMA-P)^[15]. It was registered in the international prospective register of systematic reviews, PROSPERO (under registration number CRD42017077987). The meta-analysis was based on the PICO (Patient, Intervention, Comparison, Outcome) format (P: patients with GI bleeding; I: chronic renal failure; C: normal renal function; O: mortality, blood transfusion, rebleeding). A systematic search was performed in 3 databases, Pubmed, EMBASE and the Cochrane Library, with the following terms: ("GI bleeding" OR "gastrointestinal bleeding" OR "gastrointestinal hemorrhage") AND ("chronic renal failure" OR "uremia" OR "chronic kidney failure"). The search was limited to human data and to full-text English-language articles if appropriate. The exact search term in Pubmed was: ["GI bleeding" (All Fields) OR "gastrointestinal bleeding"(All Fields) OR "gastrointestinal hemorrhage"(All Fields)] AND ["chronic renal failure"(All Fields) OR "uraemia" (All Fields) OR "uremia"(MeSH Terms) OR "uremia" (All Fields) OR "chronic kidney failure"(All Fields)] AND ["humans"(MeSH Terms) AND English(lang)]. The database search was conducted up to 10 March 2017. Reference management software (EndNote X7) was used to remove duplicates by searching overlaps between titles, authors and publication years. The reference lists in the articles obtained were also checked, and one more eligible publication was found.

Study selection

The studies were selected separately by two investigators (RH and AM). Disagreements were resolved by consulting a third reviewer (PH). Clinical studies were eligible provided they reported data on adult patients hospitalized with upper or lower GI bleeding

grouped into normal renal function and CKD or ESRD groups. Articles were eligible containing data of CKD/ESRD patients and a control group in the same study. Information on mortality, transfusion, rebleeding and length of hospitalization (LOH) was manually searched. Case reports, conference abstracts, reviews and studies on paediatric patients up to age 18 alone were excluded. We found a high number of articles in which the risk of GI bleeding in CKD patients was studied, but they were not eligible for our meta-analysis, as there were no data available on outcomes of the GI bleeding in a control population without CKD/ESRD.

Data extraction, synthesis and analysis

Mortality data, number of transfused blood units, rebleeding and length of hospitalization data were extracted to analyse the influence of CKD and/or ESRD on the outcome of GI bleeding. In Sood *et al*^[9], Tsai *et al*^[16] and Boyle *et al*^[17], the number of patients was calculated from percentages of mortality. Boyle *et al*^[17] supplied information on transfusion in mean and standard error of mean, for which statistical calculation standard deviation (SD) was computed. Tsai *et al*^[16] reported data from transfusions in the median and interquartile range (IQR), from which mean and SD were calculated with Hozo's method^[18]. All meta-analytic calculations were performed with Comprehensive Meta-Analysis software (Version 3.0, Biostat Inc.) using the random effects model (DerSimonian-Laird method^[19]). Odds ratios (OR) and 95% confidence intervals (CI) were calculated for binary outcomes. In the case of LOH and transfusion for comparing mean data, a mean difference (MD) with 95%CI was calculated. All analyses were two-tailed, with an α of 0.05.

Heterogeneity was tested using Cochrane's Q and the I^2 statistics. Based on the Cochrane Handbook, $I^2 = 100\% \times (Q - df)/Q$, with I^2 representing the magnitude of the heterogeneity (moderate: 30%-60%; substantial: 50%-90%; considerable: 75%-100%)^[20]. Only results that were available from at least 3 studies were displayed graphically with forest plots. We performed a sensitivity analysis to assess whether removing any study result in different interpretation and final conclusion^[21]. To assess the effect of the year of publication on the outcome data we performed meta-regression analysis. We calculated the regression coefficient and interpreted the data with their 95%CI and r-analog.

Quality of studies and risk of bias

Because of the low number of eligible articles, publication bias was obtained with a visual inspection of the funnel plots alone according to the Cochrane Handbook^[20]. The Newcastle-Ottawa Scale (NOS) adjusted to our study design was used^[22] to assess the quality of nonrandomized cohort studies. The

Table 1 Modified Newcastle-Ottawa Scale criteria

Adapted Newcastle-Ottawa Scale Items	High-quality items carrying a low risk of bias (green)	Low-quality items carrying a high (red) or an unknown (yellow) risk of bias
Item 1: Representativeness of the initial study population - patients with GI bleeding and CKD/ESRD	All patients with upper or lower GI bleeding and CKD/ESRD were included.	Low: any selection criteria were applied to the study population (<i>e.g.</i> , only transplanted patients). Unknown: no data on selection process.
Item 2: Representativeness of the initial study population - patients with GI bleeding without CKD/ESRD	All patients with upper or lower GI bleeding without CKD/ESRD included.	Low: any selection criteria were applied to the study population. Unknown: no data on selection process.
Item 3: Ascertainment of exposure	We defined chronic renal failure as present when eGFR was < 60 mL/min at least 3 mo. We defined end-stage renal disease as a condition where hemodialysis or chronic peritoneal dialysis is performed at least for 3 mo.	Low: CKD or ESRD is not present in all of the patients. Unknown: no definitions of the conditions mentioned are provided.
Item 4: Comparability of cohorts A	Study controls for age: no significant difference was detected.	Low: significant difference was detected. Unknown: no statement.
Item 5: Comparability of cohorts B	Study controls for taking ulcerogenic drugs: no significant difference was detected	Low: significant difference was detected between taking ulcerogenic drugs. Unknown: no comparison made by taking ulcerogenic drugs.
Item 6: Follow-up time for rebleeding	The follow-up time is clearly defined.	Low: incomplete follow-up Unknown: no follow-up time is mentioned.

CKD: Chronic kidney disease; ESRD: End-stage renal disease.

selection, comparability and outcome data were assessed based on 6 items (Table 1) with the “star system”: high-quality items with a low risk of bias received one star, while low-quality items with a high or unknown risk of bias were assigned no stars. 3 items were included during the selection process. In the case of representativeness in the study population, we assigned a star if all of the GI bleeding patients with normal or impaired renal function were included. If any selection criteria applied, we assigned no points. We used the classical definition of CKD^[23], which characterizes the disease with a glomerular filtration rate (GFR) < 60 mL/min lasting longer than 3 mo. ESRD was defined as a condition where haemodialysis or chronic peritoneal dialysis is performed for at least 3 mo. With regard to outcome, only the follow-up time for rebleeding was rated in articles that provided this information. Assessment of outcome and length of follow-up were not rated because most of the articles were retrospective.

RESULTS

Study selection

1063 articles (EMBASE: 589; PubMed: 459; Cochrane: 15) were found altogether through database searches. The flowchart (Figure 1) shows the study selection strategy. Studies in our meta-analysis were dated from 1946 to 2017. After removing duplicates, 875 publications remained. Following initial screening based on titles and abstracts, 23 articles were retrieved and screened. A further 18 were excluded because of missing outcome data or a missing control group. Patients with acute renal failure were also

included in the analysis reported in Alvarez *et al.*^[24], so we did not use the data in that publication. The remaining 5^[9,16,17,25,26] and one other^[10] eligible record which was found in reference lists were included in the meta-analysis. The basic characteristics of the 6 eligible articles in the meta-analysis are shown in Table 2. These 6 publications contained data on 406,035 patients, of whom 51315 had impaired renal function parameters and 354720 had normal renal functions. 2 articles contained data on patients with CKD and 4 on ESRD patients. There were 2 studies involving CKD and ESRD patients, with their group identified as the CKD mixed group. The number of ESRD patients analysed was 15201, the CKD group had 36035 members, and 79 patients could be classified in the CKD mixed group.

Mortality

Data on mortality was available in all of the articles included, but Zuckerman *et al.*^[26] reported no mortality data for the control group; we therefore removed it from the statistical analysis. Hung *et al.*^[25] reported mortality data from a 6-wk follow-up period, while the other articles contained data on an unknown follow-up period. In the subgroup analysis for CKD and ESRD, a higher mortality rate was detected compared to the control population (CKD: OR = 1.786, 95%CI: 1.689-1.888, $P < 0.001$; ESRD: OR = 2.530, 95%CI: 1.386-4.616, $P = 0.002$, Figure 2).

Required units for transfusion

4 studies reported data on the transfused units of red blood cells. The required transfusion was 1.8 times higher in the patients with abnormal renal function (MD

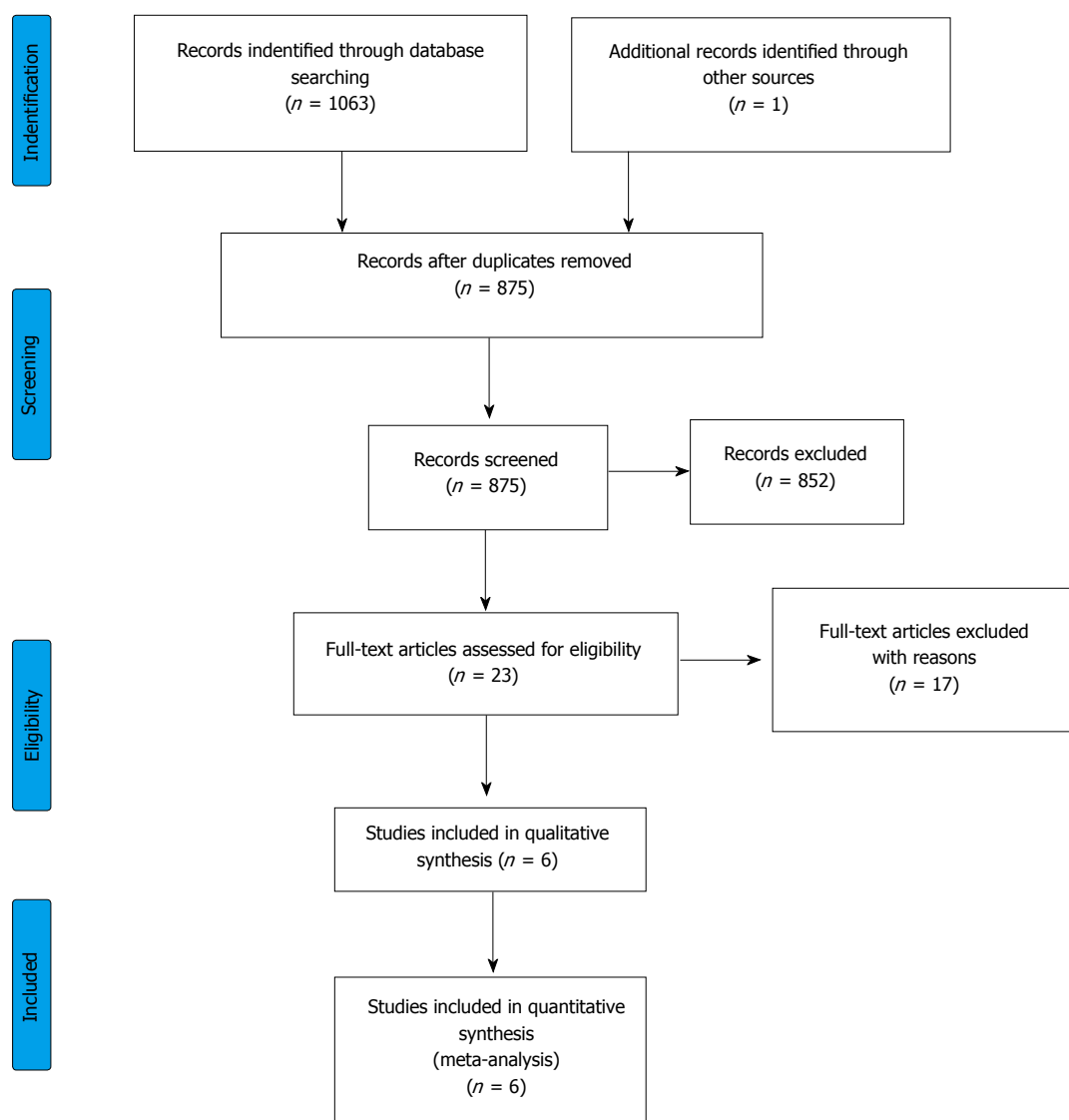


Figure 1 Flowchart of the study selection procedure.

= 1.863, 95%CI: 0.812-2.915, $P < 0.001$, Figure 3).

Rebleeding rate

It was possible to retrieve data on the rebleeding rate from 3 articles, but Cheung *et al.*^[10] contained simultaneous data from the CKD and ESDR groups, which could be analysed. Boyle *et al.*^[17] also presented data on rebleeding. However, this included cases of uncontrolled bleeding, so we excluded these data from our analysis. We found that patients with impaired renal function tend to bleed again 2.5 more times than patients with normal renal function (OR = 2.510, 95%CI: 1.521-4.144, $P < 0.001$, Figure 4).

Length of hospitalization

Two of the six articles included reported hospital stay outcomes. Patients with impaired renal function spent significantly more time in hospital after GI bleeding (MD

= 13.245, 95%CI: 6.886-19.623, $P < 0.001$, Figure 5).

Heterogeneity and quality assessment of data

High heterogeneity was detected for mortality in the ESRD group ($Q = 17.082$; $DF = 3$; $I^2 = 82.438\%$; $P < 0.001$), while the heterogeneity for CKD was low ($Q = 1.767$; $DF = 2$; $I^2 = 0\%$; $P = 0.413$). However, a low heterogeneity was detected for the transfusion requirements ($Q = 3.448$; $DF = 3$; $I^2 = 13.003\%$; $P = 0.328$), the rebleeding rate ($Q = 3.328$; $DF = 3$; $I^2 = 9.845\%$; $P = 0.344$) and LOH ($Q = 1.100$; $DF = 2$; $I^2 = 0\%$; $P = 0.577$). To ascertain publication bias, we only made a visual assessment of the funnel plot (Figure 6) because we were only able to include 6 studies in our meta-analysis. Sensitivity analysis showed no significant difference in the OR of mortality, by removing any of the articles (Supplementary Figure 1). Meta-regression showed slight significance, in the

Table 2 Basic characteristics of the studies included in the meta-analysis

Ref.	Country	Study type	Years of study	Group	Sample size	Age	Mortality	Transfusion	Rebleeding	Length of hospitalization
Boyle <i>et al</i> ^[17] , 1983	United States	Retrospective	1977-1981	Control	40	54 ± 2 ¹	√	√	-	√
Cheung <i>et al</i> ^[10] , 2010	Canada	Retrospective	2000-2006	CKD (mix)	20	59 ± 4 ¹	√	√	√	√
				CKD	50	71 ± 13				
				ESRD	50	68 ± 12				
Hung <i>et al</i> ^[25] , 2014	Taiwan	Retrospective	2007	Control	6322	54.6 ± 13.3	√	-	-	-
				ESRD	110	NR				
Sood <i>et al</i> ^[9] , 2012	United States	Retrospective	2007	Control	347245	NR	√	-	-	-
				CKD	35985	NR				
				ESRD	14983	NR				
Tsai <i>et al</i> ^[16] , 1996	Taiwan	Prospective	1991-1994	Control	640	55.7 ± 16.2 ²	√	√	√	-
				ESRD	58	64.1 ± 11.4 ²				
Zuckerman <i>et al</i> ^[26] , 1985	United States	Retrospective	1980-1983	Control	423	63 (16-96) ³	-	-	√	-
				CKD (mix)	59	57 (24-84) ³				

¹Data expressed as mean ± SEM (standard error of mean); ²Data expressed as mean ± SD (standard deviation); ³Data expressed as median (interquartile range). NR: Not reported; CKD: Chronic kidney disease; ESRD: End-stage renal disease.

Table 3 Stars based on the Modified Newcastle-Ottawa Scale

Ref.	Item 1	Item 2	Item 3	Item 4	Item 5	Item 6	Total (*)
Boyle <i>et al</i> ^[17] , 1983	*	*	-	*	*		4
Cheung <i>et al</i> ^[10] , 2010	*	*	*	*	*	*	6
Hung <i>et al</i> ^[25] , 2014	*	*	-	-	-		2
Sood <i>et al</i> ^[9] , 2012	*	*	-	-	-		2
Tsai <i>et al</i> ^[16] , 1996	*	*	*	-	*	-	4
Zuckerman <i>et al</i> ^[26] , 1985	*	*	-	-	-	*	3

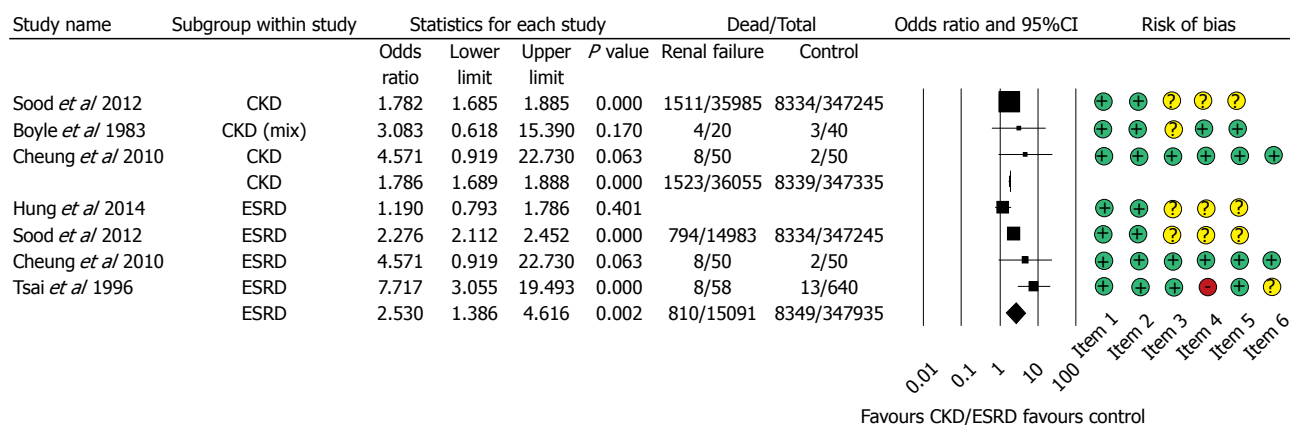


Figure 2 Forest plot representing the differences in mortality in gastrointestinal bleeding patients with normal and impaired renal function. Size of squares for risk ratio reflects weight of trial in pooled analysis. Horizontal bars represent 95%CI. CKD: Chronic kidney disease; ESRD: End-stage renal disease.

most recent articles the OR is decreasing with the time (regression coefficient: $b = -0.0548$; 95%CI: -0.0968 to -0.0128; $P = 0.0105$; r-analog: 0.2, Supplementary Figure 2A). The number of required units for transfusion has not changed since the 1980s ($b = -0.0028$; 95%CI: -0.0242 to -0.0186; $P = 0.7972$; r-analog: 0.00, Supplementary Figure 2B). Based on data from 4 articles, no difference in rebleeding rate could be observed in the last 30 years ($b = 0.0027$; 95%CI: -0.0353 to 0.03; $P = 0.8726$; r-analog: 0.00, Supplementary Figure 2C).

On the score based on the Newcastle-Ottawa Scale, articles were assigned between 2 and 6 stars out of a maximum of 6 stars (Table 3). There was a low risk of bias in representativeness in the study and the control population; it received 100% (Figure 7). With regard to ascertaining exposure, 33% of the articles represented a low risk of bias, while 66% had an unclear risk of bias. In these articles CKD and ESRD were not clearly defined, or patients were sorted based on a code system. With regard to a comparison of age, half of the articles contained no clear data on

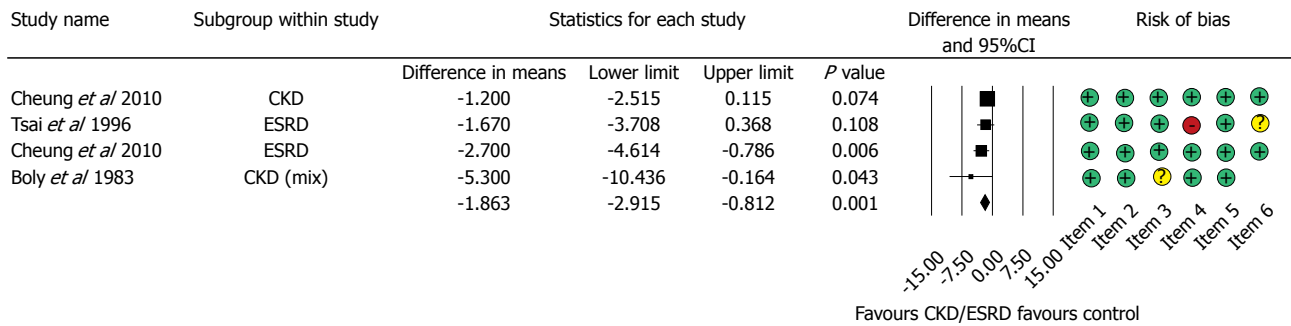


Figure 3 Forest plot representing the required units of transfusion in gastrointestinal bleeding patients with normal and impaired renal function. Size of squares for the difference in standardized mean values reflects weight of trial in pooled analysis. Horizontal bars represent 95%CI. CKD: Chronic kidney disease; ESRD: End-stage renal disease.

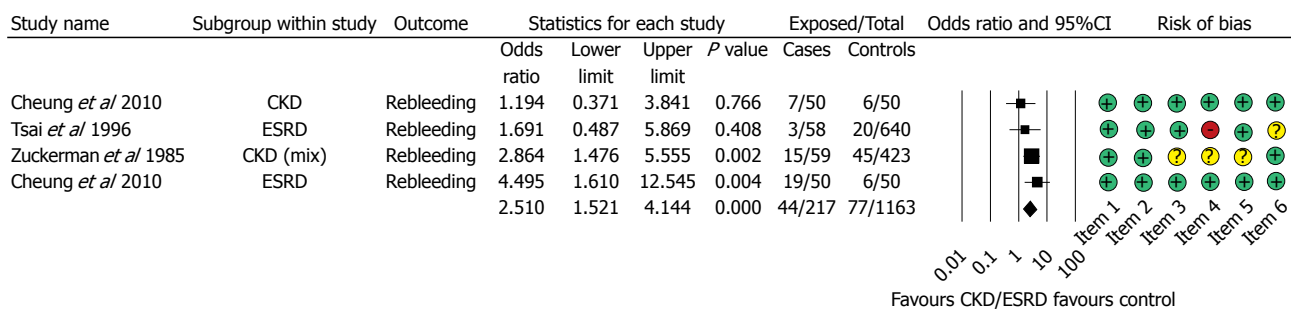


Figure 4 Forest plot representing the rebleeding rate in gastrointestinal bleeding patients with normal and impaired renal function. Size of squares for risk ratio reflects weight of trial in pooled analysis. Horizontal bars represent 95%CI. CKD: Chronic kidney disease; ESRD: End-stage renal disease.

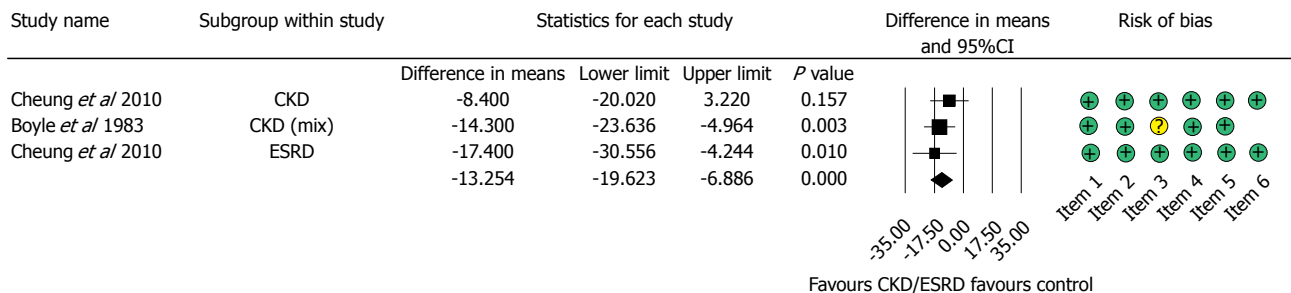


Figure 5 Forest plot representing the differences in length of hospitalization in gastrointestinal bleeding patients with normal and impaired renal function. Size of squares for the difference in standardized mean values reflects weight of trial in pooled analysis. Horizontal bars represent 95%CI. CKD: Chronic kidney disease; ESRD: End-stage renal disease.

the groups and there was a significant difference in the ages of the ESRD and control groups in Tsai *et al*^[16]. 50% of the articles reported data on taking ulcerogenic drugs; the other half represented an unclear risk of bias. The follow-up time for rebleeding was analysed in 3 articles; only one did not report this clearly.

DISCUSSION

CKD is a term that covers all degrees of decreased renal function (mild, moderate, and severe chronic kidney disease), where the GFR is lower than 60 mL/min for longer than 3 mo^[23]. CKD is a worldwide public health problem, with both incidence and prevalence rising and the main causes being diabetes mellitus and high blood pressure. ESRD patients requiring haemodialysis

or peritoneal dialysis 3 times a week represent a high burden and cost for the health care system. As the prevalence of hypertension and diabetes mellitus, the most important etiological factors for CKD and ESRD is increasing worldwide, we predict that GI bleeding with CKD will be a growing problem. According to Ohmori *et al*^[13] the number of patients on hemodialysis has tripled between 1990 and 2010. This is the first meta-analysis to report on the severity of complications after GI bleeding in patients with CKD or ESRD and normal renal function groups. Based on a systematic search in 3 databases, we were able to include 6 articles, which contained data on 406035 patients, of whom 51315 had impaired renal function. A higher prevalence of peptic ulcers was reported among ESRD patients undergoing long-term dialysis^[27,28]. The elevated risk

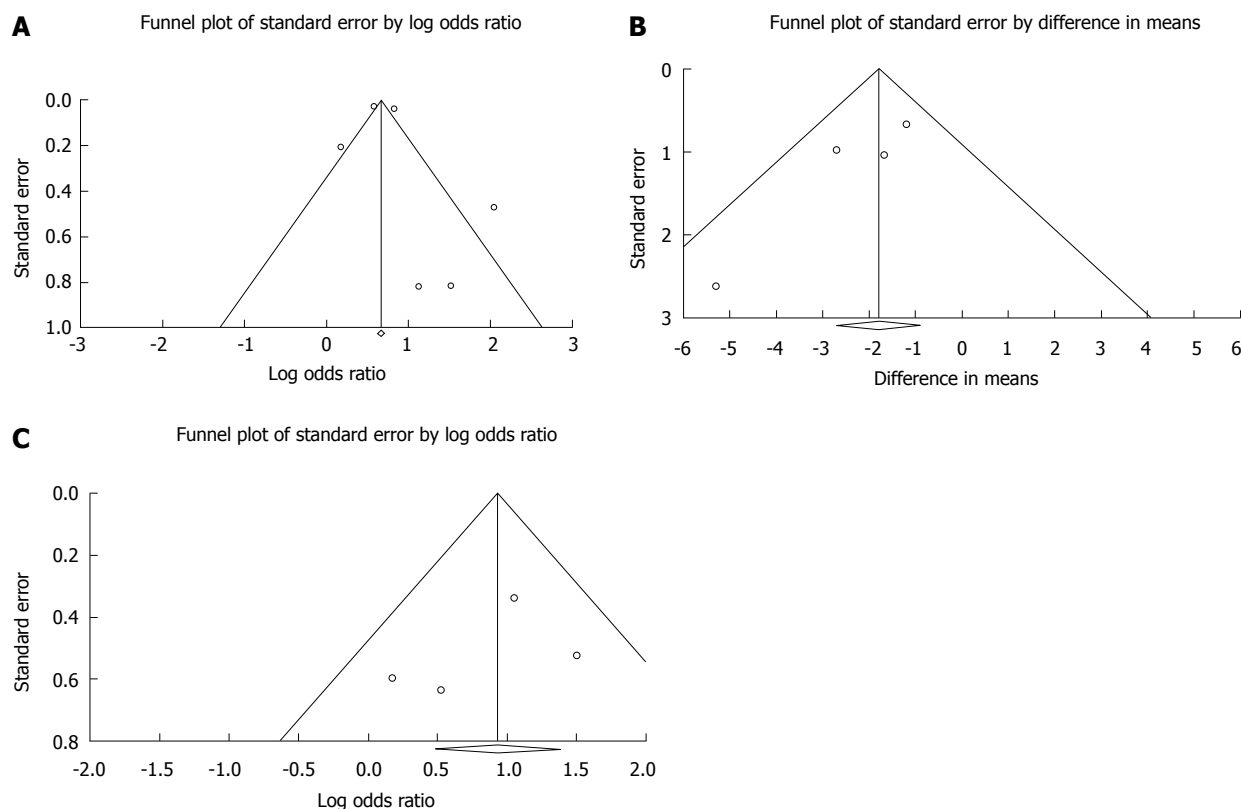


Figure 6 Funnel plot. A: Funnel plot of mortality; B: Funnel plot of required transfusion; and C: Funnel plot of rebleeding.

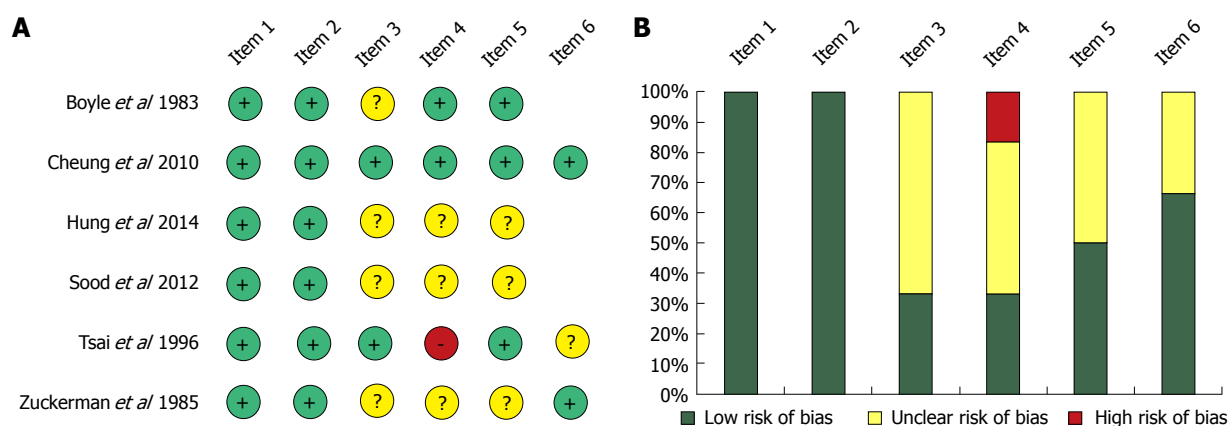


Figure 7 Risk assessment of articles included in the meta-analysis based on the modified Newcastle-Ottawa Scale (A); Risk of bias assessment graph (B).

for GI bleeding in CKD and ESRD patients is also well known^[29]. The most frequent causes of lower GI bleeding in this population have been described; diverticulosis, haemorrhoids, and ischaemic colitis have been identified in addition to angiodysplasias^[30], but no cohort study has been conducted on this topic yet. Although we did not intend to narrow our search to upper GI bleeding, the articles eligible for our inclusion criteria contained data only on patients with upper GI bleeding, and no studies with lower GI bleeding met our inclusion criteria. Only a few of the studies detailed the endoscopic findings and cause of bleeding. Cheung *et al*^[10] included only peptic ulcer bleeding

patients, while the study of Hung *et al*^[25] examined only esophageal variceal bleeding. Tsai *et al*^[16] found that erosive gastritis was significantly higher in ESRD group, while Boyle *et al*^[17] saw gastric ulcer as the most common cause of bleeding in the impaired renal function group, but it was not significant compared to controls. Zuckerman *et al*^[26] found significantly more angiodysplasia and erosive esophagitis in the impaired renal function group.

Based on the pooled data, we found that ESRD increases mortality 2.5 times while CKD increases it 1.8 times in GI bleeding compared to the controls with normal renal function, but these ORs are not

significantly different. Weng *et al.*^[31] reported that ESRD patients admitted with primary upper GI bleeding have a profoundly increased risk of in-hospital mortality. Using a large multi-centre database, Sood *et al.*^[9] reported that the in-hospital mortality risk is 50% higher in CKD patients and 3 times greater in ESRD patients. Holden *et al.*^[32] reported that the incidence rate of major bleeding episodes in haemodialyzed patients was 2.5% per person-year and that use of aspirin and/or warfarin increased this risk. Based on the result of the meta-regression the mortality-rate of GI bleeding has improved since the 1980s. It is likely one of the reasons for the heterogeneity of the data. Inhomogen patient groups also result in a significant bias. However the sensitivity analysis showed that none of the articles influences significantly the pooled OR.

Cardiovascular disease, current smoking^[33] and even haemostasis disorders^[34] may play a role in the background of higher risk for GI bleeding in ESRD patients. Unfortunately only few of the analysed articles detailed the other comorbidities of the GI bleeding patients. In the article of Cheung *et al.*^[10] there was no significant difference in the comorbidities between ESRD, CKD and normal renal function group. More people in CKD and ESRD groups suffered from hypertension, diabetes mellitus and platelet abnormalities in the study of Sood *et al.*^[9], while the cirrhosis was less common than in controls. Volume replacement and blood transfusion are important parts of the therapy of GI bleeding. This meta-analysis demonstrated that patients with chronic impaired renal function develop 2.5 times more rebleeding episodes and require almost 2 more red blood cell units for transfusion than the control group. Patients with impaired renal function spent more time in hospital than the control group.

There are several limitations to this study; therefore, the results of this meta-analysis should be regarded with caution. Unfortunately, only a low number of articles was found on this topic, with half of them written in the 1980s and 1990s. In the recent articles, CKD and ESRD groups were separated, but in the earlier publications these groups were mixed, leading to a bias in our analysis, and the definition of GFR was also not mentioned. The diagnosis was based on elevated creatinine level. Hung *et al.*^[25] only involved patients with cirrhosis and the mortality rate was monitored up to 6 wk, while hospital mortalities were presumably included in the other articles. Publications with rebleeding data did not follow patients for the same time interval, and 1 paper did not report on the follow-up time. The strength of this meta-analysis is the high number of patients.

Our results have demonstrated that patients with ESRD show higher mortality during GI bleeding. CKD patients require more transfusion, and the rebleeding rate is also more elevated than that in patients with

normal renal function. Because of these severe conditions, the LOH is also longer. Patients with ESRD or CKD should be observed more carefully due to the elevated complication rate. In this meta-analysis we wanted to highlight the importance of this clinical problem and we believe that it needs further scientific research. In order to understand the effect of CKD/ESRD and other comorbidities on the outcomes of GI bleeding in more details, observational trials, and registries on GI bleeding should be developed.

ARTICLE HIGHLIGHTS

Research background

Chronic kidney disease is a significant comorbidity, which can worsen the outcomes of gastrointestinal (GI) bleeding.

Research motivation

We wanted to understand the role of chronic kidney disease (CKD) and end-stage renal disease (ESRD) in the natural history of GI bleeding.

Research objectives

Our goal was to investigate the influence of CKD and ESRD on the outcomes of GI bleeding, based on all available data published in this topic.

Research methods

A comprehensive search was carried out in PubMed, Embase and Cochrane Library databases for studies detailing the outcomes of GI bleeding in the context of kidney functions. We used the PRISMA P protocol, registered our project through PROSPERO and assessed the quality of the included articles by using the Newcastle-Ottawa Scale, to ensure that this meta-analysis is done to the highest possible standards. The statistical calculations were performed with Comprehensive Meta-Analysis software, using the random effects model (DerSimonian-Laird method).

Research results

In this analysis 51315 patients with CKD and 354720 controls were included (6 articles). We found that the mortality of GI bleeding was significantly worse in CKD and ESRD with an OR of 1.79 and 2.53 respectively. Patients with kidney disease needed significantly more transfusion with a MD of 1.86 and the rebleeding rate was significantly worse in the group with impaired kidney function with an OR of 2.51. Patients with impaired kidney function needed significantly longer hospitalization with a MD of 13.25.

Research conclusions

This is the first meta-analysis and systematic review in this topic, which quantifies kidney disease as a negative risk factor in GI bleeding. GI bleeding in patients with chronic renal failure significantly increases the mortality rate, rebleeding rate, length of hospitalization, and require more blood transfusion compared to patients with normal kidney functions. Kidney disease significantly worsens the outlook of patients presenting with GI bleeding. Patients with chronic kidney disease will need to be treated with more caution due to the worse outcomes of GI bleeding. Close monitoring of the fluid balance and kidney functions, careful fluid therapy and prevention of acute kidney injury in these patients may improve the outcomes of GI bleeding.

Research perspectives

Although CKD, ESRD, and other comorbidities are major risk factors for unfavorable outcomes in GI bleeding, their roles are not well investigated nor understood and they need further scrutiny. We would better understand the role of CKD in ESRD in GI bleeding from analysis of extensive data from large multicenter and multinational observational studies and registries accurately recording the outcomes and the kidney functions.

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Disabling portosystemic encephalopathy in a non-cirrhotic patient: Successful endovascular treatment of a giant inferior mesenteric-caval shunt *via* the left internal iliac vein

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Abstract

Hepatic encephalopathy is suspected in non-cirrhotic cases of encephalopathy because the symptoms are accompanied by hyperammonaemia. Some cases have been misdiagnosed as psychiatric diseases and consequently patients hospitalized in psychiatric institutions or geriatric facilities. Therefore, the importance of accurate diagnosis of this disease should be strongly emphasized. A 68-year-old female patient presented to the Emergency Room with confusion, lethargy, nausea and vomiting. Examination disclosed normal vital signs. Neurological examination revealed a minimally responsive woman without apparent focal deficits and normal reflexes. She had no history of hematologic disorders or alcohol abuse. Her brain TC did not demonstrate any intracranial abnormalities and electroencephalography did not reveal any subclinical epileptiform discharges. Her ammonia level was > 400 mg/dL (reference range < 75 mg/dL) while hepatitis viral markers were negative. The patient was started on lactulose, rifaximin and low-protein diet.

On the basis of the doppler ultrasound and abdomen computed tomography angiography findings, the decision was made to attempt portal venography which confirmed the presence of a giant portal-systemic venous shunt. Therefore, mechanic obliteration of shunt by interventional radiology was performed. As a consequence, mesenteric venous blood returned to hepatopetally flow into the liver, metabolic detoxification of ammonia increased and hepatic encephalopathy subsided. It is crucial that physicians immediately recognize the presence of non-cirrhotic encephalopathy, in view of the potential therapeutic resolution after accurate diagnosis and appropriate treatments.

Key words: Non-cirrhotic patient; Portosystemic shunt; Hyperammonaemia; Interventional radiology; Mechanical embolization; Encephalopathy

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Core tip: We present the case of a non-cirrhotic female patient who first presented to the Emergency Room with acute hyperammonemic encephalopathy causing massive relapsing neurological symptoms due to a huge inferior mesenteric-caval shunt *via* the left internal iliac vein which was successfully cured by interventional radiology procedure. Therefore, the importance of accurate diagnosis and appropriate treatment of this disease should be strongly emphasized.

de Martinis L, Gropelli G, Corti R, Moramarco LP, Quaretti P, De Cata P, Rotondi M, Chiovato L. Disabling portosystemic encephalopathy in a non-cirrhotic patient: Successful endovascular treatment of a giant inferior mesenteric-caval shunt *via* the left internal iliac vein. *World J Gastroenterol* 2017; 23(47): 8426-8431 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v23/i47/8426.htm> DOI: <http://dx.doi.org/10.3748/wjg.v23.i47.8426>

INTRODUCTION

Hepatic encephalopathy (HE) most commonly occurs in patients with cirrhosis and end-stage liver disease. Non cirrhosis-related HE is, by far, less frequently encountered^[1]. The development of HE in the absence of liver disease is mainly dependent upon the presence of spontaneous portal vein thrombosis or portosystemic shunts which ultimately lead to hyperammonaemia^[1]. At difference with HE associated with end-stage liver disease, in these latter cases interventional radiology procedures provide a chance of complete remission^[1-3]. Abdominal trauma, prior surgery, post-natal viral or hepatotoxic injuries could potentially induce the occurrence of portosystemic shunts without liver disease. There are, however, shunts retaining the onphalomensenteric venous system which have in most

cases a congenital origin^[1,4-9]. Owing to overlapping neurological symptoms due to hyperammonaemia, at least some patients with HE have been misdiagnosed as harbouring psychiatric diseases (such as dementia, depression and others) with subsequent hospitalization in psychiatric institutions or geriatric facilities^[9,10]. The above cited evidences highlight the need for an accurate differential diagnosis between the spectrum of neurologic conditions and the, by far less frequent, non cirrhosis related HE, especially in view of the potential therapeutic resolution of the latter condition^[1]. We hereby report the case of a non-cirrhotic patient who first presented with acute hyperammonemic encephalopathy causing massive relapsing neurological symptoms due to a huge inferior mesenteric-caval shunt via the left internal iliac vein which was successfully cured by interventional radiology procedure.

CASE REPORT

A 68-year-old female patient presented three times to the Emergency Room with confusion, lethargy, dysarthria, nausea and vomiting. Each time the first evaluation disclosed normal vital signs where neurological examination always revealed a minimally responsive woman without apparent focal deficits but with asterix and increased tendon reflexes. The crisis resolved after conservative therapy. Brain computed tomography (CT) showed chronic cerebral vascular disease, absence of any acute intracranial lesions and electroencephalography did not reveal any subclinical epileptiform sign. Chest X-ray did not reveal any insurgent abnormalities, except for outcome signs of fractures of the IV, V and VI ribs that patients reported two months before because of an accidental fall. Normal levels of hemoglobin, leukocytes, platelets, glucose, electrolytes and creatinine were found. In the last admission serum ammonia level was finally measured and found to be far above the normal value (nv < 75 mg/dL): > 400 mg/dL. Based on these findings, the patient was eventually transferred to our Internal Medicine department and started on lactulose, rifaximin, low-protein diet. The past medical history revealed: normal growth and pubertal development with no history of congenital malformation, left quadrantectomy plus lymphadenectomy followed by radiotherapy and chemotherapy with tamoxifen for breast carcinoma in 2003 (at that time patient was declared free of disease and was not taking any anti-cancer therapy); hypertension in treatment with beta-blockers and ace-inhibitors; mechanic aortic valve replacement due to severe aortic stenosis for which was taking oral anticoagulants and pacemaker implantation in 2007; depression and migraine self-treated. She had no history of alcohol abuse, hematologic disorder or liver disease. The biochemical assessment showed: normal thyroid function, euglycemia, normal levels of ACTH and cortisol, normal GH with low IGF-1 for age (37 ng/mL

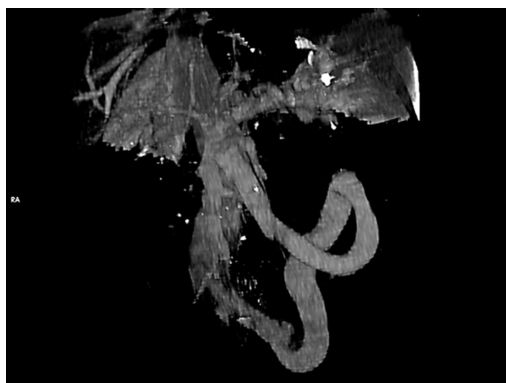


Figure 1 Volume rendering CECT (portal phase). Showing the giant portosystemic shunt, patent portal and splenic veins and no gastro-esophageal varices. The shunt extends from the inferior part of the spleno-mesenteric confluence to the left hypogastric vein. Enlarged calibre of the superior mesenteric vein is visible at the confluence.

116-353 ng/mL), INR in range (between 2.5 and 3.5 for mechanic valve), normal renal function and sodium/potassium levels. Slightly elevated transaminases (GOT 59 U/L, *nv* < 32 U/L; GPT 56 U/L, *nv* < 33 U/L) and direct/indirect bilirubin (1.3 mg/dL, *nv* < 1.2 mg/dL; 0.7 mg/dL, *nv* < 0.3 mg/dL) were also found. Viral Hepatitis markers (HAV/HBV/HCV/HEV) were negative as well as HIV1-2 serology, tumor markers (CEA/CA19.9/CA15.3/CA125/alpha-fetoprotein/NSE/CYFRA21-1) and humoral autoimmunity (ANA/ENA/pANCA/cANCA/AMA/ASMA/LKM1/SLA-LP) markers. There was not a history of hepatotoxic drugs assumption or alpha-1-antitrypsin deficiency. Hemochromatosis and Wilson's disease were ruled out based upon normal circulating levels of iron, ferritin, transferrin, copper and ceruloplasmin. The persistent presence of variable floating degree of hyperammonaemia, unresponsive to conventional treatment, prompted us to take into account more rare causes sustaining high levels of circulating ammonia in the absence of liver disease. Gastroscopy and colonoscopy did not identify any bleeding or macroscopic alteration (in particular absence of gastroesophageal varices) of the gastrointestinal tract and obstinate constipation did not affect our patient. She was not taking any drug potentially producing hyperammonemia and had not performed a high-protein diet. Coproculture was negative and excluded bacterial colonization, urine culture was positive for *E. coli* and the asymptomatic infection was easily treated. Lastly, plasmatic and urinary amino acids chromatography excluded the rare condition of urea cycle disorders. Abdominal ultrasound revealed normal liver volume and echogenicity without focal lesions and no ascites. EcoColor Doppler ruled out thrombosis of portal vein and its intrahepatic branches with hepatopetal flow. Main portal trunk diameter was within normal values (< 1.5 cm) with enlarged appearance of splenoportal confluence. Brain MRI examination was not performed because of the presence of mechanic aortic valve. Abdomen computed tomography

angiography revealed the patency of portal vein trunk with an enlarged superior mesenteric vein and a giant portosystemic shunt. The shunt presented a maximum caliber of 20 mm and showed a large retroperitoneal loop emerging from the spleno-mesenteric confluence with discharge in the left hypogastric vein (Figure 1). No collateral gastroesophageal pathways of portal circulation, normal spleen volume and no ascites were found. Then, hepatic venous pressure gradient (HVPG) measurement of portal vein pressure was performed. From right internal jugular vein the right hepatic vein was catheterized. HVPG of 1 mmHg (free flow 13 mmHg; wedge 14 mmHg) was recorded thrice with repeated occlusion balloon (Occluder, Boston Sc., United States) inflations and deflations. To test the hypothesis of a possible pre-hepatic portal hypertension, causing a misleading low HVPG, a 5 French (Fr) diagnostic catheter was negotiated through the inferior vena cava and the shunt directly in portal vein. Hepatopetal portal flow was opacified and direct portal pressure was 14 mmHg, confirming the HVPG value. Fibroscan examination revealed liver elastance of 10 KPA compatible with a low probability of clinically significant liver cirrhosis. Thus, it was not worth the risk to perform a liver biopsy taking into account that the patient was on anticoagulant therapy. Three days later transfemoral embolization of the shunt was performed. Thus, by right groin access the contralateral shunt outflow in left hypogastric vein was catheterized (Figure 2). A 45 cm long 8 Fr introducer was engaged deeply inside the left hypogastric vein and a 5 Fr catheter (Glidecath, Terumo) was advanced over a 0.035" inch glide guidewire through the loop of the shunt up to the main trunk of portal vein. Then a high flow microcatheter (Progreat, Terumo) was inserted through a side-valve into the 5 Fr catheter. Thereafter multiple coils (Ruby Coils, Penumbra INC, United States), for a total length of 974 cm, were released. Due to apparent instability of the cast of coils, the 8 Fr introducer was advanced over the 5 Fr catheter, the last was eventually removed. More proximally two large nitinol plugs were deployed near the shunt confluence in the internal iliac vein (Figure 3). Mechanical embolization was preferred to sclerosis with chemical agents to avoid the risk of reflux in the portal system with potential catastrophic complications. A control CT at 1 mo confirmed the complete shunt exclusion (Figure 4). As a consequence, mesenteric venous blood restarted to fully hepatopetally flow into the liver. The venous blood containing a high level of ammonium reached once again the liver where metabolic ammonia detoxification increased and encephalopathy subsided. After that, we observed a complete remission of symptoms, normalization of transaminases (GOT 26 U/L, *nv* < 32 U/L; GPT 22 U/L, *nv* < 33 U/L), direct/indirect bilirubin (1.1 mg/dL, *nv* < 1.2 mg/dL; 0.2 mg/dL, *nv* < 0.3 mg/dL) and ammonia level 35 mg/dL (*nv* < 75 mg/dL), in the absence of any specific therapy, and



Figure 2 Direct venography from right femoral vein approach, by a 5 Fr diagnostic catheter, of the porto-systemic shunt. Confirming the presence of the retroperitoneal loop with a hepatofugal flow to the left internal iliac vein.



Figure 3 Completion angiography. Shunt exclusion after endovascular embolization by detachable coils and two plugs and patency of the left internal iliac vein and inferior vena cava.

stable conditions after a two years follow up.

DISCUSSION

Hepatic encephalopathy is in most cases a direct consequence of cirrhosis, which generates portal-systemic venous shunts ultimately resulting in bypass of the liver. This latter aspect accounts for the fact that neurotoxic substances, such as ammonia, are not effectively detoxified by the liver and flow in high concentrations into the systemic circulation affecting the brain^[1]. However, even if, by far less frequent, cases of hyperammonemia not associated with cirrhosis were previously described. Indeed, elevated concentrations of circulating ammonia in patients with normal liver function were reported in a wide spectra of conditions which include high protein diet, severe constipation, gastrointestinal bleeding, several drugs, gastric *Helicobacter pylori* infection and urinary



Figure 4 Follow up MIP (maximum intensity projection) computed tomography at 1 mo. Pointed out the patency of the superior mesenteric, splenic and portal veins. Coils cast and plugs, proximal and distal, completely excluded shunt's flow. Platinum coils-related artifacts are evident above the nitinol plug. The course of aorta parallel to superior mesenteric vein is depicted.

tract infection with high urease-producing bacteria (*Pseudomonas*, *Proteus*), haemodialysis and enzyme deficit of urea cycle^[1-4,6-9]. In our case, the diagnostic evaluation was started by excluding common causes of cirrhosis such as chronic viral, metabolic or autoimmune hepatitis, alcohol abuse, sclerosing cholangitis, primary biliary cirrhosis, Wilson's disease, hemochromatosis, hepatotoxic drugs assumption. Subsequently, the previously mentioned conditions, potentially causing hyperammonemia in the absence of liver impairment, were ruled out. Finally, the presence of a porto-systemic shunt was established on the basis of clinical setting and CT findings. Indeed Multi-detector Computed Tomography clearly depicted the shunt characteristics and allowed the planning for subsequent diagnostic work up. As a first step, HVPG was measured and direct portal pressure was recorded to definitely exclude portal hypertension. In consideration of the clinical setting, including virus markers negativity, as well as of the endoscopic and imaging findings and the low value of liver impedance and elastography, transjugular biopsy was considered unnecessary according to the most recent expert positions^[11]. MRI can provide an accurate tool for studying in a non-invasive way the acute/chronic damage on brain^[1]. However, as mentioned before, MRI was not executable in our patient because of the mechanic aortic valve. In (Table 1) are shown the cases of non cirrhosis-related HE reported in the literature as compared to the present case^[2,3,12] and the peculiarities of each one. Although in cirrhotic patients, He *et al.*^[13,14], reported a case of inferior mesenteric vein-left gonadal vein shunt aggravating HE and a case of large paraesophageal varices causing recurrent HE. In both cases disabling encephalopathy occurred even after relief of severe portal hypertension by means of transjugular intrahepatic portosystemic shunt (TIPS) and coil embolization was necessary to achieve full

Table 1 Cases of non cirrhosis-related hepatic encephalopathy reported in the literature compared to our case report

Ref.	Patient	Presentation	Type of shunt	Treatment
Otake <i>et al</i> ^[2] , 2001	37 yr, Female, no relevant past medical history	Disturbed consciousness	Inferior mesenteric-caval shunt (left internal iliac vein)	Percutaneous transcatheter embolization (Coils)
Rogal <i>et al</i> ^[3] , 2014	58 yr, Male, gastric by-pass surgery	4 mo of confusion and violent behavior	Spontaneous splenorenal shunt (18 mm)	Percutaneous closure (Amplatzer plug)
Ali <i>et al</i> ^[12] , 2010	57 yr, Female, insulin dependent diabetes mellitus	2 wk of confusion, new onset melena	Superior mesenteric-caval shunt (left internal iliac vein) (10-20 mm)	Surgical closure
Present case	68 yr, Female, breast cancer, rib fractures	Relapsing confusion, lethargy, dysarthria	Inferior mesenteric-caval shunt (left internal iliac vein) (20 mm)	Percutaneous transcatheter embolization (Amplatzer plug and coils)

clinical recovery^[13,14]. Furthermore, there are some aspects of the present case report that need to be stressed and discussed. First of all, the relapsing and massive neurological presentation really impressed the clinicians, especially because the patient did not have previous history of similar episodes and never suffered from a neurological or hepatic disease. Although it is well known that chronic hyperammonaemia causing encephalopathy could be frequently misdiagnosed with psychiatric disorders^[10], acute presentations can be even more difficult to be rapidly identified, hence we believe that measurement of serum ammonia level should be always considered at the first clinical assessment in Emergency Unit. Secondly, the first clinical presentation occurred not only in an acute expression but also very late at 68 year-old age. This does not represent a common finding although it was reported in some cases^[12,15-18]. The causal mechanism involves spontaneous formation of a porto-systemic shunt, in this case an inferior mesenteric-caval shunt *via* the left internal iliac vein, that might be provoked by several trigger factors^[1]. Our patient reported multiple rib fractures, due to an accidental fall, just two months before the explosion of neurological symptoms and trauma is one of the several causes reported to be a possible source of spontaneous vascular shunts in human body^[1]. However shunt was too big in size to be justified by the recent trauma, supporting the hypothesis of a preexisting vessel progressively increased in size over the years, which acquired major vascular relevance. On the contrary, it seemed more likely that the trauma was the consequence of the hyperammonemia-related impairment of cerebral function. Relationship between the size of portal and shunt diameters and time of symptoms occurrence have already been reported^[19]. Lastly, the Fibroscan showed a low degree of fibrosis for a virus negative patient, not proportional to the degree of hyperammonaemia^[20]. These data, together with the mild hypertransaminasemia, suggest a minor liver impairment. Indeed, the hyperammonaemia correlates with liver damage severity in cirrhotic patients and represents a clue for the presence of portosystemic collateral veins^[21]. Moreover, mild hepatic fibrosis, fatty degeneration, infiltration of lymphocytes or intrahepatic vascular abnormalities have all been observed as

consequences of portosystemic shunts^[1]. The most likely explanation for these minimal liver involvement would be that when blood flow to the liver reduces, and it leads to lack of nutrition and fatty degeneration of hepatic cells, hepatic disfunction, cellular death and then liver atrophy/fibrosis occurs^[1]. However, there is a chance of complete biochemical, histological and clinical remission after interventional radiology procedures achieving shunt exclusion^[3]. For this reason, it is crucial that physicians initially recognize the presence of hyperammonemic encephalopathy and then, consider the rare case in which the condition is not related to cirrhosis, and can therefore be fully healed. Accurate diagnosis and subsequent appropriate treatments are able to fully revert the symptoms in most patients. However, future studies aimed at evaluating the long-term prognosis after therapy are necessary.

ARTICLE HIGHLIGHTS

Case characteristics

A 68-year-old female patient presented three times to the Emergency Room with confusion, lethargy, dysarthria, nausea and vomiting.

Clinical diagnosis

Disabling portosystemic encephalopathy due to a giant inferior mesenteric-caval shunt *via* the left internal iliac vein.

Differential diagnosis

Elevated concentrations of circulating ammonia in patients with normal liver function were reported in a wide spectra of conditions which include high protein diet, severe constipation, gastrointestinal bleeding, several drugs, gastric *Helicobacter pylori* infection and urinary tract infection with high urease-producing bacteria (*Pseudomonas*, *Proteus*), haemodialysis and enzyme deficit of urea cycle.

Laboratory diagnosis

Serum ammonia level was found far above the normal value > 400 mg/dL (nv < 75 mg/dL). Euthyroidism, euglycemia, normal levels of ACTH and cortisol, normal GH with low IGF-1 for age INR in range, normal renal function and sodium/potassium levels were observed. Slightly elevated transaminases and direct/indirect bilirubin were detected. Viral Hepatitis markers were negative as well as HIV1-2 serology, tumor markers and humoral autoimmunity markers.

Imaging diagnosis

Abdomen computed tomography angiography revealed the patency of portal vein trunk with an enlarged superior mesenteric vein and a giant portosystemic shunt. The shunt presented a maximum caliber of 20 mm and showed a large retroperitoneal loop emerging from the spleno-mesenteric confluence with

discharge in the left hypogastric vein.

Pathological diagnosis

Giant inferior mesenteric-caval shunt via the left internal iliac vein.

Treatment

Percutaneous transcatheter embolization using Amplatzer plug and coils.

Related reports

Mechanical embolization of an inferior mesenteric-caval shunt via the left internal iliac vein was described in a similar case by Otake *et al*^[2], Intern Med 2001.

Term explanation

Mechanical embolization was preferred to sclerosis with chemical agents to avoid the risk of reflux in the portal system with potential catastrophic complications. A control CT confirmed the complete shunt exclusion. As a consequence, mesenteric venous blood restarted to fully hepatopetally flow into the liver, metabolic detoxification of ammonia increased and encephalopathy subsided.

Experiences and lessons

It is crucial that physicians initially recognize the presence of hyperammonemic encephalopathy and then, even if rare, consider those case not related to cirrhosis that can therefore be fully healed. Accurate diagnosis and subsequent appropriate treatments are able to fully revert the symptoms in most patients.

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Wernicke encephalopathy in a patient after liver transplantation: A case report

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Abstract

Wernicke encephalopathy (WE) is an acute neurological disorder resulting from vitamin B1 deficiency, which is common in chronic alcoholism and is rare in acute liver failure. So far, there are 2 cases of WE reported after liver transplantation. Here, we report a case of a 45-year-old nonalcoholic male patient who developed psychiatric and neurological disturbance 15 d after receiving orthotopic liver transplantation because of hepatitis B-related cirrhosis and portal hypertension. Brain magnetic resonance imaging (MRI) showed symmetric high-signal intensities in the periaqueductal area. The patient was diagnosed with WE and given intravenous high-dose vitamin B1 immediately. His neurological disturbance resolved in 7 d after receiving the vitamin B1. Brain MRI after 5 mo showed nearly complete recovery. Most WE cases may be misdiagnosed in patients after liver transplantation, and we should pay more attention to its onset.

Key words: Liver transplantation; Thiamine deficiency; Wernicke encephalopathy; Magnetic resonance imaging; Prevention; Pharmacotherapy

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Core tip: Wernicke encephalopathy (WE) is rare in acute liver failure. This is the third case of WE after liver transplantation reported. Most WE may be misdiagnosed in patients after liver transplantation.

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INTRODUCTION

Wernicke encephalopathy (WE) was first reported by Carl Wernicke in 1881^[1] and is an acute neurological disorder resulting from vitamin B1 deficiency, which is common in chronic alcoholism. Its typical triad symptoms are ataxia, nystagmus and ophthalmoplegia, and confusion. The prevalence rate of WE has been reported as 0.4%-2.8%^[2]. It was overlooked in 68% of alcoholics and 94% of nonalcoholics^[3], and the mortality rate reached 20%^[4]. With an increasing morbidity, nonalcoholic WE is difficult to diagnose because of its various presentation. Since WE after liver transplantation can be seen in only a few reports^[5-7], we present a nonalcoholic patient who developed WE after liver transplantation.

CASE REPORT

A 45-year-old male patient was admitted to our hospital for liver transplantation because of nausea, abdominal distension for 3 mo, and unconsciousness for 3 d. His preoperative diagnoses were decompensatory cirrhosis, hepatic encephalopathy, portal hypertension and chronic hepatitis B. His hepatic encephalopathy was characterized by changes of behavior, disorientation, confusion and flapping tremor; his Glasgow coma score was 10 (E2V3M5). He underwent successful orthotopic liver transplantation with a model of end-stage liver disease score of 32.3 on January 1, 2017, and received imipenem-cilastin sodium to prevent infection, FK-506 + MMF + pred to prevent acute rejection. Because of his gastrointestinal disorder and malnutrition, parenteral nutrition was given from 3 d before transplantation till 22 d after transplantation. The components were glucose, lipids, amino acids electrolytes and insulin, while the proportion and amount differed in accordance with enteral nutrition status.

The transplanted liver recovered smoothly, without severe complications. The antibiotic therapy was discontinued 10 d after transplantation. On the 15th postoperative day, he became irritable, raving and lethargic, and appeared to gradually develop unclear enunciation, difficulty in grasping objects, and

memory loss, without nystagmus or diplopia. He had no paresthesia, muscle tremor or incontinence and no dysfunction in his cardiac, respiratory and urinary system. He had no history of alcohol consumption or psychiatric disorders. At the time, his weight was 56.0 kg, being 54.8 kg before the transplantation; his height was 172 cm and his body mass index was then 18.9 kg/m². His temperature was 36.8 °C, heart rate was 82/min, respiration was 16/min and blood pressure was 108/68 mmHg. Pupils were equal and reactive to light. In terms of laboratory examinations, blood routine test showed white blood cell count was $8.83 \times 10^9/L$, with 87.8% neutrophils and 6.2% lymphocytes, red blood cell count was $2.63 \times 10^{12}/L$, hemoglobin concentration was 81 g/L, and platelet count was $83 \times 10^9/L$. Liver function test showed alanine aminotransferase of 13.3 U/L, aspartate aminotransferase of 23.3 U/L, albumin of 39.8 g/L, and total bilirubin of 17.8 $\mu\text{mol/L}$. Besides the renal function test, the serum electrolytes test, coagulation function test, blood ammonia test and arterial blood gas were normal. The random blood glucose fluctuated from 4.9 mmol/L to 8.4 mmol/L, and the tacrolimus valley point concentration was 8.1 ng/mL.

The brain magnetic resonance imaging (MRI) showed symmetrical high T1 and T2 signal intensities in thalamus and pons (Figure 1A) and high signal intensities of T2 Flair in the paraventricular area (Figure 1B). The medical history and brain MRI examination suggested WE, even though the plasma level of thiamine was not tested, and we started intravenous vitamin B1 500 mg daily for 1 wk immediately. His difficulties in speech and grasp resolved within 3 d and other neurological symptoms recovered 7 d after thiamine treatment. Considering the side effect of thiamine, he took 3 compound vitamin B tablets orally for 3 times daily for 3 mo. His general condition was good and he underwent another brain MRI 5 mo after the surgery. It showed significant reduction of previously abnormal signal in thalamus and pons (Figure 1C and D).

DISCUSSION

Etiology and pathogenesis of WE

WE is caused by vitamin B1 (thiamine) deficiency for many reasons, such as chronic alcoholism, recurrent vomiting, parenteral nutrition, gastrointestinal surgery, cancer, liver diseases and so on. Despite some reports of WE after bone marrow transplantation^[8-11], there have been few reports about WE and liver transplantation. In this case, the patient, with a long duration of liver disease, had poor feeding before the surgery due to nausea and abdominal distension, which could be a high-risk factor for WE. It could be the parenteral nutrition given postoperatively without vitamin B1 supplement that led to his WE.

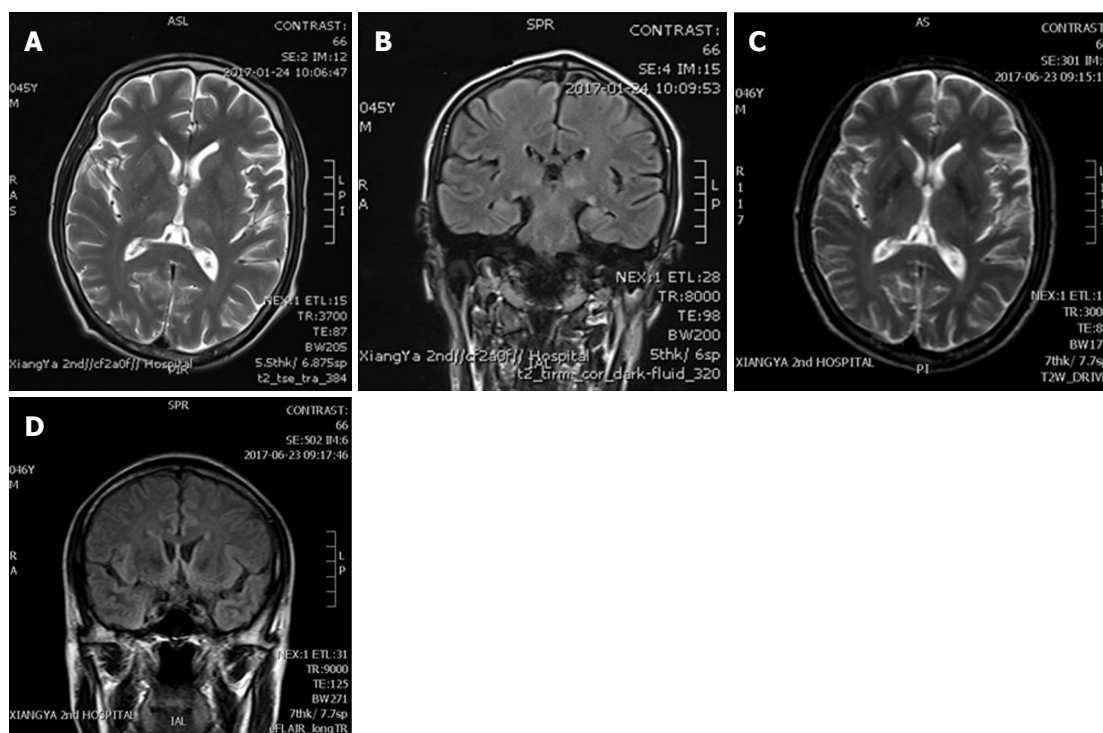


Figure 1 Brain magnetic resonance imaging. (A) and (B) are MRIs taken before the thiamine treatment, while (C) and (D) are from 5 mo after the thiamine treatment. A: Symmetrical high T2 signal intensities in pons; B: Symmetrical high T2 signal intensities in paraventricular area; C: Reduction of abnormal signal in pons; D: Reduction of abnormal signal in paraventricular area. MRI: Magnetic resonance imaging.

Vitamin B1 is water-soluble, cannot be synthesized by our bodies but can be taken in from food, with no storage in our bodies. Thiamine pyrophosphate, the biologically active form of vitamin B1, is an important coenzyme in the tricarboxylic acid cycle that is involved in energy production^[12]. Vitamin B1 deficiency will affect glucose metabolism in the brain, leading to WE because of lactic acid accumulation and acidosis, thereby interfering with neurotransmitter production, release and reuptake^[13]. Supplementation of glucose and usage of glucocorticoids can increase the consumption of vitamin B1, which may aggravate WE^[2]. The patient in our case received a large amount of glucose for energy since he developed postoperative hyperbilirubinemia and hypernatremia, and was given glucocorticoids to prevent acute rejection. These two therapies increased the consumption of vitamin B1 that led to WE.

Diagnosis of WE

The triad symptoms are well known, but only 16% of cases have complete presentations^[14]. There are one or two symptoms in the other cases, which lack specificity, making early diagnosis difficult. In WE patients, the thiamine plasma level will decrease, while the blood pyruvate level will increase. But, these tests are not carried out routinely in clinical practice because of multiple interfering factors. The examination of cerebrospinal fluid in WE patients will

be normal or with slightly elevated protein, which can help to differentiate it from other diseases. The electroencephalogram (EEG) may be abnormal in unconscious patients, with no specificity. The EEG may have a corresponding change in patients with peripheral neuropathy.

The brain MRI, which is the most important imaging examination, will show symmetric high T1, T2 and T2 Flair signal intensities in mammillary body, medial thalamus, periventricular and periaqueductal regions^[15]. It was reported that the sensitivity and specificity of brain MRI for WE diagnosis are 53% and 93%, respectively^[16]. At present, the diagnosis of WE is mainly based on the criteria proposed by the 2010 European Union of Neuroscience Association^[2], including (1) dietary deficiencies; (2) eye sign; (3) cerebellar dysfunction; and (4) either an altered mental state or mild memory impairment. We can diagnose WE clinically while conforming two of the four elements. In our case, the patient, who had experienced a long period of dietary deficiency, showed gradually deepening unconsciousness and ataxia manifestation-like inappropriate movement and dysarthrosis. His brain MRI supported WE and he recovered rapidly after supplementation of vitamin B1. Above all, we definitively diagnosed WE after liver transplantation. Besides, the differential diagnoses of WE after liver transplantation includes cerebral vascular accident, adverse effects of anti-rejection

drugs, hepatic encephalopathy and so on.

Treatment and prevention of WE after liver transplantation

WE is a clinical emergency, and once diagnosed, the patient should receive vitamin B1 treatment immediately. The initial treatment requires parenteral routes, either intramuscular or intravenous, to ensure adequate absorption^[17]. With a short half-life short, up to 96 min, vitamin B1 should be given three times a day or continuously by intravenous route. Till now, we have not reached a consensus on the dose and course of vitamin B1 therapy. So, the dose of vitamin B1 should be individualized based on the severity^[18]. In general, alcoholic WE patients need more vitamin B1 than nonalcoholics. Vitamin B1 should be given before carbohydrates and glucocorticoids because glucose metabolism consumes vitamin B1^[2].

In our case, the patient received intravenous vitamin B1 at 500 mg/d, starting immediately upon consideration of the WE diagnosis. His clinical symptoms improved and ataxia disappeared 3 d later, neurological disturbance resolved 7 d later. Then, he took compound vitamin B tablets instead of intravenous vitamin B1. The brain MRI 5 mo later revealed great improvement. The therapy of vitamin B1 supplementation was effective, without adverse reactions.

Vitamin B1 has wide safety range, since it is water-soluble and can excrete easily *via* the kidney. In order to prevent WE after liver transplantation, patients who had poor nutrition preoperation or needed a long fasting duration postoperation should receive intravenous vitamin B1 at 100 mg daily until returned to their normal diet.

Liver transplantation is a large-scale surgery, and the patients may have poor nutrition preoperation due to abdominal distension or nausea. Many factors increase the consumption of vitamin B1 postoperation, such as surgical stress, hypermetabolism, parenteral nutrition therapy, and use of glucocorticoids. The patients may suffer WE without extra supplementation of vitamin B1. We should consider WE when patients with high-risk factors develop disturbances of consciousness, diplopia or ataxia. Brain MRI can help substantially towards making the diagnosis. The effective treatment of WE is prompt supplementation of vitamin B1. To date, there are few relevant reports of WE after liver transplantation. As transplant doctors, we should improve awareness of this disease to avoid delaying the treatment.

ARTICLE HIGHLIGHTS

Case characteristics

A 45-year-old male patient received liver transplantation due to decompensatory cirrhosis because of hepatitis B and developed Wernicke encephalopathy on the 15th postoperative day.

Clinical diagnosis

The patient became irritable, raving and lethargic, and appeared to gradually develop unclear enunciation, difficulty in grasping objects, and memory loss.

Differential diagnosis

Hepatic encephalopathy and adverse effects of anti-rejection drugs.

Laboratory diagnosis

Thiamine plasma level was not tested, while the other laboratory results were close to normal.

Imaging diagnosis

Brain magnetic resonance imaging showed symmetrical high T1 and T2 signal intensities in thalamus and pons and high signal intensities of T2 Flair in the paraventricular area.

Pathological diagnosis

No pathological examination was performed.

Treatment

Intravenous vitamin B1 at 500 mg daily for 1 wk and 3 compound vitamin B tablets orally 3 times daily for 3 mo.

Related reports

Only 2 other cases of Wernicke encephalopathy after liver transplantation have been reported, and there have been case reports of Wernicke encephalopathy in bone marrow transplantation.

Term explanation

Wernicke encephalopathy is an acute neurological disorder resulting from vitamin B1 deficiency, which is common in chronic alcoholism.

Experiences and lessons

We should pay more attention to Wernicke encephalopathy after liver transplantation to avoid delaying treatment. Patients who had poor nutrition preoperation or who needed a long fasting duration postoperation should receive intravenous vitamin B1 at 100 mg daily until return to their normal diet.

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Silymarin: An option to treat non-alcoholic fatty liver disease

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Abstract

We have read with a great interest the review published by Singh *et al*, on the treatment options in alcoholic and non-alcoholic fatty liver disease, including various new targeted therapies that are currently under investigation. Recently, we described the health effects of the Mediterranean diet associated to an antioxidant complex rich in silymarin, to improve in overweight patients anthropometric parameters, glucose and lipid metabolism and intra-hepatic fat accumulation.

Key words: Mediterranean diet; Antioxidant; Silymarin; Non-alcoholic fatty liver disease

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Core tip: Supplementation of silymarin in association with an hypocaloric diet and physical activity, can be a correct approach to treat patients with non-alcoholic fatty liver disease in clinical practice.

Colica C, Boccuto L, Abenavoli L. Silymarin: An option to treat non-alcoholic fatty liver disease. *World J Gastroenterol* 2017; 23(47): 8437-8438 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v23/i47/8437.htm> DOI: <http://dx.doi.org/10.3748/wjg.v23.i47.8437>

TO THE EDITOR

We read with great interest the recent review by Singh *et al*^[1] on the treatment options in alcoholic and

non-alcoholic fatty liver disease (NAFLD), including various new targeted therapies that are currently under investigation. In the last decades the diagnosis of NAFLD was increasingly evoked in clinical practice, with a worldwide spread of NAFLD, with an estimated prevalence between 25%-30% of general population, not only in Western world but also in urban area of developing Countries^[2]. NAFLD is an umbrella term that includes different clinical pictures ranged from simple fat accumulation to steato-hepatitis, fibrosis, cirrhosis and its complications. Central obesity, dyslipidemia, insulin resistance, and diabetes in a context of metabolic syndrome are the risk factors largely associated to the development and progression of NAFLD^[3]. Recently, studies on the mechanisms involved in the pathogenesis of NAFLD, highlight the role of genetic polymorphisms enhancing oxidative stress, pro-inflammatory cytokines production and disequilibrium in the glucose and lipid metabolism^[4]. The standard of care to treat NAFLD, described by international guidelines, is focused on lifestyle modifications and in particular on starting a healthy diet and increasing physical exercise. However, no drugs are currently approved to treat NAFLD and its secondary complications by regulatory agencies^[5]. Reactive oxygen species production, including superoxide radical, hydroxyl radical, hydrogen peroxide, and lipid peroxide radicals, are involved in the pathogenesis and progression of NAFLD, in a multi-step process^[6]. *Silybum marianum*, commonly known as Milk Thistle (MT), family of Asteraceae/Compositae, has been used since the time of ancient physicians, to treat liver diseases^[7]. The active complex of MT is a lipophilic extract from the seeds of the plant and is composed of four isomer flavonolignans, collectively known as silymarin. Several pre-clinical and clinical studies have been carried out on silymarin and silibinin, its predominant and most active component. It has been described that silymarin possesses anti-oxidant, anti-inflammatory, and anti-fibrotic properties. Our study group have been reported in a randomized study, the health effects of a hypocaloric Mediterranean diet in association with an antioxidant formulation with silymarin, on liver damage, glucose metabolism and anthropometric parameters in NAFLD overweight patients^[8]. Data on the effects of silymarin in patients with NAFLD are limited. However, some studies showed that treatment with silymarin has been associated with an improvement of the oxidative profile, due to the ability of silymarin to inhibit the production of pro-inflammatory cytokines^[9]. In addition, our data can be

explained by the effectiveness of the diet associated to silymarin to reduce the extent of fat infiltration in the hepatocytes and to modulate the mitochondrial function.

In accordance with the paper by Singh *et al.*^[1], we conclude that new therapeutic targets are now under investigation for NAFLD. In this context, on the basis of our experience we support the role of silymarin, in association with lifestyle changes, to treat NAFLD patients, considering that it offers similar effects than some of the drugs listed by Singh *et al.*^[1] in terms of anti-oxidant (e.g., NOX-1/4 inhibitors), anti-fibrotic (e.g., galectin-3 antagonists, simtuzumab) and anti-inflammatory (e.g., sirtuins) properties, but with considerably lower side effects.

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