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EDITORIAL

From blood to breath: New horizons for esophageal cancer biomarkers

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Abstract

Esophageal cancer is a lethal cancer encompassing adenocarcinoma and squamous cell carcinoma sub-types. The global incidence of esophageal cancer is increasing world-wide, associated with the increased prevalence of associated risk factors. The asymptomatic nature of disease often leads to late diagnosis and five-year survival rates of less than 15%. Current diagnostic tools are restricted to invasive and costly endoscopy and biopsy for histopathology. Minimally and non-invasive biomarkers of esophageal cancer are needed to facilitate earlier detection and better clinical management of patients. This paper summarises recent insights into the development and clinical validation of esophageal cancer biomarkers, focussing on circulating markers in the blood, and the emerging area of breath and odorant biomarkers.

Key words: Breath analysis; Cancer; MicroRNA; Non-invasive; Esophageal cancer; Biomarker

Core tip: The current "gold standard" test for detection of esophageal cancer is endoscopic imaging and confirmation by biopsy. There are several barriers to endoscopy as a clinical tool to monitor patients at high risk of esophageal cancer, including high capital and personnel costs and the invasive nature of the procedure. This paper highlight new insights into the development and clinical validation of circulating and breath biomarkers of esophageal cancer.

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INTRODUCTION

Esophageal cancer is the 6th leading cause of cancer related mortality death worldwide[1,2]. Histologically it is classified into two sub-types, squamous cell carcinoma

and adenocarcinoma, each with a specific cellular origin, pathogenesis and epidemiology[3,4].

The current gold standard techniques for the detection and diagnosis of esophageal cancer, endoscopy and biopsy, are invasive. Furthermore, due to the lack of symptoms at earlier disease stages, presentation and diagnosis usually occurs late, leading to poor prognosis and 5 year survival rates as low as 15%[4,5]. Early diagnosis is associated with much higher 5 year survival rates[4], and when confined to the mucosa disease specific survival rates of up to 98% are reported[6]. Better diagnostic methods are needed to shift the majority of diagnoses to the earliest stages, and expanded access to conventional endoscopy and wider use in a screening context is not cost effective. Minimally and non-invasive biomarkers, primarily in the blood and breath, represent the most likely candidates to facilitate early detection of esophageal cancer.

Several candidate biomarkers for esophageal cancer have been proposed. However, their translation into clinical use has been slow. Biomarkers can be broadly defined as quantifiable parameters that assist in distinguishing normal from pathological processes[7,8] with applications for diagnosis, prognosis and tailoring of patient treatment[9]. In this paper we summarise recent insights into the development and clinical validation of esophageal cancer biomarkers. Whilst we recognise that there is a significant body of research which has been undertaken evaluating tissue based biomarkers in esophageal cancer, this review has deliberately focussed on minimally invasive and non-invasive methods for detection of esophageal cancer, principally circulating markers in the blood, and the emerging area of breath and odorant biomarkers. The development of robust, minimally invasive, cost effective biomarkers for early cancer will change current diagnostic, prognostic and surveillance paradigms, and could open the possibility of population screening.

SQUAMOUS CELL CARCINOMA

Esophageal squamous cell carcinoma is the most frequently diagnosed subtype of esophageal cancer worldwide[10] and typically arises in the mid and lower thirds of the esophagus[10]. The highest prevalence of squamous cell carcinoma is found within regions of Eastern Asia[11], largely attributed to the prevalence of risk factors such as tobacco smoking and the consumption of herbal tea maté and pickled vegetables[12,13]. Conversely, in Westernised societies such as the United States and Australia, incidence rates of squamous cell carcinoma have been in decline since 1998, largely due to a decline in cigarette smoking[4,12,14].

The pathogenesis of squamous cell carcinoma is highly complex, involving an accumulation of genetic modifications within the esophageal mucosa, causing progressive changes that result in invasive carcinoma[15,16]. Environmental factors, diet, smoking and alcohol consumption have been strongly implicated in the molecular mechanisms for squamous cell carcinoma; however, evidence of a causal relationship is lacking[15,17]. Genetic mutations within cyclin D1 and the tumour suppressor gene, *TP53,* are among the most frequently isolated genetic abnormalities from esophageal squamous cell carcinomas[15].

TP53 is a tumour suppressor gene with roles in DNA repair and cell cycle arrest, and is the most common mutation found in cancers, including esophageal squamous cell carcinoma[15,17,18]. *TP53* mutations have been reported in as little as 10% and up to 80% of esophageal squamous cell carcinoma[19]. Additionally, mutations to the *TP53* gene are also found in dysplastic lesions[15,16], indicating *TP53* mutations may be an event in the early stages of esophageal squamous cell carcinoma carcinogenesis. *TP53* mutations produce abnormal TP53 protein that accumulates in the nuclei of cells which may be identified by immunohistochemistry[15,19,20]. Positive *p53* staining has been demonstrated in the non-cancerous cells adjacent to tumors[19] and in cells lacking the commonly identified *TP53* mutations[20]; indicating poor specificity and sensitivity of the technique and potentially additional mutations within the gene accounting for the positive staining[20].

ESOPHAGEAL ADENOCARCINOMA

Esophageal adenocarcinoma is a highly lethal tumour usually developing in the lower third of the esophagus, or at the gastro-esophageal junction[21]. Incidence rates have risen gradually in developed countries since 1984, with a 4% increase in the incidence of adenocarcinoma in Australia between 1988 and 2005[22]. Adenocarcinoma is most prevalent in males, the elderly and the obese. However, the most significant risk factor identified for adenocarcinoma is Barrett's esophagus[12,23,24].

Barrett's esophagus is a metaplastic condition of the esophageal epithelium, affecting up to 2% of the adult population[6]. It is defined histologically by the replacement of the normal stratified squamous epithelium with a columnar epithelium with intestinal metaplasia defined by the presence of goblet cells, as a result of chronic gastro-esophageal reflux disease[24]. Gastro-esophageal reflux disease is characterised by increased acid and bile exposure to the esophageal mucosa, a consequence of extended relaxation of the lower esophageal sphincter, which may lead to esophagitis and progress to Barrett's esophagus[25]. The development of Barrett's epithelium is considered a protective mechanism, as columnar epithelium is more resistant to the harmful effects of acid and bile than the normal stratified squamous epithelium of the esophagus[26]. However, Barrett's esophagus, is also a hyper-proliferative condition, susceptible to malignant progression in some individuals[27]. Dysplasia is one of

the initial changes identified with malignant progression, characterised by cellular distortion and changes in the nuclei such as crowding and hyperchromatism[28].

Patients with Barrett's esophagus have varied risk of progression to adenocarcinoma[29], with some studies suggesting 40%-75% of cases of esophageal adenocarcinoma lack evidence of Barrett's esophagus[30,31]. It has been suggested the absence of any evidence of Barrett's esophagus could suggest an alternate, yet to be identified, pathogenic pathways[31], and that Barrett's esophagus is simply a strong risk factor in a subset of the population, but not a necessary carcinogenic step in the development of esophageal adenocarcinoma[32]. However, the absence of useful tools for the early identification and ongoing assessment of Barrett's esophagus and progression to adenocarcinoma has made assessment of this relationship challenging.

CURRENT DIAGNOSTIC METHODS OF ESOPHAGEAL CANCER

The diagnosis of esophageal cancer and its premalignant lesions is currently limited to endoscopy and subsequent biopsy analysis[11]. Endoscopy is a highly invasive and costly diagnostic procedure[33,34] and is the current gold-standard diagnostic technique for esophageal cancer and its precursor lesions[4]. Standard white light endoscopy is limited in its scope, restricted to the identification of macroscopic abnormalities that may indicate cancer, such as nodules and ulcers, consequently failing to identify early lesions that appear macroscopically normal[4]. Whilst Barrett's esophagus is visible endoscopically, dysplasia within the Barrett's segment is more difficult to identify as lesions are often flat and difficult to distinguish from surrounding non-dysplastic columnar epithelium[33].

Classification of dysplasia is subjective and studies have shown differentiation between grades of dysplasia is highly variable amongst pathologists, leading to incorrect diagnosis and un-necessary procedures[5,35]. Likewise, random biopsy protocols is prone to sampling error[36], furthering the potential for misdiagnosis.

Surveillance using endoscopy and biopsy is generally recommended for patients with Barrett's esophagus, in order to diagnose esophageal cancer at its earliest stage[37]. As a result of the low progression rate of early lesions, such as Barrett's esophagus to adenocarcinoma and the costs of endoscopy surveillance, it may not be cost effective to employ the current diagnostic procedures in surveillance programs for esophageal cancer, and screening programs have never been considered feasible[35]. Thus, there is an acute need for the development of more selective and less invasive diagnostic techniques for individuals at risk of esophageal cancer.

EMERGING BIOMARKERS OF ESOPHAGEAL CANCER

Blood biomarkers

Autoantibodies have drawn appeal as serology markers for esophageal cancer, owing to their stability and persistence in serum samples. With improvements in antibody detection technologies improving the detection limits, there is a growing interest in the utility of autoantibodies as diagnostic and prognostic biomarkers for esophageal cancer. Perhaps the most comprehensively investigated has been the tumour suppressor gene, *TP53*. The protein product of *TP53* is a nuclear phosphoprotein and in normal human plasma, the TP53 protein and anti-p53 antibodies are absent[38]. *p53* mutations can cause accumulation of non-functional protein that has increased stability and a longer half-life than the native protein[38]. The subsequent production of anti-p53 has been detected in tissue, blood and other body fluids of several cancer types, including esophageal cancer. A meta-analysis by Zhang *et al*[38] summarizing the diagnostic value of anti-p53 for esophageal cancer found that patients with esophageal cancer were seven times more likely to be positive for plasma anti-p53 compared to non-cancer controls. However, despite the high specificity, the authors reported low sensitivity, suggesting limited clinical application.

More recently, a systematic review investigated the diagnostic utility of 35 different circulating autoantibodies, both alone and in combination, as biomarkers for the early detection of esophageal cancer[39]. Although the study did not distinguish between the two esophageal cancer sub-types, the majority of the studies included in the review were esophageal squamous cell carcinoma, a greater world-wide burden of this variant compared to the adenocarcinoma sub-type[39]. Although the vast majority of studies reviewed reported positive associations between their candidate biomarker, and esophageal cancer, with high specificity reported, the sensitivity values were generally too low to be of any clinical significance[39]. However, combinations of autoantibodies did slightly improve the median sensitivity. The authors also conducted a meta-analysis on the diagnostic value of anti-p53, reporting a significant association of serum anti-p53 with esophageal cancer, with sensitivity of 91.4% and specificity of 65%[39], contrasting their previous findings for meta-analysis of anti-p53[38].

Six serum biochemical markers that included anti-p53, carcinoembryonic antigen, squamous cell cancer antigen, cytokeratin 21-1 fragment (CYFRA21-1), vascular endothelial growth factor-C and microRNA (miRNA) were reviewed in a meta-analysis by Zhang *et al*[40]. Although each biomarker candidate was associated with a positive odds ratio for esophageal cancer, and high specificity values by receiver operating characteristic curves, the sensitivity for each test was

again low, with high variability between studies[40]. Although the authors suggest that combinations of the serum markers are likely to yield better sensitivity and specificity, it is more likely that the better designed, more robust, prospective, multi-centre studies are needed to better optimise and validate candidate serum biochemical markers.

Circulating tumor cells (CTCs) originate from the primary tumor, and are released into the circulation, where they may form micro-metastases. Various assays have been developed and used to assess the diagnostic and prognostic potential of CTCs in several cancer types, including breast, colorectal, gastric and esopahgeal cancer[41]. A recent meta-analysis by Qiao *et al*[42] aimed to determine the association between CTCs and clinicopathological characteristics and prognosis (tumor stage, lymph node metastasis, distant metastasis and patient survival) in esophageal cancer. The presence of CTCs was found to correlate strongly with poor overall patient survival, and predicted poor progression free survival in Asian populations with esophageal squamous cell carcinoma[42]. CTCs also correlated with venous invasion and metastasis to local lymph nodes (N-staging). New methodologies to quantify circulating tumor DNA might also offer new diagnostic potential, but more work is needed to evaluate this possibility.

Blood biomarkers for esophageal cancer represent new tools for the early detection and prognosis. However, despite the large number of candidate markers that have been published, there remains a paucity of large, well-designed, prospective multi-centre validation studies for both esophageal squamous cell carcinoma and esophageal adenocarcinoma.

CIRCULATING miRNA

miRNA's are single stranded, non-coding RNA's that can regulate gene and protein expression[43,44]. miRNAs are abundantly expressed in a stable form, with highly consistent levels amongst individuals in a range of extracellular fluids including blood serum and plasma, and have drawn attention as biomarkers for cancer and disease[43,44]. Recent studies have reported plasma/serum circulating miRNAs to be potential diagnostic and prognostic markers in some gastrointestinal cancers - esophageal squamous, esophageal adeno-carcinoma, gastric and colorectal[43,44]. Although still an emerging area of research, recent meta-analyses have highlighted the potential of circulating miRNAs for the detection of esophageal cancer.

A review by Wang *et al*[45] of eight manuscripts investigated a total of 16 different types of miRNAs in serum and saliva of Asian esophageal squamous cell carcinoma patients. The authors reported relatively high sensitivity and specificity values for combination and single miRNA markers, suggesting some diagnostic application[45]. The prognostic utility of miRNAs have also been reviewed, with Fu *et al*[46] reporting on 39 potentially prognostic miRNAs in 25 individual studies. miR-21 and miR-375 were found to be potentially prognostic of overall survival[46]. However, the small number of manuscripts that could be included in the study, and the lack of validation studies performed using the miRNA markers limits the conclusions that can be drawn for translational application. A more comprehensive meta-analysis by Fu *et al*[47] found that although increased expression of miR-21 and decreased expression of miR-375 were significantly associated with poor overall survival in esophageal cancer, both miR-21 and miR-275 were associated with low hazard ratios.

Circulating miRNAs have also been investigated as biomarkers of esophageal adenocarcinoma and the pre-cursor condition, Barrett's esophagus. In a retrospective study of bio-banked serum samples from esophageal cancer patients, Chiam *et al*[43] identified five miRNA ratios, derived from ten unique miRNAs that were discriminatory for esophageal adenocarcinoma over non-dysplastic Barrett's esophagus and healthy controls. The predictive accuracy of the miRNA ratios was enhanced with stepwise addition of each miRNA ratio to an analysis of the cancer patient's blood sample[43], highlighting the potential for biomarker combination approaches to enhance test specificity and sensitivity.

BREATH BIOMARKERS

Breath analysis represents an attractive modality for the early detection of cancer, as it is completely non-invasive, relatively cheap compared to conventional methods, and provides a rapid result following sample collection. Breath volatile organic compounds (VOCs) as biomarkers of disease have been recognised since the time of Hippocrates in Ancient Greece, who described *fetor hepaticus* and *fetor oris* in his treatise on breath aroma and disease[48]. It is now known that a single human breath is a complex gas mixture of more than 2000 unique VOCs, representing a reservoir of potential cancer biomarkers[49]. Breath VOCs have already shown clinical utility as possible biomarkers for lung[50,51], breast[52,53], prostate[54], colorectal[55], gastric[56] and recently, esophageal cancer.

Many studies have associated breath alkanes with cancer, presumably as a bi-product of oxidative stress pathways[57]. Breath ethane has previously been investigated in late stage esophageal squamous cell carcinoma and adenocarcinoma, with no differences compared to healthy controls[58]. More advanced technologies have since been used to characterise VOCs associated with esophageal cancer. Headspace analysis of urine[59] and gastric contents[60] from esophageal cancer patients by selected ion flow tube-mass spectrometry identified several VOCs that were differentially regulated compared to healthy controls. However, there was no predominant group of VOCs in the cancer group.

The first breath analysis study to define breath VOCs in esophageal cancer identified a phenols dominant expression pattern, with phenol, methyl phenol, ethyl phenol and hexanoic acid significantly increased in esophageal cancer compared to healthy controls[61]. In the most comprehensive study to date, Kumar *et al*[62] investigated breath VOCs in esophageal squamous cell carcinoma, esophageal adenocarcinoma, Barrett's esophagus, benign conditions and gastric adenocarcinoma, compared to healthy controls. A total of 12 VOCs, comprised of phenols, aldehydes and fatty acids were identified as being discriminatory for esophageal cancer and gastric cancer compared to normal upper gastrointestinal (GI) tract[62]. Additionally, the authors found the VOC profile distinguished esophageal cancer from Barrett's metaplasia and from benign conditions of the upper GI tract (which included esophagitis, esophageal stricture, and esophageal candidiasis). Developing a risk prediction model, the authors reported eight significant predictors for adenocarcinoma: decanal, nonanal, phenol, ethyl phenol, methyl phenol, hexanoic acid, heptanal, and butyric acid, with sensitivity and specificity of 98% and 91.7% respectively when compared to normal upper GI tract[62]. Furthermore, the model accurately discriminated esophageal adenocarcinoma from non-cancer controls (benign conditions, Barrett's metaplasia and normal upper GI tract), with sensitivity and specificity of 87.5% and 82.9% respectively[62]. Interestingly, no differences in VOCs were detected between early and late stage cancers, or between tumour size and concentrations of VOCs.

Proton Transfer Reaction-Mass Spectrometry has recently been used to identify breath VOCs in a small study of Chinese esophageal cancer patients[63]. Although the study did not differentiate between esophageal squamous cell carcinoma and esophageal adenocarcinoma, the authors reported 20 ion peaks in the full mass spectra that were significantly different in cancer patients compared to healthy controls[63]. Using stepwise discriminant analysis, the authors identified seven ions that were highly discriminatory for esophageal cancer[63]. In contrast to the study by Kumar *et al*[62] the authors also suggested that their predictive model discriminated for early and late stage cancer. However, these interpretations should be carefully balanced against the small participant numbers used in the study.

CONCLUSION

Current diagnostic and surveillance procedures for esophageal cancer are invasive, expensive and ill-adapted for early detection. Recent advances have been made in the development and validation of new minimally and non-invasive biomarkers for esophageal cancer. Although several novel serology markers have been investigated, these have not translated to validated clinical tools. Circulating anti-p53 and CTCs have shown the most promise as diagnostic and prognostic markers of esophageal cancer, with recent meta-analyses supporting their use. However, the absence of well-designed, robust clinical validation trials in large patient cohorts largely limits the power of these meta-analyses. This is highlighted by the lack of differentiation between esophageal squamous cell carcinoma and esophageal adenocarcinoma, despite distinct pathologies and molecular profiles.

Circulating miRNAs have emerged as promising new biomarkers of esophageal cancers. Despite their promise, several studies have limited their focus to esophageal squamous cell carcinoma, and small clinical cohorts, with many focussing on single miRNAs rather than combined approaches. Advances in bioinformatics have facilitated analysis of large, complex miRNA microarray datasets, and future studies are likely to employ combined approaches for miRNA analysis.

The emerging field of breath and gas analysis for cancer detection represents a completely non-invasive approach to early detection, and ongoing screening of at risk individuals. With improvements in the sensitivity of VOC detection technologies, it is likely that the pool of possible breath and odorant biomarkers will significantly increase. Despite relatively few studies having investigated breath biomarkers in esophageal cancer, the initial predictive models have shown some promise. Moving forward in this rapidly developing field, it is critical that standardised approaches to the collection of breath samples are employed, to minimise study heterogeneity. Furthermore, with little evidence to support the biological origins of VOCs, mechanistic studies to better understand how VOCs are produced in cancer cells will help to improve the sensitivity and specificity of future tests.

As the incidence of esophageal cancer continues to grow world-wide, new diagnostic and prognostic tools are needed to improve survival and direct clinical management. The advances being made in new minimally and non-invasive biomarkers represents a suite of ancillary tests that could stratify patients for endoscopic and other imaging modalities, ultimately leading to improved patient care. While it is unlikely that there will ever be a single "silver bullet" biomarker, the most likely scenario will be derived from predictive algorithms based on multiple biomarkers, which could also include combinations of blood and breath analysis.

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REVIEW

Sirtuins and nonalcoholic fatty liver disease

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Abstract

Mammalian sirtuins are seven members belonging to the silent information regulator 2 family, a group of Class Ⅲ histone/protein deacetylases. Sirtuins (SIRT 1-7) have different subcellular localization and function and they regulate cellular protein function through various posttranslational modifications. SIRT1 and 3, the most studied sirtuins, use the product of cellular metabolism nicotinamide adenine dinucleotide as a cofactor to post-translationally deacetylate cellular proteins and consequently link the metabolic status of the cell to protein function. Sirtuins have been shown to play a key role in the development and rescue of various metabolic diseases including non-alcoholic fatty liver disease (NAFLD). NAFLD is currently the most chronic liver disease due mainly to high-calorie consumption and lower physical activity. No pharmacological approach is available to treat NAFLD, the current recommended treatment are lifestyle modification such as weight loss through calorie restriction and exercise. Recent studies have shown downregulation of sirtuins in human as well as animal models of NAFLD indicating an important role of sirtuins in the dynamic pathophysiology of NAFLD. In this review, we highlight the recent knowledge on sirtuins, their role in NAFLD and their unique potential role as novel therapeutic target for NAFLD treatment.

Key words: SIRT1; SIRT3; Sirtuins; Non-alcoholic fatty liver disease

Core tip: Non-alcoholic fatty liver disease (NAFLD) is a leading cause of chronic liver disease with no effective pharmacological therapy. The discovery of treatment is hindered by the insufficient understanding of the pathophysiology of the disease. Sirtuins are key players in hepatic carbohydrate and lipid metabolism, insulin signaling, and inflammation and hence may represent a novel therapeutic target for NAFLD. However, the particular role for each sirtuin, the cross talk between sirtuins in different cell compartments or within a given organelle, and the development of selective sirtuins

activators/inhibitors still need further investigation.

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INTRODUCTION

Non-alcoholic fatty liver diseases (NAFLD) is emerging as the leading cause of chronic liver diseases affecting one billion of people in the world. The current model for NAFLD pathophysiology, "the multiple-hit hypothesis", characterizes NAFLD as the manifestation of both genetic and environmental factors, dysfunction of various organs and organelles, as well as the complex interaction between hepatocytes and other cells (*e.g.*, Kupffer and stellate cells) in the liver[1]. Moreover, the liver is a hub for many metabolic pathways making NAFLD a multistage, progressive disease with systemic consequences. NAFLD is commonly associated with obesity, insulin resistance and enhanced risk of cardiovascular disease and mortality[2-6]. Importantly, cardiovascular diseases are the main cause of morbidity in NAFLD patients. High-calorie consumption and lower physical activity have contributed to the rise in the prevalence of NAFLD. To date, no approved pharmacological approaches are available to treat NAFLD, the current confirmed recommendations for NAFLD are lifestyle modifications such as weight loss through caloric restriction (CR) and increased physical activity[7-9]. Therefore, a pressing need for developing new novel pharmacological treatments, is still remaining. An inclusive pharmacological approach would be one that addresses the pathogenic complexity of NAFLD. Currently, sirtuins have been under intense investigation as a novel therapeutic target for the treatment of NAFLD. In this review, we summarize the current knowledge on the pathophysiology of NAFLD and on the sirtuins as a potential target for the treatment of NAFLD.

NAFLD PATHOPHYSIOLOGY

NAFLD is a spectrum of liver diseases that occurs in the absence of excessive alcohol intake or viral infection. It includes hepatic steatosis (> 5% of fat in the liver), nonalcoholic steatohepatitis (NASH, fat deposit with inflammation), cirrhosis and hepatocellular carcinoma[9-12]. NAFLD is currently the most widespread form of liver disease affecting 10%-30% of all ages from childhood to adult population, and is predicted to be the leading cause of liver pathology and liver failure in the coming years[13,14]. NAFLD is more prominent in obese and insulin resistant individuals affecting 70%-90% in these populations[15,16]. NAFLD is also present in 10%-20% of the general pediatric population; this proportion increases to 50% in obese children in western society[13,17-22]. A more recent study suggests that metabolic derangements may start early in life, even in utero. Exposure to excess fuel in fetal life may result in NAFLD in the offspring[23,24].

Our understanding of the mechanisms involved in the pathophysiology of NAFLD are insufficient to pinpoint the major determinants involved in the development and progression of the disease and to develop therapeutic strategies for NAFLD. Studies on genetic and molecular factors involved in NAFLD clearly implicate lipid and glucose metabolism in the development of the disease. Moreover, functional studies implicate the different cell population in the liver as well as interaction between the liver, adipose tissue, gut and the muscle in the pathogenesis of NAFLD. In contrast to the "two-hit hypothesis" proposed by Day[25] in which hepatic accumulation of triglyceride (TG) ("1st hit") sensitizes the liver to additional insults such as oxidative stress and pro-inflammatory cytokines ("2nd hit") resulting in NASH. The current understanding, "the multiple parallel hypothesis", refers to NAFLD as a systemic, multifactorial disease involving multiple organs, such as adipose tissue, muscle and the intestine, and organelles such the endoplasmic reticulum and the mitochondria.

Hepatic steatosis

Hepatic steatosis, which is previously considered as the benign form of NAFLD, results from an imbalance between influx of fatty acids to the liver from the diet, adipose tissue lipolysis or *de novo* lipogenesis; and their oxidation or export in the circulation as very low density lipoproteins (VLDL)[9]. Failure of insulin to suppress lipolysis in insulin resistant adipose tissue is commonly associated with NAFLD[26,27]. Moreover, it is estimated that in NAFLD patients, roughly 60% of fatty acids in the liver originate from adipose tissue, 25% from *de novo* lipogenesis, and 15% from the diet[28]. Interestingly, both β-oxidation of fatty acids in the liver and VLDL secretion, are initially upregulated in non-alcoholic fatty liver in an attempt to compensate for the rise in fatty acids in the liver[29-32]. However, this short term compensatory mechanism is insufficient to sustain the ongoing influx of fatty acid to the liver leading to liver injury[30-32]. NASH patients have lower VLDL secretion and lower fatty acid oxidation (FAO) than patients with fatty liver[30,31].

Non-alcoholic steatohepatitis and fibrosis

Non-alcoholic steatohepatitis (NASH) is a more severe form of NAFLD that is generally defined by the presence of steatosis with inflammation and cellular damage. Fibrosis is commonly described as an irreversible scarring of liver tissue with excessive presence of

extracellular matrix. The presence of fibrosis is one of the most important predictors of NAFLD related mortality[10,33]. The current understanding of NASH pathogenesis follows a multiple hits model[34,35] that implicate multiple stressors. Lipotoxicity, endoplasmic reticulum stress, adipose tissue derived adipokines (TNFα and IL6), gut endotoxins and LPS produced by gut microbiota that drift into to the liver through the portal vein due to changes in the intestinal permeability in NAFLD, and oxidative stress trigger inflammatory response and progressive liver damage. Inflammation can sometimes precede steatosis, and patients with NASH can present without much steatosis suggesting that inflammation can sometimes occur first. Recent studies have also shown that individuals with hepatic steatosis may progress to fibrosis in a relatively short period of time (3-7 years)[36,37]. NAFLD patients may be classified into two categories, slow and fast progressors. The slow progressors may develop NASH but no fibrosis while the fast progressors may develop fibrosis and sometimes skip NASH stage of the disease[38]. Changes in mitochondrial function is an important mechanism that may drive the switch from hepatic steatosis to NASH. Several reports indicate that mitochondrial respiration is elevated in NAFLD patients[29,30]. However, in humans with NASH, respiration may be uncoupled from ATP production, causing significant increases in reactive oxygen species (ROS)[30]. Importantly, elevated ROS production was associated with an increase in detoxification and antioxidant capacity in hepatic steatosis, but not in NASH, indicating that mechanisms to cope with excess ROS generation may be insufficient in NASH[30].

ROLE OF SIRTUINS IN NAFLD

Sirtuins are a group of proteins that belong to the family of silent information regulator 2. Sirtuins have been shown, in recent years, to play an important role in the pathophysiology of various metabolic diseases including NAFLD[39]. Sirtuins are implicated in many cellular and physiological functions including hepatic glucose and fatty acid metabolism, mitochondrial function, hepatic gluconeogenesis, insulin secretion and the maturation of fat cells[40,41] as illustrated in Figure 1. Sirtuins regulate protein function through a growing list of posttranslational modification including deacetylation, succinylation and malonylation[42,43]. Seven mammalians sirtuins (SIRT1-SIRT7) have been identified and shown to share the same conserved NAD binding site and catalytic core domain but with different N and C termini[44]. The different sirtuins have various subcellular localization and expression[44]. SIRT 1, 6, and 7 are localized mainly in nucleus while SIRT 3, 4 and 5 are localized to the mitochondrial matrix and SIRT2 predominantly cytoplasmic[44]. Recent studies have shown reduced levels of most sirtuins in NAFLD. Direct evidence came from Wu *et al*[45] who demonstrated decreased expression of SIRT1, SIRT3, SIRT5, and SIRT6 in NAFLD patients compared to the control group. This was associated with increased expression of lipogenic genes including sterol regulatory element binding protein-1, fatty acid synthase, and acetyl-CoA carboxylase. In contrast to the other sirtuins, the expression of SIRT4 was upregulated in NAFLD patients[45]. Interestingly, in a recent study, Bruce *et al*[46] indicated that exposure to excess dietary fat during early and post-natal life increases the susceptibility to develop NASH in adulthood and this was associated with reduced sirtuin abundance. Offspring fed a high fat diet (HFD) developed NAFLD while HFD-fed offspring of mothers fed a HFD diet developed NASH in combination of reduced NAD^+/NADH, SIRT1, SIRT3 and increased expression of genes involved in lipid metabolism[46]. SIRT1 and SIRT3 are the most studied sirtuins; we will focus mainly on these two sirtuins, their mode of action and their role in NAFLD.

Both SIRT1 and SIRT3 are NAD^+-deacetylase that use NAD as a cofactor to deacetylate cellular proteins. Lysine acetylation is a reversible, dynamic reaction of adding acetyl groups to lysine residues. Acetylation affects all proteins in the cell and has recently been shown to be abundant in the mitochondria where it plays a key role in the dynamic regulation of proteins and thereby cell metabolism[43,47-54]. Dysregulation of lysine acetylation plays a pathogenic role in diverse conditions such as metabolic syndrome, aging, cancer and NAFLD[55-58].

SIRT1 and NAFLD

Studies from our group and others document strong involvement of the mitochondria in the pathogenesis of NAFLD[59-62]. SIRT3 is the most investigated mitochondrial sirtuin, while SIRT1 has been shown to be expressed in various metabolic tissues including liver, adipose tissue, skeletal muscle, pancreas and brain. SIRT1 plays a key role in the development of NAFLD through its involvement in the regulation of both lipid and carbohydrate metabolism[45,46,63-66]. Studies in mice and in cultured cells have characterized SIRT1 as a metabolic sensor that has the potential to improve NAFLD.

Inhibition of SIRT1 signaling in human fetal hepatocytes resulted in an increase in intracellular glucose and lipid levels with upregulation of *de novo lipogenesis* and gluconeogenesis related genes[66]. In mice, liver specific deletion of SIRT1 as well as SIRT1 downregulation using small hairpin RNA resulted in hepatic steatosis, inflammation and endoplasmic reticulum stress[67,68]. Hepatocyte-specific deletion of SIRT1 impaired PPARα signaling and decreased FAO. However, SIRT1 overexpression increased levels of PPARα and increased FAO[67].

SIRT1 is reduced by HFD while CR resulted in an increase in hepatic SIRT1 expression and improvement in NAFLD histology[69]. Overexpression

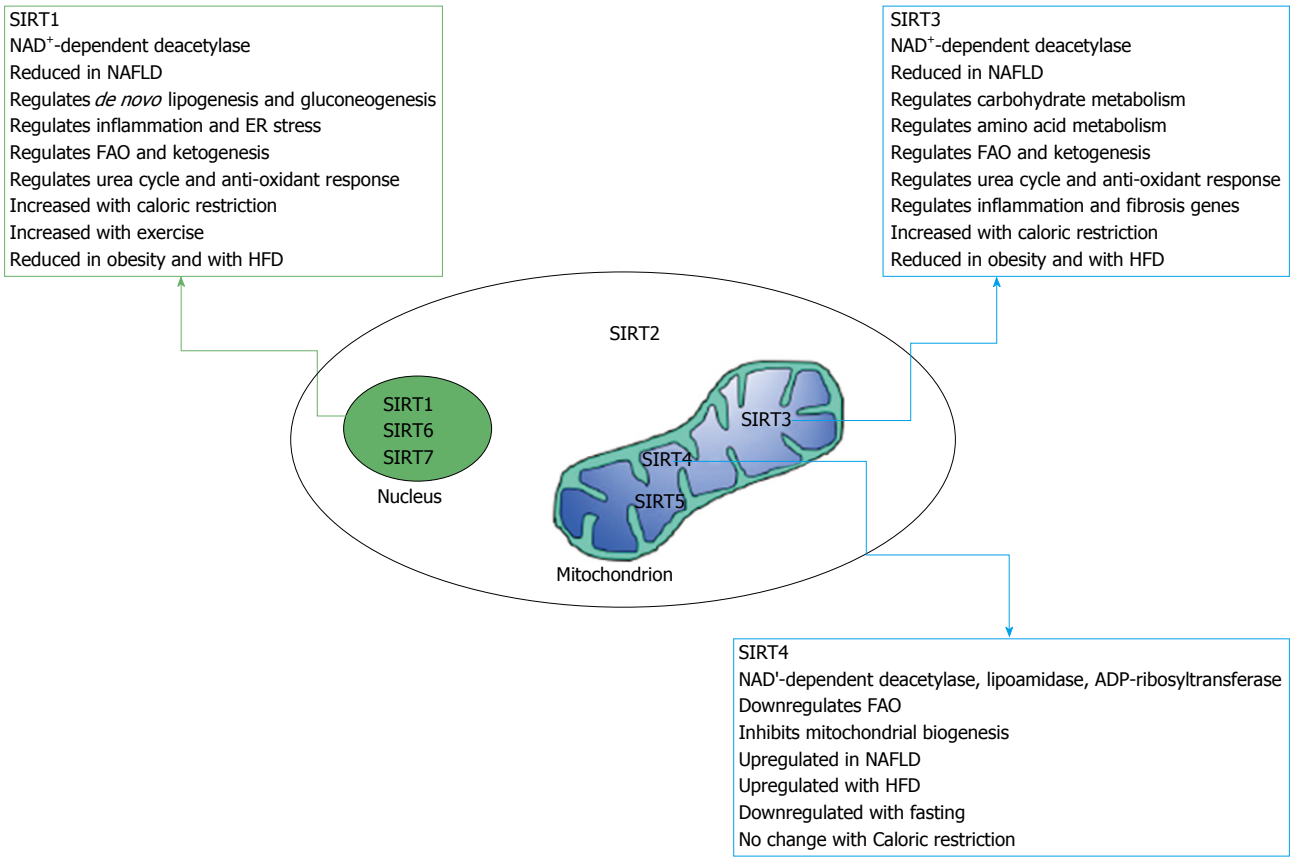

Figure 1 An illustration representing various sirtuins with summary findings for SIRT1, SIRT3, and SIRT4. NAFLD: Non-alcoholic fatty liver diseases; FAO: Fatty acid oxidation; HFD: High fat diet.

of SIRT1 in mice provided protection against HFD induced hepatic steatosis through upregulation of FAO and downregulation of lipogenesis[64]. Moreover, treatment of mice fed a HFD with resveratrol (RSV), a polyphenol found in red wine and other plants, improved lipid metabolism, and decreased NAFLD and inflammation in the liver[70]. Interestingly, it has been documented that inhibition of SIRT1 signaling in human fetal hepatocytes resulted in an increase in intracellular glucose and lipid levels[66]. SIRT1 is also modulated in obesity. Recent studies have shown a correlation between plasma SIRT1 and NAFLD in obese patients. SIRT1 was significantly lower in an obese group with severe liver steatosis compared to a group with mild steatosis, and both groups had lower SIRT1 in the plasma compared to control lean patients[71]. Phenotypic similarities exist between CR and SIRT1 overexpression. Mice overexpressing SIRT1 are leaner and resistant to hepatic steatosis and insulin resistance[72]. Together, these studies indicate a potential therapeutic use of SIRT1 in hepatic steatosis[66].

SIRT3 and NAFLD

SIRT3 is a soluble protein located in the mitochondrial matrix and has been shown as a major regulator of mitochondrial protein acetylation and function[44,73]. SIRT3 regulates carbohydrate metabolism, ketogenesis, β-oxidation, and amino-acid metabolism and stress-related pathways[73-77]. The protein is encoded by the nuclear genome and is translated as a 45-kDa protein with an N-terminal mitochondrial targeting sequence that is cleaved to give the 28-kDa enzymatically active protein[78]. SIRT3 is expressed in many tissues including the liver, adipose tissue, heart, brain and kidney[44]. Although SIRT3-KO mice are metabolically undistinguishable from WT controls under basal conditions, they show increased hyperacetylation of mitochondrial proteins in the liver and the heart[54,74,75,79]. About 65% of all mitochondrial proteins have at least one acetylated lysine[48,54,73]. SIRT4 and SIRT5 are also localized to the mitochondria and unlike SIRT3-KO mice, SIRT4 and SIRT5-KO mice did not display the global increase in hepatic mitochondrial acetylation observed in SIRT3-deficient animals.

Mitochondria play a key role in the adaptation to CR and SIRT3 has been identified as an important regulator in CR-associated metabolic changes[54]. The expression of SIRT3 is considerably increased in response to CR or prolonged fasting[75,80,81]. SIRT3 regulates the function of several mitochondrial proteins involved in oxidative phosphorylation, FAO, the urea cycle, and the antioxidant response system[73,75,82-85]. Unlike wild-type mice where FAO is upregulated with fasting, fasted SIRT3 deficient mice display reduced FAO and ATP production with increased hepatic TG

Table 1 Published SIRT1 activators

SIRT1 activators	Ref.
Resveratrol	Howitz *et al*[109], 2003
	Wood *et al*[102], 2004
	Timmers *et al*[104], 2011
	Smith *et al*[105], 2009
	Milne *et al*[107], 2007
	Amiot *et al*[113], 2013
	Yoshino *et al*[100], 2012
	Chachay *et al*[112], 2014
SRT1720	Feige *et al*[111], 2008
	Funk *et al*[110], 2010
	Yamazaki *et al*[101], 2009
	Pacholec *et al*[106], 2010
SRT2104	Libri *et al*[108], 2012
	Venkatasubramanian *et al*[103], 2013
	Hoffmann *et al*[114], 2013

content[75].

SIRT3 also regulates the acetylation levels of mitochondrial electron transport complex I and regulates ATP synthesis[77]. ATP levels were reduced by more than 50% in the heart, liver and kidney of mice lacking SIRT3[77]. Succinate dehydrogenase (SDH) (one of complex II subunits of the electron transport chain) has been identified as a direct target of SIRT3, suggesting a role of SIRT3 in the regulation of complex II[86,87]. Increased succinate concentrations is involved in hepatic stellate cells (HSCs) activation. The expression of SIRT3 and SDH activity are decreased in isolated liver and HSCs from methionine- and choline-deficient (MCD) diet-induced NAFLD. Suppression of SIRT3 using siRNA exacerbated HSC activation while SIRT3 overexpression attenuated HSC activation *in vitro*[88]. Interestingly, liver- and muscle-specific SIRT3-KO mice show no detectable changes in their metabolic phenotype in response to HFD[89] suggesting more studies are needed to ascertain the role of tissue specific function of SIRT3[76,89].

Published studies document that both obesity and chronic HFD reduce SIRT3 activity, induce hyperacetylation of various mitochondrial proteins and impair mitochondrial function[58,75,90]. HFD has been shown to induce SIRT3 expression and FAO early after initiation of high-fat feeding[58]. However, chronic HFD suppress SIRT3 expression, increase mitochondrial protein acetylation, and ultimately reduce FAO. Wild type mice fed a HFD develop obesity, hyperlipidemia, type 2 diabetes mellitus, and NASH[91-93]. These effects of HFD feeding are significantly accelerated in SIRT3 deficient mice[58]. Our unpublished data also show that overexpression of SIRT3 rescues NAFLD in mice heterozygous for the mitochondrial trifunctional protein, an animal model of mitochondrial dysfunction generated by our group[94].

SIRT3-KO mice subjected to MCD diet exhibit increased serum ALT levels, increased hepatic content, higher expression of inflammatory and fibrogenic genes, and reduced (SOD2) activity. However, overexpression of SIRT3 resulted in opposite effects suggesting that SIRT3 ablation aggravates MCD induced NASH while SIRT3 overexpression alleviates the MCD induced phenotype[95].

Palmitate modulated oxygen consumption and enhanced ROS levels and apoptosis in SIRT3 deficient mouse primary hepatocytes and SIRT3 siRNA-depleted hepatocytes[96]. Recent studies using HFD induced NAFLD in mice identified a differentially expressed microRNA (miRNA) in livers of NAFLD mice compared with controls. The expression of miRNA-421 was significantly upregulated in mice with NAFLD and SIRT3 was identified as target for this micro-RNA. Overexpression of miRNA-421 in hepatocytes decreased SIRT3 and FOXO3 protein levels, and reduced oxidative damage while suppression of this miRNA had opposite effects[97]. Interestingly, exposure of fetuses to maternal obesity contributes to early perturbations in whole body and liver energy metabolism, and this was associated with reduced SIRT3 and reduced hepatic FAO. These findings suggest that changes in SIRT3 activity precedes the development of obesity associated insulin resistance and NAFLD in the offspring[98].

Sirtuins activators and inhibitors

Weight loss through CR and exercise have been shown to improve insulin resistance and inflammation. Based on the beneficial effect of CR on NAFLD and other diseases and the associated increase in sirtuins levels or activity, the development of molecules that activate or inhibit sirtuins is of great interest[99].

The discovery of selective and potent sirtuins activators and inhibitors is still in its early stages. A list of Sirt1 activators that were tested in human and animal NAFLD is shown in Table 1[100-114]. RSV, a natural polyphenol found in grapes and other plants, mimicks CR and enhances sirtuins activity[102,109]. However, due to its poor bioavailability, reformulated forms of RSV-related compounds have been developed such as resVida, Longevinex®, SRT50 along with other RSV unrelated molecules such as SRT1720, SRT2104, and SRT2379. The formulated form of RSV resVida (150 mg/d RSV) showed beneficial effects, similar to CR effect, in healthy obese men including reduced intrahepatic lipid, plasma glucose, TG, alanine-aminotransferase and inflammation markers[104]. SRT1720 was the most potent SIRT1 activator; it enhanced SIRT1 activity by 750% at 10 μmol/L although other studies by Pacholec *et al*[106] concluded that neither SRT1720 nor RSV are direct activators of SIRT1 and one study reported that RSV does not have beneficial effects in NAFLD patients[112]. Administration of SRT1720 to diet-induced obesity rodent models protected from obesity and insulin resistance by enhancing oxidative metabolism in the liver, muscle, and adipose tissues[105,107,111]. As in CR, SIRT1720 induced mitochondrial biogenesis, increase mitochondrial respiration and ATP levels[110].

Moreover, SRT1720 reduced levels of hepatic liver content and aminotransferase and the expressions of lipogenic genes[101]. Recent studies, however, indicate that the activation of SIRT1 by RSV is indirect and is mediated by activation of AMPK[40,115]. Sirtuins are themselves regulated by the cofactor NAD^+ as well as their reaction product nicotinamide (NAM) from NAD^+. NAM (the amide form of vitamin B3, nicotinic acid) is a water-soluble sirtuin inhibitor. NAM binds to a conserved region in the sirtuin catalytic site and favors a reverse reaction instead of the deacetylation reaction[116]. Computational studies indicate that NAM inhibition of SIRT3 involves apparent competition between the inhibitor and the enzyme cofactor NAD^+ while the inhibition of other sirtuins activity was non-competitive[117]. More detailed review on sirtuins inhibitors and activators is found in[99,118]. More studies are needed to develop more potent and specific activators and inhibitors of sirtuins activity.

CONCLUSION

Sirtuins represent potential targets for treatment of NAFLD due to the role they play in cellular pathways involved in hepatic lipid and carbohydrate metabolism, insulin signaling, and inflammation. Additional studies are urgently needed to further our understanding of the interaction among various sirtuins in NAFLD and to develop selective activators/inhibitors of sirtuins.

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REVIEW

Can probiotics benefit children with autism spectrum disorders?

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Abstract

Children with autism are commonly affected by gastrointestinal problems such as abdominal pain, constipation and diarrhea. In recent years, there has been a growing interest in the use of probiotics in this population, as it hypothetically may help to improve bowel habits and the behavioral and social functioning of these individuals. The gut microbiome plays an important role in the pathophysiology of organic as well as functional gastrointestinal disorders. Microbial modification with the use of antibiotics, probiotics, and fecal transplantation have been effective in the treatment of conditions such as recurrent *Clostridium difficile* infection, pouchitis, and irritable bowel syndrome. The present review presents a number of reported clinical, immunological and microbiome-related changes seen in children with autism compared to normally developed children. It also discusses gut inflammation, permeability concerns, and absorption abnormalities that may contribute to these problems. Most importantly, it discusses evidence, from human and animal studies, of a potential role of probiotics in the treatment of gastrointestinal symptoms in children with autism.

Key words: Microbiome; Gastrointestinal; Inflammation; Functional bowel disease; Probiotics; Autism

Core tip: Important new information has identified an abnormal intestinal microbial community in children with autism, an abnormality reported in many gastrointestinal (GI) conditions, including inflammatory bowel disease and irritable bowel syndrome (IBS). There is a complex interplay in these conditions between GI function (motility, secretion, permeability), the immune system, and the microbiota. Many parents of children with autism complain of GI symptoms, and they administer probiotics, a treatment which has been found to be safe and effective for adults with IBS. Future investigations are needed to determine if

probiotic treatment would benefit the symptoms and behavior of these children.

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INTRODUCTION

The influence of the enteric microbiota on the human body has only started to be unveiled. Its impact is wide, as it has been shown to affect a number of processes including the immune response, metabolism, and neurologic function[1-3]. The disruption of the normal commensal microbial community in humans, also called "dysbiosis", is associated with an increasing number of disorders such as inflammatory bowel disease, irritable bowel syndrome, obesity, hypertension, diabetes, and autism[4-8]. The aim of the present review is to synthetize current data on the association between microbiota dysbiosis and autism, and to assess if its modification could have a beneficial effect in children with autism.

GASTROINTESTINAL ABNORMALITIES IN AUTISM

Autism is a neurodevelopmental disorder which affects social interaction, verbal and non-verbal communication, and behavior. A recent report from the Centers for Disease Control and Prevention indicates a rise in the prevalence of autism in children to one in 68 children in the United States (78% increase since 2007)[9].

Children with autism spectrum disorders (ASD) are among the populations that are most often referred to the Pediatric Gastroenterology clinic. During a two-year period, 3% (121/4013) of children seen by 4 pediatric gastroenterologists for various abdominal complaints in our clinic had an underlying ASD (C. Bearden, U.T. Bioinformatics, personal communication 9-24-2016). The true prevalence of gastrointestinal symptoms (GIS) in ASD is not known, but available data suggest a figure approximately 40%[10]. Wang *et al*[11] reported data obtained from families with children with ASD registered in the Autism Genetic Resource Exchange (AGRE). In their study of 589 affected children, 42% had GIS. Increased autism symptom severity was associated with higher odds of having GIS[11]. Abdominal pain, constipation, diarrhea, nausea, and bloating were the most common symptoms. In the largest study, Mazurek *et al*[12] reported that of 2973 children in an ASD network, 42% reported GIS lasting > 3 mo. A wide range of gastrointestinal (GI) problems have been reported, including feeding abnormalities, gastroesophageal reflux, abdominal pain, diarrhea, fecal incontinence, constipation, and alternating diarrhea and constipation have been reported in one out of three children in the autism spectrum[13,14]. More recently, based on a large epidemiological study, eosinophilic esophagitis in children with ASD and dysphagia has been added to the list of disorders with increased risk in this population, compared to the general population[15]. This group of children with autism reportedly also has severe anxiety, irritability and social withdrawal symptoms, which may overshadow their GI complaints[16].

Some researchers such as Pusponegoro *et al*[17] have reported no differences between children with autism and controls with regard to gastrointestinal symptoms, intestinal inflammation (based on fecal calprotectin), microbiota (based on urinary D-lactate) or intestinal permeability (based on urinary lactulose/mannitol ratio). However, this group reported an increased urinary I-FABP (marker of enterocyte damage) in children with autism who had severe behavioral abnormalities, compared with autistic children with mild maladaptive behavior and compared with normal children[17].

INFLAMMATION HYPOTHESIS

A number of recent studies have suggested that the GIS in ASD may be a manifestation of an underlying inflammatory process. Systemic inflammation has been suggested by an excessive accumulation of receptors for advanced glycation end products (RAGE) in blood and their proinflammatory ligand S100A9 in the plasma of individuals with ASD[18]. The level of S100A9 in plasma correlated with the autism severity score. Another study hypothesized that the inflammation may be pathophysiologically related to an abnormal microbiota. They compared the metagenomic profile of ileal and colonic biopsies in children with ASD, ulcerative colitis (UC), and Crohn's disease (CD). These investigators found that the transcriptome profiles of these tissues of children with ASD segregated apart from normal controls and alongside those with CD and UC when they used principal components analysis, as would be seen with an inflamed colon[19]. However, the authors did not identify why these tissues of ASD children had different transcriptional profiles; for example, they did not look for evidence of inflammation by assessing serum cytokines or fecal inflammatory markers such as calprotectin or interleukin-8. Other groups studying ASD have failed to show changes in gut biopsy cytokine levels[20] or changes in fecal calprotectin[21]. One must keep in mind that these studies were small, and measurable abnormalities were observed in a significant subset of with ASD (approximately 25% of those studied).

Enhanced T cell activation, heightened immunoglobulin and cytokine profiles, as well as histologic changes assessed in intestinal biopsies such as infiltra-

tion of lymphocytes, monocytes, natural killer cells and eosinophils have been described in children with autism[22-26]. These findings can be present in other gastrointestinal conditions such as food allergies and immunodeficiency[27]. In contrast, other laboratory measures of intestinal health, such as fecal levels of calprotectin, lactoferrin, secretory IgA, and elastase have found to be normal in children with autism[21,28]. In addition, reports of intestinal permeability (IP) in children with autism have been conflicting. Studies have reported abnormal IP in these children compared to controls[29,30]. Some have also reported increased IP to occur in first degree relatives of patients with autism *et al*[31]. In contrast, our group as well as others (mostly in small series) have found that the intestinal permeability of children with autism was not different from normal controls[17,32-34].

A recent report indicated that children with autism also have an abnormal carbohydrate digestion based on significant decrease in the expression on their intestinal biopsies of disaccharidases (sucrose-isomaltase, maltase-glucoamylase, and lactase), as well as the hexose transporters (SGLT1 and GLUT-2)[35], a finding which agreed with a previous uncontrolled study[36]. This finding was not supported by extensive observations of Kushak *et al*[37] from a center that performs many intestinal biopsies. These investigators had originally found that more than half of a group of children with autism had low levels of the enzyme lactase in duodenal biopsies[38]. However, in a follow-up study which included neurotypical controls, mucosal disaccharidase activity was not different comparing autistic and nonautistic individuals. Interestingly, even though the disaccharidases were within the normal range, the investigators found that children with ASD had evidence of mucosal inflammation on intestinal biopsy. Standard fecal indicators of gut inflammation, fecal calprotectin and lactoferrin were similar in both groups. A measure of gut permeability, lactulose/rhamnose ratio in urine after oral administration, was also not statistically different in patients with and without autism. Larger controlled studies are required to determine if the gastrointestinal symptoms in children with autism are in fact related to reproducible, "organic" findings, such as intestinal inflammation, to differences in nutrient digestion, or to an abnormal intestinal permeability[27].

FUNCTIONAL BOWEL DISEASE HYPOTHESIS

Gastrointestinal symptoms in ASD may be simply a reflection of sensory over-responsivity to abdominal signals. However, in the authors' opinion, the most common gastrointestinal complaints in children with ASD resemble those of adults and teens with functional bowel diseases such as irritable bowel syndrome (IBS). Irritable bowel syndrome is characterized by symptoms of diarrhea and/or constipation, typically with the relief of pain accompanying the passage of a stool, symptoms which fulfill the Rome Ⅲ criteria[39]. Many children with ASD have diffuse abdominal pain and an irregular stool pattern with either diarrhea or constipation, or alternating diarrhea and constipation. We have postulated that a significant proportion of children with ASD and chronic GIS, have a form of IBS. However, the Rome Ⅲ criteria are validated in adults with normal IQ but are somewhat difficult to apply to normal children, and even more so in those with ASD. When compared to GI symptom scores in ASD, which have been useful but are not validated, there is much broader experience in quantifying autistic behavior changes, such as irritability as measured by the Aberrant Behavior Checklist[40]. As mentioned, studies have shown that the presence and severity of GI symptoms correlate with the severity of underlying autism[11,28,41].

GUT MICROBIOME IN AUTISM

Trillions of microbes and 500-1000 species of microorganisms are natural inhabitants of our gastrointestinal tract, wherein the phyla *Firmicutes*, *Bacteriodetes*, and *Actinobacteria* are the most common. Anaerobic bacteria, yeasts, viruses, and bacteriophages (viruses which reside and proliferate within bacteria) also influence the gut microbial diversity[42,43]. The gut microbiome has a symbiotic interaction with the various organ systems of our body, and it is known to contribute to many GI functions, such as maintaining the integrity of the epithelial barrier, stimulating immune interactions, participating in gastrointestinal motility, and regulating drug and nutrient metabolism[44]. This normal interaction can be disturbed by a number of events, such as infections, gastrointestinal diseases, dietary changes, and neurologic disorders. Drugs such as acid suppressants, antibiotics, and corticosteroids have also been reported to perturb this homeostatic equilibrium. This dysbiosis contributes to the pathophysiology of many gastrointestinal conditions such as inflammatory bowel disease, functional gastrointestinal disease, food allergy, obesity, and liver disease[45].

The enteric microbiome of children with ASD is different from that of typically developed children. Abnormal colonization could be related to diverse factors, including a more restricted diet and exposure to more antibiotic early in life. For example, two studies found that children with ASD were more likely to be treated with antibiotics for otitis media[46,47]. Finegold *et al*[48] reported different levels of bacterial phyla in children with ASD by pyrosequencing. When comparing autistic children with controls there were changes in phyla *Firmicutes* (63% *vs* 39%, respectively), *Bacteriodetes* (30% *vs* 51%), *Actinobacteria* (0.7% *vs* 1.8%), and *Proteobacteria* (0.5% *vs* 3.1%)[48]. In a different study, this same group also reported the presence of non-spore-forming anaerobes and microaerophilic bacteria

in gastric and duodenal aspirates from children with autism, organisms which were not present in control children[48].

As mentioned, a less diverse microbial community in gut of children with autism with lower levels of some genera (*Prevotella, Coprococcus* and *Veillonellaceae*) has been reported. Interestingly, these particular species are known to be versatile carbohydrate metabolizers; and in a controlled trial, reduced colonization correlated with autistic symptoms but not with diet pattern[49]. Other differences in individuals with ASD include the overgrowth of *Clostridium* species, including *Clostridium histolyticum (*linked to the presence of GI symptoms in one study), and low levels of *Bifidobacteria*, a species known to have anti-inflammatory effects[48,50,51].

Overgrowth of other bacteria such as *Desulfovibrio* species has also been found in children with autism and their relatives, compared to controls[52]. Additionally, higher levels of *Caloramator*, *Sarcina*, *Alistipes*, *Akkermansia*, *Sutterellaceae* and *Enterobacteriaceae* were found in children with autism compared with typically developed children[53,54]. Kang *et al*[49] reported a less diverse fecal microbiome by pyrosequencing of 16S rDNA in children with autism. Despite these studies, it should be noted that when bacteria tag-encoded pyrosequencing was used, Gondalia *et al*[55] did not find differences in the gut microbiome, comparing children with autism with their siblings.

Much work needs to be done in determining the metabolic consequences of an abnormal microbiota in ASD. Bacterial by-products are the likely mediators of systemic effects that could lead to alterations in the children's behavior. Some investigators have hypothesized that the abnormal microbiota in children with ASD produces changes in behavior *via* a mechanism involving excessive production of short chain fatty acids (SCFA), such as propionate and butyrate, which represent the major anions of human feces. These SCFA can produce behavioral changes in rodents when injected into the brain ventricles or systemically *via* intermediates such as p-cresol that alter dopamine metabolism[56]. Ongoing investigations have begun to highlight the importance of SCFA in ASD[57,58].

TARGETING THE GUT MICROBIOME AS A POTENTIAL TREATMENT FOR CHILDREN WITH AUTISM

Probiotics

The internationally accepted definition of probiotics is "live microorganisms which when administered in adequate amounts confer a health benefit on the host". Dietary prebiotics are "selectively fermented ingredients that allows specific changes, both in the composition and/or activity in the gastrointestinal microflora that confers benefits upon host well-being and health". The potentially synergistic combinations of pro- and prebiotics are called synbiotics[59]. Functional bowel disorders (including IBS, functional abdominal pain, functional dyspepsia, and cyclic vomiting syndrome) are the most common conditions leading to referral of children to the pediatric gastroenterology clinic[60]. Recent evidence suggests that an abnormal fecal microbiota may play a causal or contributory role to IBS in adults[61] and children[62].

In adults with a functional GI disorders, there is accumulating evidence for a beneficial effect of probiotics. Evidence for probiotic efficacy in IBS now includes 23 randomized controlled trials (RCTs) (2575 patients) and the demonstration of improvement in global symptoms, abdominal pain, bloating and flatulence; however there was heterogeneity among the studies and authors concluded the optimal probiotic has not been identified[63]. In the most recent meta-analysis, which included 21 RCT's, a 1.82-fold (CI: 1.27-2.60) relative rate of improvement *vs* placebo was noted[64]. Fewer studies have been done in children; the only systematic review concluded that 4 probiotics were associated with improvement in symptoms in children with IBS: *L. rhamnosus GG*, *L. reuteri DSM 17938*, VSL#3, and a combination probiotic containing 3 *Bifidobacteria*[65].

The differences in the gut microbiome comparing autistic and typically developed children described in the previous section may provide a clue to the cause for GI symptoms. One early study of vancomycin, a poorly absorbed antibiotic known to destroy *Clostridia* and other gram positive organisms, demonstrated an improvement in diarrhea and more normal behavior, as evidenced by videotape, when vancomycin was given short-term[66]. As mentioned, the gut microbiome can be altered by the use of antibiotics, prebiotics, probiotics, or synbiotics (prebiotics plus probiotics) administered by physicians or parents to ameliorate symptoms in children with ASD[57,67-69].

Virtually all of the GI functions postulated to be impaired in ASD have been shown to be improved by probiotics in animal studies. For example, we previously found that a human breast milk and gut commensal, *Lactobacillus. reuteri,* when fed daily, reduced lipopolysaccharide (LPS)-induced intestinal inflammation[70]. In newborn rat pups, another probiotic, *Bifidobacterium bifidum* reduced gut permeability across the tight junctions that "seal together" the epithelial cells in a model of necrotizing enterocolitis[71]. A recent study by Buffington *et al*[72], which aimed to study mechanisms of abnormal behavior in autism, utilized a maternal high fat diet to induce abnormal social (withdrawal) behavior in the offspring. It is worthy to mention that in humans, too, maternal obesity[73,74], and maternal diabetes[75] been shown to be linked to autism in the offspring. In the mice, high-fat maternal diet produced changes in neurotransmission in the hypothalamus of the newborns. Abnormal behavior was found to be correctable by co-housing "autistic pups" with normal infant pups whose mothers did not take a high fat diet,

Table 1 Evidence supporting a role for probiotics in treating gastrointestinal symptoms in autism spectrum disorders

Clinical symptoms	Ref.
Children with ASD have an abnormal fecal microbiota	[28,35,48,51,54,98-100]
GI symptoms common in ASD are similar to those in IBS	[11,12]
IBS also is associated with an abnormal fecal microbiota	[61,62,101]
Meta-analysis shows IBS symptoms are improved by probiotic treatment. (Preliminary evidence suggests potential benefits in ASD in children and rodents models.)	[65,72,102-104]
Mild inflammation in the GI tact may be seen in children with ASD. (There is evidence to support or refute this contention: abnormal duodenal and ileal biopsies and high plasma S100A9 but normal fecal calprotectin and lactoferrin levels)	[19,22-26,31,37]
Probiotics reduce gut inflammation (Shown in animal models and in human diseases)	[70,105-108]
Systemic inflammation can be also seen in children with ASD	[18,109-111]
Immune modulation of children with ASD may reduce clinical symptoms	[41,112]

ASD: Autism spectrum disorders; GI: Gastrointestinal; IBS: Irritable bowel syndrome.

indicating a microbial effect which was evidenced by a change in microbiota. Following this hypothesis, the authors found that by administering a probiotic, *Lactobacillus reuteri*, the antisocial behaviors and aberrant neurotransmission could be reversed[72].

The lay press and internet have certainly embraced the concept that gut bacteria are linked to autism. A particularly fascinating recent publication from Pärtty *et al*[76] randomized 75 infants at birth to a supplement of *Lactobacillus rhamnosus GG (LGG)* or placebo for the first 6 mo of life and measured microbiota and psycho-behavioral diagnoses 2 and 13 years later. They found no major changes in microbiota. However, at the age of 13, 17% of the children treated with placebo had attention deficit disorder or Asperger's syndrome, compared to none who received *LGG*.

Recent reviews concluded that probiotics should be studied in children with ASD[50,77]. Our interpretation of the rationale for probiotic investigation in ASD is summarized in Table 1. However, it is controversial whether oral probiotics can produce positive effects in such a complex condition. Currently available probiotics are mainly aerobic, derived from milk cultures, not normally a significant part of the human gut microbiome which are primarily anaerobic; and they are short-lived in the human gut. Kristensen *et al*[78] looked at normal humans given probiotics and showed in a meta-analysis of 6 RCTs limited to adults that there was no change in alpha-diversity (number of species) or evenness with probiotic treatment. One trial did show a change in beta-diversity (relative contributions of the various species)[78]; however, virtually all studies which have shown changes in fecal microbial composition during probiotic administration were done in babies, for example preterm infants[79,80]. One study that did show that a probiotic could alter the fecal microbiota focused on older children with cystic fibrosis[81] and another showed changes in adults with alcoholic cirrhosis[82]. Most of these trials used quantitative polymerase chain reaction (PCR), rather than 16S ribosomal RNA gene sequencing. Using 16S rRNA techniques, we[83] and others[78] have not shown differences in microbial composition in adults treated with probiotics. The same lack of effect on the infant's fecal microbiome was observed in a number of studies of infants whose mothers were treated with probiotics before birth and/or during breast feeding[84-86].

Therefore, alternative mechanisms may account for potentially beneficial effects of probiotics in IBS and possibly ASD. An important alternative mechanism by which a probiotic be beneficial is *via* the metabolites that these organisms release in the gut lumen which may reach the circulating blood. A number of studies have shown abnormal fecal metabolites, such as short chain fatty acids (SCFA) related to changes in microbiota[87]. Para-cresol (a phenolic compound) has been suggested to be a urinary marker for autism[88], especially in those with constipation and ASD[89]. In a mouse model of autism induced by maternal immune activation, autistic behaviors such as communication abnormalities, stereotypies, and anxiety behaviors were associated with abnormal serum metabolities produced by the microbiota, including 4-ethylphenyl sulfate (the major metabolite) and p-cresol (to a lesser extent)[57]. These abnormalities and some of the behaviors were improved by giving orally a human commensal *B. fragilis* (not traditionally viewed as a probiotic). In a biomarker discovery study in 52 young children with ASD who were compared to neurotypical controls, a number of plasma markers were found to be altered, many of them were directly related to mitochondrial metabolism. These included elevated succinic acid, aspartate, glutamate, and aminoisobutyrate and decreased citric acid, isoleucine, and creatinine[90].

Despite these gaps in our knowledge regarding "if and why" probiotics may work in autism, in a recent survey of more than 500 physicians who treat children with autism, 19% reported using probiotics[91]. Many autism websites also advocate treatment of children with ASD with probiotics. These recommendations are not evidence-based. A recent review summarized the existing 4 trials of probiotics for ASD[92]. There were methodological difficulties in most; for example, one was a case-control study that had a high risk of selection bias which showed improvement in mental concentration (but not in behavior) in ASD patients treated with *Lactobacilus acidophilus*[93]. Another manuscript which was included as part of a retrospective

case-cohort analysis, reported that probiotic treatment improved an autism treatment evaluation checklist, although the authors did not report which probiotics were given and which dose[28]. A third study was a double-blind placebo-controlled crossover trial which reported reduced disruptive behavior, anxiety and communicative disturbance when the children were on probiotic (*Lactobacillus plantarum)* but is not readily available in reference libraries[94]. A 4th study reported beneficial effects of a 4-mo treatment with a combination probiotic (comprising 3 *Lactobacilli*, 2 *Bifidobacilli*, and 1 *Streptococcus* species). In this latter study, the probiotic increased the qPCR-determined ratio of fecal *Bifidobacilli* to *Firmicutes* and total *Lactobacilli,* while reducing fecal *Clostridia* and fecal tumor necrosis factor (TNF)-alpha levels. This latter study did suggest beneficial effects on the microbiome, although effects of this combination probiotic on autistic behaviors were not reported[77].

Fecal microbiota transplantation

In children and adults with severe gastrointestinal diseases, such as *Clostridium difficile (C. difficile)*-associated colitis or inflammatory bowel disease, fecal microbiota transplantation (FMT) had the potential for more significant and prolonged effects. FMT was effective in many cases of antibiotic-associated *C. difficile* colitis and is now used around the world for severe or multiply recurrent *C. difficile* infection, and it may have a role in the treatment of inflammatory bowel disease (particularly Crohn's disease) and autoimmune conditions. However, fecal transplantation carries many risks, including aspiration, transmission of norovirus, bacteremia, induction of obesity, and possible transmission of autoimmune conditions, including rheumatoid arthritis and Sjogren's syndrome[95,96]. We do not believe this treatment will have a role in the treatment of gastrointestinal symptoms in autism, although there may be successful reductionist approaches, for example combinations of defined communities of culturable commensal organisms, such as those used in the "RePOOPulate" studies in Canada, in which 33 carefully selected isolates from healthy donors were able to eradicate *C. difficile* from patients who had encountered multiple recurrences[97].

CONCLUSION

Gastrointestinal symptoms in children with autism are common and are often linked to the children's abnormal behavior and social interactions. Probiotics are hypothesized to positively impact gut microbial communities and alter the levels of specific potentially harmful metabolites in children with ASD. Whether probiotics improve behavior and these markers has yet to be determined. Although the evidence presented in this review does not confirm benefit of probiotics in this population, it provides a solid rationale for the design of larger prospective trials.

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REVIEW

Thiopurines and inflammatory bowel disease: Current evidence and a historical perspective

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Abstract

The use of thiopurines in inflammatory bowel disease (IBD) has been examined in numerous prospective, controlled trials, with a majority demonstrating a clinical benefit. We conducted this review to describe the historical and current evidence in the use of thiopurines in IBD. A systematic search was performed on MEDLINE between 1965 and 2016 to identify studies on thiopurines in IBD. The most robust evidence for thiopurines in IBD includes induction of remission in combination with anti-tumor necrosis factor (anti-TNF) agents, and maintenance of remission and post-operative maintenance in Crohn's disease. Less evidence exists for thiopurine monotherapy in induction of remission, maintenance of ulcerative colitis, chemoprevention of colorectal cancer, and in preventing immunogenicity to anti-TNF. Evidence was often limited by trial design. Overall, thiopurines have demonstrated efficacy in a broad range of presentations of IBD. With more efficacious novel therapeutic agents, the positioning of thiopurines in the management of IBD will change and future studies will analyze the benefit of thiopurines alone and in conjunction with these new medications.

Key words: Inflammatory bowel disease; Ulcerative colitis; Crohn's disease; Thiopurines; Mercaptopurine; Azathioprine

Core tip: In this review, we systematically describe the historical and current evidence in the use of thiopurines in inflammatory bowel disease (IBD). The most robust evidence for thiopurines in IBD includes induction of remission in combination with anti-tumor necrosis factor agents, and maintenance of remission and post-operative maintenance in Crohn's disease. With more effective and newer therapeutic agents, the positioning of thiopurines in the management of IBD

will change. Future studies should examine the benefit of thiopurines alone and in conjunction with these novel agents.

Axelrad JE, Roy A, Lawlor G, Korelitz B, Lichtiger S. Thiopurines and inflammatory bowel disease: Current evidence and a historical perspective. *World J Gastroenterol* 2016; 22(46): 10103-10117 Available from: URL: http://www.wjgnet.com/1007-9327/full/v22/i46/10103.htm DOI: http://dx.doi.org/10.3748/wjg.v22.i46.10103

INTRODUCTION

Historically, the use of thiopurines, mercaptopurine and azathioprine, purine antagonists which inhibit DNA and RNA synthesis (Figure 1), in the treatment of inflammatory bowel disease (IBD) was based upon their efficacy in other autoimmune disorders, including systemic lupus erythematous and rheumatoid arthritis[1]. The efficacy of thiopurines in both Crohn's disease (CD) and ulcerative colitis (UC) has been documented in prospective, double-blind, placebo-controlled trials, with data supporting their beneficial therapeutic effects in inducing and maintaining disease remission, postoperative maintenance in CD, and chemoprevention of colorectal cancer (CRC)[2-6]. In addition, the medication has taken on an important role in conjunction with anti-tumor necrosis factor (TNF) therapy by interfering with antibody production[7].

Despite this evidence demonstrating the efficacy of thiopurine agents, there exists a hesitation with their clinical use. This may be based upon the fact that some of the trials were withdrawal designed[8], pediatric-based[9], recruited a small number of patients, or utilized a scoring system not universally accepted[10]. Furthermore, their role in infectious as well as malignant complications has been scrutinized.

In this review, undertaken after the passing of Dr. Daniel Present, we will review the historical basis, current evidence, and clinical experience in the use of thiopurines at various stages of IBD. We will also comment on how its use has changed over time and postulate on its positioning in the future. Lastly, this review will be accompanied by an experience overview by Dr. Daniel Present and Dr. Burton Korelitz, co-investigators on the seminal paper on the use of thiopurines in IBD[10].

For completeness, we conducted a systematic electronic search for relevant full-text articles in English using the MEDLINE database between January 1, 1965 and January 1, 2016. We used search terms associated with IBD and thiopurines, including "inflammatory bowel disease", "Crohn's disease", or "ulcerative colitis" in combination with "thiopurines", "azathioprine", "mercaptopurine", and "6-mercaptopurine". Reference lists from retrieved studies and review articles were examined to identify additional studies of relevance. Preference was given to high impact articles with randomized trial designs.

THIOPURINES FOR INDUCTION OF REMISSION IN CD

A number of controlled clinical trials have investigated the efficacy of thiopurines in the treatment of active CD. The results of the four earliest trials were published in the 1970s[11-14]. The first three studies investigating azathioprine were small (enrolling 12-16 patients), utilized varying doses of drug (ranging from 2 to 4 mg/kg/d), and followed patients for a maximum of 24 wk. The response rates in these studies varied from 36%-100%. The largest of these initial trials was reported by Summers *et al*[13] in 1979, and involved a 17-wk randomized, double-blind, placebo-controlled trial of azathioprine 2.5 mg/kg/d in 136 patients with active CD (defined as a Crohn's Disease Activity Index (CDAI) score > 150). The rates of remission (CDAI < 150 at week 17) with azathioprine (36%; 21/59) were superior to placebo (26%; 20/77), although not at the level of statistical significance.

The first long-term study to demonstrate the efficacy of mercaptopurine to induce remission was reported by Present *et al*[10] in 1980. Eighty-three chronically ill patients with CD were entered into a two-year double-blind study comparing mercaptopurine 1.5 mg/kg with placebo. Crossover data showed that improvement occurred in 67% of courses of mercaptopurine compared with 8% of courses of placebo ($P < 0.001$). Mercaptopurine was also found to be more effective than placebo in fistula closure and steroid reduction and discontinuation. Importantly, this trial was the first to establish the notion of the delayed onset of action, as the mean time to response was 3.1 mo, with 89% of responders doing so within 4 mo of starting mercaptopurine.

In the last two decades, all studies investigating the efficacy of thiopurines in inducing remission in CD have utilized active comparator groups rather than placebo alone. In a three-arm randomized, double-blind study comparing mercaptopurine 50 mg daily, oral methotrexate 12.5 mg weekly, and placebo in patients with active CD and Harvey-Bradshaw Index (HBI) $\geqslant$ 7, Oren *et al*[15] showed that the rates of remission (HBI $\leqslant$ 3 without steroids) using mercaptopurine or placebo were equivalent (9/32 in the mercaptopurine arm *vs* 6/26 in the placebo arm). This remission rate was not significantly different when compared to the methotrexate arm. In a similar study involving methotrexate, Maté-Jiménez *et al*[16], studied 38 patients with steroid-dependent CD who were randomized to mercaptopurine 1.5 mg/kg/d, methotrexate 15 mg/wk, or 5-aminosalicylic acid (5-ASA) 3 g/d. Compared with the 5-ASA group (14% remission), patients in both the mercaptopurine (93.7%) and methotrexate (80%) arms had statistically higher rates of remission. Finally,

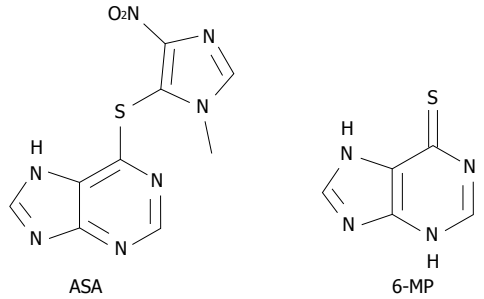

Figure 1 Chemical structures of azathioprine and mercaptopurine. AZA: Azathioprine; 6-MP: 6-mercaptopurine.

in a recent trial comparing azathioprine to methotrexate, patients with steroid-dependent CD with a CDAI $\geqslant$ 200 were treated with either intravenous methotrexate 25 mg/wk or oral azathioprine 2 mg/kg/d for a 6-mo period (in addition to a 12-wk prednisolone taper starting at 40 mg daily). The primary outcome - the proportion of patients entering first remission (CDAI < 150 without steroids) at 3 and 6 mo of therapy - was statistically similar between the two treatment groups (44% remission rate at 3 mo with methotrexate *vs* 33% with azathioprine; 56% remission rate at 6 mo with methotrexate *vs* 63% with azathioprine)[17].

A recent trial evaluating the effectiveness of thiopurines for the induction of remission in Crohn's disease was reported in 2010 by Colombel *et al*[18] in the Study of Biologic and Immunomodulator Naive Patients in Crohn's Disease (the SONIC trial). The results of this study showed azathioprine to be less effective than infliximab as an induction agent for CD. Patients with active CD (CDAI 220-450) were randomized to one of three treatment arms: infliximab 5 mg/kg, azathioprine 2.5 mg/kg/d, or a combination of infliximab and azathioprine. Thirty-two percent (54/170) of azathioprine patients achieved clinical remission (CDAI < 150) at week 26 compared to 48% (81/169) of infliximab patients [risk ratio (RR) = 0.66, 95%CI: 0.51-0.87]. Similarly, significantly more infliximab patients than azathioprine patients achieved the primary study outcome of steroid free remission (44% *vs* 30%, respectively, $P = 0.006$). When assessing the combination of azathioprine and infliximab, significantly more patients in the combination therapy group (60%; 102/169) achieved clinical remission compared to patients treated with infliximab alone (48%) or azathioprine alone (32%, $P < 0.001$). Although, patients with heterozygous thiopurine methyl transferase (TPMT) activity were excluded, potentially minimizing the success of azathioprine monotherapy.

In addition, two randomized trials have found azathioprine therapy ineffective in achieving sustained corticosteroid-free remission. In an open-label trial of adults with CD for less than 6 mo at risk for disabling disease. patients randomly assigned to treatment with azathioprine 2.5 mg/kg/d were no more likely to experience clinical remission compared to patients who received azathioprine only in cases of corticosteroid dependency, chronic active disease with frequent flares, poor response to corticosteroids, or development of severe perianal disease[19]. In a prospective double-blind trial of patients with CD for less than 8 wk, patients randomly assigned to azathioprine 2.5 mg/kg/d were no more likely to achieve sustained corticosteroid-free remission compared to patients randomized to placebo (44.1% *vs* 36.5%), however, azathioprine was more effective in preventing moderate to severe relapse in a *post hoc* analysis[20].

The most recent Cochrane analysis (2013) evaluating the efficacy of thiopurines for induction of remission in CD compiled the results from 13 randomized control trials including 1211 patients: 9 comparing thiopurines to placebo and 6 using active comparators[21]. This analysis found no statistically significant difference in clinical remission rates between thiopurines and placebo (48% *vs* 37%, respectively, when combining the data from 5 studies with 380 total patients; RR = 1.23, 95%CI: 0.97-1.55). Thiopurine therapy was found to be no better at inducing steroid-free remission compared to methotrexate (RR = 1.13, 95%CI: 0.85-1.49) and 5-ASA or sulfasalazine (RR = 1.24, 95%CI: 0.80-1.91). Lastly, azathioprine was found to be significantly inferior to infliximab for induction of steroid-free clinical remission (RR = 0.68, 95%CI: 0.51-0.90). The only benefit of azathioprine for inducing remission in CD was found when it was used in combination with infliximab, as combination therapy was significantly superior to infliximab alone for induction of remission.

THIOPURINES FOR MAINTENANCE OF REMISSION IN CD

Dating back to the 1970s, multiple controlled trials have evaluated the efficacy of azathioprine and mercaptopurine for maintenance of remission in CD. While several earlier studies compared azathioprine or mercaptopurine to placebo, the more recent trials have used active comparators.

The first randomized, double-blind, placebo-controlled trial of azathioprine for therapy of CD was reported by Willoughby *et al*[14], in 1971. This small study aimed to determine the effect of azathioprine 2 mg/kg/d on maintaining remission of CD once it had been induced by prednisolone alone or in combination with azathioprine. At the end of the 24-wk study period, 4/5 patients on azathioprine maintained remission compared to 2/5 on placebo.

Over the course of the following three decades, multiple additional trials compared azathioprine to placebo[6,13,14,22-25]. Although these trials varied in their duration of therapy and dose of azathioprine (ranging from 1 mg/kg/d to 2.5 mg/kg/d), each added to the growing body of literature exploring the use of azathioprine for maintaining remission in CD. A

2015 Cochrane review presented a pooled analysis of the results from these seven studies[6]. In total, 532 patients were included, and there were a statistically higher proportion of patients who maintained remission over 6 to 18 mo with azathioprine compared to placebo. While only 58% (168/288) of patients on placebo maintained remission at study endpoints, 72% (175/244) of azathioprine patients were in remission (RR = 1.25, 95%CI: 1.11-1.42).

Two studies have compared azathioprine or mercaptopurine to 5-ASA or sulfasalazine for the maintenance of remission in CD. In 1979, Summers *et al*[13], reported the results from 86 patients with medically or surgically induced CD remission who were randomized to placebo, sulfasalazine, prednisone, or azathioprine (1 or 2.5 mg/kg/d). Over the course of one year, both patients in the azathioprine arm and sulfasalazine arm had similar rates of maintaining remission: 76% (53/73) azathioprine *vs* 68% (52/77) placebo. A second study by Maté-Jiménez *et al*[16] in 2000 reported the results from a maintenance trial in which CD patients in remission were randomized to either mercaptopurine 1 mg/kg/d or 5-ASA 3 g/d for 45 wk of therapy. Though mercaptopurine was superior to 5-ASA in maintaining remission (53% *vs* 0%), the numbers of patients in each study arm were very small. A pooled analysis of the results of these two trials found no difference between azathioprine or mercaptopurine and 5-ASA or sulfasalazine in the proportion of patients who maintained remission at 12 mo (RR = 1.09, 95%CI: 0.88-1.34)[6].

One study each has compared thiopurines to budesonide and methotrexate. In 2009, Mantzaris *et al*[24] suggested azathioprine (2.0-2.5 mg/kg/d) to be superior to budesonide (6-9 mg/d) in maintaining CD remission. In this prospective, randomized, controlled one-year trial including patients with steroid dependent CD in remission (CDAI < 150), 76% (29/38) of patients in the azathioprine arm maintained remission at one year compared to 46% (18/39) in the budesonide arm (RR = 1.65, 95%CI: 1.13-2.42). In contrast to these positive findings, Maté-Jiménez *et al*[16] found no difference in the rates of maintaining remission between mercaptopurine (50%; 8/16) and methotrexate (53%; 8/15) at 76 wk.

Finally, the most recent trial investigating the role of azathioprine in maintaining remission in CD utilized azathioprine in combination with infliximab and compared maintenance rates with infliximab alone[25]. In this study reported by Mantzaris *et al*[24], 47 patients with active, steroid-dependent CD were induced with tapered steroids along with infliximab 5 mg/kg at weeks 0, 2, and 6, or combination infliximab and azathioprine 2.5 mg/kg for 6 wk. Those entering remission were then continued into the maintenance phase of the trial where they were treated with either infliximab alone (5 mg/kg every 8 wk) or combination infliximab and azathioprine 2.5 mg/kg for 12 mo. The rates of maintaining remission at one year were statistically similar in the combination therapy group (81%; 13/16) and the infliximab monotherapy group (80%; 16/20) (RR = 1.02, 95%CI: 0.74-1.40).

Given the cumulative results of the trials that have been conducted, a recent Cochrane review concluded that azathioprine is more effective than placebo for maintenance of remission in CD[6]. Similarly, azathioprine may be superior to budesonide for maintenance of remission, although this conclusion is based on the results of only one small study. Finally, thiopurines have not yet been rigorously compared to other active maintenance therapies, including infliximab, and more adequately powered trials are necessary to allow for definitive conclusions.

THIOPURINES FOR MAINTENANCE OF SURGICALLY INDUCED REMISSION IN CD

Approximately two thirds of CD patients will require at least one intestinal resection, and more than 50% will still require at least one additional surgery in their lifetime[23,26,27]. It is thus imperative to optimize post-operative prophylactic management strategies to reduce this risk. The role of thiopurines in post-operative prevention of CD recurrence will be discussed here.

A Cochrane analysis by Gordon *et al*[5] in 2014 embodies the most up to date, extensive review of thiopurine use as a preventative measure following intestinal resection. However, heterogenous study design by the trials in question makes the data less clear. Seven randomized controlled trials involving thiopurines were identified - four compared to 5-ASA[28-33], one compared to 5-ASA and adalimumab[34], one to infliximab[27], and one to placebo alone[35].

Thiopurine use overall appears to reduce post-operative recurrence risk when compared to placebo (12 mo endoscopic recurrence risk of 43.7% for azathioprine compared to 69% for placebo)[35]. However, efficacy outcomes when compared to 5-ASA agents were less clear, and did not demonstrate superiority of one modality over the other[28-30,33]. However, Reinisch *et al*[33] noted that perceived lack of efficacy may relate to suboptimal dosing strategies (with dosing based on metabolite levels providing better remission rates). Only two controlled trials addressed the utility of anti-TNF as compared to thiopurines, and results were conflicting[27,34].

Subsequent to the Cochrane analysis above, three randomized-controlled trials sought to examine the impact of anti-TNF therapy on post-operative disease recurrence, and indirectly addressed questions of thiopurine efficacy and optimization. Regueiro *et al*[36] randomized patients to either anti-TNF therapy or placebo after resection, and patients were instructed to otherwise continue pre-operative therapy. Forty-six

percent were taking a thiopurine throughout. Within the placebo arm, 100% of patients without thiopurine exposure experienced endoscopic disease recurrence compared to 71.4% of those on thiopurine monotherapy, with a 28.6% reduction in recurrence (P = 0.08; the study was not powered to assess thiopurine efficacy). Of note, combination therapy of thiopurine and infliximab was not significantly more effective than infliximab monotherapy, with endoscopic recurrence rates of 27.8% and 18.5% respectively.

De Cruz *et al*[37] published data from the Post-Operative Crohn's Endoscopic Recurrence study in which patients at medium or high risk patients for postoperative recurrence of disease were then treated with adalimumab, thiopurine, or none of these agents. All patients received metronidazole initially and disease assessment followed with a "standard" or "active" assessment pathway, the comparison of which was the basis of the study[37]. Adalimumab did not appear significantly more effective at reducing short-term disease recurrence risk (43% endoscopic recurrence in adalimumab arm compared to 61% recurrence in mercaptopurine arm at 12 mo, P = 0.17). Notably, patients in the post-operative thiopurine who were on thiopurines prior to resection had similar outcomes to those who were pre-operatively thiopurine naïve, suggesting that "failing" a thiopurine preoperatively is not necessarily a contraindication for post-operative thiopurine use.

Finally, at the 2016 European Crohns and Colitis Organization annual meeting, initial data from the Trial of Prevention of Post-operative Crohn's Disease was presented; the largest double-blind placebo-controlled trial to date, comparing mercaptopurine to placebo for up to 36 mo postoperatively in 240 patients. The primary endpoint, CDAI evidence of recurrence, was reached in 23.2% of the placebo arm compared to 12.5% of the mercaptopurine arm (adjusted P = 0.073, unadjusted 0.046). Out to week 157, a higher proportion of mercaptopurine patients maintained endoscopic remission (Rutgeerts i0) than placebo (22.5% *vs* 12.5%, P = 0.041). In subset analysis, superiority of mercaptopurine over placebo was seen in smokers [hazard radio (HR) = 0.127, 95%CI: 0.04-0.46; number-needed-to-treat (NNT) = 3], but not in non-smokers.

Thiopurines appear to have a role in the prevention of recurrence of disease following intestinal resection. Consistently more effective than placebo, the utility of thiopurines when compared to mesalamine therapy is less clear, and may reflect a need to dose adjust according to serum thiopurine metabolite levels. The benefits of thiopurines use when compared to or in conjunction with anti-TNF would ideally be stratified in clinical practice according to the patient's risk of recurrence and prior management strategies.

THIOPURINES FOR INDUCTION OF REMISSION IN UC

While there are several randomized controlled studies regarding the efficacy of thiopurines in inducing remission in CD, there are considerably fewer high quality controlled studies in UC. The first controlled trials evaluating the effectiveness of thiopurines in UC were published in the 1970s[38,39]. In a small study of 20 patients with active proctocolitis, azathioprine 2.5 mg/kg/d produced significant improvement in clinical symptoms, inflammatory markers, and endoscopic and biopsy findings, but was not superior to sulfasalazine over a 3 mo period[38]. A similar study in 80 patients with flare of UC showed no benefit from the addition of azathioprine 2.5 mg/kg/d over 1 mo compared to a standard course of corticosteroids[39].

In a study by Sood *et al*[40], 83 patients with severe UC, steroid dependent on prednisone 1 mg/kg/d and sulfasalazine 6-8 g/d, were randomized to azathioprine or placebo with similar remission rates between azathioprine and placebo arms (68% and 64%, respectively). In a small trial on 34 patients with UC receiving prednisone for induction therapy, subjects randomized to additionally receive 1.5mg/kg/d of mercaptopurine were more likely to achieve steroid-free remission and a Mayo Clinic score less than 7 (78.6%) compared to patients randomized to additionally receive 3 g/d of 5-ASA (25%) over a 7.5 mo induction phase[16].

In an investigator-blind study by Ardizzone *et al*[41], 72 patients with steroid dependent, clinically and endoscopically active UC on prednisolone 40 mg/d were randomized to azathioprine 2 mg/kg/d or oral 5-ASA 3.2 g/d. At 6 mo, patients in the azathioprine group were more likely to experience corticosteroid-free, clinical and endoscopic remission compared to 5-ASA, both in intention-to-treat [odds ratio (OR), 4.78; 95%CI: 1.57-14.5] and per-protocol (OR = 5.26; 95%CI: 1.59-18.1) analysis[41].

In a meta-analysis of thiopurine induction therapy for remission of UC, the efficacy rate was 73% (95%CI: 63%-83%) for azathioprine and 64% (95%CI: 53-75%) for placebo or 5-ASA with an insignificant OR, of 1.59 (95%CI: 0.59-4.29)[42]. This efficacy rate decreased slightly to 65% (95%CI: 55-75%) based on large number of heterogeneous, open uncontrolled, and retrospective studies. Most of these studies, both controlled and uncontrolled, examined very small numbers of patients and differed significantly in study design, patient selection, classification of steroid dependence, thiopurine dosage, duration of induction, evaluation of response, follow up, and handling of concomitant treatments[42-46].

More recently, thiopurines are being positioned as part of combination therapy with TNF-α antagonists as induction therapy. In the UC SUCCESS randomized, double-blind trial of 239 patients with moderate to

severe UC, 39.7% (31 of 78) of patients receiving infliximab 5 mg/kg intravenous at weeks 0, 2, 6, and 14 with azathioprine 2.5 mg/kg/d, achieved corticosteroid-free remission at 16 wk compared with 22.1% (17 of 77) of patients receiving infliximab alone (P = 0.017) and 23.7% (18 of 76) of patients receiving azathioprine alone (P = 0.032)[47]. Rates of mucosal healing were also greater in patients exposed to combination therapy with infliximab and azathioprine compared to azathioprine monotherapy[47].

In lieu of the above studies, there is not clear evidence for the use of thiopurine monotherapy in UC induction, however, there may be evidence for thiopurines in combination with other immunosuppressive agents to improve the likelihood of inducing remission.

THIOPURINES FOR MAINTENANCE OF REMISSION IN UC

In one of the first controlled trials of azathioprine for maintenance, Jewell *et al*[39] (1974) followed 80 patients admitted for their first attack of UC, deriving no benefit from one-year maintenance with azathioprine 2.5 mg/kg/d[39]. Though azathioprine therapy appeared to reduce the relapse rate in patients who presented with flare of established UC, data failed to reach statistical significance.

In 1992, Hawthorne *et al*[48] examined the role of azathioprine in the maintenance of remission of UC in 67 patients and in 12 patients with active UC or corticosteroid dependence over the course of a year. Patients in remission and randomized to receive or continue azathioprine at a median dose of 100 mg/d had a relapse rate of 36% (12/33) compared with 59% (20/34) for patients given placebo (HR = 0.5; 95%CI: 0.25-1.0). After adjusting for sex, age, duration of remission and treatments prior to study entry, continued azathioprine therapy demonstrated a statistically significant benefit over withdrawal (HR = 0.43; 95%CI: 0.2-0.93)[48]. For patients with active disease at randomization, there was no benefit from continued azathioprine therapy[48].

In another study of 34 patients with UC, patients who achieved remission after induction with corticosteroids and maintained on mercaptopurine monotherapy for 76 wk were more likely to maintain remission (63.6%) compared to methotrexate (MTX) 15 mg/wk (14.3%) or 3 g/d of 5-ASA (0%)[16]. In a small study of 45 patients with steroid-dependent UC, patients randomized to sulfasalazine 6-8 g/d, oral prednisolone 1 mg/kg/d, and azathioprine 2 mg/kg/d had fewer relapses of disease over one year compared to sulfasalazine 6-8 g/d and oral prednisolone 1 mg/kg/d as corticosteroids were tapered over 12-16 wk[40].

In a similar study, 35 patients with newly diagnosed severe UC and randomized to sulfasalazine with azathioprine were significantly less likely to experience relapse of disease (4; 23.5%) compared to sulfasalazine with placebo (10; 55.6%) over one year[49]. Moreover, maintenance of remission was significantly longer in patients given azathioprine with sulfasalazine compared to controls[49]. However, in a follow up prospective, randomized, open-label study from the same institution on 25 patients with severe UC on oral corticosteroids tapers, no difference was detected in relapse rates between patients given azathioprine 2.5 mg/kg/d compared to sulfasalazine 6 g/d[50].

There are several other observational, retrospective, and nonrandomized trials evaluating thiopurine therapy in UC maintenance therapy[41,51-57]. Data from a meta-analysis demonstrated an overall mean efficacy of 65% (95%CI: 62%-67%), with a mean efficacy of 66% (95%CI: 59%-73%) for steroid-resistant patients and 71% (95%CI: 66%-77%) for steroid-dependent patients[42]. In evaluating fairly homogenous controlled studies encompassing 124 patients, mean efficacy was 60% (95%CI: 51%-69%) for thiopurine therapy and 37% (95%CI: 28%-47%) for placebo and 5-ASA with an OR, of 2.56 (95%CI: 1.51-4.34) for maintaining remission[42]. The authors also calculated an absolute risk reduction of 23% with a NNT with thiopurines of 5 to prevent one relapse of disease[42].

According to a Cochrane analysis (2012) designed to assess failure to maintain clinical or endoscopic remission at 12 mo, based on 4 studies and 232 patients, there is low-quality evidence that azathioprine is superior to placebo for maintenance of remission in UC with a RR, of 0.68 (95%CI: 0.54-0.86)[2]. However, given varied treatment schedules, there was not enough evidence to determine an effect by dose or with combining medications. In this analysis, this pooled risk ratio suggested a NNT with azathioprine of 5 to prevent one relapse of disease, with an attributable risk reduction of 21%[2]. The authors of this analysis did not feel the existing data supported any meaningful evidence for thiopurines over 5-ASA agents or sulfasalazine, with an insignificant RR, of 1.52 (95%CI: 0.66-3.50)[2].

Overall, there is a lack of high quality trials evaluating the use of thiopurine therapy in maintenance of remission in UC. Existing evidence does not support thiopurine use alone or in combination with standard 5-ASA as compared to standard maintenance with 5-ASA therapies alone for remission in UC. Thiopurines may, however, be useful in patients who cannot tolerate 5-ASA therapies or in patients who require repeated courses of corticosteroids to induce remission. Considerably more data is required to further evaluate the use of thiopurines for maintenance of remission in UC compared to standard maintenance therapy, particularly in the era of biologics.

THIOPURINES IN CHEMOPREVENTION

In patients with IBD, chronic intestinal inflammation is a major risk factor for the development of gastrointestinal malignancy. In a meta analysis, quantitative

estimates of CRC risk in UC have been reported to be 2% after 10 years, 8% after 20 years, and 18% after 30 years of disease[58]. More recent data (2015) has found a cumulative risk of advanced neoplasia in UC of 2%, 5.3% and 14.7% at 10, 20 and 30 years, respectively[59]. In addition, numerous cohort studies on CRC in UC have noted a relationship between CRC risk with the extent of disease, with a standardized incidence ratio (SIR) of 1.7 for proctitis, 2.8 for left-sided colitis, and 14.8 for pancolitis[60]. In a population-based cohort study, patients with CD were also shown to be at an increased risk of CRC, with a pooled SIR of 1.9 (95%CI: 1.4-2.5)[61].

Although it has recently been suggested that the risk of CRC in IBD may be overestimated and more recent population-based studies in UC have demonstrated a decreasing risk of CRC over the past several decades, it is clear that colonic inflammation is a major risk factor for CRC[62]. Based on this risk, many studies have the examined the potential chemopreventive benefits of medications that reduce inflammation in IBD, with thiopurines being the most reported in the literature. However, there is conflicting data regarding the chemoprophylaxis effects of thiopurines on the risk of dysplasia and CRC in IBD.

The first study to address this question was published in 1994 from a prospective registry of 755 patients with IBD from the St. Mark's Hospital, London[63]. In this cohort, 15 patients with UC developed CRC with no significant modification by thiopurine exposure. In a larger study of 364 cases of CRC and 1172 matched controls, thiopurine use in the 12 mo preceding a diagnosis of CRC was not protective compared to unexposed controls (OR = 1.35, 95%CI: 0.92-1.98)[64]. In another retrospective study on 315 patients from the Mount Sinai Hospital, New York, who underwent surveillance colonoscopy, 96 (30.5%) were exposed to thiopurines for an average duration of 7.4 years at an average dose of 60.6 ± 19.5 mg/d of mercaptopurine equivalents. There was no protective effect of thiopurine exposure on colorectal neoplasia with 16% of patients exposed to thiopurines and 18% of those unexposed progressing to any neoplasia (HR = 1.06, 95%CI: 0.59-1.93) with 5% of patients in each group developing advanced neoplasia (HR = 1.30, 95%CI: 0.45-3.75)[65]. Similarly, in a study of 188 patients with UC-related colorectal cancer and matched controls, there was no association between thiopurine use and colorectal cancer (OR = 2.1, 95%CI: 0.7-7.2)[66]. Several other studies have produced similar findings, confirming a lack of benefit for thiopurines in preventing CRC[43,67-74].

More recent data, however, has suggested a potential role for thiopurines in chemoprevention. In a nationwide nested case-control study from a Dutch pathology database of 173 cases of IBD-related CRC and 393 matched IBD controls, patients treated with thiopurines were less often diagnosed with CRC compared with those never treated with thiopurines with an OR, of 0.36 (95%CI: 0.16-0.36)[75]. In a Dutch insurance-based cohort study of 2578 patients with IBD comprising 16289 person-years of follow-up, those who had used $\geqslant$ 50 mg of thiopurines per day for at least 6 mo had a significantly decreased risk of developing advanced neoplasia (adjusted HR = 0.10, 95%CI: 0.01-0.75)[76].

Further support for a protective effect of thiopurines was established using data from the ENEIDA registry (Estudio nacional en Enfermedad Inflamatoria Intestinal sobre determinantes genéticos y ambientales), a nationwide, hospital-based, prospectively maintained, Spanish database of incident and prevalent IBD patients[59]. In this study of 831 patients with UC with 26 cases of CRC and 29 cases of high-grade dysplasia, use of thiopurines (OR = 0.21, 95%CI: 0.06-0.74, P = 0.015) and being in a surveillance colonoscopy program (OR = 0.33; 95%CI: 0.16-0.67; P = 0.002) were independent protective factors for advanced neoplasia[59].

In a meta-analysis by Jess *et al*[4] based on two population-based studies and 13 Clinic- and insurance-based studies, there was no significant overall protective effect of thiopurines on colorectal neoplasia in IBD (OR = 0.87, 95%CI: 0.71-1.06). There was, however, a tendency toward a protective effect of thiopurines in studies using both colorectal dysplasia and CRC as the outcome instead of CRC alone (OR = 0.72, 95%CI: 0.50-1.05). In this analysis, a meta-regression suggested a trend toward a protective effect of thiopurines in more recent studies, but was not statistically significant (meta-regression; P = 0.16). Another meta analysis, however, based on nine case-control and ten cohort studies demonstrated that the use of thiopurines was associated with a significant decreased incidence of colorectal neoplasm in IBD (RR = 0.71, 95%CI: 0.54-0.94), even after adjustment for duration and extent of the disease[3].

Although the data is not overwhelmingly clear given the heterogeneity in the abovementioned trials, it is likely that there is some benefit to thiopurine therapy in reducing the risk of colitis-associated CRC in IBD. Future prospective studies would be useful to clarify if simply control of inflammation reduces the risk of CRC rather than a direct effect of thiopurines and the appropriate dosing and duration for maximizing a potential chemoprotective benefit.

THIOPURINE ADVERSE DRUG REACTIONS AND THE RISK OF INFECTION

It is well established that thiopurines are effective in treating IBD. This effectiveness, however, must be weighted against various adverse reactions with up to 60% of patients discontinuing thiopurine therapy during their disease course[43,77-81]. Multiple studies have cited intolerable dose-dependent and idiosyncratic

adverse events, such as hepatotoxicity, myelosupression and pancreatitis as primary reasons for discontinuation. Adverse effects tended to occur within the first three months of thiopurine initiation and longer duration of use appears to be associated with a lower risk of discontinuation[53,78,81]. In a Dutch cohort study of 363 patients over eight years of follow up, 32% experienced hepatotoxicity, 19% gastrointestinal effects, 12% myelosuppression, 11% pancreatitis, 11% fever, 9% general malaise, and 8% arthralgia[78].

Given that thiopurines have broad suppressive effects on the immune system, benign and opportunistic infectious complications are a serious concern to both patients and providers. This risk is further compounded in patients who require multiple immunosuppressive agents such as corticosteroids and biologic therapy. Studies have demonstrated increased rates of viral, fungal, parasitic, bacterial, and mycobacterial infections in patients exposed to thiopurine therapy[82,83].

Retrospective analyses of patients with IBD treated with thiopurines have showed rates of infection ranging from 7.4% to 14.1%[82,83]. Viral infections are of particular concern with a predisposition to cytomegalovirus, varicella zoster virus and Epstein-Barr virus (EBV) infections as a result of thiopurine induced T lymphocyte apoptosis[84-88]. In a study from the Mayo Clinic, thiopurine use increased the risk of an opportunistic infection by 2-3 fold (OR = 3.8, 95%CI: 2.0-7.0) and when combined with corticosteroids, greatly increased the risk (OR = 17.5, 95%CI: 4.5-68)[87]. Moreover, data from the Crohn's Therapy, Resource, Evaluation, and Assessment Tool registry found that the concomitant use of infliximab did not increase the risk of serious infection compared to conventional immunomodulator therapy alone over 2 years of follow-up[86].

Based on the above evidence, it is clear that the increased risk of serious infection associated with thiopurine use must be carefully balanced with the therapeutic benefits. This increased risk of infection requires appropriate prevention, prompt diagnosis, and effective treatment.

THIOPURINES AND THE RISK OF MALIGNANCY

In addition to the risk of infection, thiopurines also increase the risk of cancer. Thiopurines promote the development of cancer by a variety of mechanisms including direct alteration in DNA, activation of oncogenes, reduction in immunosurveillance of malignant cells, and impaired control of oncogenic viruses[89-91]. Several population-based cohort and meta-analyses have demonstrated that current use of thiopurines for IBD is associated with a 1.3 to 1.7 overall relative risk of cancer[71,92]. Specific cancers linked to long-standing thiopurine use in the setting of IBD include lymphomas, myeloid disorders, and skin cancers.

Multiple studies have demonstrated an increased risk of Non-Hodgkin Lymphoma following thiopurine exposure, with standardized incidence ratios ranging from 1.6 to 37.5, with no excess risk attributed to IBD itself[93-95]. The majority of lymphoma associated with thiopurine exposure is EBV-associated, resulting from the loss of immune control of EBV-infected B lymphocytes[96]. More concerning, there are several cases in the literature of fatal post-mononucleosis lymphoma in young men who previously tested seronegative for EBV[93]. Furthermore, Hepatosplenic T-cell Lymphoma, though extremely rare, is associated with thiopurine use in combination with TNF-α antagonists in adolescent and young males[97]. Despite the increased risks, recent data suggest no differences of survival with lymphoma between patients with IBD and expected survival for the general population[98].

In terms of myeloid disorders, in a study from the Cancers Et Surrisque Associé aux Maladies inflammatoires intestinales En France (CESAME) cohort, the risk of myeloproliferative disease was not increased among patients with IBD or ongoing thiopurine treatment (SIR = 1.54; 95%CI: 0.05-8.54)[92]. However, patients with past exposure to thiopurines had an increased risk of myeloid disorders (SIR = 6.98; 95%CI: 1.44-20.36)[92]. For skin cancers, there is considerable evidence that thiopurines increase the risk of basal cell and squamous cell carcinomas[99-101]. In another study from the CESAME cohort, an increased risk of basal cell and squamous cell carcinomas was demonstrated in the patients with IBD and associated with ongoing thiopurine exposure (HR = 5.9; 95%CI: 2.1-16.4) and past thiopurine exposure (HR = 3.9; 95%CI: 1.3-12.1)[101].

Although there is clear evidence for a risk of primary cancers associated with thiopurine use, several retrospective and prospective cohort studies have demonstrated no increased risk of new or recurrent cancer in patients with a history of cancer exposed to thiopurine therapy. Although data is limited, the CESAME group found that exposure to thiopurines did not increase the risk of new or recurrent cancer in patients with a history of cancer[92]. In a study by the New York Crohn's and Colitis Organization, exposure to TNF-α antagonists, antimetabolites (thiopurines or methotrexate), or the combination of these agents, was not associated with an increased risk of new or recurrent cancer within 5 years following a diagnosis of cancer (Log-rank P = 0.14)[102]. Furthermore, after adjusting for the risk of recurrence of prior cancer, there was still no difference in risk of new or recurrent cancer between exposure groups (anti-TNF-α HR = 0.32, 95%CI: 0.09-1.09; anti-TNF-α with an antimetabolite HR = 0.64, 95%CI: 0.26-1.59; antimetabolite HR = 1.08, 95%CI: 0.54-2.15)[102].

THIOPURINE DRUG METABOLISM AND BLOOD LEVEL MONITORING

Over the last decade, research has demonstrated that

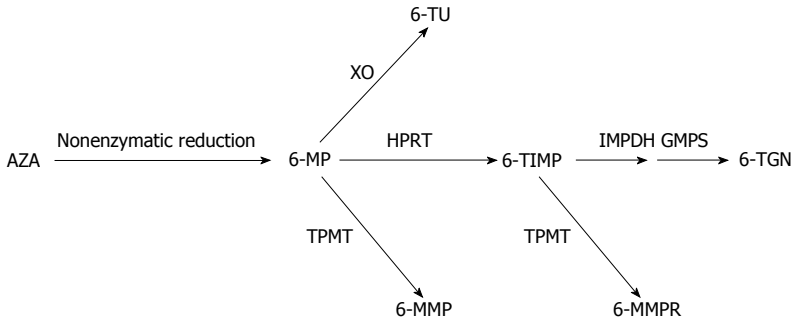

Figure 2 Metabolic pathway for azathioprine and mercaptopurine. AZA: Azathioprine; 6-MP: 6-mercaptopurine; 6-TU: Thiouric acid; 6-MMP: 6-methylmercaptopurine; 6-TIMP: 6-thioinosine-monophosphate; 6-MMPR: Methyl-mercaptopurine ribonucleotide; 6-TGN: Thioguanine nucleotide; XO: Xanthine oxidase; TPMT: Thiopurine methyltransferase; HPRT: Hypoxanthine phosphoribosyl transferase; IMPDH: Inosine monophosphate dehydrogenase; GMPS: Guanosine monophosphate synthetase.

thiopurine efficacy is dependent upon a therapeutic blood value of 6-thioguanine nucleotide (6-TGN), the metabolic product of the parent drug[18]. Azathioprine is metabolized into 6-mercaptopurine in the liver by a non-enzymatic pathway[103]. After conversion into 6-mercaptopurine, different metabolic pathways compete, leading to the formation of 6-TGN by hypoxanthinephosphoribosyl transferase and 6-methylmercaptopurine by the TPMT enzymatic system (Figure 2). The therapeutic metabolite 6-TGN inserts itself into the DNA of leukocytes as a fraudulent base, thereby preventing T-cell proliferation, leading to subsequent immunosuppression[104]. In addition, studies have also demonstrated that azathioprine and its metabolites induce T cell apoptosis by modulation of Ras-related C3 botulinum toxin substrate 1 (Rac1) activation[105]. 6-methylmercaptopurine is associated with hepatotoxicity.

TPMT activity, ranging from the rare complete deficiency in 0.3% of adults to homozygous (normal) activity in 90% of adults, determines the breakdown to 6-methylmercaptopurine, the metabolic product causing hepatotoxicity. Prior to the assay for the presence of TPMT, the initial dose of mercaptopurine was 50 mg/d and then complete blood counts were followed with titration of the dose to the white blood count. With the advent of an assay for TPMT enzyme levels, it is now standard of care to measure its presence prior to initiation of therapy to identify patients at risk for toxicity. However, TPMT screening does not obviate the need for periodic hematologic monitoring.

Moreover, several studies have shown that 6-TGN levels greater than 235 pmol/8 × 10^8 red blood cells (RBCs) correlate with therapeutic efficacy[54,104,106,107]. This level is not weight based and although it is recommended that 6-TGN levels are monitored, especially in nonresponsive patients, many gastroenterologists initiate weight based dosing followed by dose titration dictated by clinical factors and leukocyte count.

The concomitant use of 5-ASA increases 6-TGN levels, improving therapeutic potential, however, the combination of these medications may also lead to greater risk of toxicity, especially myelotoxicity[108,109]. In patients with increased TPMT activity leading to high levels of 6-methylmercaptopurine and lower levels of 6-TGN, the addition of allopurinol has been shown to inhibit xanthine oxidase activity resulting in higher therapeutic 6-TGN levels and lower 6-methylmercaptopurine levels. In addition, allopurinol upregulates aldehyde oxidase and therefore 6-thioxanthine production, which then inhibits TPMT[110]. Several studies have reproduced these findings and dual therapy with allopurinol can improve the therapeutic effect and decrease hepatotoxicity[111,112]. Practically, the addition of 100 mg of allopurinol should lead to decreasing the thiopurine dose by 50% and complete blood counts must be followed closely for myelosuppression[111,113].

More recently, several studies have examined the impact of low-dose weight-based azathioprine in combination with allopurinol in patients with normal TPMT activity. In a small prospective cohort, 69.6% patients with IBD randomized to low-dose azathioprine in combination with allopurinol 100mg were in clinical remission without the need for steroid or biologic treatment, and with less adverse events, at 24 wk compared to 34.7% of the patients treated with azathioprine monotherapy[114]. In an uncontrolled, retrospective, observational cohort of patients treated with low-dose weight-based azathioprine in combination with allopurinol, 69% with CD and 61% with UC had a clinical response at a median of 19 mo with 52% and 54% in clinical remission, respectively, with the highest response rates for thiopurine-naïve patients[115]. These studies suggest that low-dose weight-based azathioprine in combination with allopurinol may be effective therapeutic strategy.

In patients treated with 5-ASA or allopurinol with thiopurines, periodic therapeutic drug monitoring is necessary in order to minimize toxicity. In addition, in patients intolerant to thiopurines secondary to preferential 6-methylmercaptopurine metabolism, it is possible to achieve therapeutic 6-TGN levels while reducing 6-methylmercaptopurine levels by splitting the dose or changing to 6-TGN as primary therapy[116].

COMBINATION THERAPY: THIOPURINES WITH TNF-α ANTAGONISTS

Studies have also demonstrated the importance of thiopurines in combination with anti-TNF therapies. In an ad hoc analysis of A Crohn's Disease Clinical Trial Evaluating Infliximab in a New Long-term Treatment Regimen (ACCENT I) trial, it was apparent that patients receiving concomitant immunomodulators had an improved remission and response rate at week 52[117]. This was the first indicator that combination therapy was more effective than monotherapy, likely attributed to immunomodulators decreasing antibody response to infliximab. Subsequently, numerous studies have supported initial evidence that immunogenicity contributes to increased formation of antibodies to anti-TNF, leading to lower trough levels, and eventual loss of response[118,119].

The effect of combination therapy in preventing antibody formation has been reproduced in various studies, including both the SONIC trial and UC SUCCESS[18,47]. Both of these trials compared infliximab or azathioprine monotherapy to combination therapy in patients naïve to both anti-TNF and thiopurine therapy. In both CD and UC, patients treated with combination therapy were more likely to achieve corticosteroid free remission, response, as well as mucosal healing, and there is now ample data to suggest that combination therapy is associated with higher anti-TNF trough levels[120]. In UC SUCCESS, 19% of patients receiving infliximab monotherapy developed antibodies *vs* 3% in patients receiving combination therapy[47]. However, sub-analysis of randomized control trials on anti-TNF use in patients who were not naïve to either an anti-TNF or thiopurine have failed to show an impact of combination therapy on clinical outcomes, despite less formation of antibodies. Similarly, Jones *et al*[121] studied the effect of adalimumab monotherapy *vs* combination therapy in patients not naïve to both drugs, and the data failed to show a higher remission or response rate with combination therapy at 52 wk. Similarly, in employing combination therapy with adalimumab, only a modest improvement was noted in those receiving combination therapy, with less need for dose escalation in this sub-group[122].

There have been various other trials examining the effect of combination therapy administered for 3, 6, or 9 mo with the suggestion that the initial combination decreases the long-term antibody formation thereby improving long-term clinical outcomes[123,124]. All of the aforementioned trials incorporated weight based azathioprine or mercaptopurine dosage.

A recent study assessed the correlation between levels of 6-TGN with both infliximab trough and antibody levels to infliximab[125]. Seventy-two patients who received combination maintenance therapy had levels of 6-TGN that significantly correlated with infliximab trough levels and antibody levels. Contrary to 6-TGN levels greater than 235 pmol/8 × 10^8 RBCs, which is considered therapeutic under monotherapy, those treated with combination therapy only required a 6-TGN level of 125 pmol/8 × 10^8 RBCs in order to decrease antibody formation and attain therapeutic levels of infliximab. This is the first study to suggest that prevention of antibodies to anti-TNF may require minimal doses of an immunomodulator, which theoretically may decrease long-term adverse effects.

Many observational trials have attempted to answer whether combination therapy is more effective than monotherapy. There are conflicting studies and henceforth, still some uncertainty as to whether and when to initiate thiopurines therapeutically as well as in preventing antibody formation. With the advent of more biologics and small molecules as therapy for IBD, thiopurines may be administered in smaller doses, and continue to play an integral role in maintaining therapeutic response.

EXPERIENCE OVERVIEW BY DRS. BURTON KORELITZ AND DANIEL PRESENT: INTERVIEWED BY DR. SIMON LICHTIGER

Induction of remission with thiopurines in CD can usually be achieved within 4 to 6 wk, with steroids serving as a bridge. For induction of remission in ulcerative colitis, although no randomized, placebo-controlled trial has confirmed their efficacy, we know well from our practice that thiopurines are effective and possibly work faster than in CD. For postoperative prevention, although our own studies did show statistical significance, the results are less robust than for anti-TNFs.

In lieu of enzyme testing or levels, we have always started patients on 50 mg per day of mercaptopurine and monitored the white blood cell count for leukopenia and the mean corpuscular volume for macrocytosis. Leukopenia would typically occur very quickly, so labs by week two would often detect these patients. Abnormalities in liver function are rare and we accept mildly elevated transaminases up to 200 with clinical efficacy. These laboratory values are also useful surrogates to assess medication compliance. For patients with mild side effects such as rash, fever, or arthralgia, we have found slow desensitization to be relatively simple and very effective starting with 1/8^{th} the dose of mercaptopurine followed by a slow escalation. However, in patients with pancreatitis, desensitization is rarely successful and not recommended.

In our experience, opportunistic infections are rare and more often found in patients on concomitant steroids. We also feel that solid malignancies, such as breast, lung, liver, pancreas, and kidney are not more common in those treated with thiopurines. We had not even heard of hepatosplenic T-cell lymphoma until the advent of biologics. Dr. Present always felt his practice

Table 1 Current evidence and dosing for thiopurines in inflammatory bowel disease

Thiopurine indication	Evidence and azathioprine dose
Crohn's disease induction	Monotherapy, less robust evidence: 1.5-2.5 mg/kg/d[11-17] Combination therapy with infliximab, more robust evidence: 2.5 mg/kg/d[18,19]
Crohn's disease maintenance	Monotherapy, more robust evidence: 1.5-2.5 mg/kg/d[6,13,14,22-25]
Postoperative maintenance in Crohn's disease	Monotherapy, more robust evidence: 2-2.5 mg/kg/d[5,28-36]
Ulcerative colitis induction	Monotherapy, less robust evidence: 1.5-2.5 mg/kg/d[16,38-42] Combination therapy with infliximab, more robust evidence: 2.5 mg/kg/d[47]
Ulcerative colitis maintenance	Monotherapy, less robust evidence: 2-2.5 mg/kg/d[2,16,40,42,50]
Chemoprevention	Monotherapy, less robust evidence: dose not established[3,4,59,74-76]
Preventing immunogenicity to anti-TNF	Combination therapy with anti-TNF, less robust evidence: dose not established[47,117-125]

TNF: Tumor necrosis factor.

did not demonstrate any increased risk of lymphoma with only 2 cases after more than 45 years of thiopurine use, but data demonstrates that lymphoma is statistically increased, albeit very rare.

CONCLUSION

Based on the above evidence and clinical experience overview by Drs. Korelitz and Present, thiopurines have demonstrated efficacy in a broad range of presentations of IBD (Table 1). Although extensive evidence for thiopurines has often been limited by trial methodology and design, over 50 years of clinical experience has demonstrated its efficacy and relative safety[126,127]. With the advent of more efficacious novel therapeutic agents with a wide variety of immunologic targets, the positioning of thiopurines in the management of IBD will undoubtedly change. Future studies will analyze the benefit of thiopurines in conjunction with these new medications, both as an individual synergistic adjunct and as a preventive agent for the production of antibodies to biologics. Notwithstanding, it continues to be a useful therapeutic option as monotherapy and in combination with other medications for inducing and maintaining durable remission in IBD.

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MINIREVIEWS

Role of *dupA* in virulence of *Helicobacter pylori*

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Abstract

Helicobacter pylori (*H. pylori*) is a gastric human pathogen associated with acute and chronic gastritis, 70% of all gastric ulcers, 85% of all duodenal ulcers, and both forms of stomach cancer, mucosal-associated lymphoid tissue (MALT) lymphoma and adenocarcinoma. Recently, attention has focused on possible relationship between presence of certain virulence factor and *H. pylori*-associated diseases. Some contradictory data between this bacterium and related disorders has been observed since not all the colonized individuals develop to severe disease. The reported diseases plausibility related to *H. pylori* specific virulence factors became an interesting story about this organism. Although a number of putative virulence factors have been identified including cytotoxin-associated gene a (*cagA*) and *vacA*, there are conflicting data about their actual participation as specific risk factor for *H. pylori*-related diseases. Duodenal ulcer promoting gene a (*dupA*) is a virulence factor of *H. pylori* that is highly associated with duodenal ulcer development and reduced risk of gastric cancer. The prevalence of *dupA* in *H. pylori* strains isolated from western countries is relatively higher than in *H. pylori* strains from Asian countries. Current confusing epidemiological reports will continue unless future sophisticated and molecular studies provide data on functional and complete *dupA* cluster in *H. pylori* infected individuals. This paper elucidates available knowledge concerning role of *dupA* in virulence of *H. pylori* after a decade of its discovery.

Key words: *Helicobacter pylori*; *dupA*; Bacterial virulence; Infection; Clinical outcome

Core tip: *Helicobacter pylori* (*H. pylori*) is one of the most common bacterial infections worldwide. Ten years ago, *virB4* homologue was identified as a new virulence factor, *dupA* "duodenal ulcer promoting gene A" by Lu and her colleagues. Nowadays, new genetical analysis using available sequences can help scientists to draw a better conclusion about *dupA* and its actual role in pathogenesis of *H. pylori*-related diseases. In this paper, we aim to draw a new shaped overview regarding *H. pylori* and its virulence factors with emphasis of *dupA*.

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INTRODUCTION

Due to the difficulty in diagnosis and fastidious condition of an optimal growth, *Helicobacter pylori* (*H. pylori*) was an unculturable and thus forgotten microorganism for many years[1]. Following the clinical and histological observations in gastritis and duodenal ulcer patients, Marshall and Warren were able to isolate and characterize this bacterium around thirty-three years ago[1,2]. New era had been started after this groundbreaking discovery and revealed as a publication in *Lancet* written by those Australian scientists[1]. As most of other human bacteria, *H. pylori* is mainly acquired during childhood and persists for the whole life of the colonized individual if not treated efficiently[3]. From bacteriologic point of view, *H. pylori* is a rod-shaped, microaerophilic Gram-negative organism which colonizing more than half of the world population[4]. Bacterial colonization induces acute inflammation in the gastric mucosa, a clinical manifestation which can be followed by diverse gastroduodenal disorders, but noted that only a minority of infected individuals develop severe diseases include duodenal ulcer and gastric cancer[4-8]. Many virulence-associated genes of *H. pylori*, including outer inflammatory protein a (*OipA*), vacuolating cytotoxin gene a (*vacA*), cytotoxin-associated gene a (*cagA*) and blood-group antigen-binding adhesion (*babA2*) are believed to have a critical role in determining the final clinical manifestation of the infection[9,10]. Therefore, various studies have conducted to discover better insights into the role of these proposed virulence factors in pathogenesis of digestive diseases[11-14]. None of the mentioned virulence factors have distinguished as discriminating factor in the development of peptic ulcer disease and gastric cancer. The main rationale for different diseases outcome observed among colonized individuals is still under debate, though scientists proposed different array of virulence biomarkers in this bacterium as regular answer to this question. In this paper, we aim to open a new window for defining a better description of a specific *H. pylori* virulence factor duodenal ulcer promoting gene a (*dupA*) based on current available knowledge.

VIRULENCE OF *H. PYLORI*

The definition of a virulence factor is referring to the ability of a bacterium to induce and develop a disease with a spectrum of severity[15]. Strains possessing these virulence factors are isolated more frequently from patients with the more serious clinical manifestations. It is logic to consider that for increase the chance of survival within harsh gastric condition *H. pylori* needs such smart strategies to keep the colonization. However, virulence factors can induce more cell damage with infiltrate immune cells to the location and thus inflammation will be the high priority event in epithelial cells[3]. Due to the chronic characteristic of *H. pylori* infection, scientists should expect to have particular definition of virulence factors for this bacterium. Virulence factors of *H. pylori* play an inevitable role in the development of gastroduodenal diseases through mucosal inflammation[10]. Basically, the criteria for being a virulence factor are (1) biologic rationale; (2) epidemiologic consistency; and (3) enough evidences for being linked with certain disease[15,16]. In order to define a virulence factor for each bacterium, it should pass many *in vivo* and *in vitro* experiments[17-20]. However, it is worthwhile to emphasize that only a limited number of proposed virulence factors had been successfully confirmed for *H. pylori*[17-19]. It had been well-documented that all *H. pylori* strains have several virulence factors such as flagella and urease enzyme since they have a critical role in bacterial colonization[4]. Urease enzyme (as cytoplasmic protein) is necessary to establish primary bacterial colonization in the gastric mucosa. *H. pylori* flagella provide sufficient ability to quickly penetrate the gastric mucosa layer to avoid exposure with harsh acid condition in the stomach[4]. In addition, some adhesines such as *babA2*, *iceA1* and Sialic acid-binding adhesin (*sabA*) are mostly present in *H. pylori* strains, and these factors help the bacterium to attach properly to the epithelial cells and serve as a unique virulence factor[9,21]. Clinically, gastric cancer and duodenal ulcer are standing in quite opposite sides of *H. pylori*-related disease spectrum. It brings a big query in the mind about disease plausibility which only can be explained with existence of diverse, but, specific virulence factors in this microorganism.

cagA

cagA is located at the end of the cag pathogenicity island (PAI), which is a 39-kb region transferred horizontally from an unknown bacterial source. The "pathogenicity islands" include *cagA* encode proteins contributing in signal transduction cascades that result in cytoskeletal rearrangement *via* actin polymerization and host cell protein phosphorylation[4]. Virulent strains of *H. pylori* possess the *cagPAI*. Many of *H. pylori* strains from patients with peptic ulcer or gastric cancer carry *cagA*, whereas many of those strains from asymptomatically infected persons lack this gene[4]. Currently, we identify two major types of *H. pylori* isolates: *cagA* gene-negative and *cagA* gene-positive strains. Counting a virulence factor for *cagA* needs another classification which is based on polymorphism in Glu-Pro-Ile-Tyr-Ala (EPIYA) motifs[4]. In *cagA* positive strains, there is a region contains the EPIYA motifs, which contains a tyrosine phosphorylation site[4]. Brief-

ly, two major types (Western and Eastern *cagA*) were determined according to this polymorphism. Though, we need more biologic rationale to be consistent with clinical evidences to present better information on how to interoperate this classic virulence factor in *H. pylori*.

vacA

To now, *vacA* is the second most extensively investigated virulence factor of *H. pylori*. Virtually all *H. pylori* strains have a functional *vacA* gene which codes for the secreted pore-forming protein *vacA*[22]. The main difference in bacteria carrying *vacA* is expression levels and disease severity which are associated with sequence variation in different domains of secreted protein[4]. There is a big gap on our knowledge regarding biologic function of this protein since still many contradictory findings are exist[23-26]. So we need more investigation to determine how to count on *vacA* as useful *H. pylori* virulence factor.

dupA

As first time, in 2005, it has been described that a new virulence factor which was located in the plasticity region of the *H. pylori* genome. PR or "plasticity region" where composed the dupA, has a relatively high rate of allelic diversity in *H. pylori* genomic DNA[27,28]. Whole genome analysis of J99 and 26695 revealed regions where G + C content was lower than rest of the *H. pylori* genome (34% against 40%)[29]. Later, since high variability was observed in this region, it termed as "plasticity region". Currently, we know that more than 60% of strain-specific genes of H. pylori are located in this area. In J99 and 26695 strains, two regions with lower G + C content and 45 kb and 69 kb long has been named as plasticity zones[30]. More than 50% of strain specific open reading frames (ORFs) are located in plasticity zone which are 46% and 48% unique genes from 26695 and J99, respectively. Interestingly, in comparison with 26695, the strain J99 has 33 more ORF in plasticity region (*jhp914-jhp951*)[30]. Lu *et al*[31] investigated this region and reported a continuous gene covering *jhp0917* and *jhp0918* genes for first time which is a risk factor for duodenal ulcer diseases. Accordingly, they named the *jhp0917-jhp0918* gene the dupA gene. To date, many of putative *H. pylori* genes have been suggested to be linked with increasing risk of digestive diseases, while none have been confirmed to be actually associated with unique and specific *H. pylori*-related disease such as gastric cancer or duodenal ulcer. Therefore, *dupA* can be named as first candidate to have achieved this distinction. Following the primary study by Lu *et al*[31], a large number of controversial examinations has been published[32-42]. The global prevalence of *dupA* in patients with gastritis was reported around 45% which is highly differed among subjects with various nationality (31% in Asian and 64% in Western countries)[43,44]. Therefore, among most of Asian countries, a significant association between disease development and *dupA* status can be reported[38,45-54]. In two studies, first by Imagawa *et al*[37] patients infected with *dupA*-positive strains showed higher risk to suffer from duodenal ulcer than *dupA*-negative patients. In second study, we have found that higher acid resistance of the *dupA*-positive strains can explain the adaptation of those strains to human stomach with high gastric acid output[35]. Indeed, Lu *et al*[31] described that infections with *H. pylori dupA*-negative strains can increase the risk for duodenal ulcer, but it reduce the chance of occurrence for gastric[31]. Antral induction of IL-8 production is a main character of *dupA* pathogenesis causing predominant gastritis[46]. The mentioned mucosal inflammation and polymorphonuclear leukocytes (PMN) infiltration can lead to the occurrence of duodenal ulcer[31]. In a systematic review by Shiota *et al*[55] with more than 2466 patients, they confirmed an association between certain clinical outcomes and the *dupA* status. Moreover, presence of an extra 600 bp in *dupA* ORF in *H. pylori* strains such as g27 showed that the length of the *dupA* is differ among various strain, mostly declared that *dupA* has two main genotypes accordingly, (long and short type)[35,38,55]. Unfortunately, most of studies in past did not consider this two types of *dupA* and thus the final results by them might be cautiously useful. Another interesting topics about *dupA* is existence of several mutations in gene length[38,56]. At different positions, these mutations can create a premature stop codon with considerable effects on its produced proteins function[56]. Strains isolated from patients with duodenal ulcer mostly carrying *dupA* without stop codon in comparison with other diseases types[27]. Notwithstanding, without frameshift mutation *dupA* which called intact long-type *dupA* rather short-type *dupA* is highly associated with gastric cancer[57]. It has been extensively reported that there is an association between increased expression levels of IL-8 and *dupA* in the gastric mucosa of *H. pylori*-colonized individuals. As expected, many reports are indicating on gastric mucosal inflammatory cell infiltration was significantly higher in patients with *dupA*-positive *H. pylori* than in patients with *dupA*-negative strain[56,57]. As such, current data suggesting that only intact long type *dupA* can produce DupA protein and also serve as real virulence factor for *H. pylori* strains. In brief, current knowledge about *dupA* positive strain and its subsequent diseases vulnerability insist on significant associations between the *dupA* gene and an increased risk for duodenal ulceration rather gastric cancer. As final remarks about *dupA*, we can mention to these sentences as follow: (1) Additional tests of the *dupA* DNA sequence are necessary to determine actual importance of intact *dupA*; also in level of proteins with immunoblotting techniques; (2) Similar to the *cagA*, it has been asserted that *dupA* is forming a Type 4 secretion system (T4SS) as a full gene cluster. Noted that *virB4* and *dupA* as homologous genes together

are the major constituents of T4SS where located in plasticity region[52]; (3) Jung *et al*[38] recently examined South American population from Colombia to see association between *dupA* and *virB* gene homologs and clinical outcomes. In total, we concluded that intact *dupA* without shift mutation can serve as actual virulence factor with consistent results worldwide. It is no doubt that evaluation of various genes located in plasticity region are required and new data in close future can enrich our knowledge about this mysterious region of *H. pylori* genome; and (4) Broadly defined, virulence of *H. pylori* play an essential role in the development of severe gastroduodenal diseases such as duodenal ulcer through mucosal inflammation. With this regard, *dupA* as one of important risk factor was in focus of many researches in last years. The discrepancy observed among the epidemiologic studies can be explained by using various methods to determine existence of *dupA*, variation in ORF and different population's bias. Thus, despite advances in our understanding of the development of *H. pylori*-related diseases, further work is required to clarify the roles of *H. pylori* virulence factors.

CONCLUSION

H. pylori plays a critical role in the development of severe digestive diseases; though, the main virulence determinant acting in this field are still not completely defined. Now the question is to find the determining item to represent this interesting disease pattern. For sure, we admitted that *H. pylori* is involved in pathogenesis of both gastric cancer and duodenal ulcer while they are in quite opposite side of digestive diseases, again, how we can still accept a crucial role for *H. pylori* in these gastroduodenal diseases? Many studies had been performed to elucidate actual biologic role of *dupA* in development of severe gastroduodenal diseases such as gastric cancer[46-48]. The observed discrepancy of *dupA* link with disease outcomes might be associate with the plasticity region of *H. pylori* or the limitation of PCR to detect the various forms of *dupA* gene; however, in order to draw a better conclusion further experiments are required[58,59]. Interestingly, the presence of *dupA* was significantly associated with *H. pylori* eradication failure with no biologic explanation[60-62]. In conclusion, it sounds that rather than promoting gastric cancer or duodenal ulceration in all populations, dupA is an effective factor for some of populations. Because of microarray analysis as new technology many new genes can be proposed as novel virulence biomarker for *H. pylori*.

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MINIREVIEWS

Dilemma of first line regimens in metastatic pancreatic adenocarcinoma

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Abstract

Pancreatic cancer is one of the deadliest cancers, ranking fourth among cancer-related deaths. Despite all the major molecular advances and treatment breakthroughs, mainly targeted therapies, the cornerstone treatment of metastatic pancreatic cancer (mPC) remains cytotoxic chemotherapy. In 2016, more than 40 years after the introduction of gemcitabine in the management of mPC, the best choice for first-line treatment has not yet been fully elucidated. Two main strategies have been adopted to enhance treatment efficacy. The first strategy is based on combining non-cross resistant drugs, while the second option includes the development of newer generations of chemotherapy. More recently, two new regimens, FOLFIRINOX and gemcitabine/nab-paclitaxel (GNP), have both been shown to improve overall survival in comparison with gemcitabine alone, at the cost of increased toxicity. Therefore, the best choice for first line therapy is a matter of debate. For some authors, FOLFIRINOX should be the first choice in patients with an Eastern Cooperative Oncology Group score (0-1) given its lower hazard ratio. However, others do not share this opinion. In this paper, we review the main comparison points between FOLFIRINOX and GNP. We analyze the two pivotal trials to determine the similarities and differences in study design. In addition, we compare the toxicity profile of the two regimens as well as the impact on quality of life. Finally, we present studies revealing real life experiences and review the advantages and disadvantages of possible second-line therapies including their cost effectiveness.

Key words: Review; Metastatic pancreatic cancer; FOLFIRINOX; Gemcitabine/nab-paclitaxel; Pivotal trials

Core tip: This paper is a mini-review that compares the design of the two pivotal trials studying the role of FOLFIRINOX and gemcitabine/nab-Paclitaxel in the management of metastatic pancreatic cancer. It also

analyzes the effects these regimens have on toxicity profile, quality of life, real life experiences, choice of second-line therapy and cost.

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INTRODUCTION

Adenocarcinoma of the pancreas is one of the most aggressive human cancers, ranking fourth among cancer related deaths[1]. Recent biomolecular progress has led to a better comprehension of pancreatic carcinogenesis; however, the revolutionary targeted and immune therapies have not shown any significant results[2]. Subsequently, cytotoxic drugs remain the backbone of treatment for metastatic pancreatic cancer (mPC). Gemcitabine has been the standard of care in mPC since 1996, providing a limited survival of six months due to the intrinsic capacity of cancer cells and the surrounding microenvironment to resist cytotoxicity[3-5]. More aggressive regimens were developed to overcome these resistance mechanisms. The combination of non-cross resistant agents, GTX (gemcitabine, docetaxel and capecitabine) and PEFG (cisplatin, epirubicin, fluorouracil and gemcitabine), enhanced tumor shrinkage by acting on different stages of cell cycle and bypassing mechanisms of drug resistance[6-8].

In 2011, French investigators from the Groupe Tumeurs Digestives of Unicancer and the PRODIGE Intergroup published the results of a phase Ⅱ/Ⅲ trial that revealed a clinically significant survival benefit and better quality of life for a regimen combining 5-FU/leucovorin, oxaliplatin and irinotecan (FOLFIRINOX) at the expense of increased toxicity[9]. Another option is nab-paclitaxel, which is a second generation chemotherapy agent that exploits the ability of albumin to deliver the hydrophobic molecule, paclitaxel, to targeted tissues. Nab-paclitaxel was combined with gemcitabine in the multinational MPACT phase Ⅲ trial and added an OS benefit of 2.6 mo compared to single agent Gemcitabine[10,11]. Table 1 summarizes the efficacy of the FOLFIRINOX and gemcitabine/nab-paclitaxel (GNP) as published in the pivotal studies of ACCORD/PRODIGE and MPACT trials, respectively.

The best choice for first-line therapy is a matter of debate. The National Comprehensive Cancer Network (NCCN) panel considers FOLFIRINOX as the first choice for *Eastern Cooperative Oncology Group (*ECOG) 0-1 patients given its lower HR for death, whereas nab-paclitaxel should be reserved for ECOG 2 patients (NCCN 2016). Conversely, ASCO and ESMO consider both regimens as acceptable treatment options for ECOG 0-1 patients[12,13]. Indirect comparisons using the ESMO magnitude of clinical benefit scale show a higher score for the FOLFIRINOX regimen when compared to GNP (5/5 *vs* 2/5, with a higher score indicating a better regimen in terms of survival benefit and quality of life)[14]. In addition, a Bayesian meta-analysis comparing multiple systemic protocols in advanced pancreatic cancer showed a trend toward better survival with FOLFIRINOX compared to GNP[15]. In view of this debate, we conducted this review to discuss the main comparison points between FOLFIRINOX and GNP, including the design of the two pivotal trials, toxicity profiles, quality of life, real life experiences, choice of second-line therapy and cost effectiveness.

TRIAL DESIGN: PRODIGE *VS* MPACT

The PRODIGE and MPACT trials were both randomized controlled trials (RCTs) based on an intent to treat principle and included 342 and 861 patients with mPC, respectively. Both trials had nearly the same tumor characteristics[9,16]. Additionally, the median age (61 years for both trials) and sex ratio (1.6 for PRODIGE and 1.3 for MPACT) were nearly identical. However, the French trial included only patients less than 76 years old with good performance status based on the ECOG evaluation system (ECOG 0-1). In contrast, the MPACT trial did not specify an age limit (age ranged from 27 to 86 years) and included patients with intermediate performance status based on the KPS system (KPS < 90 in nearly 42% of patients). In addition, the PRODIGE trial only included patients from French centers while the MPACT trial was a multinational study including patients from North America (63%), Australia (14%), and Eastern (15%) and Western Europe (9%). In addition, patients in the Gemcitabine arm of the PRODIGE trial received only 6 mo of therapy even if they were not progressing (17%), nearly half of whom did not continue. While some authors do not consider these differences important given that the survival curves of the gemcitabine arm in the two trials are "superimposable", others do not share this opinion. In fact, Gemcitabine is a well-known drug that is tolerated in the elderly population, even in intermediate health systems such as that of Eastern Europe. The same is not true when a new drug such as nab-paclitaxel is added to gemcitabine. In fact, the forest plot in the MPACT study clearly shows an effect of age and country on hazard ratio. In the same sense, Tehfe *et al*[17] published an analysis of patients from Canada (a subgroup of the MPACT trial) and showed an OS equal to 11.9 mo in the GNP arm compared to 7.1 mo in the gemcitabine arm with a hazard ratio of 0.76. However, this subgroup analysis included only 63 patients and was underpowered to detect a statistically significant result.

Table 1 Comparison of the pivotal studies approving FOLFIRINOX and gemcitabine/nab-paclitaxel in metastatic pancreatic cancer

		ACCORD/PRODIGE trial (FOLFIRINOX)[9]	MPACT trial (GNP)[10]
Study characteristics	Duration	December 2005-October 2009	May 2009-April 2012
	Location	France	Multinational
	Number of patients	342	861
	Study design	Phase 2-3	Phase 3
	Control arm	Gemcitabine	Gemcitabine
Patient and tumor characteristics	Median age	61 years	62 years
	Sex distribution	Male (62%)	Male (57%)
	ECOG	PS 0 (37.4%)	KPS 100 (16%)
		PS 1 (61.9%)	KPS 80-90 (77%)
		PS 2 (0.6%)	KPS 60-70 (7%)
	Tumor stage	Metastatic	Metastatic
	Metastatic sites	Liver (87.6%)	Liver (85%)
		Lung (19.4%)	Lung (35%)
		Peritoneum (19.4%)	Peritoneum (4%)
	Tumor location	Head (39.2%)	Head (44%)
Response	ORR (%)	31.6	23
	PR (%)	31	23
	SD (%)	38.6	27
	DCR (%)	70.2	48
	PFS (mo)	6.4	5.5
	OS (mo)	11.1	8.5
	1-yr OS (%)	48.4	35
Safety (Grade 3-4 toxicities)	Neutropenia	45.7	38
	Febrile neutropenia	5.4	3
	Thrombocytopenia	9.1	13
	Anemia	7.8	13
	Fatigue	23.6	17
	Peripheral neuropathy	9	17
	Diarrhea	12.7	6
Side effects	Toxic death	0.6	4
	Alopecia	11.2	50
	G-CSF use	42.5	26

DCR: Disease control rate; GNP: Gemcitabine/nab-paclitaxel; PR: Partial response; ORR: Overall response rate; OS: Overall survival; SD: Stable disease.

TOXICITY AND QUALITY OF LIFE

The toxicity profile of a chemotherapy regimen is a major contributor in its adoption. Based on the two trials, hematologic toxicity is in favor of the FOLFIRINOX regimen and includes a lower incidence of neutropenia (45% *vs* 38%) (although the use of G-CSF was more common), anemia (7.8% *vs* 13%), and thrombocytopenia (9.1% *vs* 13%). The remaining toxicities are listed in Table 1[9,16]. Peripheral neuropathy attributed to Nab-paclitaxel is a particular debilitating toxicity; grade 3 peripheral neuropathy was encountered in 17% of the patients but improved to grade 1 toxicity or less in a median of 29 d[10]. Real-life studies with a closer follow-up of patients showed fewer side effects compared to those reported in the clinical trials[18]. Chemotherapy-induced hair loss is often a major determinant of the treatment regimen selected and was more commonly encountered in the GNP regimen (50% *vs* 11.2%)[9,16].

Overall, FOLFIRINOX remarkably improved global health status, emotional functioning and many of the symptoms of mPC, such as pain and anorexia (although FOLFIRINOX did not relieve diarrhea), in the first two months of treatment. It also showed significantly increased time to physical or cognitive deterioration[19]. On the other hand, quality of life was not assessed in the MPACT trial. In contrast, GNP showed significant improvement in quality-adjusted survival in comparison to gemcitabine alone using the Quality-Adjusted Time Without Symptoms or Toxicities (Q-TWiST) methodology, despite the limitations of the Q-TWiST analysis and the lack of prospective quality of life data from the MPACT trial[8].

Because significant toxicity was not uncommon, more tolerable treatment regimens were created by modifying the administration or drug dosing schedule. In the modified FOLFIRINOX regimens, either the 5-fluorouracil bolus was omitted or the dose of irinotecan was reduced. Stein *et al*[20] published solid data in a prospective study, enrolling both locally advanced and mPC patients who received a modified FOLFIRINOX regimen including a 25% dose reduction in 5-FU or irinotecan. These modifications successfully maintained the efficacy of the drugs while significantly decreasing the toxicity profile (decreased neutropenia, vomiting and fatigue). Additional exploratory analyses of the MPACT trial showed that patients who had dose delays or reductions (71% and 41%, respectively) had better outcomes[8]. These practical changes are capable of modifying the tolerance profile of the drugs while preserving efficacy. Tables 2 and 3 compare the

Table 2 Comparison of the FOLFIRINOX and modified FOLFIRINOX trials

		ACCORD/PRODIGE trial (FOLFIRINOX)[9]	Stein *et al*[20] Modified FOLFIRINOX	Mahaseth *et al*[21] (Modified FOLFIRINOX)	Ghorani *et al*[22] (Modified FOLFIRINOX)
	Location	France	United States	United States	United Kingdom
	Number of patients	342	44	36	18
	Study design	Phase 2-3 Prospective	Phase 2 Prospective	Phase 2 Prospective	Retrospective
Study design	Dosing		25% reduction in bolus 5-FU and irinotecan doses	No 5-FU bolus	No 5-FU bolus and 25% reduction in irinotecan doses
Patient and tumor characteristics	Median age	61 years	62	63	60
	Sex distribution	Male (62%)	Male (56.8%)	Male (56.8%)	Male (44.6%)
	ECOG	PS 0 (37.4%)	PS 0 (46%)	PS 0 (22%)	PS 0 (56.6%)
		PS 1 (61.9%)	PS 1 (54%)	PS 1 (76%)	PS 1 (44.4%)
		PS 2 (0.6%)		PS 2 (1%)	
	Tumor stage	Metastatic	Metastatic	Metastatic	Locally advanced and metastatic
	Metastatic sites	Liver (87.6%)	Liver (54.1%)		
		Lung (19.4%)	Lung (32.4%)		
		Peritoneum (19.4%)	Peritoneum (37.8%)		
	Tumor location	Head (39.2%)	Head (54.8%)	NA	Head (566%)
Response	ORR (%)	31.6	35.1	30	47
	PR (%)	31	35.1	NA	47
	SD (%)	38.6	51.5	NA	23
	DCR (%)	70.2	86.6	NA	80
	PFS (mo)	6.4	6.1	8.5	7.2
	OS (mo)	11.1	10.2	9	9.3
	1-yr OS (%)	48.4	38	NA	NA
Safety (grade 3-4 toxicities)	Neutropenia	45.7	12.2	3	0
	Febrile N.	5.4	4.1	0	5.6
	Thrombocytopenia	9.1	9.5	4	0
	Anemia	7.8	5.4		0
	Fatigue	23.6	12.2	13	5.6
	Peripheral neuropathy	9	2.7	4	0
	Diarrhea	12.7	16.2	13	16.7
	Toxic death	0.6	0	0	0
Additional information			Pegfilgastrim on each cycle	Pegfilgastrim on each cycle	Pegfilgastrim on each cycle

DCR: Disease control rate; PR: Partial response; ORR: Overall response rate; OS: Overall survival; SD: Stable disease.

classical to the modified form of FOLFIRINOX and GNP respectively[20-23].

CHOICE OF SECOND-LINE

The optimal treatment sequence dictates the choice of first-line treatment for mPC. In fact, in the PRODIGE trial, only 47% of the patients were fit enough to receive second-line therapy while only 12.5% of patients received a second-line therapy after initially receiving a gemcitabine-based combination, yet the median OS was limited to 4.4 mo among those receiving second-line treatments. On the other hand, in the MPACT trial, 40% of the patients received additional therapy after GNP[24]. According to these data, similar numbers of patients were able to receive second-line therapy after either FOLFIRINOX or GNP.

Data on the administration of GNP after FOLFIRINOX failure in the literature is limited to a few retrospective studies with conflicting data. The AGEO trial, a prospective multicenter study, evaluated the use of GNP in the second-line setting after FOLFIRINOX failure. The disease control rate was 58% with a 17.5% overall response rate and OS of 8.8 mo. Twelve patients (21%) had an ECOG of 2, and 40% had grade 3-4 toxicities without any treatment-related deaths[25]. In another retrospective study by Zhang *et al*[26], 28 patients treated with the same regimen showed less satisfactory results, with an OS of 23 wk.

Small retrospective studies assessed the efficacy of FOLFIRINOX in the second line setting with a modest improvement in OS, but none evaluated its efficacy after GNP[27,28]. In fact, the only data available is from the exploratory analyses of the second line treatment of the MPACT trial, where FOLFIRINOX (despite demonstrating interesting data) was only administered to 18 patients (10.5% of the whole population), calling the use of this treatment sequence into question[16].

Consequently, definitive recommendations concerning the optimal sequence of therapy cannot be made. The prospective data from the AGEO trial makes GNP a better and more plausible option as a second-line option after FOLFIRINOX administration. However, large RCTs are needed to create newer guidelines.

Table 3 Comparison of the gemcitabine/nab-paclitaxel and modified gemcitabine/nab-paclitaxel trials

		MPACT trial[10] (GNP)	Krishna *et al*[23] (Modified GNP)
Study design	Location	International	United States
	Number of patients	861	49
	Dosing		Omission of Day 7 doses
	Study design	Phase 3	Retrospective
Patient and tumor characteristics	Median age (yr)	62	65
	Sex distribution	Male (57%)	Male (57%)
	Tumor stage	Metastatic	Metastatic
	Metastatic sites	Liver (85%)	Liver (57%)
		Lung (35%)	Lung (27%)
		Peritoneum (4%)	Peritoneum (43%)
	Tumor Location	Head (44%)	Head (51%)
	PFS (mo)	5.5	4.8
	OS (mo)	8.5	11.1
Safety (Grade 3-4 toxicities)	Neutropenia	38	10
	Thrombocytopenia	13	4
	Anemia	13	15
	Fatigue	17	6
	Peripheral neuropathy	17	2
	Diarrhea	6	0

GNP: Gemcitabine/nab-paclitaxel; OS: Overall survival; PFS: Progression free survival.

COST-EFFECTIVENESS

In addition to weighing efficacy and safety, oncologists must evaluate financial considerations to choose the optimal chemotherapy regimen. In fact, the NCCN shows a tendency toward incorporating the financial burden of cancer drugs into its decision-making strategy. Cost-effectiveness of each regimen is largely dependent on the societal willingness-to-pay (WTP) threshold set by each country. For instance, setting the WTP in Canada at $130000 makes the FOLFIRINOX regimen the optimal strategy in mPC. However, decreasing the limit to $80000 renders Gemcitabine monotherapy the only possible therapeutic choice[29]. Similarly, the increased WTP threshold in Greece rendered the GNP protocol a potential option in the treatment of patients with mPC[30].

Both FOLFIRINOX and GNP showed consistent cost-effectiveness and cost-utility with superior survival efficacy in independent analytical studies[31,32]. However, it is not until recently that the values of each regimen were compared. The value of the different regimens in mPC was compared based on Medicare rates, which take into consideration the cost and administration of the drug, hospitalization and management of associated adverse events. The monthly costs of FOLFIRINOX and GNP were $7234 and $12221 respectively. However, the cost of the overall treatment based on progression free survival in each protocol was estimated at $46289 and $67216. FOLFIRINOX seemingly exhibits higher cost-effectiveness than GNP according to these results. However, it is worth mentioning that the cost of the FOLFIRINOX regimen is mainly due to its toxicity profile. Dosing modifications could limit the incidence of serious side effects and thus further increase the cost-effectiveness of this protocol (Monthly cost of FOLFIRINOX is $763 versus $9008 for the GNP protocol). Consequently, in September 2015, the National Institute for Health and Care Excellence recommended against the use of GNP in patients with mPC due to the limited benefits in comparison to the cost of the drug. An alternative cheaper option that might be considered is modified GNP (which is yet to be validated), which has an overall treatment cost of $36226[33].

CONCLUSION

Overall, both FOLFIRINOX and GNP result in better overall survival and quality of life. In the absence of direct comparison, the treatment choice for patients with mPC is determined by physical toxicity and financial cost, both of which favor the FOLFIRINOX regimen. Further studies should aim to evaluate the modified schedules and dosing of both regimens in multinational RCTs and search for biomarkers that predict response to treatment[34]. In addition, the choice of first-line therapy in the future may not be limited to these two regimens, as newly developed drugs/therapeutic strategies should be tested in clinical trials to find more efficacious options for patients with good performance status.

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ORIGINAL ARTICLE

Basic Study

Characterization of smooth muscle, enteric nerve, interstitial cells of Cajal, and fibroblast-like cells in the gastric musculature of patients with diabetes mellitus

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Abstract

AIM

To investigate histologic abnormalities in the gastric smooth muscle of patients with diabetes mellitus (DM).

METHODS

Full-thickness gastric specimens were obtained from patients undergoing surgery for gastric cancer. H&E stain and Masson's Trichrome stain were performed

to assess the degree of fibrosis. Immunohistochemical staining using various antibodies was also performed [antibodies against protein gene product 9.5 (PGP9.5), neuronal nitric oxide synthase (nNOS), vasoactive intestinal peptide (VIP), neurokinin-1 (NK1) receptor, c-Kit, and platelet-derived growth factor receptor-alpha, (PDGFRα)]. Immunofluorescent staining and evaluation with confocal microscopy were also conducted.

RESULTS

Twenty-six controls and 35 diabetic patients (21 short-duration patients and 14 long-duration patients) were included. There were no significant differences in basic demographics between the two groups except in mean body mass index (BMI) (higher in the DM group). Proportions of moderate-to-severe intercellular fibrosis in the muscle layer were significantly higher in the DM group than in the control group ($P < 0.01$). On immunohistochemical staining, c-Kit- and PDGFRα-positive immunoreactivity were significantly decreased in the DM group compared with the control group ($P < 0.05$). There were no statistically significant differences in PGP9.5, nNOS, VIP, and neurokinin 1 expression. On immunofluorescent staining, cellularity of interstitial cells of Cajal (ICC) was observed to decrease with increasing duration of DM.

CONCLUSION

Our study suggests that increased intercellular fibrosis, loss of ICC, and loss of fibroblast-like cells are found in the smooth muscle of DM patients. These abnormalities may contribute to changes in gastric motor activity in patients with DM.

Key words: Diabetes mellitus; Interstitial cells of Cajal; Fibroblast-like cell; Gastroparesis; Enteric nerve system

Core tip: In this study, we discovered that increased intercellular fibrosis, loss of interstitial cells of Cajal, and loss of fibroblast-like cells are found in the smooth muscle of diabetes mellitus (DM) patients. These abnormalities may contribute to changes in gastric motor activity in patients with DM.

Park KS, Cho KB, Hwang IS, Park JH, Jang BI, Kim KO, Jeon SW, Kim ES, Park CS, Kwon JG. Characterization of smooth muscle, enteric nerve, interstitial cells of Cajal, and fibroblast-like cells in the gastric musculature of patients with diabetes mellitus. *World J Gastroenterol* 2016; 22(46): 10131-10139 Available from: URL: http://www.wjgnet.com/1007-9327/full/v22/i46/10131.htm DOI: http://dx.doi.org/10.3748/wjg.v22.i46.10131

INTRODUCTION

Diabetes mellitus (DM), a metabolic disease caused by failure of blood sugar control, is a very common disorder, with a prevalence of 8.7% in adults[1]. It is well known that a lack of treatment of DM results in critical damage by causing acute complications (such as diabetic ketoacidosis or nonketotic hyperosmolar coma) along with chronic complications including nephropathy, angiopathy, neuropathy, and ophthalmopathy[2]. It is also known that the majority of DM patients suffer one or more gastrointestinal (GI) symptoms, which involve abdominal pain, early satiety, constipation, diarrhea, nausea, vomiting, and fecal incontinence, and that these symptoms result in a lower quality of life for patients[3-8]. Although the mechanisms of GI complications in DM patients are still not completely understood, GI motor disturbance appears to play a critical role. Because factors including GI smooth muscle, intrinsic or extrinsic enteric nervous system (ENS), and GI hormones are involved in the control of GI motility, it is possible to hypothesize that damage to these factors causes GI dysmotility, and various GI symptoms might occur according to the sites involved[9].

Gastroparesis, a kind of GI complication of DM, is characterized by delayed gastric emptying[10], and occurs as a result of a problem in postprandial gastric contraction activity[9,11,12]. The major symptoms of gastroparesis include postprandial fullness, early satiety, nausea, vomiting, abdominal distension, and abdominal pain. Although many other diseases and circumstances such as medication, connective tissue disorders, neurologic disorders, and tumors can also be related to gastroparesis, DM is the most common cause[3,9,11,13]. In the past, diabetic gastroparesis was regarded as an ambiguous and rare condition that was caused by the irreversible damage of the vagal nervous system, which occurs after an extremely long presence of type 1 DM; however, since the introduction of the "gastric transit time" concept, many studies have been conducted into the pathophysiology of diabetic gastroparesis[5,11,12,14].

For an intact gastric emptying, synergic and appropriate movements of the proximal stomach, distal stomach, pylorus, and small intestine play critical roles. The role of the nervous system, which controls the gastric smooth muscle, is extremely important during gastric emptying[15]. However, recent studies show that the intragastric motor neurotransmission process causing gastric contraction is more complex than a simple process in which the neurotransmitters from nerve endings combine with the receptors of smooth muscle cells (SMC), and it is well known that the interstitial cells of Cajal (ICC) play a very important role during this neurotransmission process[16-20]. Although there is not enough information about the roles in this neurotransmission process, fibroblast-like cells (FLCs) also show network connections to SMC *via* gap junctions. Therefore, it is reasonable to assume that FLCs perform some role in the GI contraction process[21]. We can estimate the degree of expression of FLCs by immunohistochemical or immunofluorescent staining,

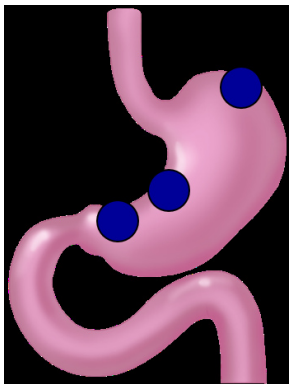

Figure 1 Tissue-sampled sites. Full-thickness tissue samples (2 cm × 1 cm size) were obtained from tumor-free sites in fundus, less curvature of corpus, and less curvature of antrum.

because they are widely stained with antibody to platelet-derived growth factor receptor α (PDGFRα)[22].

Research into the pathophysiology of diabetic gastroparesis has hitherto been performed by means of animal models; not only is research using human gastric tissue rare, but the results also do not match well with those of animal models. Therefore, we intend to investigate how SMC, ENS, ICC, and FLCs are affected in DM using human gastric smooth muscle tissue.

MATERIALS AND METHODS

Subjects and tissues

Gastric specimens were obtained from gastric cancer patients who had been admitted to four university hospitals (Keimyung University Dongsan Hospital, Youngnam University Hospital, Kyungpook National University Hospital, Catholic University of Daegu Hospital) in Daegu province, South Korea, for surgery. Shortly after gastrectomy, entire layered tissues of 1 cm × 2 cm in size were taken from areas free of cancer infiltration and used for various microscopic evaluations.

The tissues were taken from fundus, lesser curvature of corpus, and lesser curvature of antrum in the cases of total gastrectomy, and were taken from lesser curvature of corpus, and lesser curvature of antrum in the cases of subtotal gastrectomy (Figure 1). The tissues were obtained shortly after surgery, and removed tissues were fixed in formalin immediately.

The study protocol was reviewed and approved by the Institutional Review Board at Keimyung University Dongsan Hospital, Daegu, South Korea. A precise explanation of the protocol was given to each patient by a coordinator, and all the patients provided written informed consent before inclusion in this study.

H/E and Masson's Trichrome stain

Tissue samples were fixed in formalin and embedded in paraffin. Sections (4-μm thick) were stained with H&E (hematoxylin and eosin) and Masson's Trichrome to evaluate the degree of fibrosis of the muscularis propria layer. Each microscopic evaluation was performed by the same pathologist who was blind to the group to which the patient belonged. The degree of fibrosis was estimated by consulting the criteria which are used for the estimation of hepatic fibrosis in chronic hepatitis[23]: mild fibrosis means minimal fibrosis without bridging; moderate fibrosis means bridging fibrosis without encirclement; severe fibrosis means muscle fiber-encircling fibrosis (Figure 2). The degree of fibrosis was compared between the two groups (no or mild fibrosis *vs* moderate or severe fibrosis).

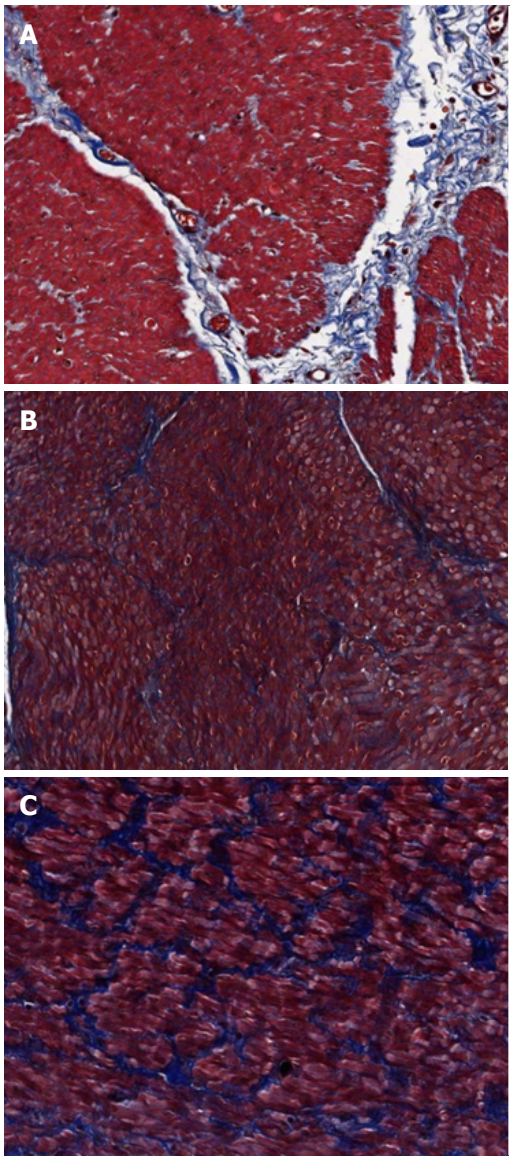

Figure 2 Degree of intercellular fibrosis. A: Mild fibrosis means minimal fibrosis without bridging; B: Moderate fibrosis means bridging fibrosis without encirclement; C: Severe fibrosis means muscle fiber-encircling fibrosis. Masson's Trichrome stain (× 200).

Immunohistochemical staining

Sections (4-μm thick) from tissue embedded in a paraffin block were mounted on Superfrost Plus® glass slides (VWR Scientific, West Chester, PA, United States) and incubated at 60 ℃ for 15 min. Slides were deparaffinized in xylene, rehydrated in graded alcohol,

Table 1 Patient demographics *n* (%)

	DM (*n* = 35)	Control (*n* = 26)	*P* value
Age (years)	62.3 ± 8.9	59.7 ± 10.4	0.297
Gender			
Male	24 (68.6)	15 (57.7)	0.428
Female	11 (31.4)	11 (42.3)	
BMI (kg/m^2)	24.2 ± 3.1	22.6 ± 3.0	0.045
UGI symptoms			
Yes	26 (74.3)	22 (84.6)	0.366
No	9 (25.7)	4 (15.4)	

UGI: Upper gastrointestinal; DM: Diabetes mellitus; BMI: Body mass index.

and washed in tap water. Endogenous peroxidase activity was blocked by incubating the sections with 3% H_2O_2. Slides were placed in a steam cooker that was filled with 10 mmol/L sodium citrate buffer (pH 6.0) for antigen retrieval. After treatment with protein block (DAKO, Carpinteria, CA, United States) for 10 min to block nonspecific protein binding, the rabbit monoclonal or polyclonal antibody for PDGFR-α (sc-338, Santa Cruz Biotechnology, Dallas, TX, United States), neuronal nitric oxide synthase (nNOS) (EP1855Y, Abcam, MA, United States), neurokinin-1 (NK1) receptor (NB100-74469, Nobus, CO, United States), protein gene product 9.5 (PGP9.5) (318A-16, Cell Marque, CA, United States), vasoactive intestinal peptide (VIP) (NB100-6568, Nobus, CO, United States), and c-kit (sc-5535, Santa Cruz Biotechnology, Dallas, TX, United States) were applied for 1 h, respectively. After reaction with a biotinylated anti-mouse antibody for 30 min, antigen-antibody complexes were visualized using a streptavidin-horseradish peroxidase conjugate (DAKO LSAB kit; DAKO, Los Angeles, CA, United States) and diaminobenzidine as a chromogen. Slides were counterstained with Mayer's hematoxylin for 3-5 min. The results were expressed as stained cell numbers under high magnification (× 400). Each value was calculated from a mean of three different sites.

Immunofluorescent staining

After washing sections (4-μm thick) from tissue embedded in paraffin block with phosphate-buffered saline (PBS, pH 7.4) and 3% dehydroxide solution for 5 min, sections were preincubated with blocking solution (Invitrogen, Carlsbad, CA, United States) for 30 min before being incubated with the anti-ICC (ab5506, Abcam, Cambridge, United Kingdom). After the sections were incubated for 90 min with the primary antibodies, they were washed with PBS again before being incubated with secondary antibody (Alexa Fluor 488 goat anti-rabbit antibody, Invitrogen, CA, United States) for 90 min at 24 ℃. After rewashing with PBS, the specimens were counterstained with 4',6-diamidino-2-phenylindole (DAPI) and mounted. The immunostained tissues were evaluated with confocal laser scanning microscopy (LSM 5 EXCITER; Carl Zeiss, Jena, Germany), using a C-Apochromat objective lens (× 40). Image analysis was completed with LSM software (version 3.98, Carl Zeiss, Jena, Germany). Intensity of fluorescence in the DM group was compared with that in the control group and expressed as a percentage.

Statistical analysis

Values were compared between the DM and control groups. If necessary, subgroup analyses of the DM group between long-term (prevalent 10 or more years) and short-term (prevalent less than 10 years) groups were performed. The SPSS statistical package ver. 20.0 (SPSS Inc., Chicago, IL, United States) was used for statistical analyses. All data are presented as the mean ± SD for continuous variables and as frequency or percentage for categorical variables. Student's *t*-test was used for the comparison of continuous variables and a Pearson's χ^2 test for that of categorical variables. *P* values less than 0.05 were considered statistically significant.

RESULTS

Patient characteristics

From four university hospitals, 61 patients were registered: 39 were male and 22 were female. Thirty-six patients underwent total gastrectomy and 25 received subtotal gastrectomy. The number of patients in the control group was 26 and that of DM patients was 35; among the DM patients, 14 had suffered DM for 10 or more years. Age, gender, and frequency of upper-GI symptoms did not significantly differ between the two groups; however, patients in the DM group had a body mass index (BMI) of 24.2 ± 3.1 kg/m^2, which was higher than the average BMI of the control group, which was 22.62 ± 3.0 kg/m^2 (*P* = 0.045) (Table 1).

Fibrosis

Frequency of moderate or severe fibrosis appeared to be 80.0% in the antrum, 85.7% in the body, and 81.3% in the fundus for the DM group. However, in the control group, the frequency of moderate or severe fibrosis was 30.8% in the antrum, 42.3% in the body, and 28.6% in the fundus. Therefore, the degree of fibrosis was statistically higher in the DM group in all areas of the stomach (Table 2). However, the DM duration did not affect the degree of fibrosis in any part of the stomach.

Immunohistochemical staining

When observed at high-power magnification (× 400), the number of c-Kit (+) cells, indicating ICC, appeared to be 11.6 ± 3.6 in the antrum, 12.3 ± 3.8 in the body, and 12.1 ± 3.4 in the fundus of the control group, whereas the antrum, body, and fundus of the DM group yielded c-Kit (+) cell numbers of 8.4 ± 2.9, 8.0 ± 2.8, and 8.4 ± 2.4, respectively. Therefore, the

Table 2 Degree of intercellular fibrosis *n* (%)

Degree of fibrosis	DM (*n* = 35)	Control (*n* = 26)	*P* value
Antrum			< 0.001
Moderate to severe	28 (80.0)	8 (30.8)	
None or mild	7 (20.0)	18 (69.2)	
Body			0.001
Moderate to severe	30 (85.7)	11 (42.3)	
None or mild	5 (14.3)	15 (57.7)	
Fundus			0.003
Moderate to severe	13 (81.3)	6 (28.6)	
None or mild	3 (18.8)	15 (71.4)	

DM: Diabetes mellitus.

Table 3 Results of immunohistochemical stain

	DM (*n* = 35)	Control (*n* = 26)	*P* value
c-Kit			
Antrum	8.35 ± 2.89	11.63 ± 3.64	0.001
Body	7.98 ± 2.84	12.29 ± 3.84	0.000
Fundus	8.27 ± 2.40	12.16 ± 3.38	0.001
PDGFRα			
Antrum	7.45 ± 1.96	7.49 ± 2.58	0.965
Body	6.73 ± 2.37	9.13 ± 4.00	0.010
Fundus	5.33 ± 2.73	7.16 ± 1.90	0.021
PGP9.5			
Antrum	18.39 ± 5.16	18.64 ± 6.09	0.930
Body	15.47 ± 3.94	17.38 ± 4.98	0.090
Fundus	12.89 ± 5.76	14.22 ± 5.84	0.656
nNOS			
Antrum	9.01 ± 4.01	8.17 ± 3.06	0.393
Body	7.75 ± 2.22	8.06 ± 3.79	0.976
Fundus	5.67 ± 2.61	6.08 ± 2.42	0.747
VIP			
Antrum	4.73 ± 2.25	4.79 ± 2.90	0.720
Body	5.19 ± 2.14	5.33 ± 2.40	0.952
Fundus	3.79 ± 2.09	5.05 ± 2.94	0.256
NK1R			
Antrum	0.65 ± 0.74	0.36 ± 0.38	0.253
Body	0.77 ± 0.78	0.59 ± 0.63	0.460
Fundus	0.54 ± 0.40	0.57 ± 0.60	0.705

DM: Diabetes mellitus; PDGFRα: Platelet-derived growth factor receptor α; PGP9.5: Protein gene product 9.5; nNOS: Neuronal nitric oxide synthase; VIP: Vasoactive intestinal peptide; NK1R: Neurokinin 1 receptor.

number of c-Kit (+) cells was lower in the DM group than in the control group in all areas of the stomach ($P < 0.001$) (Table 3; Figure 3A and B).

However, the average PDGFRα (+) cell numbers in the control group were found to be 7.5 ± 2.6 in the antrum, 9.1 ± 4.0 in the body, and 7.2 ± 1.9 in the fundus, whereas the antrum, body, and fundus of the DM group yielded PDGFRα (+) cell numbers of 7.5 ± 2.0, 6.7 ± 2.4, and 5.3 ± 2.7, respectively. Therefore, fewer PDGFRα (+) cells were found in the body ($P = 0.010$) and the fundus ($P = 0.021$) of the DM group compared to those of the control group (Table 3; Figure 3C and D).

There were no significant differences between both groups with regard to degree of expression of PGP9.5, nNOS, VIP, or NK1 receptor in any areas of stomach (Table 3).

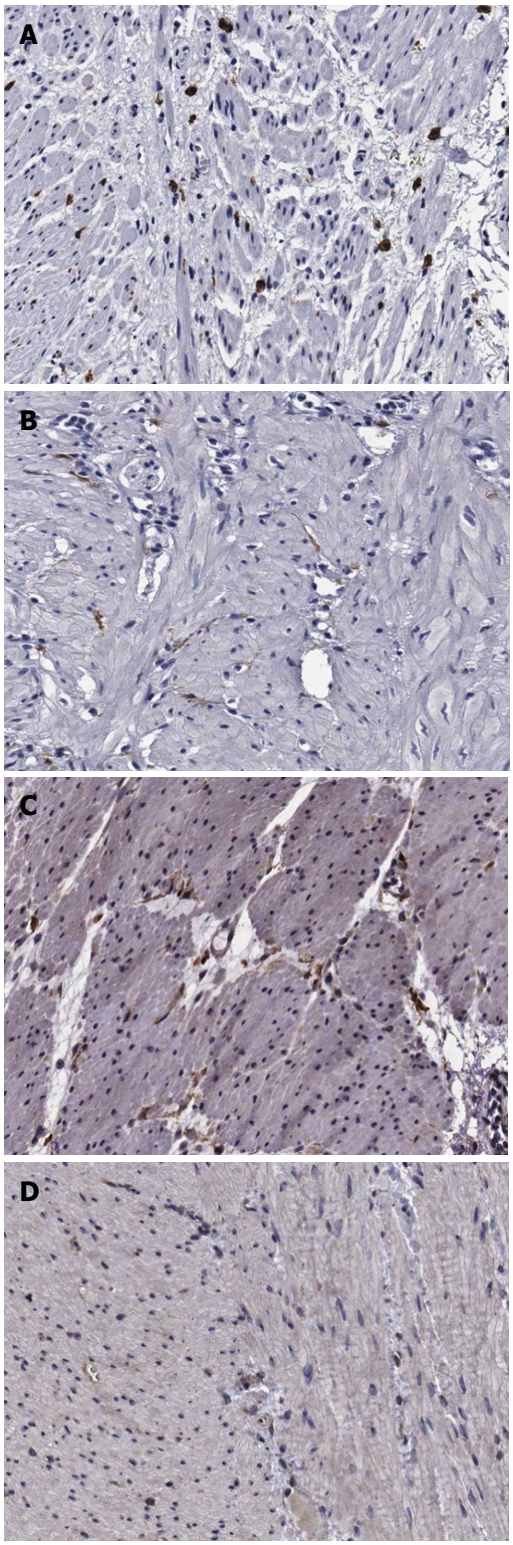

Figure 3 Immunohistochemical staining of interstitial cells of Cajal (upper panel) and platelet-derived growth factor receptor α-positive fibroblast-like cells (lower panel) in the human gastric corpus (× 200). Cellularity of ICC is higher in the control group (A) than in the DM group (B). Cellularity of FLCs is higher in the control group (C) than in the DM group (D). DM: Diabetes mellitus; FLCs: Fibroblast-like cells.

Immunofluorescent staining

Immunofluorescence intensity of c-Kit (+) cells was 100.0% ± 13.2% in the control group, 64.1% ± 0.7% in the DM group of < 10 years' duration, and 36.1%

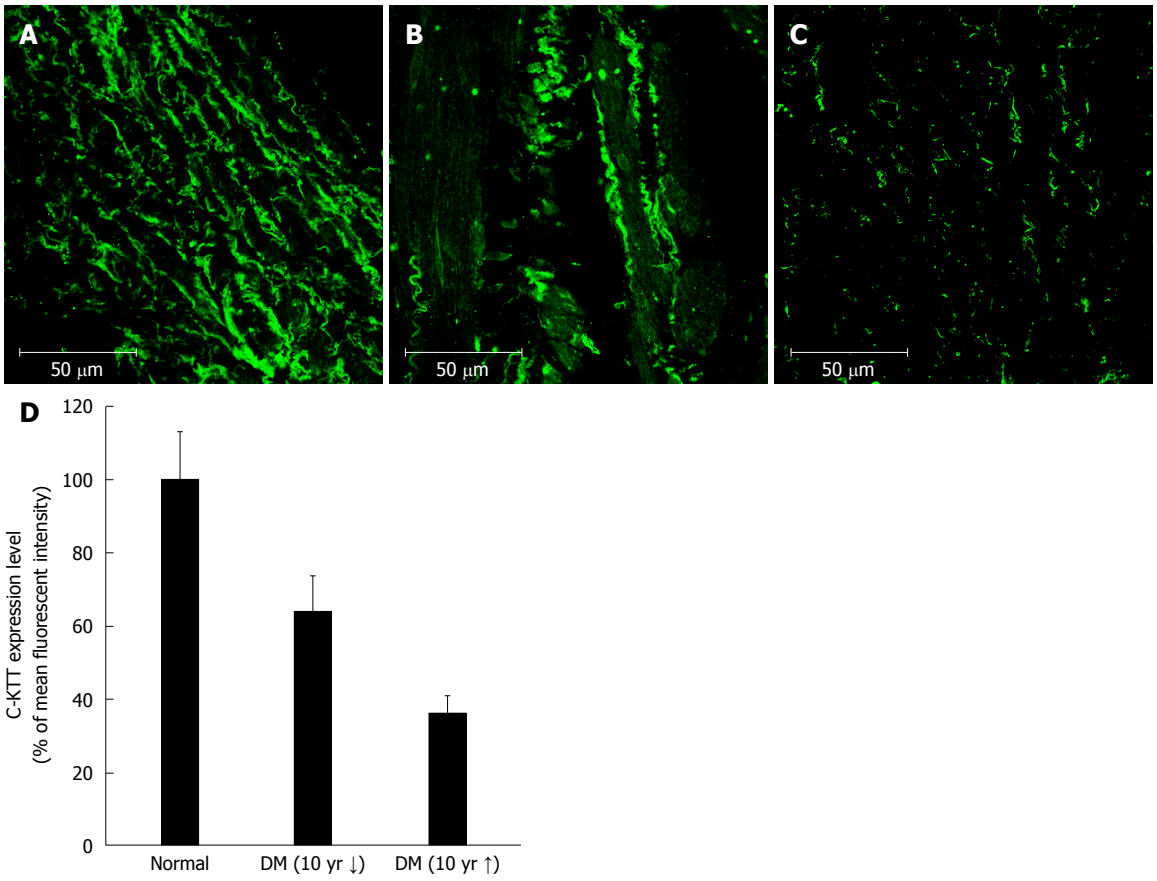

Figure 4 Immunofluorescent staining of interstitial cells of Cajal in the human gastric corpus. Panels (A), (B), and (C) show representative images from the control, DM of < 10 years' duration, and DM of > 10 years' groups, respectively. Cellularity of ICC decreases with increasing duration of DM (D). DM: Diabetes mellitus; ICC: Interstitial cells of Cajal.

± 5.1% in the DM group with > 10 years' duration. Therefore, with increasing DM duration, the density of c-Kit (+) cells appeared to decrease (Figure 4).

DISCUSSION

In this research, the effects of DM on SMC, ENS, ICC, and FLCs (all related to GI motility) were investigated. Results showed that DM patients have excessive amounts of fibrosis in their gastric smooth muscles; decreased density of ICC and PDGFRα was also found in these patients.

Because formerly reported studies related to the gastric smooth muscle were achieved by means of animal experimentation and only a few studies used human gastric tissue, human gastric smooth muscle samples excised during cancer surgery were used in this study.

The tissues used in this study were taken from regions isolated from the cancer foci. Although the duration of gastric cancer was not consistent in each patient and the possibility that the cancer itself might affect the structure of adjacent smooth muscle cannot be disregarded, we included cancer surgery cases because it is difficult in practice to obtain gastric smooth muscle tissue samples from cases other than these[16,24].

Extrinsic nerve (such as vagus or sympathetic nerve) dysfunction[25,26], ICC dysfunction[16,19,27], intrinsic enteric nerve dysfunction[16,28], and smooth muscle dysfunction itself[29] have been consistently suggested as factors that affect gastric motility disorder during the causation of gastroparesis in DM patients. In this study, markers for these components were also observed by means of immunohistochemical and immunofluorescent staining.

Although several reports indicate that DM patients showing gastroparesis also show dysfunction of extrinsic nerve cells[25,26], this could not be observed in this study because the tissues excised were too small. Because it is unrealistic to stain entire specimens for observation, it seems more appropriate to measure pancreatic or gastric secretory function after stimulation of the vagal nerve to evaluate the function of the gastric extrinsic nervous system.

Results from previous studies regarding changes in the gastric smooth muscle in DM patients are not consistent. Several studies report the degeneration and fibrosis of smooth muscle[30]; however, one report demonstrated no relationship of early DM with fibrosis[31]. These results suggest that fibrosis of the gastric smooth muscle might indicate an advanced state of

diabetic complication. In our study, the ratio of moderate or severe fibrosis was significantly higher in the DM group than in the control group, but there was no difference according to the prevalent duration of DM. This result suggests that fibrosis of the gastric smooth muscle begins during the early stage of DM.

Since the role of ICC is accepted to be extremely critical for proper GI motility, several GI motility disorders have been confirmed to be caused by ICC damage[32-34]. The pathophysiology of diabetic gastroparesis has also been established to involve damage to ICC, not only in animal experiments but also in a human study[27]. This damage includes both a decrease in the number of ICC and microstructural abnormality[35]. Recent studies have shown that Ano-1 is the most important protein involved in the electrophysiological role of ICC, and that abnormalities in Ano-1 are involved in the development of diabetic gastroparesis[36]. In this study, we also proved that the number of ICC is decreased in all gastric areas of DM patients, and that these numbers are more severely decreased in long-term cases of DM. However, we could not investigate the degree of Ano-1 expression and microstructural abnormality of ICC; further investigations into this are necessary.

FLCs that express PDGFRα are interstitial cells that are assumed to have a particular role in GI motility and are connected to SMC through gap junctions. Although located very close to ICC, ultrastructural and functional aspects of FLCs are distinct from those of ICC[21]. Located very close to nerve endings, FLCs are considered to have a role in neurotransmission, especially within purinergic neurotransmission[37,38]. In this study, whereas a decrease in FLCs was observed in the gastric body and fundus of the DM group, no difference in the numbers of FLCs was observed in the antrum of the DM group compared to that of the control group. Considering that the fundus and upper body of the stomach play important roles in gastric accommodation through postprandial relaxation, it can be hypothesized that the damage in gastric accommodation caused by the FLCs decrease might be the major element causing gastroparesis in DM patients. Further research will be necessary after considering the functional aspects of FLCs.

The damage caused to not only the extrinsic nervous system but also the intrinsic ENS in the diabetic animal model has long been investigated and has led to the elucidation of the impairment of nonadrenergic noncholinergic neurotransmission, impaired postreceptor response to adrenalin[39,40], and especially impaired NO-mediated neurotransmission[33,41]. One study showed the impairment of several kinds of neurotransmitters including nNOS in colonic smooth muscle of DM patients[42], while another report showed decreased expression of both nNOS and NK-1 in the gastric smooth muscle of DM patients[16]. Therefore, reduced expression of nNOS was anticipated in this research as well; however, no difference in nNOS expression was observed between the DM group and control group. The result also did not exhibit any difference between the two groups with regard to expressions of NK-1 receptor, PGP9.5 (neuronal marker), and VIP. Further investigation using immunofluorescence may be helpful in providing more clarity.

There are several limitations of our study. First, the symptom intensity and serial glucose level of each patient from the DM group were not analyzed. Because DM patients do not always show symptoms of gastroparesis, further study for the identification of pathologic factors associated with the presence or degree of symptoms will be necessary. Second, physiologic studies for investigation of gastric smooth muscle function and mechanism of muscular fibrosis were not performed. Additional studies on how the pathologic abnormalities observed in this study and gastric smooth muscle dysfunction affect each other might be helpful in the discovery of the mechanism of gastric dysmotility and the subsequent symptoms. Lastly, according to recent animal studies, the differentiation process of macrophages plays an important role in the causation of diabetic gastroparesis[43,44]; however, experiments on this process could not be performed in this study. Because very little research into the role of the macrophage differentiation process in the causation of diabetic gastroparesis has been performed in human tissue, future study on this topic is needed.

Despite these limitations and the necessity for future research, this study is valuable because abundant human tissues were used to identify effects on SMC, ICC, and FLCs in DM patients and the findings considered the prevalent duration of DM.

COMMENTS

Background

Gastroparesis, a kind of gastrointestinal (GI) complication of diabetes mellitus (DM), is characterized by delayed gastric emptying, and occurs as a result of a problem in postprandial gastric contraction activity. Although many other diseases and circumstances such as medication, connective tissue disorders, neurologic disorders, and tumors can also be related to gastroparesis, DM is the most common cause. Although the mechanisms of diabetic gastroparesis are still not completely understood, gastric motor disturbance appears to play a critical role. Because factors including gastric smooth muscle, intrinsic or extrinsic enteric nervous system (ENS), and GI hormones are involved in the control of gastric motility, it is possible to hypothesize that damage to these factors causes gastric dysmotility and gastroparetic symptoms.

Research frontiers

Research into the pathophysiology of diabetic gastroparesis has hitherto been performed by means of animal models; not only is research using human gastric tissue rare, but the results also do not match well with those of animal models.

Innovations and breakthroughs

This is a unique study that investigated the histologic abnormalities in the gastric smooth muscle of patients with DM using human gastric tissues.

Applications

Increased intercellular fibrosis, loss of interstitial cells of Cajal (ICC), and loss

of fibroblast-like cells were found in the gastric smooth muscle of DM patients. These findings suggest that changes in gastric motor activity in patients with DM may be caused by these abnormalities.

Terminology

The ICC is a kind of interstitial cell that is located in the GI tract. Many ICC communicating with each other form network systems and serve as electrical pacemakers. As a result, spontaneous electrical slow waves are generated in the GI tract. Since the role of ICC was accepted to be extremely critical for proper GI motility, several GI motility disorders have been confirmed to be caused by ICC damage.

Peer-review

In this study the authors aimed to investigate histologic abnormalities in the gastric smooth muscle of patients with DM and showed that DM patients have excessive amounts of fibrosis on their gastric smooth muscles, which may contribute to changes in gastric motor activity in patients with DM. They used histologic and staining techniques to identify the proposed changes of tissue samples. Since most of the published findings have been obtained from animal research, using human tissues makes this study distinguished and important.

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ORIGINAL ARTICLE

Basic Study

Correlation between colonic secretion and colonic motility in rats: Role of ghrelin

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Abstract

AIM

To explore the relationship between colonic secretory function and colonic motility.

METHODS

Using a rat model chronically implanted with intracere-

broventricular (ICV) and cecal catheters, we validated the correlation between colonic secretion and colonic motor functions, as well as the role of ICV injection volume.

RESULTS

Compared to saline controls (5 μL/rat), ICV acyl ghrelin at 1 nmol/5 μL enhanced the total fecal weight, accelerated the colonic transit time, and increased the fecal pellet output during the first hour post-injection, while ICV des-acyl ghrelin at 1 nmol/5 μL only accelerated the colonic transit time. These stimulatory effects on colonic motility and/or secretion from acyl ghrelin and des-acyl ghrelin disappeared when the ICV injection volume increased to 10 μL compared with saline controls (10 μL/rat). Additionally, the ICV injection of 10 μL of saline significantly shortened the colonic transit time compared with the ICV injection of 5 μL of saline. The total fecal weight during the first hour post-injection correlated with the colonic transit time and fecal pellet output after the ICV injection of acyl ghrelin (1 nmol/5 μL), whereas the total fecal weight during the first hour post-injection correlated with the fecal pellet output but not the colonic transit time after the ICV injection of des-acyl ghrelin (1 nmol/5 μL).

CONCLUSION

Colonic secretion does not always correlate with colonic motility in response to different colonic stimulations. Acyl ghrelin stimulates colonic secretion.

Key words: Colonic transit time; Fecal pellet output; Ghrelin; Intracerebroventricular injection; Secretion; Transit

Core tip: The colokinetic effects of acyl ghrelin and des-acyl ghrelin depend on the intracerebroventricular (ICV) injection volume, and the acute increase of the ICV volume accelerates the colonic transit time. In addition, acyl ghrelin, rather than des-acyl ghrelin, stimulates colonic secretion. Colonic secretion does not always correlate with colonic motility in response to different colonic stimulations.

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INTRODUCTION

The colonic motor function is assessed by the colonic transit time (CTT), geometric center of colonic motility, and fecal pellet output. The fecal pellet output is a simple and easy method to measure colonic motor function[1,2]. Recently, the geometric center method has been validated as a good measure to evaluate colonic transit; however, it requires the use of radiochromium which limits its application. The CTT using trypan blue dye is another option to measure the entire colonic motor function. This method has the advantage of no radioactivity. Our recent study showed that human/rat corticotropin releasing factor (h/r CRF)[3,4], in addition to ghrelin as demonstrated by other studies, accelerated the CTT in conscious fed rats, using the trypan blue dye method. Peripheral CRF injection was also shown to increase the fecal output and secretion[5].

Ghrelin is a 28-amino acid peptide that is mainly synthesized in the gastric oxyntic glands[6,7]. Acyl ghrelin and des-acyl ghrelin are the two major molecular forms of ghrelin found in the stomach and plasma. Acyl ghrelin is a ghrelin peptide acylated by ghrelin *O*-acyl transferase[8,9], and des-acyl ghrelin is produced with lacking *O*-*n*-octanoylation at serine 3[10,11]. Acyl ghrelin is well known as an orexigenic gut-brain peptide[12] and has the ability to regulate the gastrointestinal motility[13] and energy balance[14,15]. In contrast, des-acyl ghrelin is known to decrease food intake and disrupt the gastric motility[16,17]. Intracerebroventricular (ICV) injection of acyl ghrelin has been reported to speed the CTT[18], but the impact of des-acyl ghrelin through ICV injection on the colonic motor function is still unexplored.

In the current study, first, we aimed to investigate the effects of des-acyl ghrelin on colonic secretory and motor functions, as well as to validate the relationship between colonic secretion and motility. Second, we aimed to investigate the role of ICV injection volume in our unique rat model which can simultaneously measure the colonic motility and secretion in conscious rats.

MATERIALS AND METHODS

Animals

Male Sprague-Dawley rats (National Laboratory Animal Center, Taipei, Taiwan) weighing 250-320 g at the initial period of the experiment were used and housed in group cages under controlled illumination (light cycle: 08:00-20:00), humidity and temperature of 22.5 ± 1.5 ℃, and free access to water and laboratory chow pellets (LabDiet®, Brentwood, MO, United States). All experiments were performed starting at 9 a.m. in freely moving conscious rats, in accordance to guidelines which have been approved by the Institutional Animal Care and Use Committee, Taipei Veterans General Hospital.

Surgery

Implantation of the ICV catheter: For ICV implan-

tation, the rats were anesthetized with sodium pentobarbital (50 mg/kg, Nembutal; Abbott Laboratories, Abbott Park, IL, United States) by intraperitoneal injection, placed in a stereotaxic apparatus (Benchmark™, myNeuroLab, St. Louis, MO, United States), and implanted with a guide cannula (25-gauge; Eicom, Kyoto, Japan), which reached the right lateral ventricle. The stereotaxic apparatus was placed 0.8 mm posterior to the bregma, 1.4 mm right lateral to the midline, and 4.5 mm below the outer surface of the skull using a stereotaxic frame with the incisor bar set at the horizontal plane passing through the bregma and lambda[19,20]. The guide cannula was secured, a dummy cannula (Eicom) was inserted into the guide cannula, and a screw cap (Eicom) was placed as described in our previous study[19,20]. The rats were allowed 7 d for full recovery before food intake measurement after the implantation of the ICV catheters. If the rats did not increase their body weight 7 d after the operation, they were considered to have been injured during the operation and were excluded. All ICV injections with a total volume of 10 μL were administered over 60 s *via* the AMI-5 (Eicom).

Implantation of the colonic catheter: The rats were anesthetized with sodium pentobarbital (50 mg/kg, Nembutal; Abbott) by intraperitoneal injection. After laparotomy of the lower abdomen, the proximal colon was exposed and a catheter (3 Fr, 1-mm diameter; ATOM) was implanted into the proximal colon, 2 cm distal from the cecocolonic junction[3,4,21]. The catheter was fixed with a purse-string suture at the colonic wall and routed subcutaneously to the interscapular region, exteriorized through the skin, and secured together with an intravenous catheter for intracolonic administration of the dye marker[3,4,21]. The animals were allowed to recover for 7 d before simultaneous measurement of colonic motor and secretory functions.

Preparation of drugs

Rat *O-n*-octanoylated ghrelin (Peptides International, Inc., Luisville, KY, United States) and rat des-acyl ghrelin (Peptides International) were kept in powder form at -20 ℃, and dissolved in sterile, pyrogen-free 0.9% saline (Otsuka, Tokyo, Japan) immediately before use.

Colonic motor and secretory function tests

Measurement of the CTT: The measurement of colonic motor and secretory function was modified from our previous studies[3,4,21]. The CTT was calculated using an enteral non-absorbable dye marker, trypan blue (Sigma Chemical Co., St. Louis, MO, United States). The dye (0.2 mL) was injected through the catheter positioned in the proximal colon, followed by a 0.2 mL saline flush 10 min after the ICV injection of acyl ghrelin and des-acyl ghrelin (1.0 nmol/rat). The CTT was defined as the time interval between the dye injection and the discharge of the first blue pellet.

Measurements of fecal pellet output and total fecal weight: The rats were accustomed to single housing for 7 d before the experiment. The total number of pellets was recorded every hour for 2 h following an intracolonic injection of trypan blue. The total fecal material was collected every hour for 2 h following the intracolonic injection of trypan blue, and its content was weighed as the total fecal weight[3,4,21].

Statistical analysis

All the results are expressed as mean ± standard error of the mean. One-way analysis of variance followed by the Student-Newman-Keuls post-hoc test was used to detect the differences among groups. The relationship between total fecal weight, CTT, and the fecal pellet output in response to the ICV injection of either saline, acyl ghrelin, or des-acyl ghrelin was analyzed by Spearman's nonparametric correlation. Differences were considered statistically significant when $P < 0.05$.

RESULTS

The ICV injection of acyl ghrelin (1 nmol/5 μL/rat) significantly accelerated the CTT, and increased the fecal pellet output and total fecal weight during the first hour post-injection, but des-acyl ghrelin (1 nmol/5 μL/rat) only significantly accelerated the CTT

As compared with saline controls (5 μL/rat), ICV acyl ghrelin (1 nmol/5 μL/rat) and des-acyl ghrelin (1 nmol/5 μL/rat) significantly accelerated the mean CTT from 292 to 236 and 234 min, respectively ($P < 0.05$, Figure 1A). During the first hour post-injection, ICV acyl ghrelin (1 nmol/5 μL/rat) also significantly increased the fecal pellet output and total fecal weight ($P < 0.05$, Figure 1B and D). ICV acyl ghrelin (1 nmol/5 μL/rat) did not affect the fecal pellet output and total fecal weight during the second hour post-injection ($P > 0.05$, Figure 1C and E). ICV des-acyl ghrelin (1 nmol/5 μL/rat) did not affect either the fecal pellet output or total fecal weight during first and second hour post-injection ($P > 0.05$, Figure 1C and E).

An increased ICV injection volume shortened the CTT, which led to the disappearance of the colokinetic effects of acyl ghrelin and des-acyl ghrelin (1 nmol/10 μL/rat)

An ICV injection of 10 μL of saline significantly shortened the mean CTT from 292 to 191 min compared to the ICV injection of 5 μL of saline ($P < 0.05$, Figure 1A and F). Moreover, the ICV injection of acyl ghrelin (1 nmol/10 μL/rat) and des-acyl ghrelin (1 nmol/10 μL/rat) did not have any effects on the CTT, fecal pellet output, and total fecal weight, compared to saline controls (10 μL/rat, Figure 1F-J).

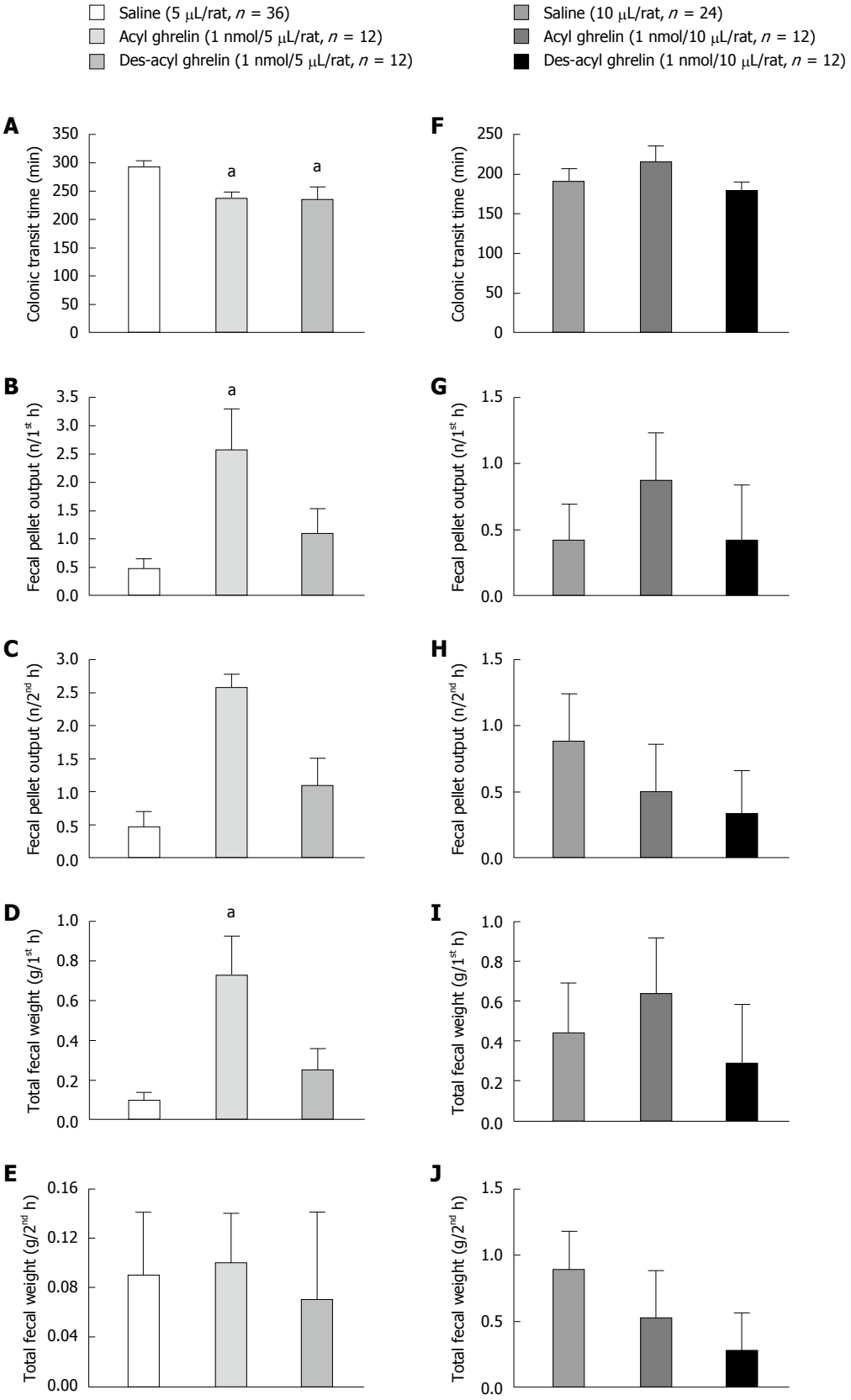

Figure 1 Effects of acyl ghrelin and des-acyl ghrelin on the colonic transit time (A and F), fecal pellet output (B, C, G, and H), and total fecal weight (D, E, I, and J) during the first and second hour post-injection at different intracerebroventricular injection volumes (5 μL/rat: A-E *vs* 10 μL/rat: F-J). Data are presented as mean ± standard error of the mean. $^{a}P < 0.05$, *vs* saline controls.

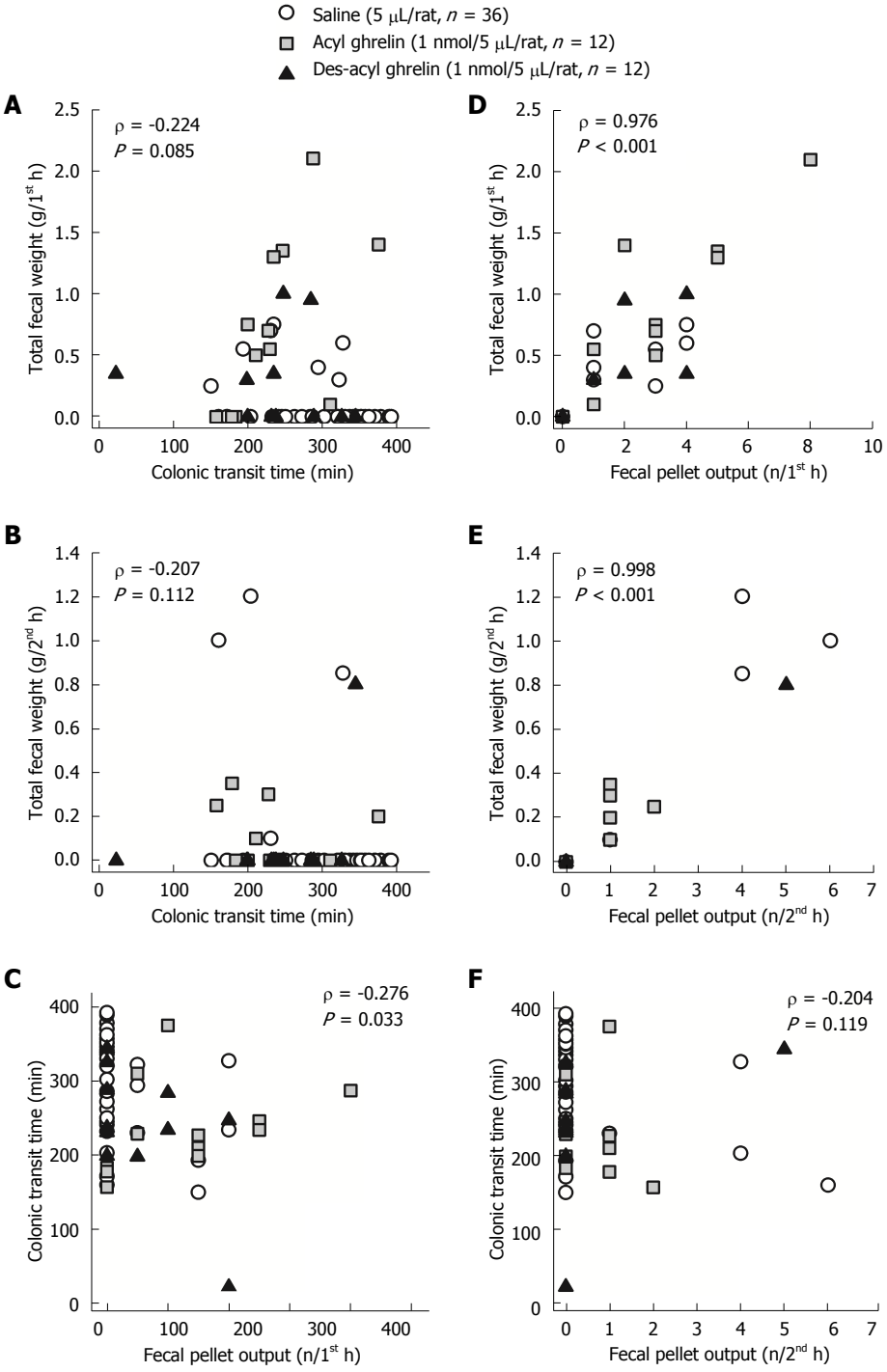

Figure 2 Pooled data demonstrating the relationships among total fecal weight (A-D), colonic transit time (A, B, C, and F), and fecal pellet output (C-F) stimulated by saline, acyl ghrelin, and des-acyl ghrelin with the intracerebroventricular injection volume at 5 μL.

Relationships between total fecal weight, CTT, and fecal pellet output with an ICV injection volume of 5 μL

We pooled the data from the ICV injection of saline, acyl ghrelin, and des-acyl ghrelin at 5 μL, and analyzed the correlations among total fecal weight, CTT, and fecal pellet output. The total fecal weight did not correlate with the CTT during the first hour and second hour post-injection ($P > 0.05$, Figure 2A and B), whereas the total fecal weight exhibited a significantly positive correlation with the fecal pellet output ($P < 0.001$, Figure 2D and E). The CTT had a negative correlation with the fecal pellet output during the first hour ($P < 0.05$, Figure 2C) but not the second hour post-injection ($P > 0.05$, Figure 2F).

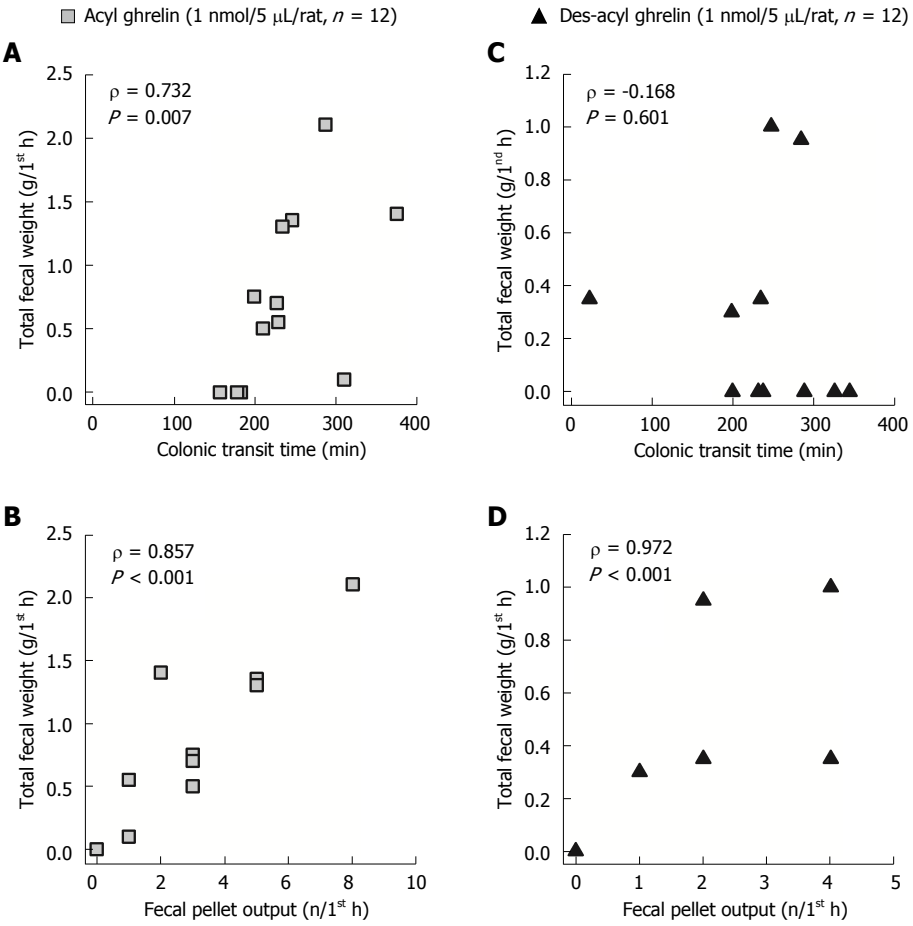

Figure 3 The relationships among total fecal weight, colonic transit time, and fecal pellet output during the first hour post-injection stimulated by either acyl ghrelin (A and B) or des-acyl ghrelin (C and D) with the intracerebroventricular injection volume at 5 μL.

Relationships between total fecal weight, CTT, and fecal pellet output during the first hour post-injection stimulated by acyl ghrelin and des-acyl ghrelin, respectively, with an ICV injection volume at 5 μL

We analyzed the correlations between total fecal weight, CTT, and fecal pellet output during the first hour post-injection, in response to ICV injection of either acyl ghrelin or des-acyl ghrelin with the volume at 5 μL. The total fecal weight significantly correlated with the CTT and fecal pellet output during the first hour after ICV acyl ghrelin (1 nmol/5 μL/rat) injection ($P < 0.01$, Figure 3A and B). The total fecal weight significantly correlated with the fecal pellet output, but not the CTT, during the first hour after ICV des-acyl ghrelin (1 nmol/5 μL/rat) injection ($P < 0.001$, Figure 3C and D).

DISCUSSION

In the present study, we first demonstrated the *in vivo* effects of ICV des-acyl ghrelin on colon motor and secretory functions. ICV injection of des-acyl ghrelin at 1 nmol/5 μL accelerated the CTT without altering the fecal pellet output and total fecal weight during the first hour and second hour post-injection. Acyl ghrelin and ghrelin mimetics have been previously shown to exhibit colokinetic effects such as shortening the CTT[22] and decreasing the time to the first bowel movement[23], and may have the clinical implication in relieving diet-induced constipation in a rat model[24]. In addition to accelerating the CTT and increasing the fecal pellet output during the first hour post-injection, we also showed that the ICV injection of acyl ghrelin at 1 nmol/5 μL enhanced the total fecal weight during the first hour post-injection, which is consistent with the results that intrathecal but not intravenous application of acyl ghrelin to the L6-S1 region of the spinal cord increased the fluid output through the anal cannula[25]. Therefore, in our current study, the stimulatory properties of acyl ghrelin on the colonic secretion and motility are confirmed.

Although an acute increase of the ICV pressure has been reported to immediately suppress the amplitude of gastric and duodenal contractions in rabbits[26], the effects of the ICV injection volume on colonic secretion and motility still remain obscure. Our study was the first to demonstrate that the increased ICV injection volume (from 5 μL to 10 μL) shortened the CTT in saline controls. Because the CTT has been shortened in the saline controls, the stimulating effects on colonic motility and/or secretion by either acyl ghrelin or des-acyl ghrelin disappeared when the ICV injection vol-

ume increased to 10 μL at the same dose (1 nmol/rat). Acute moderate to severe head injury patients have been shown to have prolonged gastric emptying[27]. An increased intracranial pressure is proposed to be the major cause of gastric motility dysfunction[28]. The finding that an acutely increased ICV injection volume shortened the CTT may be explained by the increased intracranial pressure in a limited intracerebroventricular space, though we did not measure the increased intracranial pressure in our current study.

The CTT is considered a reflection of the motor function in the entire colon, while the fecal pellet output is the reflection of the distal colonic motor function[29]. This means that the acceleration of the colonic transit is not always equal to the increase of fecal pellet output. A recent rodent study indicated that the central administration of CRF and restraint stress accelerate the colonic transit but do not always correlate with the increase of fecal pellet output[28]. Our results are in accordance with this point (Figure 2B and C). We also showed that the ICV injection of acyl ghrelin enhanced the CTT, fecal pellet output, and total fecal weight, while the ICV injection of des-acyl ghrelin only accelerated the CTT. Therefore, we propose that acyl ghrelin accelerates the entire colon and distal colonic motor functions, whereas des-acyl ghrelin, lacking *O-n*-octanoylation at serine 3, may have greater effects in stimulating the proximal colon motor function without altering the distal colonic motility. In addition, our findings provide new information regarding the relationship between colonic secretion and motility. The finding that the total fecal weight during the first hour post-injection correlated with the CTT stimulated by the ICV injection of acyl ghrelin (Figure 3A) but not des-acyl ghrelin (Figure 3B) suggests that colonic secretion does not always correlate with colonic motility in response to different colonic stimulations.

In conclusion, the colokinetic effects of acyl ghrelin and des-acyl ghrelin depend on the ICV injection volume, and the acute increase of the ICV volume accelerates the CTT. In addition, acyl ghrelin, rather than des-acyl ghrelin, stimulates colonic secretion. Colonic secretion does not always correlate with colonic motility in response to different colonic stimulations.

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COMMENTS

Background

Acyl ghrelin is well known as an orexigenic gut-brain peptide and has the ability to regulate the gastrointestinal motility and energy balance. In contrast, des-acyl ghrelin is known to decrease food intake and disrupt the gastric motility.

Research frontiers

The intracerebroventricular (ICV) injection of acyl ghrelin has been reported to speed the colonic transit time (CTT), but the impact of des-acyl ghrelin through ICV injection on the colonic motor function is still unexplored.

Innovations and breakthroughs

This is the first study to investigate the effects of des-acyl ghrelin on colonic secretory and motor functions, as well as to validate the relationship between colonic secretion and motility. This is also the first study to evaluate the role of ICV injection volume on the colonic motility and secretion in conscious rats.

Applications

Using a rat model chronically implanted with ICV and cecal catheters, the authors validated the correlation between colonic secretion and colonic motor functions, as well as the role of ICV injection volume.

Terminology

The colokinetic effects of acyl ghrelin and des-acyl ghrelin depend on the ICV injection volume, and the acute increase in ICV volume accelerates the CTT. In addition, acyl ghrelin, rather than des-acyl ghrelin, stimulates colonic secretion. Colonic secretion does not always correlate with colonic motility in response to different colonic stimulations.

Peer-review

Colonic secretion does not always correlate with colonic motility in response to different colonic stimulations in rats: role of intracerebroventricular injectional volume. The aims of this study were to investigate the influences of des-acyl ghrelin on colonic secretory and motor functions, as well as to validate the relationship between colonic secretion and motility. Also the authors aimed to appraise the role of ICV injectional volume in our unique rat model which can simultaneously measure the colonic motility and secretion in conscious rats.

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ORIGINAL ARTICLE

Basic Study

C5a/C5aR pathway is essential for up-regulating SphK1 expression through p38-MAPK activation in acute liver failure

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Abstract

AIM

To investigate the role of the complement 5a (C5a)/C5a receptor (C5aR) pathway in the pathogenesis of acute liver failure (ALF) in a mouse model.

METHODS

BALB/c mice were randomly assigned to different groups, and intraperitoneal injections of lipopolysaccharide (LPS)/D-galactosamine (D-GalN) (600 mg/kg and 10 μg/kg) were used to induce ALF. The Kaplan-Meier method was used for survival analysis. Serum alanine aminotransferase (ALT) levels, at different time points within a 1-wk period, were detected with a biochemistry analyzer. Pathological examination of liver tissue was performed 36 h after ALF induction. Serum complement 5 (C5), C5a, tumor necrosis factor-α (TNF-α), interleukin (IL)-1β, IL-6, high-mobility group protein B1 (HMGB1) and sphingosine-1-phosphate

levels were detected by enzyme-linked immunosorbant assay. Hepatic morphological changes at 36 h after ALF induction were assessed by hematoxylin and eosin staining. Expression of C5aR, sphingosine kinase 1 (SphK1), p38-MAPK and p-p38-MAPK in liver tissue, peripheral blood mononuclear cells (PBMCs) and peritoneal exudative macrophages (PEMs) of mice or RAW 264.7 cells was analyzed by western blotting. C5aR mRNA levels were detected by quantitative real-time PCR.

RESULTS

Activation of C5 and up-regulation of C5aR were observed in liver tissue and PBMCs of mice with ALF. Blockade of C5aR with a C5aR antagonist (C5aRa C5aRa) significantly reduced the levels of serum ALT, inflammatory cytokines (TNF-α, IL-1β and IL-6) and HMGB1, as well as the liver tissue damage, but increased the survival rates ($P < 0.01$ for all). Blockade of C5aR decreased SphK1 expression in both liver tissue and PBMCs significantly at 0.5 h after ALF induction. C5aRa pretreatment significantly down-regulated the phosphorylation of p38-MAPK in liver tissues of ALF mice and C5a stimulated PEMs or RAW 264.7 cells. Moreover, inhibition of p38-MAPK activity with SB203580 reduced SphK1 protein production significantly in PEMs after C5a stimulation.

CONCLUSION

The C5a/C5aR pathway is essential for up-regulating SphK1 expression through p38 MAPK activation in ALF in mice, which provides a potential immunotherapeutic strategy for ALF in patients.

Key words: Acute liver failure; C5a/C5aR; p38-MAPK; Sphingosine kinase 1

Core tip: Recent studies and our work show that SphK1 and complement activation play an important role in systemic inflammation in acute liver failure (ALF). It has been shown that C5a activates sphingosine kinase 1 (SphK1) in macrophages. However, the mechanism of C5a-induced SphK1 activation is unknown. In this study we found that excessive activation of C5 and up-regulation of C5aR in liver tissue, and the C5a/C5aR pathway is essential for potentiating SphK1 expression through p38 MAPK activation in ALF. To our knowledge, this is the first report of the mechanism of C5a-induced SphK1 activation, which provides a potential immunotherapeutic strategy for ALF in patients.

Lei YC, Lu CL, Chen L, Ge K, Yang LL, Li W, Wu YH. C5a/C5aR pathway is essential for up-regulating SphK1 expression through p38-MAPK activation in acute liver failure. *World J Gastroenterol* 2016; 22(46): 10148-10157 Available from: URL: http://www.wjgnet.com/1007-9327/full/v22/i46/10148.htm DOI: http://dx.doi.org/10.3748/wjg.v22.i46.10148

INTRODUCTION

Despite availability of efficient antiviral drugs and artificial liver support system, acute liver failure (ALF) remains a largely intractable clinical problem, with high mortality rates (about 80%)[1]. It often requires urgent liver transplantation due to the limited therapeutic options[1-3]. Growing evidence suggests that ALF can trigger systemic inflammation through release of pro-inflammatory cytokines, such as tumor necrosis factor-α (TNF-α), interleukin (IL)-1β, and IL-6[4,5].

Sphingosine kinase 1 (SphK1) is an intracellular signaling enzyme that generates the lipid mediator sphingosine-1-phosphate (S1P)[6]. Several pro-inflammatory stimuli, including complement 5a (C5a), activate SphK1 on macrophages, and blockade of SphK1 attenuates inflammatory responses[7-9]. Previous studies have shown that SphK1 plays a critical role in sepsis-induced inflammatory responses[10], and our recent work showed that expression and activation of SphK1 play an important role in ALF in a mouse model[11,12].

The C5a fragment is the most powerful pro-inflammatory anaphylatoxin generated during complement activation[13]. Increasing evidence suggests that excessive C5a can cause deleterious exaggeration of the innate immune responses during bacterial infections[14-16]. In animal models of sepsis, blockade of the C5a/C5aR pathway attenuates organ injury and increases survival rates in mice[16]. Recent studies indicate that complement activation plays an important role in lipopolysaccharide (LPS)/D-galactosamine (D-GalN)- and acetaminophen (APAP)-induced ALF in mice[17,18]. Moreover, C5a is over-produced during ALF, and inhibition of C5aR signaling alleviates liver injury in an animal model of ALF[17].

Based on the above results, we speculated that activation of the C5a/C5aR pathway plays an important role in SphK1 activation in ALF. Here, we report that SphK1 activation relies on C5a/C5aR interactions, which involve the mitogen-activated protein kinase (MAPK) signaling pathway. Blocking C5a/C5aR interactions effectively prevents LPS/D-GalN-induced ALF in mice, indicating that intervention of complement activation may be a useful immunotherapeutic strategy for ALF in patients.

MATERIALS AND METHODS

Animal model of ALF and treatment

Male BALB/c mice, weighing 20 ± 0.5 g, were obtained from the Experimental Animal Center of Nanchang University (Nanchang, China). Specific pathogen-free male mice around 6-wk-old were used for all experiments. Mice were handled and treated in accordance with the strict guiding principles of the National Institution of Health for experimental care and use of animals and approved by the animal care and use committee of Zhejiang Hospital. After ALF was

induced, mice were sacrificed at the indicated time points as described previously[12]. Mice were randomly assigned to five groups (12 mice per group): PBS group, ALF group, C5aR antagonist (C5aRa) or cobra venom factor (CVF) pretreatment group, and C5aRa + CVF pretreatment group. All of the groups were observed for 1 wk.

Blockade of C5aR and complement depletion

For blockade of C5aR, mice were intraperitoneally injected with 1 mg/kg C5aRa (GL Biochem Ltd., Shanghai, China) 30 min before D-GalN/LPS challenge or treatment with PBS as a mock control. Complement was depleted by two intraperitoneal injections (7.5 U each in 200 μL of saline/mouse) of CVF (Quidel Corporation, San Diego, CA, United States). The first injection was given 24 h before D-GalN/LPS or saline administration, and the second injection was given 5 h after the first CVF injection. This was done to prevent any adverse effects of rapid loss of complement.

Quantification of alanine aminotransferase and detection of serum cytokines, high-mobility group protein B1, C5, C5a and S1P

Serum alanine aminotransferase (ALT) levels were measured using an Olympus AU5400 automatic biochemistry analyzer, and the levels of serum cytokines (TNF-α, IL-1β and IL-6), high-mobility group protein B1 (HMGB1) and S1P were measured by enzyme linked-immunosorbant assay (ELISA) as described previously[12]. Serum C5a and C5 levels were measured with commercial ELISA kits according to the manufacturer's instructions.

Western blot analysis and histological study of liver tissue

Western blot analysis was performed as previously described[11,12]. Briefly, 40 μg of protein from total tissue or cell lysate were used, and the blots were probed using polyclonal anti-mouse SphK1, C5aR, p38-MAPK and phospho-p38-MAPK antibodies (Santa Cruz Biotechnology, Dallas, TX, United States), with β-actin detected with anti-β-actin antibody (Santa Cruz Biotechnology) as a loading control. For histological study, liver tissue was fixed in 40 g/L phosphate-buffered formalin, and 3-5 μm tissue sections were cut and stained with hematoxylin and eosin (HE) before microscopic evaluation at × 200 or × 400 magnification.

Quantitative real-time polymerase chain reaction

Liver tissues were obtained from mice at the indicated time points and total RNA was extracted using TRIZOL (Invitrogen, Carlsbad, CA, United States) and then reverse transcribed into cDNA using ReverTra Ace qPCR RT kit (Toyobo, Osaka, Japan). The cDNA was then amplified by PCR. Quantitative PCR with SYBR Green Realtime PCR Master Mix-Plus (Toyobo) was performed using a Prism 7000 (Applied Biosystems Inc, Foster City, CA, United States) sequence detection system, and mRNA levels were normalized to that of the housekeeping gene β-actin. Primer sequences for PCR amplification were as follows: C5aR forward, 5′-TGGACCCCATAGATAACAGCAG-3′ and C5aR reverse, 5′-GGAACACCACCGAGTAGATGAT-3′; β-actin forward, 5′-TGGAATCCTGTGGCATCCATGAAAC-3′ and β-actin reverse, 5-AAAACGCAGCTCAGTAACAGTCCG-3′.

Peritoneal exudative macrophage isolation and cell culture

Peritoneal exudative macrophages (PEMs) were harvested from BALB/c mice. Cells were re-suspended in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS) at 5×10^6 cells/mL in a 6-well plate and incubated for 4 h; 500 ng/mL of C5a (Biovision, Milpitas, CA, United States) and 10 nmol/L of C5aRa (60-min pre-incubation) were used. PEMs were harvested and lysed for western blot analysis of SphK1. RAW 264.7 cells were obtained from the Institute of Cell Biology of the Chinese Academy of Sciences (Shanghai, China) and were cultured in 6-well plates and propagated in DMEM supplemented with 10% FBS, 100 U/mL penicillin, and 100 mg/mL streptomycin. Cells were stimulated with C5a (500 ng/mL) or C5a + C5aRa (pre-incubated for 60 min with 10 nmol/L of C5aRa in the presence of C5a). Inhibition of p38-MAPK activity was achieved with SB203580 (10 mmol/L).

Statistical analysis

Data are expressed as the mean ± standard error of the mean. Statistical significance was determined by a two-tailed Student's *t*-test or one-way analysis of variance (ANOVA), and, specifically, a log-rank test for survival analysis. A P value < 0.05 was considered statistically significant. Statistical image analysis was performed after determining that the data fit a normal distribution. A two-tailed Student's *t*-test was employed after the exclusion of outliers that were less or greater than two standard deviations away from the median. All statistical analyses were performed using SPSS 13.0 for Windows.

RESULTS

Excessive activation of C5 and up-regulation of C5aR in liver tissue of mice with ALF

To address the relevance of C5 activation in ALF in mice, we first challenged BALB/c mice with D-GalN/LPS and examined C5 activation over a time course. Upon D-GalN/LPS challenge, C5a levels in serum rapidly increased within 6 h and peaked at 12 h (Figure 1A). Although serum C5a levels began to decrease after 24 h of ALF induction (Figure 1B), the serum C5a level at 36 h was still higher than that of the unchallenged mice (Figure 1B). Compared to the controls, expression of C5aR mRNA (> 15-fold) and protein was elevated significantly in liver tissue of

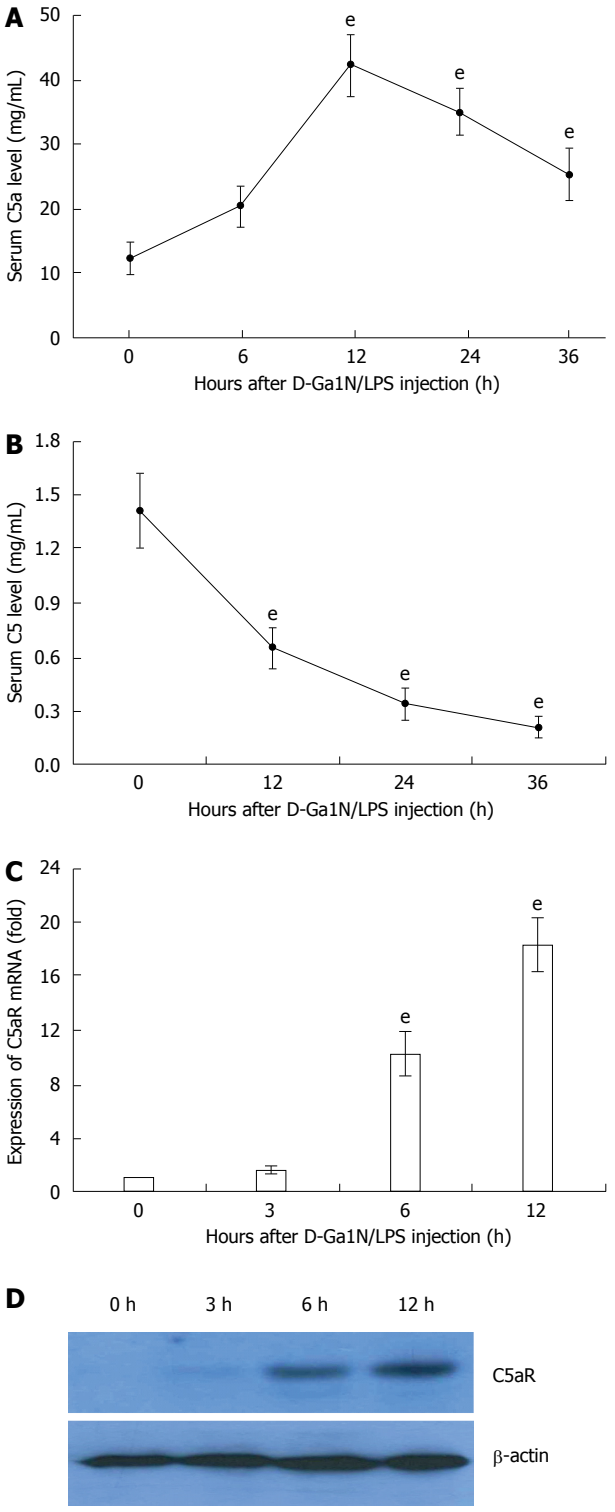

Figure 1 Excessive activation of C5 and up-regulation of C5aR in liver tissue of mice with acute liver failure. Acute liver failure was induced in BALB/c mice using D-GalN (600 mg/kg) and LPS (10 μg/kg). A: Serum levels of C5a at 12, 24 and 36 h increased significantly compared with that at 0 h (42.8 ng/mL ± 4.77 ng/mL, 35.22 ng/mL ± 3.62 ng/mL, 25.52 ng/mL ± 4.02 ng/mL *vs* 12.23 ng/mL ± 2.55 ng/mL, t = 19.31, 17.2, and 9.67, respectively, $^{e}P < 0.001$); B: Serum levels of C5 at 12, 24 and 36 h increased significantly compared with that at 0 h (0.65 mg/mL ± 0.117 mg/mL, 0.343 mg/mL ± 0.09 mg/mL, 0.211 mg/mL ± 0.06 mg/mL *vs* 1.413 mg/mL ± 0.209 mg/mL, t = 11.03, 16.29, and 19.15, respectively, $^{e}P < 0.001$); C and D: Expression of C5aR mRNA and protein in liver tissue. The mean ± SE of three independent experiments is shown (error bar indicates standard error).

ALF mice (Figure 1C and D). These results suggest that excessive complement activation occurs during D-GalN/LPS-induced ALF in mice.

Blockade of the C5a/C5aR pathway attenuates D-GalN/LPS-induced ALF in mice

To achieve a blockade of C5aR signaling during ALF, we chose to use a C5aRa. Blockade of C5aR apparently attenuated ALF, as demonstrated by a significant reduction in serum levels of ALT (Figure 2A), inflammatory cytokines (Figure 2B and C) and the liver tissue damage (Figure 2D), as well as an increase in survival rates (Figure 2E) in mice after D-GalN/LPS challenge. In addition, depleting complement by CVF pretreatment also resulted in a significant decrease in susceptibility to D-GalN/LPS challenge, as evidenced by a reduction in serum ALT levels (Figure 3A) along with an increase in survival rates (Figure 3B) compared with the control. Furthermore, treatment with C5aRa further lessened the pathogenic effect on D-GalN/LPS challenged mice receiving CVF pretreatment, as evidenced by lower levels of serum ALT (Figure 3C), inflammatory cytokines (TNF-α, IL-1β and IL-6) and HMGB1 (Figure 3D and E). These data clearly demonstrate that abrogating the C5a/C5aR pathway can alleviate the severity of ALF.

C5a/C5aR pathway is required for the expression of SphK1 during ALF

Given the critical role of SphK1 in D-GalN/LPS-induced ALF[11,12], we measured SphK1 expression in C5aRa pretreated mice. These mice exhibited a significant reduction in SphK1 expression in both liver tissue and peripheral blood mononuclear cells (PBMCs) at 0.5 h after ALF induction (Figure 4A and B). Blockade of SphK1 activity *in vivo* was confirmed by reduced S1P level (Figure 4C). Moreover, treatment with CVF appeared to be capable of further reducing SphK1 expression in ALF mice receiving C5aRa (Figure 4A and B).

Previous evidence has suggested that macrophages and PBMCs are the major source of SphK1 expression during D-GalN/LPS-induced ALF in mice[11,12] and C5aR is highly expressed in macrophages and the macrophages-derived cell line RAW 264.7[19]. To further explore whether C5a could directly induce SphK1 expression in macrophage, we stimulated murine PEMs with recombinant C5a. Compared to the controls, the C5a stimulated PEMs exhibited a significant increase in SphK1 expression, and this effect was abolished by C5aRa pretreatment (Figure 4C), indicating that C5a/C5aR interactions directly modulate SphK1 production in macrophages. Furthermore, C5a treatment led to an increase in SphK1 expression in RAW 264.7 cells and this effect was also abolished by C5aRa pretreatment (Figure 4D). These results indicate that the C5a/C5aR pathway associates with SphK1 expression and the pathogenesis of ALF in mice.

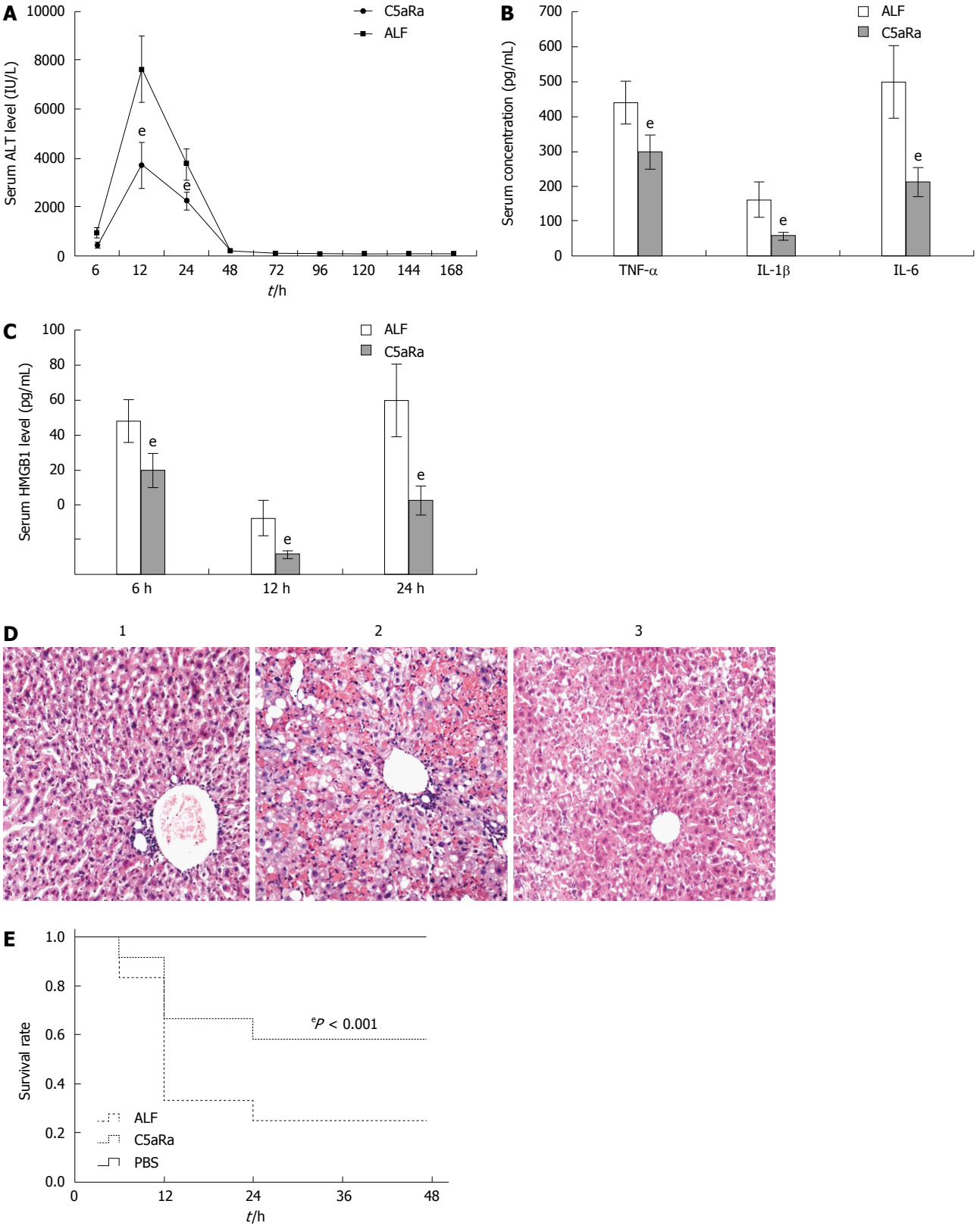

Figure 2 C5aRa attenuates D-GalN/LPS induced acute liver failure in mice. A: C5aRa decreased serum levels of ALT at 12 h and 24 h significantly (3736.12 IU/L ± 937.98 IU/L *vs* 7612.78 IU/L ± 1379.21 IU/L, 2225.07 IU/L ± 381.99 IU/L *vs* 3741.74 IU/L ± 637.53 IU/L, t = 8.05 and 7.07, respectively, $^{e}P < 0.001$); B: C5aRa reduced serum levels of TNF-α, IL-1β and IL-6 at 12 h (299.35 pg/mL ± 50.61 pg/mL *vs* 439.33 pg/mL ± 63.59 pg/mL, 57.42 pg/mL ± 12.98 pg/mL *vs* 106.69 pg/mL ± 49.87 pg/mL, 213.52 pg/mL ± 42.69 pg/mL *vs* 500.87 pg/mL ± 104.14 pg/mL, t = 5.96, 6.94, and 8.84 respectively, $^{e}P < 0.001$); C: C5aRa reduced HMGB1 levels at 6, 12 and 24 h (18.14 ng/mL ± 4.08 ng/mL *vs* 60.23 ng/mL ± 5.47 ng/mL; 16.21 ng/mL ± 5.11 ng/mL *vs* 67.14 ng/mL ± 14.27 ng/mL; 15.42 ng/mL ± 6.23 ng/mL *vs* 48.71 ng/mL ± 15.6 ng/mL, t = 9.13, 11.64, and 6.85, respectively, $^{e}P < 0.001$); D: Immune cell infiltration and tissue damage were detected by HE staining at 36 h after onset of ALF (1 = normal mice; 2 = ALF mice; 3 = C5aRa treated mice; magnification, × 100); E: Kaplan-Meier analysis of the effect of C5aRa on survival rates of animals ($^{e}P < 0.001$, log-rank test, F = 14.06). The mean ± SE of three independent experiments is shown (error bar indicates standard error). ALF: Acute liver failure. TNF-α: Tumor necrosis factor-α; IL: Interleukin.

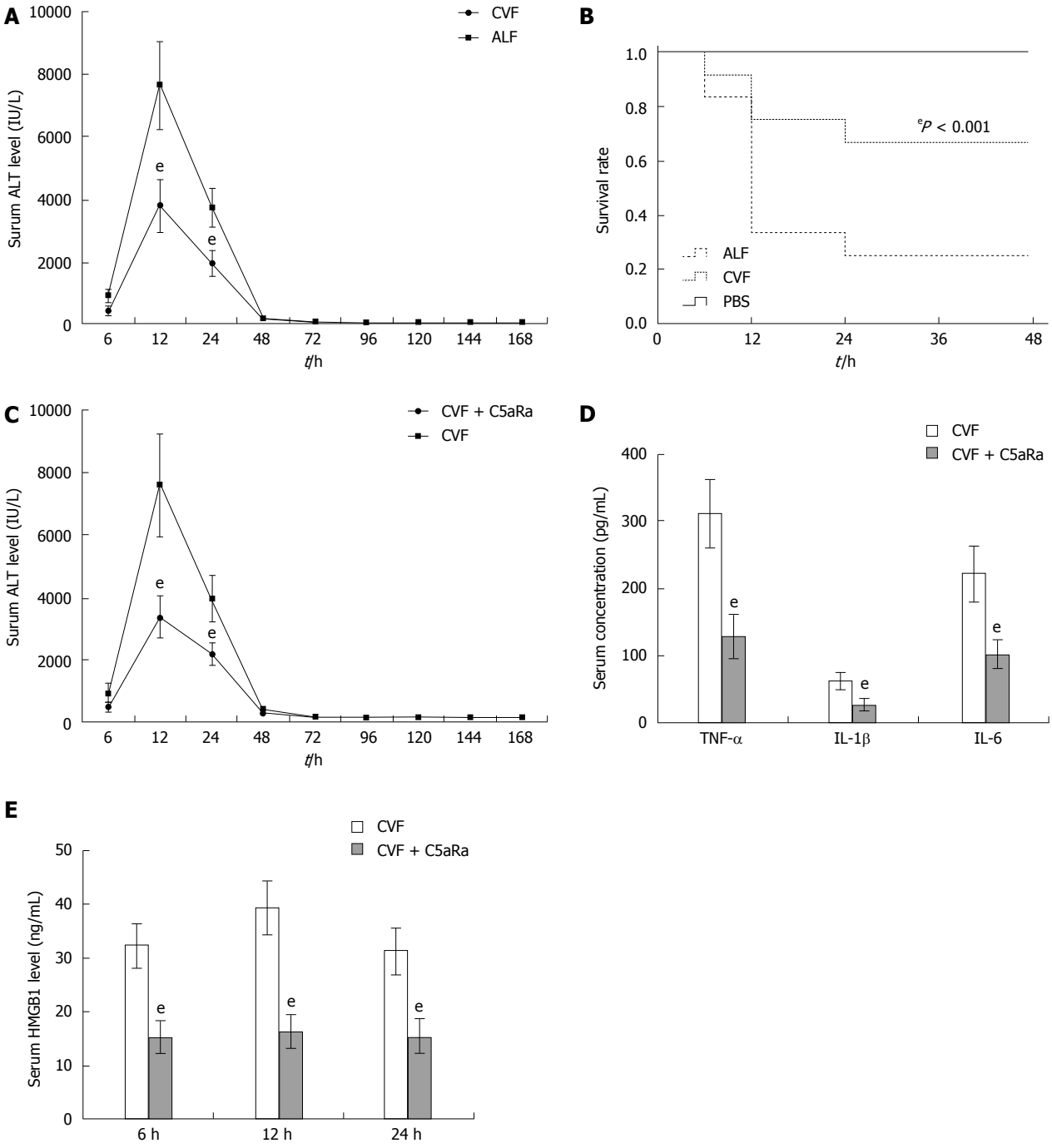

Figure 3 C5aRa further lessens the pathogenic effect on D-GalN/LPS challenged mice receiving cobra venom factor pretreatment. A: Cobra venom factor (CVF) treatment decreased serum levels of ALT at 12 h and 24 h significantly (3798.28 IU/L ± 839.68 IU/L *vs* 7612.78 IU/L ± 1379.21 IU/L, 1965.93 IU/L ± 371.74 IU/L *vs* 3798.28 IU/L ± 839.68 IU/L, t = 8.34 and 8.18, respectively, $^{e}P < 0.001$); B: Kaplan-Meier analysis of the effect of CVF on survival rates of animals ($^{e}P < 0.001$, log-rank test, F = 14.84); C: C5aRa further decreased serum levels of ALT at 12 h and 24 h significantly compared with CVF (1668.4 IU/L ± 339.68 IU/L *vs* 3798.28 IU/L ± 839.68 IU/L, 1069.69 IU/L ± 171.74 IU/L *vs* 1965.93 IU/L ± 371.74 IU/L, t = 8.14 and 7.58, respectively, $^{e}P < 0.001$); D: C5aRa further reduced serum levels of TNF-α, IL-1β and IL-6 at 12 h significantly compared with CVF (129.67 pg/mL ± 32.79 pg/mL *vs* 312.19 pg/mL ± 51.25 pg/mL; 27.73 pg/mL ± 8.78 pg/mL *vs* 63.28 pg/mL ± 13.27 pg/mL; 103.66 pg/mL ± 22.33 pg/mL *vs* 223.67 pg/mL ± 41.77 pg/mL, t = 10.39, 7.74, and 8.78, respectively, $^{e}P < 0.001$); E: C5aRa further reduced HMGB1 levels at 6, 12 and 24 h in ALF mice (15.14 ng/mL ± 3.08 ng/mL *vs* 33.23 ng/mL ± 4.17 ng/mL; 16.21 ng/mL ± 3.11 ng/mL *vs* 39.44 ng/mL ± 5.07 ng/mL; 15.42 ng/mL ± 3.23 ng/mL *vs* 31.33 ng/mL ± 4.36 ng/mL, t = 11.49, 13.53, and 10.16, respectively, $^{e}P < 0.001$). ALF: Acute liver failure. TNF: Tumor necrosis factor; IL: Interleukin; ALT: Alanine aminotransferase; HMGB1: High-mobility group protein B1.

C5aR signaling induces SphK1 expression through activation of p38-MAPK

Recent studies show that C5a has a critical role in regulating macrophage functions and p38-MAPK is activated in macrophages during C5a stimulation[20]. Whether C5a induced SphK1 expression through p38-MAPK activation needs further investigation. To explore the relation of C5a/C5aR interactions with p38-MAPK phosphorylation *in vivo*, we first examined the tyrosine phosphorylation status of p38-MAPK after

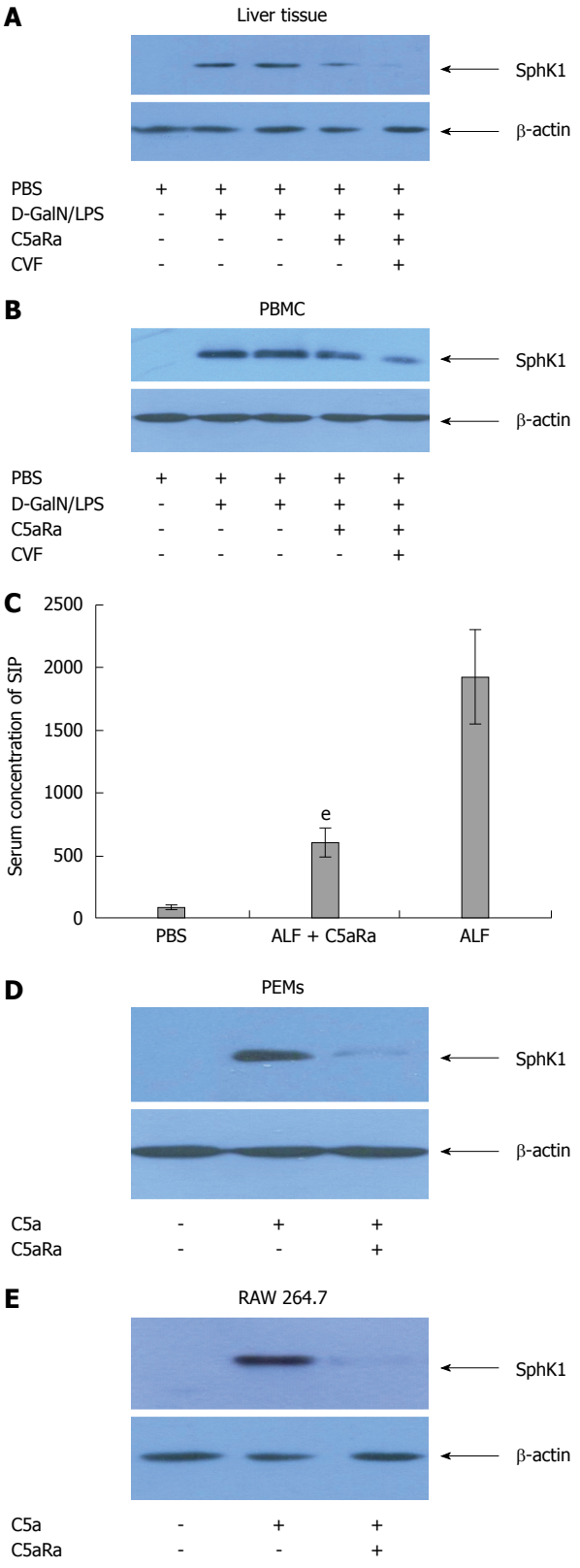

Figure 4 C5a/C5aR signaling is required for the expression of SphK1 during acute liver failure. A and B: Blocking of C5aR with C5aRa reduced SphK1 expression in liver tissue and peripheral blood mononuclear cells (PBMCs) of acute liver failure (ALF) mice; C: Blocking of C5aR by C5aRa reduced S1P level in liver tissue significantly than that of control (ALF group), $^{e}P < 0.01$; D, E: Recombinant murine C5a (500 ng/mL) induced SphK1 expression in peritoneal exudative macrophages (PEMs) and RAW 264.7 cells, and was abolished by incubation for 60 min with 10 nmol/L C5aRa. The data shown are representative of three separate experiments.

C5aRa pretreatment. As expected, the liver tissues presented with remarkably lower levels of p38-MAPK phosphorylation at 6 h and 12 h in C5aRa pretreated mice compared to controls (Figure 5A). In line with the *in vivo* result, a faster and persistent phosphorylation of p38-MAPK was clearly observed in PEMs stimulated with C5a, and C5aRa abolished this effect (Figure 5B). These results indicated that the C5a/C5aR pathway is involved in mediating C5a-induced p38-MAPK phosphorylation.

To investigate whether p38-MAPK activation mediates C5aR-dependent SphK1 induction, we used SB203580 to directly inhibit p38-MAPK activity. Pre-incubation with SB20380 significantly reduced SphK1 production in PEMs upon C5a stimulation (Figure 5C). Under our experimental condition, SB203580 showed no cytotoxicity, as confirmed by > 95% of the cells with trypan blue exclusion after incubation for 6 h and 24 h (data not shown). These data indicate that the p38-MAPK signaling pathway mediates C5a/C5aR-induced SphK1 production in macrophages.

DISCUSSION

D-GalN/LPS-induced ALF in mice efficiently reproduces the clinical syndrome of ALF in humans. Although it has been noticed that complement activation plays an important role in LPS/D-GalN- and APAP-induced ALF[17,18], the possible role of complement activation in promoting ALF has not been investigated previously. Here, we found that ALF strongly activates the complement system, leading to a C5a increase. Attenuation in wild-type mice treated with C5aRa upon LPS/D-GalN-induction clearly validated the role of the C5a/C5aR pathway in the pathogenesis of ALF. *In vivo* and *in vitro* experiments also suggest that C5a/C5aR interactions up-regulate the expression and activation of SphK1 in macrophages through p38-MAPK activation.

Extensive expression and activation of SphK1 are a hallmark for LPS/D-GalN-induced ALF[11,12]. Our data show that upon LPS/D-GalN challenge, inhibition of C5aR signaling with C5aRa can substantially reduce SphK1 production, thus protecting the animals from ALF. These results demonstrate that the C5a/C5aR pathway, *via* induction of SphK1 expression, plays a key role in ALF. It also implies that the excessive activation of complement, particularly C5a levels, may serve as a clinical criterion for disease diagnosis and prediction of severity in patients with ALF.

It has been shown that bacterial infections and LPS can quickly activate the complement system[16,21]. In accordance with the previous results[17,18], the complement system is rapidly activated in ALF. This is confirmed by the observation of serum C5a level upsurge at the early stage of ALF development, indicating that C5a is an early mediator of ALF. This excessive complement activation and consumption at the early stage appear to explain the drop in serum

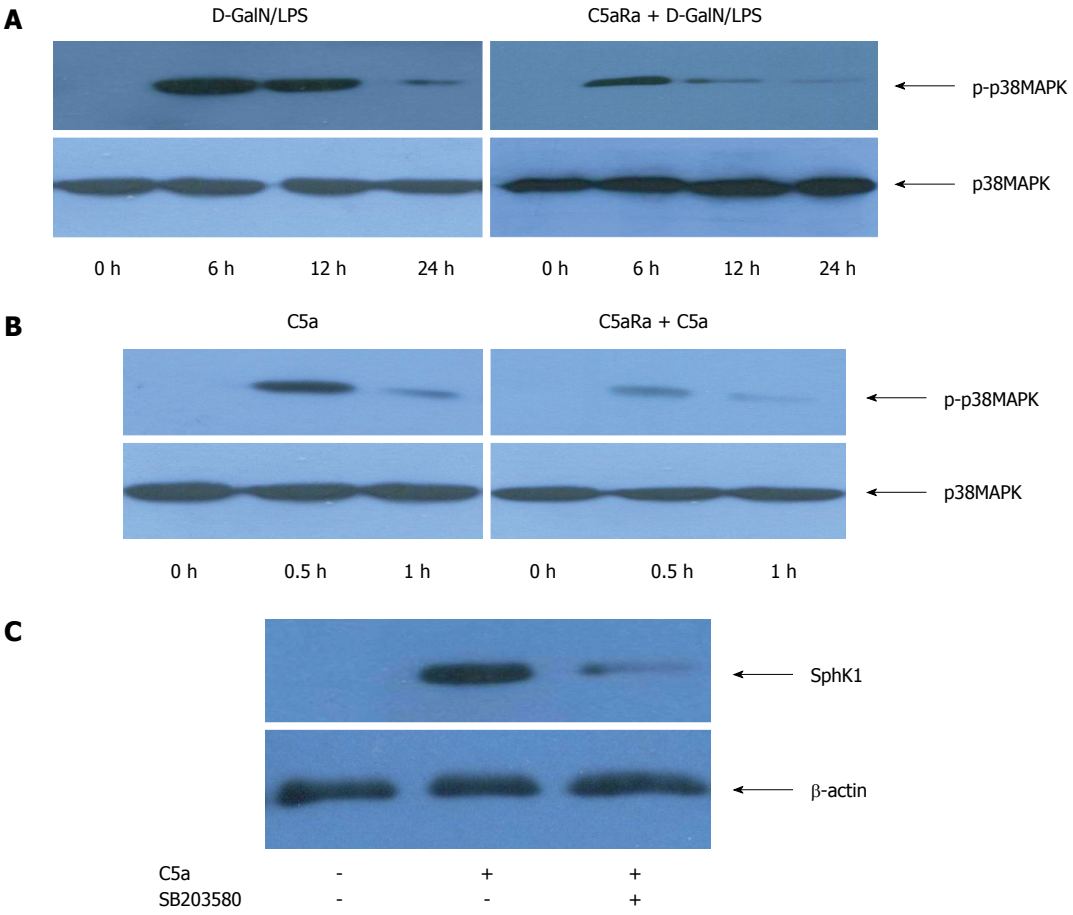

Figure 5 C5aR signaling induces SphK1 expression through activation of p38-MAPK. A: C5a induced p38-MAPK activation in liver tissue of acute liver failure (ALF) mice; B: PEMs were stimulated with C5a (500 ng/mL) or C5a + C5aRa (pre-incubated 60 min with 10 nmol/L of C5aRa in the presence of C5a); C: Inhibition of p38-MAPK activity with SB203580 down-regulated SphK1 expression in PEMs stimulated with C5a.

C5 levels and C5 exhaustion in LPS/D-GalN-induced ALF. Although serum C5a levels in these mice began to decrease at 24 h after challenge, likely due to C5 exhaustion, the serum C5a level at 36 h was still higher than that of control, implying that C5a may also play a role in pathogenesis at the late stage of disease.

Excessive expression and activation of SphK1 are critical to the pathogenesis of ALF and inhibition of SphK1 with a specific inhibitor (N,N-dimethylsphingosine, DMS) attenuates mouse liver injury and increases the survival rate, supporting a critical role for SphK1 in ALF[17]. Our study clearly showed that if C5aR signaling was inhibited, LPS/D-GalN failed to induce massive expression of SphK1 in the affected liver, indicating that C5a/C5aR interactions participate in the expression and activation of SphK1 for causing the disease.

Several proinflammatory stimuli, including anaphylatoxin C5a and TNF-α, activate SphK1 on human macrophages, and blockade of SphK1 inhibits several pro-inflammatory responses triggered by these stimuli[7-9]. As a result, we speculated that C5a/C5aR interactions are essential for SphK1 expression and activation. It has been shown that C5a is able to activate the three major MAPK pathways in most of inflammatory cells, including macrophages[22,23], and a recent study indicated that the C5a/C5aR pathway is necessary for p38-MAPK phosphorylation in macrophages[20]. Furthermore, MAPK activation plays a key role in C5a-induced production of inflammatory cytokines[15,22]. In accordance with these results, we found that *in vitro*, C5a-induced SphK1 expression is largely dependent on activation of the p38-MAPK pathways. Our result identified that C5 activation and C5a/C5aR interactions play an important role in mediating p38-MAPK activation in liver tissue that leads to the pathogenesis of ALF.

Although the specific mechanism of complement activation during ALF is not very clear, the observation that C5a/C5aR signaling play an important role in the development of experimental ALF sheds light on the new strategies for treating ALF patients. Further research is needed to investigate the potential role of C5a in ALF in humans, and C5aR antagonist or C5a-neutralizing antibodies appear to have existing advantages for their usage in clinical treatment of ALF.

In conclusion, our results provide direct evidence that C5a/C5aR signaling plays a critical role in the pathogenesis of LPS/D-GalN-induced ALF in mice. Moreover, it is the first time we found that the C5a/C5aR pathway participates in the expression and activation of SphK1 through p38-MAPK activation.

We have reasons to believe that interfering in the C5a/C5aR signaling pathway can become a promising immunotherapeutic strategy for ALF in patients.

COMMENTS

Background

Recent studies and our work show that sphingosine kinase 1 (SphK1) and complement activation play an important role in systemic inflammation in acute liver failure (ALF). It has been shown that complement 5a (C5a) activates SphK1 in macrophages. However, the mechanism of C5a-induced SphK1 activation is unknown.

Research frontiers

Systemic inflammation is an important feature of ALF, in which macrophages and inflammatory cytokines released by macrophages play a critical role. C5a is the most powerful pro-inflammatory mediator, and excessive C5a can cause exaggeration of the inflammatory responses while blockade of C5a/complement 5a receptor (C5aR) interactions increases survival rates in mice with sepsis. Moreover, complement activation also plays an important role in ALF.

Innovations and breakthroughs

In this study the authors found excessive activation of C5 and up-regulation of C5aR in liver tissue of ALF mice. The C5a/C5aR pathway is essential for potentiating SphK1 expression through p38-MAPK activation in ALF in mice. This is the first report of the mechanism of C5a-induced SphK1 activation.

Applications

Blockade of the C5a/C5aR pathway may represent a valuable immunotherapeutic strategy for ALF in patients.

Peer-review

Very interesting study. The authors investigated the role of the C5a/C5aR pathway in ALF in a mouse model. BALB/c mice were randomly assigned to different groups, intraperitoneal injection of D-galactosamine/lipopolysaccharide were used to induce ALF. Serum C5, C5a, tumor necrosis factor-α, interleukin (IL)-1β, IL-6, high-mobility group protein B1 and sphingosine-1-phosphate were detected by enzyme-linked immunosorbent assay. Hepatic morphological changes at 36 h were assessed, and the expression of C5aR, SphK1, p38-MAPK and p-p38-MAPK in liver tissue, peripheral blood monocytes and peritoneal exudative macrophages of mice or RAW 264.7 cells were analyzed. The authors found that the C5a/C5aR pathway is essential for potentiating SphK1 expression through p38-MAPK activation in ALF, providing a potential immunotherapeutic strategy for ALF in patents.

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ORIGINAL ARTICLE

Basic Study

NADPH oxidase-1 deficiency offers little protection in *Salmonella typhimurium*-induced typhlitis in mice

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Author contributions: Chu FF and Esworthy RS designed the study, performed the experiments, analyzed the data, and drafted the manuscript; Doroshow JH and Shen B edited the manuscript and provided financial support for the studies.

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Institutional animal care and use committee statement: Care and use of mice in this study conformed to NIH (USA) and Association for the Assessment and Accreditation of Laboratory Animal Care (AAALAC) standards and were performed under protocol 11043 approved by the City of Hope BRI Institutional Animal Care and Use Committee on 1/12/12 and renewed 1/15/14. Animals were bred and reared in the Animal Resources Center at the City of Hope based on standards and guidelines set by the United States Department of Agriculture; approved by the National Institutes of Health, Office for Laboratory Animal Welfare; and accredited by the AAALAC International.

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Abstract

AIM

To test whether Nox1 plays a role in typhlitis induced by *Salmonella enterica serovar* Typhimurium (S. Tm) in a mouse model.

METHODS

Eight-week-old male wild-type (WT) and Nox1 knockout (KO) C57BL6/J (B6) mice were administered metronidazole water for 4 d to make them susceptible to S. Tm infection by the oral route. The mice were given plain water and administered with 4 different doses of S. Tm by oral gavage. The mice were followed for another 4 d. From the time of the metronidazole application, the mice were observed twice daily and weighed daily. The

ileum, cecum and colon were removed for sampling at the fourth day post-inoculation. Portions of all three tissues were fixed for histology and placed in RNAlater for mRNA/cDNA preparation and quantitative real-time PCR. The contents of the cecum were recovered for estimation of S. Tm CFU.

RESULTS

We found Nox1-knockout (Nox1-KO) mice were not more sensitive to S. Tm colonization and infection than WT B6 mice. This conclusion is based on the following observations: (1) S. Tm-infection induced similar weight loss in Nox1-KO mice compared to WT mice; (2) the same S. Tm CFU was recovered from the cecal content of Nox1-KO and WT mice regardless of the inoculation dose, except the lowest inoculation dose (2×10^6 CFU) for which the Nox1-KO had one-log lower CFU than WT mice; (3) there is no difference in cecal pathology between WT and Nox1-KO groups; and (4) there are no S. Tm infection-induced changes in gene expression levels (IL-1b, TNF-α, and Duox2) between WT and Nox1-KO groups. The Alpi gene expression was more suppressed by S. Tm treatment in WT than the Nox1-KO cecum.

CONCLUSION

Nox1 does not protect mice from S. Tm colonization. Nox1-KO provides a very minor protective effect against S. Tm infection. Using NOX1-specific inhibitors for colitis therapy should not increase risks in bacterial infection.

Key words: Knockout mouse; NADPH Oxidase-1; *Salmonella typhimurium*; Goblet cells; Reactive oxygen species

Core tip: Using Nox1-knockout mice (Nox1-KO), we examined the role of cecum Nox1 in *Salmonella typhimurium* (S. Tm) infection. Mice were rendered susceptible to infection with oral metronidazole. Four days after S. Tm inoculation, Nox1-KO mice had equal or slightly lower CFU/g cecum contents and equal or slightly less pathology by histological assessment than wild-type (WT) mice. Quantitative real-time PCR measure of mRNA levels for inflammatory cytokines Il-1β and TNF-α were significantly higher in S. Tm treated WT *vs* untreated mice but not in S. Tm treated Nox1-KO mice. Since Nox1 may have a role in inflammatory bowel disease, treating subjects with Nox1 inhibitors may not make patients vulnerable to pathogens.

Chu FF, Esworthy RS, Doroshow JH, Shen B. NADPH oxidase-1 deficiency offers little protection in *Salmonella typhimurium*-induced typhlitis in mice. *World J Gastroenterol* 2016; 22(46): 10158-10165 Available from: URL: http://www.wjgnet.com/1007-9327/full/v22/i46/10158.htm DOI: http://dx.doi.org/10.3748/wjg.v22.i46.10158

INTRODUCTION

The generation of reactive oxygen species (ROS) during infection is an important part of host defense to fend off bacterial invasion. A well-known source of ROS is produced by NADPH oxidase (NOX)-2 in innate immune cells to kill bacteria engulfed in phagosomes[1]. Mutations in genes encoding for the NOX2 complex lead to chronic granulomatous disease due diminished bactericidal activity. Nox2-KO mice are highly susceptible to *Salmonella enteric serovar* Typhimurium (S. Tm) colonization and mucosal inflammation[2]. S. Tm has been widely used as a mouse colitis model to study strategies by which enteropathogenic bacteria break colonization resistance[3]. Diminished ROS generation by the NOX2 complex also is a risk factor for early-onset pediatric pancolitis and Crohn's-like disease[4].

Two other members of NADPH oxidases, NOX1 and DUOX2, expressed in the epithelium of the intestine are linked to very-early-onset inflammatory bowel disease[5]. Both *Nox1* and *Duox2* gene are barely expressed in the intestine of germ-free mice and are highly elevated when colonized with commensal bacteria[6,7]. Duox2 and Duoxa2 (DUOX maturation factor-2) expression is highly elevated in *Helicobacter felis*-infected mouse gastric epithelium compared to uninfected mice[8]. Lack of Duox activity in *Duoxa*-KO mice increased *Helicobacter felis* colonization[8]. Duox2 protein is highly elevated in the ileum and colon mono-associated with segmented filamentous bacterium compared that in germ-free mice[7]. However, Duox2 does not appear to protect mice against S. Tm infection since there is no difference in S. Tm colonization between wild-type (WT) and Duoxa-KO mice[7].

Nox1-generated ROS can regulate intestinal epithelial cell proliferation, apoptosis, and migration[9-11]. Recently, it was shown that deficiency in Cyba/p22^{Phos} (an obligatory partner of Nox1, Nox2 and Nox4) in intestinal epithelium resulted in protection from *Citrobacter rodentium* and *Listeria monocytogenes*[1]. The epithelium-specific *Cyba*-KO (*Cyba*ΔIEC-KO) mice are considered to be equivalent to Nox1ΔIEC-KO because Nox2 and Nox4 are virtually unexpressed in the intestinal epithelium. Because *C. rodentium*-induced Duox2 gene expression only occurs in the WT mice but not *Cyba*ΔIEC-KO mice, it was proposed that Nox1 regulates Duox2 expression in the intestinal epithelium[1].

We have previously shown that *GPx1*-KO and *GPx2*-KO mice (deficient in antioxidant glutathione peroxide-1 or -2) are more susceptible to S. Tm infection than WT mice[12]. Because Nox1-deficiency may protect mice against *C. rodentium* infection, we hypothesize that Nox1-produced ROS exacerbates S. Tm infection. In this manuscript, we report that Nox1-knockout (Nox1-KO) mice are equally susceptible to S. Tm colonization and infection as WT mice. We concluded that Nox1 does not play a role in S. Tm-

induced colitis.

MATERIALS AND METHODS

Mice

WT and *Nox1*-KO (generated by Karl-Heinz Krause, Geneva University, Switzerland) mice were derived from strain C57BL/6 (B6)[11]. Animal were bred and reared in the Animal Resources Center at the City of Hope based on standards and guidelines set by the United States Department of Agriculture, approved by the National Institutes of Health (NIH), Office for Laboratory Animal Welfare, and accredited by the AAALAC. Weaned mice were fed Lab diet 5061 (LabDiet, St Louis, MO, United States), *ad lib*, and received water *via* an automated water purification system until the beginning of the study at 8 wk of age. Care and use of mice in the study conformed to NIH (United States) and AAALAC standards and were performed under protocol 11043 approved by the City of Hope BRI Institutional Animal Care and Use Committee on 1/12/12 and renewed 1/15/14. Eight-week-old male WT and *Nox1*-KO B6 mice were given the antibiotic metronidazole (0.75 g/L in drinking water) for 4 d to facilitate oral S. Tm infection[13]. To mask the metallic taste of metronidazole, 1 g of sucralose (Splenda®) was added to 450 mL water to prevent dehydration during the treatment. Metronidazole facilitates bacteria colonization by reducing anaerobic bacteria populations and by thinning the mucus layer in the gut[14]. After 4 d the mice were switched to regular water and orally gavaged with S. Tm in a volume of 50-100 μL phosphate buffered saline. One-inch-long 22-gauge plastic gavage needles were used to deliver the bacteria and to minimize the possibility of injury to the mice. As part of the study data collection and to ensure that the animals were not in distress prior to the endpoint all mice were observed and weighed daily from the time of placement on metronidazole to euthanasia, *i.e.*, 4 d post-inoculation. The high risk of systemic infection in B6 mice precluded a longer study duration. Mice treated with metronidazole and then water gained about 5% body weight over the interval. Mice inoculated with S. Tm lost up to 18% body weight regardless of inoculation dose. There was no statistical difference in weight lost between the Nox1-KO and WT groups. Mice were euthanized by CO_2 exposure, the recommended method under NIH and AAALAC guidelines.

Salmonella enteric S. Tm

A virulent strain of S. Tm, IR715, was obtained from Dr. Andreas J. Baumler (University of California, Davis, CA, United States), who derived this strain from isolate 14028 (American Type Culture Collection)[12]. S. Tm was grown aerobically at 37 ℃ in lysogeny broth (LB) containing 50 mg/mL nalidixic acid (Sigma) overnight. Cells were harvested, resuspended in PBS with 10% glycerol, then stored in aliquots at -80 ℃ without freeze-thaw. Between 2 × 10^6 to 6.2 × 10^8 colony-forming units (CFUs) of S. Tm were used to inoculate mice as shown (Figure 1). The titer was determined on LB agar plates containing 50 mg/mL nalidixic acid.

S. Tm colonization and disease parameters

Cecum luminal contents were collected sterilely, weighed, serially diluted, and plated on nalidixic acid-containing LB agar to allow detection down to approximately 1 × 10^6 CFU/g. The luminal contents have 2-4 log higher number of S. Tm than cecal tissue and are widely used as an indicator for bacterial colonization[12,15,16]. The inoculated S. Tm was the only bacteria capable of producing large-size colonies on nalidixic acid-containing LB agar under standard aerobic conditions[11]. To verify that the colonies were S. Tm, we performed automated-ribosomal-intergenic-spacer analysis (ARISA) PCR using ITSF (5′-GTCGTAACAAGGTAGCCGTA-3′) and ITSReub (5′-GCCAAGGCATCCACC-3′) primers on randomly selected colonies[17]. ARISA PCR produces a pattern of products with aggregate sizes characteristic of bacterial groups. We also sequenced ARISA PCR products to confirm the S. Tm identity.

The ileum, colon and cecum were excised for histology analysis. The tissues were fixed in phosphate buffered formalin. The processed slides were stained with H&E for pathology analysis and with Alcian blue counterstained with nuclear fast red for goblet cell counts. The slides were photographed, and Alcian blue stained goblet cells were counted from the sections containing full-length glands. Pathology was scored according to a 14-point system that accounts for mucin depletion, apoptosis, abscesses, and distortion of the glands[12].

Messenger RNA was prepared from the cecum, where S. Tm colonization is most reliably detected. Quantitative real-time PCR (qRT-PCR) was performed on mouse cecal mRNA. A segment of the cecum isolated from mice treated with 2 × 10^6 CFU of S. Tm was treated with RNALater (Ambion), and then RNA was isolated using the Triazol Kit (Thermo Scientific). Two μg of RNA was used to make cDNA with reagents from Promega and random hexamer primers (0.4 μg) from Invitrogen. PCR primers and probes (Thermo Scientific) used are shown in Table 1. The qRT-PCR was performed on a BioRad CFX96 instrument for 40 cycles. The ΔΔCt method was used to analyze differences in mRNA levels among groups, normalized with β-actin.

Statistical analysis

GraphPad Prism version 6 was used for statistical analysis. All groups were compared in pair-wise *t*-tests except the cecum pathology score, which was analyzed by pair-wise Mann-Whitney tests. S. Tm CFU from cecal contents was analyzed from log 10 transformed

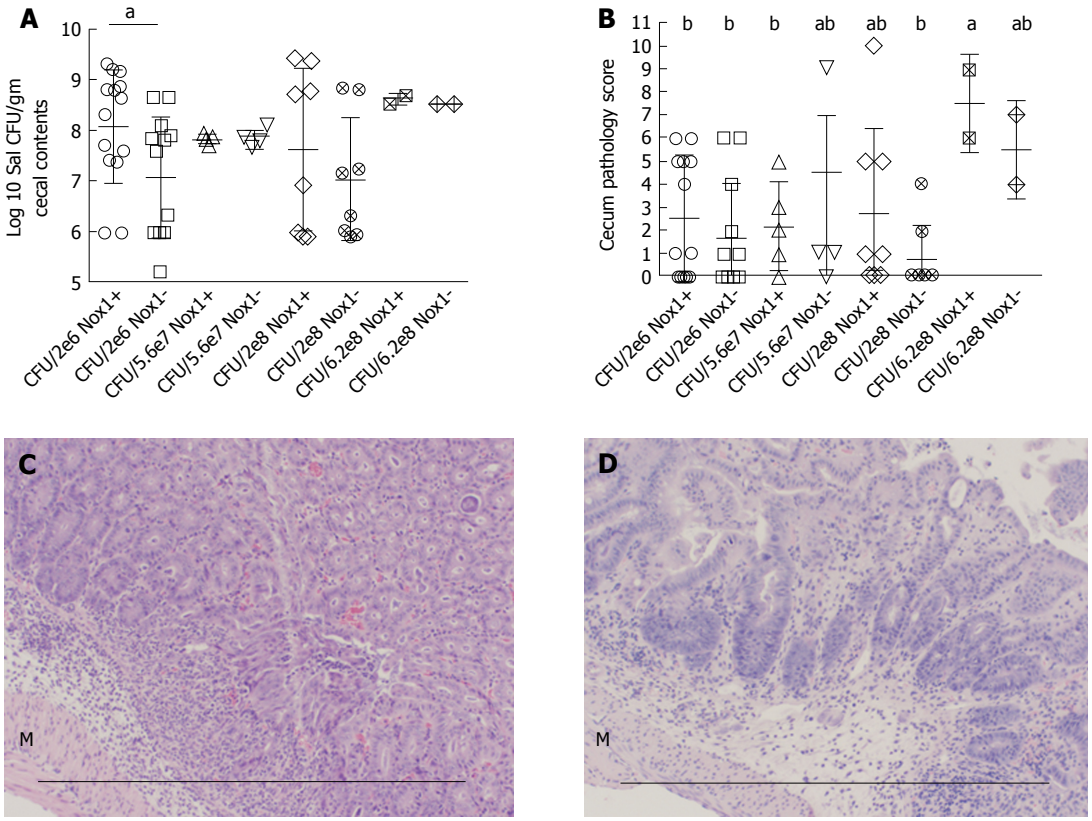

Figure 1 The CFU and pathology in the cecum between Nox1-knockout and wild-type mice with 4 different doses of S. Tm. A: Scatter plot of log10 transformed S. Tm CFU per gram recovered from cecal contents from WT (Nox1+) and Nox1-KO mice inoculated with 2×10^6, 5.6×10^7, 2×10^8 and 6.2×10^8 of S. Tm. The contents were plated so that 1 colony would yield a count $\geqslant 1 \times 10^6$ CFU/g. Zero colonies were assigned to 1×10^6 to include all mice analyzed in the panel. "a" indicates a significant difference between the Nox1 and WT groups treated with 2×10^6 S. Tm (colon-P = 0.0068; cecum-P = 0.0098); B: Scatter plot of pathology scores of mice from the groups shown in A. The groups with different letter designations in each figure are different, where a > b (α = 0.05). The groups sharing a same letter are not different; *e.g.*, ab is not different from a or b group. Horizontal bars indicate mean ± SD; C, D: Show the worst pathology identified in Nox1-KO and WT cecum, respectively (score = 6), for mice inoculated with the lowest (2×10^6) CFU. Both groups show edema and infiltration between the muscular layers (M) and the glands. The glands are devoid of goblet cells and generally distorted. Scale bars are approximately 0.5 mm. Nox1-KO: Nox1-knockout; WT: Wild-type.

Table 1 Taqman primer and probe IDs for quantitative real-time polymerase chain reaction

Gene name (gene symbol)	Catalog number[1]	Amplicon size
β-Actin (*Actb*)	Mm00607939_s1	115
Dual oxidase-2 (*Duox2*)	Mm01326247_m1	65
Il1b	Mm00434228_m1	90
Intestinal alkaline phosphatase (*Alpi*)	Mm01285814_g1	60
Tnfα	Mm00443258_m1	81
Villin-1 (*Vil1*)	Mm004944146_m1	55

[1]Catalog number from Thermo Fisher Scientific Inc.

data, which renders the data into parametric sets. The statistical analysis was reviewed and approved by Lianlian Du, MSci, biostatistician, Beijing Rehabilitation Hospital of Capital Medical University, Beijing, China.

RESULTS

Nox1-KO cecum and colon has a 7.5% and 20% higher number of goblet cells than WT counterparts

Nox1 is highly expressed in the colon, and Nox1-generated ROS can activate Notch1 signaling presumably by activating the metalloproteases (Mmp2 and Mmp9), which are involved in generation of Notch ligands[18]. Notch1 signaling promotes differentiation of epithelial cells into absorptive cells. Compared to WT mouse colon, Coant *et al*[18] reported that Nox1-KO colon had a 2-fold higher number of goblet cells and a 50% reduction of colonocytes due to decreases in Notch1 signaling. Because goblet cells are specialized to produce a mucus layer over the epithelial surface that acts as a barrier to intestinal microbes, changing the number of goblet cells could affect cell resistance to bacterial colonization. Therefore, we compared the number of goblet cells in the cecum and colon of Nox1-KO and WT mice (Figure 2). We found that Nox1-KO colon has a 20% increase in goblet cells compared to WT colon; however, we observed only a 7.5% increase in the Nox1-KO cecum, which is the major site of inflammation. This difference may have been overlooked in *Cyba*ΔIEC-KO mice, which do not have Nox1 activity[1].

Nox1 does not significantly affect S. Tm colonization and inflammation in mice

We inoculated mice with 4 different doses of S. Tm (2

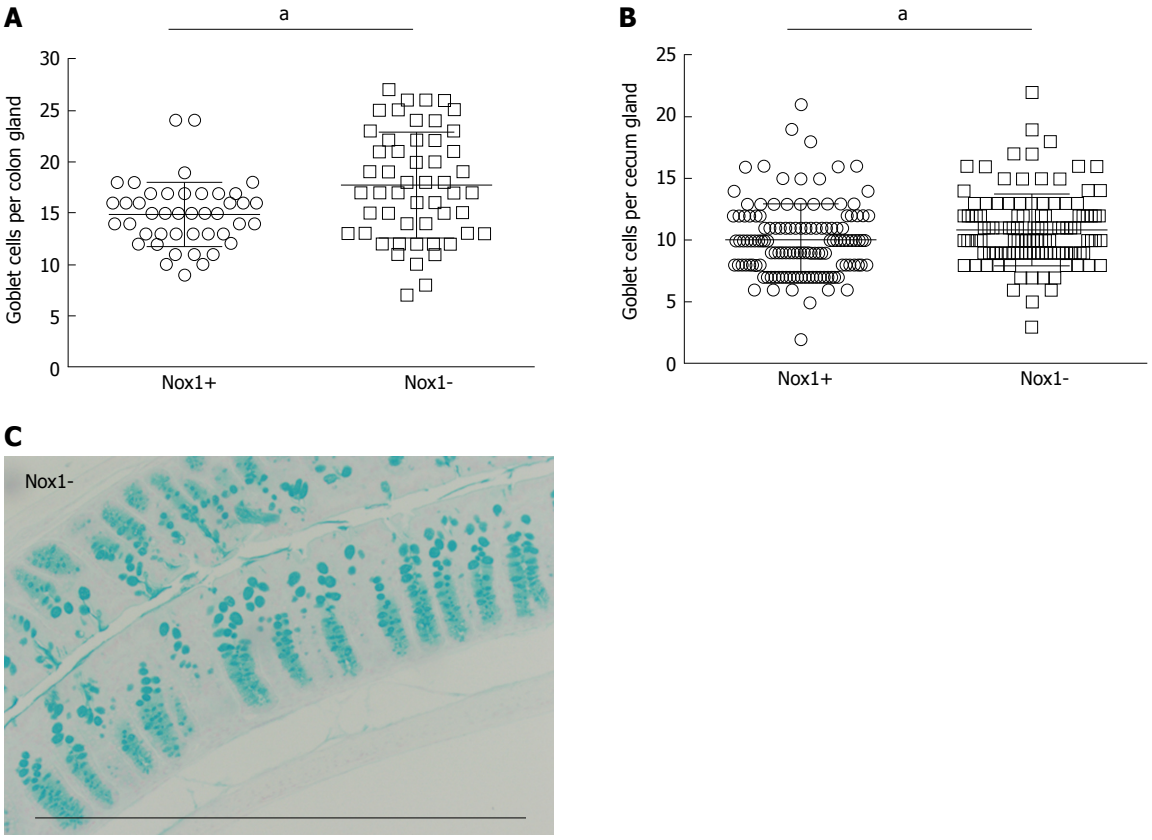

Figure 2 Goblets per colon (A) and cecum gland (B) counted from cross sections stained with Alcian blue and nuclear fast red (C). Each point in panels A and B represents a separate gland. Between 3-15 and 7-14 glands (obtained from 8 WT and 9 Nox1-KO mice) were counted from colon and cecum, respectively. Scale bar approximately 0.5 mm. "a" indicates a statistically significant difference between the 2 groups.

$\times 10^6$, 5.6×10^7, 2×10^8, and 6.2×10^8) to compare the CFU and pathology in the cecum between Nox1-KO and WT mice (Figure 1). When inoculated with the lowest number of S. Tm (2×10^6), Nox1-KO mice had a significantly lower number of S. Tm CFUs in the cecum than WT mice ($P = 0.034$). No differences in the cecal S. Tm CFUs were found between Nox1-KO and WT mice when inoculated with higher doses of S. Tm (Figure 1A). Also, the CFUs of S. Tm in mouse cecum remained the same regardless of the inoculation doses, which is consistent with previous work[19].

We analyzed cecum pathology scores to determine the effect of Nox1. Scores of 0-6 generally reflect with presence of crypt apoptosis, hyperproliferation, and mucin depletion without overt signs of inflammation. Scores of above 6 are considered inflamed, showing immune infiltration, crypt distortion, goblet cell depletion, and elevated apoptosis in the epithelium. The worst cecal histology from a Nox1-KO and a WT mouse inoculated with a low dose of S. Tm (2×10^6) is shown in Figure 1C and 1D. Only WT mice inoculated with the highest dose (6.2×10^8) of S. Tm had a median score above 6, which is significantly higher than four groups of WT and Nox1-KO mice inoculated with lower doses of bacteria ($P \leqslant 0.025$) (Figure 1B). However, there was no difference between the WT and Nox1-KO groups inoculated with the same high dose. These results indicate that Nox1 does not have significant impact on S. Tm infection.

Nox1 does not affect mucosal gene expression altered by S. Tm infection

Although the pathology scores are low in the mice inoculated with 2×10^6 CFU of S. Tm, these mice tend to have higher IL-1β and TNF-α mRNA levels compared to control mice that received metronidazole only (Figure 3A and B). However, we observed no difference in IL-1β and TNF-α mRNA levels between Nox1-KO and WT mice treated with metronidazole and S. Tm or untreated.

S. Tm infected intestine has decreased brush-border enzyme activities and gene expression levels including intestinal alkaline phosphatase (Alpi), sucrose-isomaltase, and maltose[19]. We found that S. Tm infected WT mouse cecum had decreased levels of *Alpi* gene expression ($P \geqslant 0.044$), while the Alpi mRNA levels in the S. Tm-infected Nox1-KO mice were not significantly lower than the un-treated control mice (Figure 3C). Villin mRNA levels were not affected by S. Tm (data not shown). Villin is an actin-binding protein that regulates actin dynamics and organization of the brush border of enterocytes[20]. Loss of Villin mRNA is indicative of gross destruction of the gland surface architecture, reflected by pathology scores of 8 and above as observed in S. Tm treated Gpx1-KO and Gpx2-KO mice[12]. The overall trend indicates that the

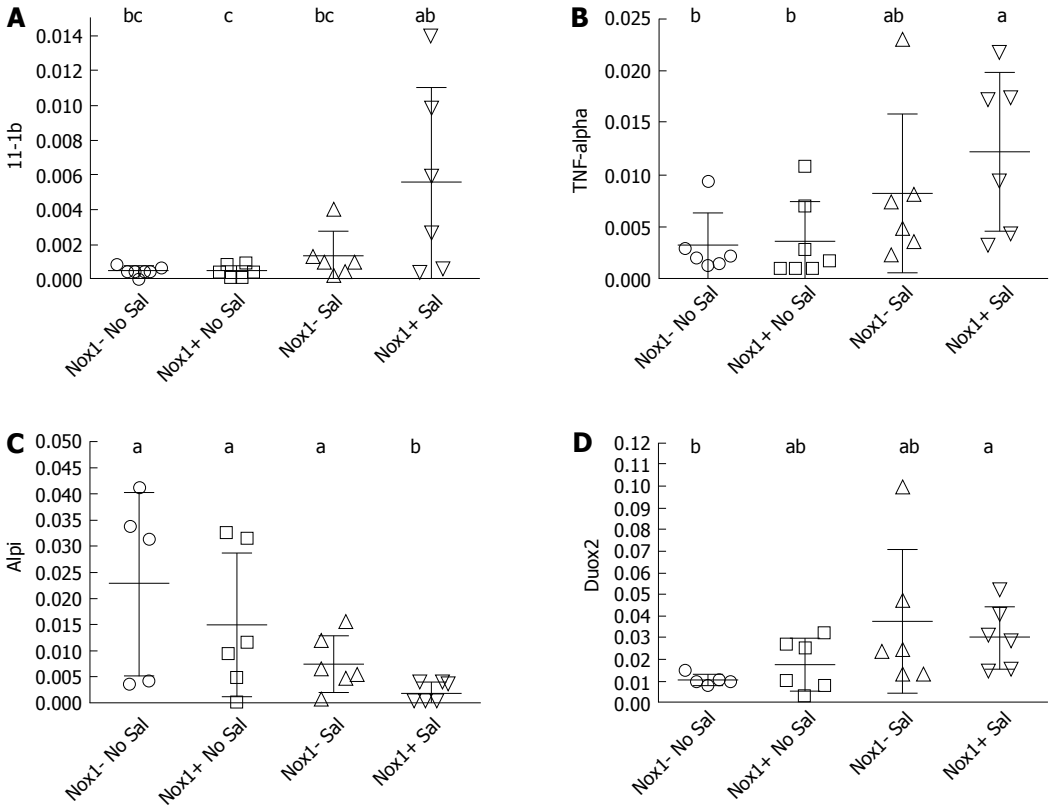

Figure 3 Quantitative real-time polymerase chain reaction analysis of cecum mRNA from the 2 × 10^6 CFU-treated wild-type and Nox1-knockout mice to measure inflammation markers IL-β (A), TNF-α (B), a brush border marker for the integrity of the epithelium, Alpi (C); and Duox2 levels (D). Horizontal bars are mean ± SD. The groups with different letter designations in each figure are different, where a > b > c (α = 0.05). The groups sharing a same letter are not different; *e.g.*, bc is not different from b or ab group. Nox1-KO: Nox1-knockout; WT: Wild-type.

Nox1-KO mice tend to respond more mildly to S. Tm infection than WT mice, although not in a significant or uniform way.

We have previously shown that *Duox2* gene expression was elevated 30-fold in the cecum of S. Tm-inoculated *GPx1*-KO mice (mean pathology scores of 10) compared to infected WT mice[12]. Here, the S. Tm-infected WT and Nox1-KO mouse cecum had 2- and 4-fold higher *Duox2* gene expression than the non-infected respective controls (Figure 3D). No difference in the *Duox2* mRNA levels between the WT and Nox1-KO cecum were observed.

DISCUSSION

Nox1 expressed in intestinal epithelium plays an important role in cell signaling, regulating many cellular events, including differentiation, proliferation, apoptosis, and migration[10,11,18]. Recently, Nox1 was shown to exacerbate pathogenic bacterial infection, including *C. rodentium* and *L. monocytogenes* studied in *Cyba*ΔIEC-KO mice[1]. In the present study, we compared S. Tm colonization and S. Tm-induced colitis in Nox1-KO and WT mice inoculated at different doses and found that Nox1 plays a very minor role in *S.* Tm infectivity. Because we found that Nox1-KO mice have a significant increase of goblet cells in the cecum and colon and because goblet cells secrete mucus to form a barrier to fend off bacterial infection[14,16], the protective effect of Nox1-deficiency is likely due to the increase in goblet cells rather than a direct effect of ROS production. The strong protective effect of Cyba deficiency against *C. rodentium* and *L. monocytogenes* may have contributions from multiple Nox deficiencies, because Nox2 is induced in the intestinal epithelium by serotonin, a neuroendocrine secreted by entero-chromaffin cells[21].

We have confirmed that S. Tm infection suppresses intestinal alkaline phosphatase (Alpi) expression in WT mice and likely in Nox1-KO mice. Mice deficient in Alpi suffer from dysbiosis[22]. The antibiotic-induced susceptibility to S. Tm or *Clostridium difficile* can be prevented by oral supplement of calf Alpi[15]. It remains unclear how S. Tm inhibits Alpi gene expression and activity.

We have reported that intestinal inflammation, as observed in mice deficient in GPx1 and GPx2 [GPx1/2-double knockout (DKO)], have elevated Nox1 gene expression in the ileum[11]. Nox1 deletion completely abolished GPx1/2-DKO intestinal inflammation. Because NOX1 and NOX2 are the major sources of ROS in the artery wall for conditions such as hypertension, hypercholesterolemia, and diabetes, NOX inhibitors are being developed to treat ROS-associated diseases[23]. A clinical relevance of this study is that when targeting Nox1, it is unlikely that the anti-Nox1

therapy will increase risks of bacterial infection.

Nox1 is important for symbiotic-lactobacilli-induced cell proliferation in the ileum[10]; it also promotes restitution of colons damaged by dextran sulfate sodium[9,24]. Whether anti-Nox1 therapy has adverse effects other than bacterial infection needs to be further investigated.

In conclusion, we demonstrated that Nox1-KO mice are not more susceptible to S. Tm colonization than WT mice. The clinical relevance of this and other studies is that anti-Nox1 therapy should not present a major risk for bacterial infection, such as by S. Tm, *Citrobacter rodentium* or *Listeria monocytogenes*. However, because Nox1 also has a positive role in cell proliferation and tissue restitution, more studies are needed to clarify other potential risks of anti-Nox1 therapy.

ACKNOWLEDGMENTS

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COMMENTS

Background

Generation of reactive oxygen species (ROS) is implicated in the pathology of inflammatory bowel disease, fibrosis, hypertension, stroke, and atherogenesis and may play role in tumorigenesis. NADPH oxidase 1 (Nox1) appears to be a major generator of ROS in many of these cases. Therefore, identifying specific Nox1 inhibitors may lead to new therapeutic agents. ROS can also be generated by Nox2 as part of the innate immune response to pathogenic microflora, and it has been speculated that Nox1 participates in the control of gut microflora. The authors and others are testing the idea that Nox1 may play a vital role in defense against gut pathogens by colonizing Nox1-KO mouse gut with pathogens, such as *Salmonella enterica serovar* Typhimurium (S. Tm) in this study.

Research frontiers

The role of Nox1 in disease has just recently been explored, and the search for potent and specific inhibitors is emerging as a major research focus. In support of using Nox1 inhibition as a therapy, it is important learn if there are major risks from pathogens or other complications such as existing damage to the gut. Wound healing and epithelial restitution may be impaired by inhibition of Nox1.

Innovations and breakthroughs

The study shows that Nox1 expression levels do not significantly affect colonization by S. Tm in the gut. Together with studies on *Listeria* and *Citrobacter*, this work suggests that inhibition of Nox1 activity poses little risk to the subject for bacterial infection.

Applications

Studies of this type will help define the risks inherent in the use of Nox1 inhibitors as therapeutic agents.

Terminology

Nox1 is a member of a family of oxidases that generate either superoxide or hydrogen peroxide (ROS) using NADPH as the electron donor. Nox1 generates superoxide. S. Tm is an enteropathogenic bacteria commonly used to explore microbial pathology in rodent models.

Peer-review

The authors investigated the role of Nox1 in S. Tm colonization and infection in a mouse model. The paper is interesting and adds to our general understanding of S. Tm infection and demonstrates that Nox1 does not play an important role in S. Tm colonization.

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ORIGINAL ARTICLE

Basic Study

Non-invasive evaluation of liver stiffness after splenectomy in rabbits with CCl_4-induced liver fibrosis

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Abstract

AIM

To investigate the diagnostic performance of liver stiffness measurement (LSM) by elastography point quantification (ElastPQ) in animal models and determine the longitudinal changes in liver stiffness by ElastPQ after splenectomy at different stages of fibrosis.

METHODS

Liver stiffness was measured in sixty-eight rabbits with CCl_4-induced liver fibrosis at different stages and eight healthy control rabbits by ElastPQ. Liver biopsies and blood samples were obtained at scheduled time points to assess liver function and degree of fibrosis. Thirty-one rabbits with complete data that underwent splenectomy at different stages of liver fibrosis were then included for dynamic monitoring of changes in liver stiffness by ElastPQ and liver function according to blood tests.

RESULTS

LSM by ElastPQ was significantly correlated with histologic fibrosis stage (r = 0.85, P < 0.001). The optimal cutoff values by ElastPQ were 11.27, 14.89, and 18.21 kPa for predicting minimal fibrosis, moderate fibrosis, and cirrhosis, respectively. Longitudinal

monitoring of the changes in liver stiffness by ElastPQ showed that early splenectomy (especially F1) may delay liver fibrosis progression.

CONCLUSION

ElastPQ is an available, convenient, objective and non-invasive technique for assessing liver stiffness in rabbits with CCl_4-induced liver fibrosis. In addition, liver stiffness measurements using ElastPQ can dynamically monitor the changes in liver stiffness in rabbit models, and in patients, after splenectomy.

Key words: Fibrosis stages; Splenectomy; Elastography point quantification; Liver stiffness; Non-invasive technique

Core tip: Elastography point quantification (ElastPQ) is a non-invasive technique for assessing tissue stiffness, and was used in this study. Splenectomy is a surgical intervention for liver cirrhosis patients with hypersplenism. The aim of the current study was to evaluate the diagnostic accuracy of liver stiffness measurement by ElastPQ in animal models and determine the longitudinal changes in liver stiffness by ElastPQ after splenectomy at different stages of fibrosis. We conclude that liver stiffness measurements using ElastPQ can be used to dynamically monitor the changes in liver stiffness in rabbit models, and in patients, after splenectomy.

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INTRODUCTION

Liver fibrosis, which is characterized by encapsulation or replacement of injured tissue by a collagenous scar[1], represents a common pathological process in chronic liver injury of varying etiologies. Cirrhosis, which is morphologically described as abnormal liver architecture encompassing fibrous bands surrounding regenerative nodules, is the end stage of liver fibrosis and has clinical complications, including liver failure, portal hypertension, and ultimately, hepatocellular carcinoma. A growing body of clinical evidence has indicated that liver fibrosis can reverse and possibly return to normal following the development of effective treatments for chronic hepatitis infection (B and C)[2-6], autoimmune hepatitis[7], and primary biliary cirrhosis[8].

In addition, improved results on the molecular mechanisms associated with the pathogenesis of hepatic fibrosis has led to growing acceptance of liver fibrosis as a potentially reversible process[9,10]. Hepatic stellate cells (HSCs) are a worldwide research focus based on their activation and transdifferentiation to myofibroblasts, which ultimately results in liver fibrosis in response to a variety of injuries; more interestingly, previous studies have indicated that macrophages can influence the process of liver fibrosis *via* different mechanisms[11,12]. Circulating macrophages arise from monocytes in the bone marrow (BM)[13], and Swirski *et al*[14] and other researchers[15,16] have indicated that numerous monocytes in the spleen could be mobilized in the pathological state such that the spleen can be considered a monocyte reservoir. BM cell infusion can improve liver function[17] and decrease liver fibrosis[18], while splenectomy can result in liver function improvements for patients with liver cirrhosis[19-21]. Furthermore, a previous study indicated that splenectomy attenuated murine liver fibrosis when accompanied by hypersplenism[22].

On the other hand, liver biopsy is traditionally regarded as the gold standard for staging fibrosis. Nevertheless, as an invasive procedure, liver biopsy is unwelcome in patients who need repeated examination to monitor fibrosis progression. Furthermore, liver biopsy is limited by serious complications[23,24], sampling errors[25], and both inter-pathologist and intra-pathologist variability[26]. Shear wave elastography, a reliable, rapid and non-invasive technique, has been used to evaluate tissue stiffness for many years and is increasingly important in the diagnosis of liver fibrosis[27-29]. Furthermore, an acoustic radiation force impulse (ARFI) technique, elastography point quantification (ElastPQ)[30], has been developed to measure the tissue[31-34]. However, no data are available on the changes in fibrotic liver stiffness after splenectomy at different pathological stages using ElastPQ.

We took advantage of a CCl_4-induced liver fibrosis model in rabbits, from which liver biopsies were obtained at scheduled time points and ElastPQ was easily performed, to evaluate the correlation between liver fibrosis histological staging and liver stiffness measured by ElastPQ before splenectomy (Experiment 1). In addition, we determined the longitudinal changes in liver stiffness using ElastPQ after splenectomy at different pathological stages (Experiment 2).

MATERIALS AND METHODS

Animals

One hundred and eight male New Zealand White rabbits weighing 2000-2500 g on arrival at the laboratory were purchased from the Experimental Animal Center of West China Medical Center, Sichuan University (Chengdu, China). All rabbits were acclimatized for one week to adapt to the new environment. Daily evaluation of rabbit health status was performed for one week to ensure the animals were clinically healthy prior to the experiments. The

animals were individually housed in cages under a set temperature (22 ± 1 ℃) and relative humidity (45% ± 10%) with a 12-h light/12-h dark cycle. Each animal was allowed free access to a standard diet for rabbits and fresh water. The experimental procedures were approved by the Institutional Animal Ethical Committee of Sichuan University (Chengdu, China), and all animals received humane care according to the Guide for the Care and Use of Laboratory Animals published by the United States National Institutes of Health (NIH Publication No. 85-23, revised 1996).

CCl_4-induced liver fibrosis

Liver fibrosis was induced by intraperitoneal injection of CCl_4, as described previously[35]. Unfortunately, in a pilot experiment (10 rabbits), using the regimen reported by Zhang *et al*[35], a mortality rate of 60% (6 of 10 rabbits) was observed. The pilot study was stopped, and a modified method for induction of liver fibrosis was explored and eventually adopted. The injection started with 50% CCl_4, which was diluted in olive oil, in doses of 0.10 mL/kg body weight twice per week for the first two weeks, which allowed the rabbits to gradually adapt to the toxic agent. Then, 50% CCl_4 was given intraperitoneally in doses of 0.20 mL/kg body weight twice a week for another 18 wk in Experiment 1, and the liver injury induced by 50% CCl_4 lasted for ten weeks from the first operation in Experiment 2. This method was sufficient to produce all stages of liver fibrosis. Humane endpoints were established in the modeling process according to the guidelines for assessing discomfort in experiment animals[36]. No animals died in Experiment 1.

Ultrasound-based examinations

On the same day, just before surgery and blood collection, eight rabbits were chosen at random for preoperative examinations after at least four hours of fasting. The rabbits were anesthetized with a 40 mg/kg dose of pentobarbital *via* ear border vein injection and were then placed in the supine position with whole abdominal skin preparation. Liver stiffness measurements were then performed in or close to the subxiphoid region by two experienced examiners *via* ElastPQ with a 4-cm depth and a 0.5 cm × 1.5 cm region of interest on vessel-free areas at the end-inspiration phase with an iU22 ultrasound system (Royal Philips Electronics, the Netherlands) equipped with an ElastPQ feature and two transducers, C5-1 (1-5 MHz) (used in this study) and L9-3 (3-9 MHz) (not used in this study). Both examiners were blinded to the clinical, serological, and histological data. The results are expressed in kilopascals. ElastPQ results were obtained with 10 valid measurements from each operator; a success rate of at least 60% and an interquartile range of all successful measurements less than 30% of the median values were considered reliable. The successful measurements obtained by each operator were used for inter-examiner agreement analysis, while the median measurement obtained by both operators for each rabbit were used for other analyses in the current study.

Serum parameters

After ultrasound-based examinations, peripheral blood was collected *via* the ear border vein. Levels of the following parameters were determined: (1) class Ⅰ biomarkers of liver fibrogenesis, including type Ⅳ collagen, and hyaluronic acid[37], were quantified using a standardized and optimized commercial radioimmunoassay kit (Haiyan Biotechnology Center, Shanghai, China) and (2) conventional liver function tests, including total bilirubin (TB), albumin, alanine aminotransferase (ALT), and aspartate aminotransferase (AST) levels (Leadman Biochemistry Co., Ltd, Beijing, China).

Surgical procedure

In Experiment 1, after 4-wk of modeling, the eight rabbits were randomly divided into two equal groups following preoperative examinations (ultrasound-based examinations and the blood test mentioned above). After disinfection, the operation began with a midline abdominal incision. In one group (Group S, splenectomy group), total splenectomy was performed by ligature of the splenic vascular pedicle with 4-0 chromic catgut; then, a 1-cm × 1-cm piece of hepatic tissue from the subxiphoid region of the liver was cut for biopsy. In another group (Group L, liver biopsy group or sham group), the same process was performed with the exception of total splenectomy. The abdominal cavity was closed after confirming that there was no active hemorrhage in all rabbits. To obtain different stages of liver fibrosis at different time intervals, the same surgical process was repeated for the remaining rabbits every two weeks until the 20th wk. Due to humane endpoints and failed liver stiffness measurements, only seven rabbits underwent surgery at the 8th, 14th, 18th, and 20th wk (Table 1). In this case, four rabbits randomly underwent splenectomy plus liver biopsy, while the remaining three underwent liver biopsy alone.

In Experiment 2, after the first operation in each rabbit, a 1 cm × 1 cm piece of hepatic tissue was cut to dynamically monitor the changes in histological features according to the aforementioned ultrasound-based examinations and blood tests every two weeks for 10 wk. To avoid adhesions, chitosan (0.5 mL/surgery) was used. However, due to the increase in operation times, it was difficult to acquire liver tissue along the original midline incision. In this case, a left or right subcostal incision was needed. In Experiments 1 and 2, all animals were given penicillin intramuscularly at a dose of 40 U/rabbit to prevent infection during surgery, which was repeated once daily for a further two days. To reduce bias, only the hepatic tissue obtained in or very close to the subxiphoid region was included for analysis. In addition, due to humane

Table 1 Detailed information on the experimental process

Modeling time	Distribution of operation	Humane Termination[1]	Rabbits left[2]	POW 2	POW 4	POW 6	POW 8	POW 10	Humane Termination[3]	Exclusion	Death	Rabbits left[4]
0W	/	0	90	/	/	/	/	/	/	/	/	/
2W	/	0	90	/	/	/	/	/	/		/	/
4W	AAAA	0	82	AAAA	AAAA	AAA[5]	AA	AA	1	1	1	5
	BBBB			BBB	BBB	BBB	BBB	BBB				
6W	AAAA	0	74	AAA	AAA	AAA	AA	AA	2	0	2	4
	BBBB			BBBB	BBB	BBB	BB	BB				
8W	AAAA	2	64	AAA	AA	AA	AA	AA	2	0	1	4
	BBBB[5]			BBB	BBB	BB	BB	BB				
10W	AAAA	3	53	AAA	AAA	AA	AA	AA	2	1	1	4
	BBBB			BBBB	BBB	BBB[5]	BB	BB				
12W	AAAA	3	42	AAA	AAA	AA	AA	AA	3	0	1	4
	BBBB			BBB	BB	BB	BB	BB				
14W	AAAA	2	32	AAA	AA	AA	AA	A	5	0	0	2
	BBBB[5]			BBB	BBB	BB	B	B				
16W	AAAA	4	20	AAAA	AAA	AAA	AA	AA	4	0	1	3
	BBBB			BBB	BB	BB	BB	B				
18W	AAAA	3	9	AAA	AAA	AAA	AA	AA	4	0	0	3
	BBBB[5]			BBB	BB	BB	B	B				
20W	AAAA	2	0	AA	AA	AA	AA	A	4	1	0	2
	BBB			BBB	BBB	BB	BB[5]	B				
Total	68	19	/	/	/	/	/	/	27	3	7	31

[1]Number of rabbits with humane termination during Experiment 1; [2]Number of rabbits left excluding rabbits with humane termination and failed liver stiffness measurement *via* ElastPQ during Experiment 1; [3]Number of rabbits with humane termination during Experiment 2; [4]Number of rabbits left excluding rabbits with humane termination, failed liver stiffness measurement *via* ElastPQ, and death during Experiment 2; A[5] and B[5] indicate rabbits with failed LSM *via* ElastPQ. POW: Postoperative weeks; LSM: Liver stiffness measurement; ElastPQ: Elastography point quantification; A: Rabbits receiving splenectomy and liver biopsy; B: Rabbits receiving only liver biopsy.

endpoints and failed liver stiffness measurement (LSM) and death during the surgical procedure, only thirty-one rabbits with complete experimental data were available for analysis after the 10-wk surveillance period (Table 1).

Liver histological assessment

Liver biopsy samples taken at the time of the operation were fixed in formalin and embedded in paraffin. Sections (4 μm) were stained with hematoxylin and eosin and Masson trichrome. A biopsy sample with a minimum of 5 portal tracts was required for diagnosis. Two doctors with significant experience, who were blinded to all animal characteristics, were responsible for evaluating liver fibrosis, which was staged on a scale of 0-4 according to METAVIR[38] (F0, no fibrosis; F1, portal fibrosis without septa; F2, portal fibrosis and a few septa; F3, numerous septa without cirrhosis; and F4, cirrhosis). The fibrosis stage was independently assessed on each histological section by both doctors. In the case of discrepancies, histological sections were simultaneously reviewed again by the two doctors to reach a final consensus. Typical liver fibrosis stages (F1-F4) are illustrated in Figure 1.

Statistical analysis

The quadratic-weighted k coefficient of Cohen was used to assess the consistency of the two doctors who were in charge of the pathological examinations, while the ICC (interclass correlation coefficient) was used to evaluate the agreement between the two examiners who performed the liver stiffness measurement *via* ElastPQ.

The median LSM obtained by both operators for each ElastPQ was calculated and used for further analyses. Because the LSM values were not normally distributed, the Kruskal-Wallis nonparametric analysis of variance test was used to compare these values with the categories of the consensus fibrosis stage. Correlations between the LSM and histologic fibrosis stage were further analyzed using Spearman correlation coefficients. The diagnostic performance of ElastPQ and serum fibrosis markers, including type Ⅳ collagen and hyaluronic acid, was assessed using receiver operating characteristic curves (ROC). The optimal cutoff values for predicting different fibrosis stages were chosen to maximize the sum of the sensitivity and specificity, and the corresponding positive predictive values (PPVs) and negative predictive values (NPVs) were computed. The AUC (area under ROC) values for the different diagnostic criteria for the same data were compared using the nonparametric DeLong test.

Quantitative data were presented as the mean ± SD or median (quartile), while categorical data were expressed as the number of cases with/without percentage. Statistical analyses also included the nonparametric Mann-Whitney *U* and Student's *t* tests.

All statistical analyses were performed using SPSS 19.0 (SPSS, Chicago, IL, United States) for Windows

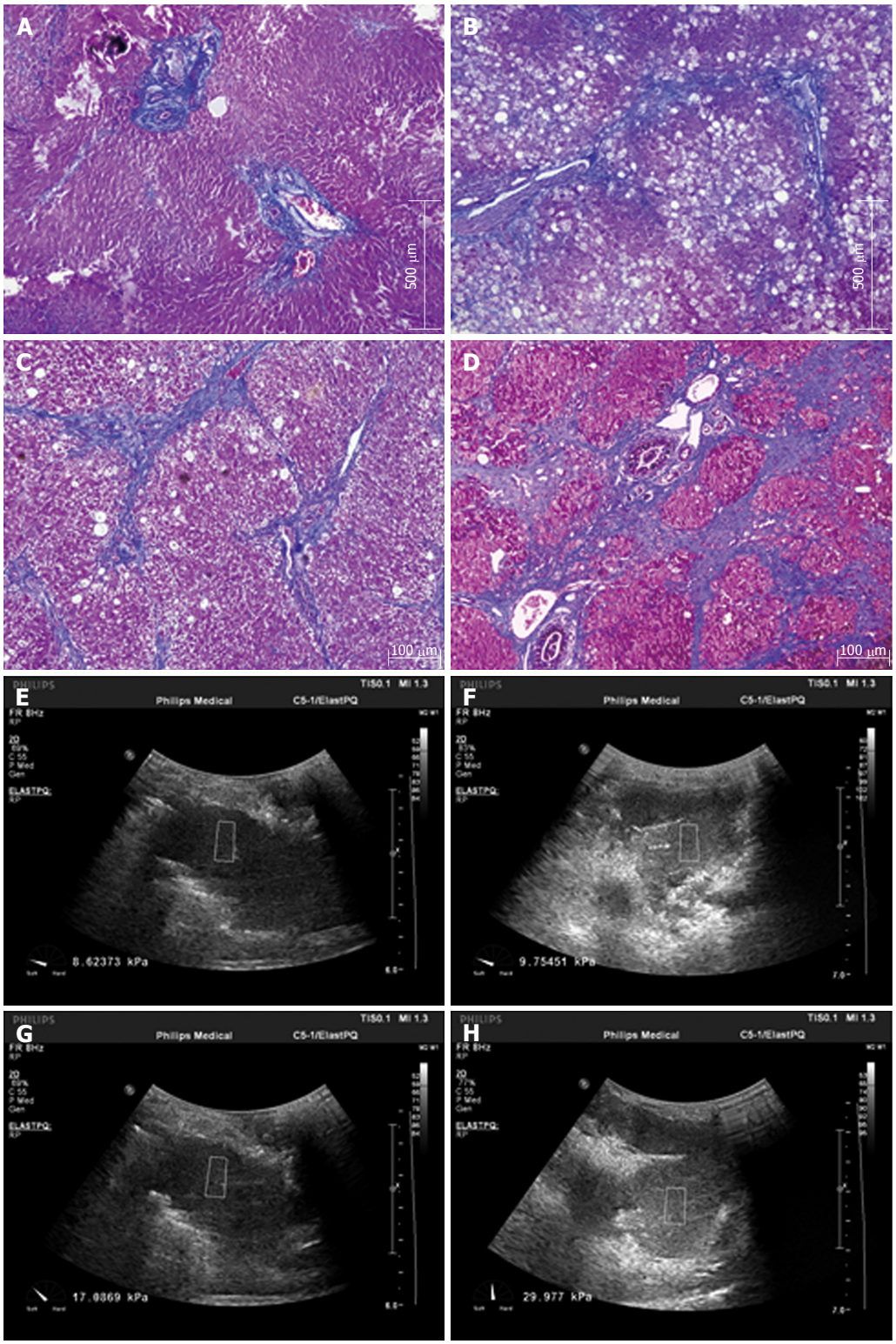


Figure 1 Masson trichrome staining for assessment of liver fibrosis stages according to METAVIR (A: F1, B: F2, C: F3, and D: F4; 100 ×) and the corresponding ElastPQ images (E: F1; F: F2; G: F3; and H: F4).

and significance was set at a P value < 0.05.

RESULTS

Experimental details

The experimental details are presented in Table 1. In addition to eight controls, ninety rabbits were planned for inclusion. As mentioned in the Materials and methods section due to humane termination (n = 19) and failed LSM (n = 3) during Experiment 1, information on sixty-eight rabbits was available for analysis. Similarly, 10 wk after splenectomy or sham

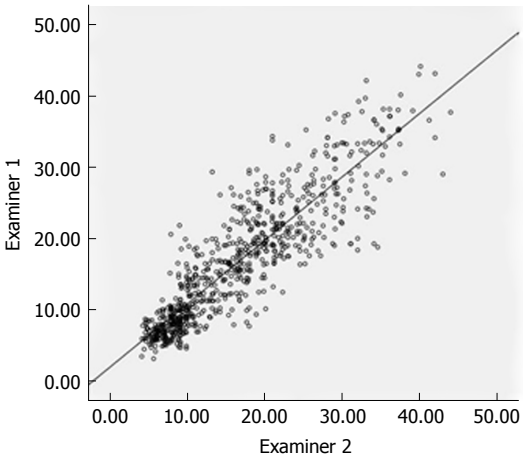


Figure 2 Graph shows correlation of elastography point quantification results between two examiners (ICC value of 0.888, r^2 = 0.788, P < 0.05).

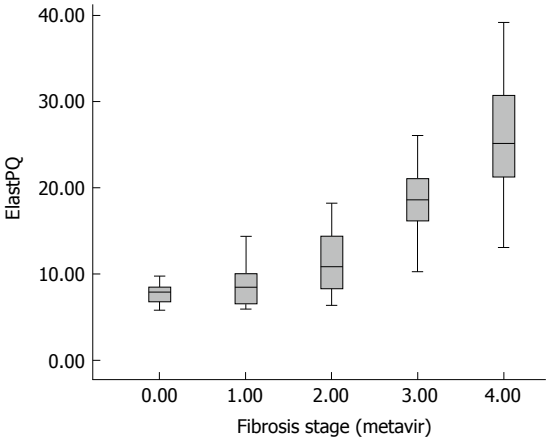


Figure 3 Boxplot shows the elastography point quantification results for each fibrosis stage. The top and bottom of the boxes are the first and third quartiles, respectively. Accordingly, the length of the box plot represents the interquartile range within which 50% of the values were located. The lines through the middle of the boxes indicate the median values. ElastPQ: Elastography point quantification.

operation, complete data for only thirty-one rabbits were available for comparable analyses.

Agreement between observers

The two doctors responsible for pathological diagnosis were initially in agreement for 197 (85.3%) of the 231 liver samples (231 = 76 + 5 × 31) (k coefficient = 0.792, P < 0.01), and 100% agreement was reached after final reviews. The ElastPQ results identified by the two examiners were strongly correlated with an ICC value of 0.888, and are illustrated in Figure 2.

Basic characteristics of the included rabbits

After 20 wk of medication, all fibrosis stages confirmed by pathological examinations were observed. As shown in Table 2, F1 was diagnosed in 11 cases (14.5%), F2 in 16 (21.1%), F3 in 16 (21.1%), and F4 in 25 (32.9%), and eight healthy rabbits (F0, n = 8, 10.4%) were included as controls. Table 3 includes the basic information on rabbits with different stages of fibrosis. Except for body weight and TB, AST, ALT, and albumin levels, a trend for a stepwise increase in liver fibrosis progression was found in the parameters, including type Ⅳ collagen (F0: 200.8 ± 131.5 μg/L, F1: 427.1 ± 226.2 μg/L, F2: 683.4 ± 332.5 μg/L, F3: 1161.4 ± 482.5 μg/L, and F4: 1292.0 ± 689.7 μg/L), hyaluronic acid (F0: 225.6 ± 117.1 μg/L, F1: 475.7 ± 296.4 μg/L, F2: 676.2 ± 274.8 μg/L, F3: 724.0 ± 264.5 μg/L, and F4: 1182.3 ± 1091.3 μg/L), and LSM [F0: 7.88 kPa (6.60-8.46 kPa), F1: 8.46 kPa (6.22-10.35 kPa), F2: 10.89 kPa (8.09-14.46 kPa), F3: 18.62 kPa (16.03-21.16 kPa), and F4: 25.10 kPa (20.28-30.95 kPa)].

Table 2 Distribution of liver fibrosis stages at different time intervals in Experiment 1

	F0	F1	F2	F3	F4
0 wk	98	/	/	/	/
2 wk		/	/	/	/
4 wk		5	3	0	0
6 wk		3	4	1	0
8 wk		2	4	1	0
10 wk		1	2	3	2
12 wk		0	2	1	5
14 wk		0	1	2	4
16 wk		0	0	4	4
18 wk		0	0	3	4
20 wk		0	0	1	6
Total	8	11	16	16	25

Relationship between histological findings and LSM by ElastPQ

The median liver stiffness measured with ElastPQ in the eight controls was 7.88 kPa (6.60-8.46 kPa). The liver stiffness measured in the rabbits with fibrosis ranged from 5.86 kPa to 39.12 kPa. Based on the different fibrosis stages, the median liver stiffness values in the animals with F1 to F4 were 8.46 kPa (6.22-10.35 kPa), 10.89 kPa (8.09-14.46 kPa), 18.62 kPa (16.03-21.16 kPa), and 25.10 kPa (20.28-30.95 kPa), respectively, indicating a gradual increase in fibrosis progression, which is shown in Figure 3, with a Spearman correlation coefficient of 0.85 (P < 0.001). Given that the distributions of ElastPQ results for F0 and F1 were comparable and only eight F0 rabbits were included, F0 and F1 rabbits were combined as a single group for further analyses. Significant differences in the LSM by ElastPQ between each fibrosis stage were observed (F0-1 *vs* F2, P < 0.01; F2 *vs* F3, P < 0.01; and F3 *vs* F4, P < 0.01).

Relationship between the LSM by ElastPQ and fibrosis blood tests

ROC curves of ElastPQ, hyaluronic acid, and type Ⅳ collagen for predicting minimal fibrosis (F0-F1 *vs* F2-F4), moderate fibrosis (F0-F2 *vs* F3-F4), and cirrhosis (F0-F3 *vs* F4) are shown in Figure 4A-C. The

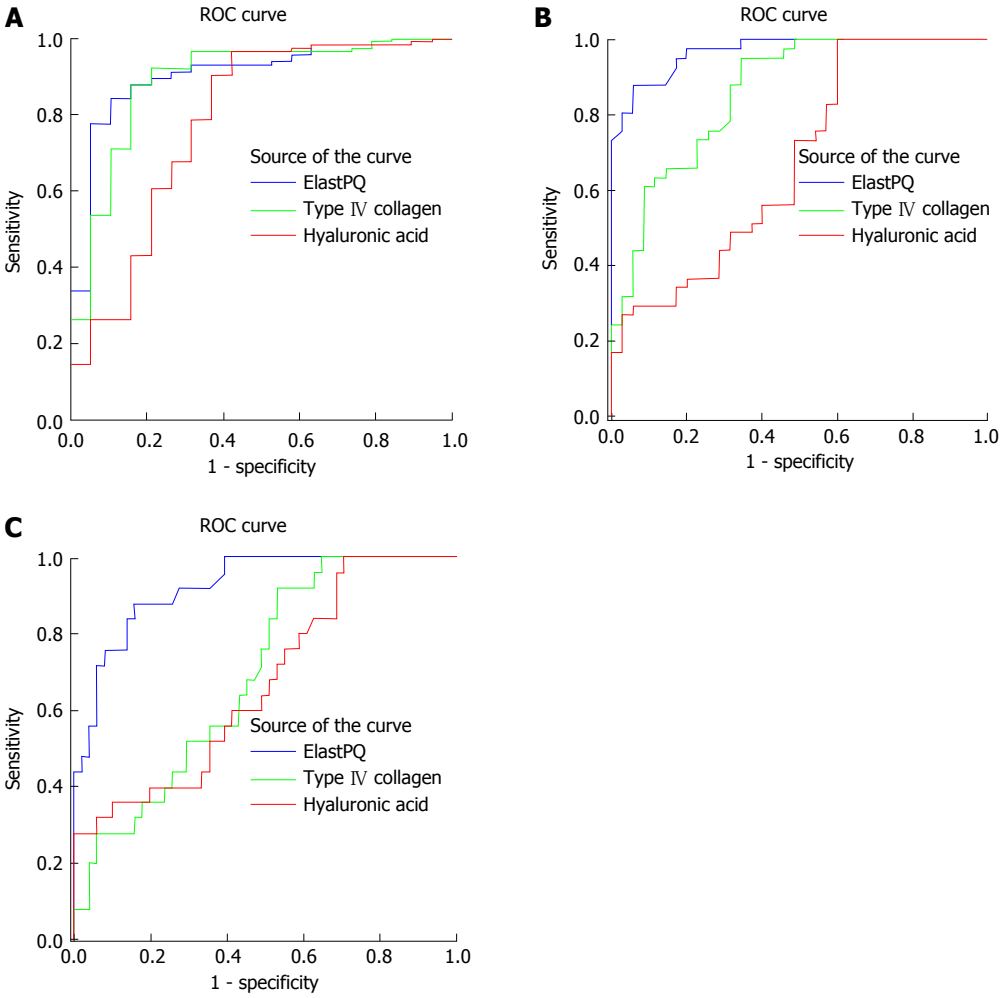


Figure 4 Receiver operating characteristic curves of elastography point quantification and serum fibrosis markers for diagnosis of (A) minimal fibrosis (F0-F1 *vs* F2-F4), (B) moderate fibrosis (F0-F2 *vs* F3-F4), and (C) cirrhosis (F0-F3 *vs* F4). ElastPQ: Elastography point quantification.

Table 3 Basic characteristics of rabbits with different liver fibrosis stages in Experiment 1

Parameters	F0 (n = 8)	F1 (n = 11)	F2 (n = 16)	F3 (n = 16)	F4 (n = 25)
Body weight (kg)	2.34 ± 0.24	2.28 ± 0.27	2.39 ± 0.14	2.30 ± 0.38	2.24 ± 0.33
Type Ⅳ collagen (μg/L)	200.8 ± 131.5	427.1 ± 226.2	683.4 ± 332.5	1161.4 ± 482.5	1292.0 ± 689.7
Hyaluronic acid (μg/L)	225.6 ± 117.1	475.7 ± 296.4	676.2 ± 274.8	724.0 ± 264.5	1182.3 ± 1091.3
TB (μmol/L)	0.98 ± 0.53	1.91 ± 0.63	2.20 ± 0.85	1.81 ± 0.82	1.64 ± 0.91
AST (IU/L)	26.8 ± 14.7	345.0 ± 295.9	449.2 ± 304.7	666.4 ± 428.3	616.1 ± 609.2
ALT (IU/L)	14.0 ± 3.7	254.7 ± 194.0	301.5 ± 210.7	456.3 ± 316.0	486.5 ± 295.8
Albumin (g/L)	42.7 ± 4.9	40.4 ± 4.6	36.5 ± 4.3	32.3 ± 6.4	35.3 ± 5.9
LSM (kPa)	7.88 (6.60-8.46)	8.46 (6.22-10.35)	10.89 (8.09-14.46)	18.62 (16.03-21.16)	25.10 (20.28-30.95)

ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; LSM: Liver stiffness measurement; TB: Total bilirubin.

AUROC (area under ROC) of ElastPQ for predicting minimal fibrosis (0.931, 95%CI: 0.849-0.977) was comparable to those of hyaluronic acid (0.807, 95%CI: 0.700-0.889) and type Ⅳ collagen (0.919, 95%CI: 0.833-0.969), while the ElastPQ for predicting moderate fibrosis and cirrhosis (0.969, 95%CI: 0.901-0.995; 0.925, 95%CI: 0.841-0.973) was significantly superior to hyaluronic acid (0.677, 95%CI: 0.560-0.780; 0.670, 95%CI: 0.553-0.774) and type Ⅳ collagen (0.861, 95%CI: 0.762-0.930; 0.695, 95%CI: 0.578-0.795), which is summarized in Table 4. The ElastPQ critical values for differentiating fibrosis stages were subsequently confirmed by the ROC, and the corresponding specificities, sensitivities, PPVs, and NPVs are listed in Table 5.

Longitudinal change in the LSM by ElastPQ and liver function following splenectomy

The longitudinal ElastPQ and laboratory data for rabbits with different stages of fibrosis after splenectomy and

Table 4 Comparison between elastography point quantification and fibrosis blood tests

AUROC	F0-F1 *vs* F2-F4	F0-F2 *vs* F3-F4	F0-F3 *vs* F4
ElastPQ	0.931 (0.849-0.977)	0.969 (0.901-0.995)	0.925 (0.841-0.973)
Hyaluronic acid	0.807 (0.700-0.889)	0.677 (0.560-0.780)[a]	0.670 (0.553-0.774)[a]
Type Ⅳ collagen	0.919 (0.833-0.969)	0.861 (0.762-0.930)[a]	0.695 (0.578-0.795)[a]

Comparison of AUROC between ElastPQ and hyaluronic acid or type Ⅳ collagen, [a]$P < 0.05$. ElastPQ: Elastography point quantification.

Table 5 Cutoff and performance values of elastography point quantification for diagnosis of liver fibrosis stages

Parameter	F0-F1 *vs* F2-F4	F0-F2 *vs* F3-F4	F0-F3 *vs* F4
AUROC	0.931 (0.849-0.977)	0.969 (0.901-0.995)	0.925 (0.841-0.973)
Optimal cutoff value	11.27	14.89	18.21
Sensitivity (%)	82.5 (70.1-91.3)	87.8 (73.8-95.9)	88.0 (68.8-97.5)
Specificity (%)	94.7 (74.0-99.9)	94.3 (80.8-99.3)	84.3 (71.4-93.0)
PPV (%)	97.9 (88.9-99.9)	94.7 (82.3-99.4)	73.3 (54.1-87.7)
NPV (%)	64.3 (44.1-81.4)	86.8 (71.9-95.6)	93.5 (82.1-98.6)

PPV: Positive predictive values; NPV: Negative predictive value.

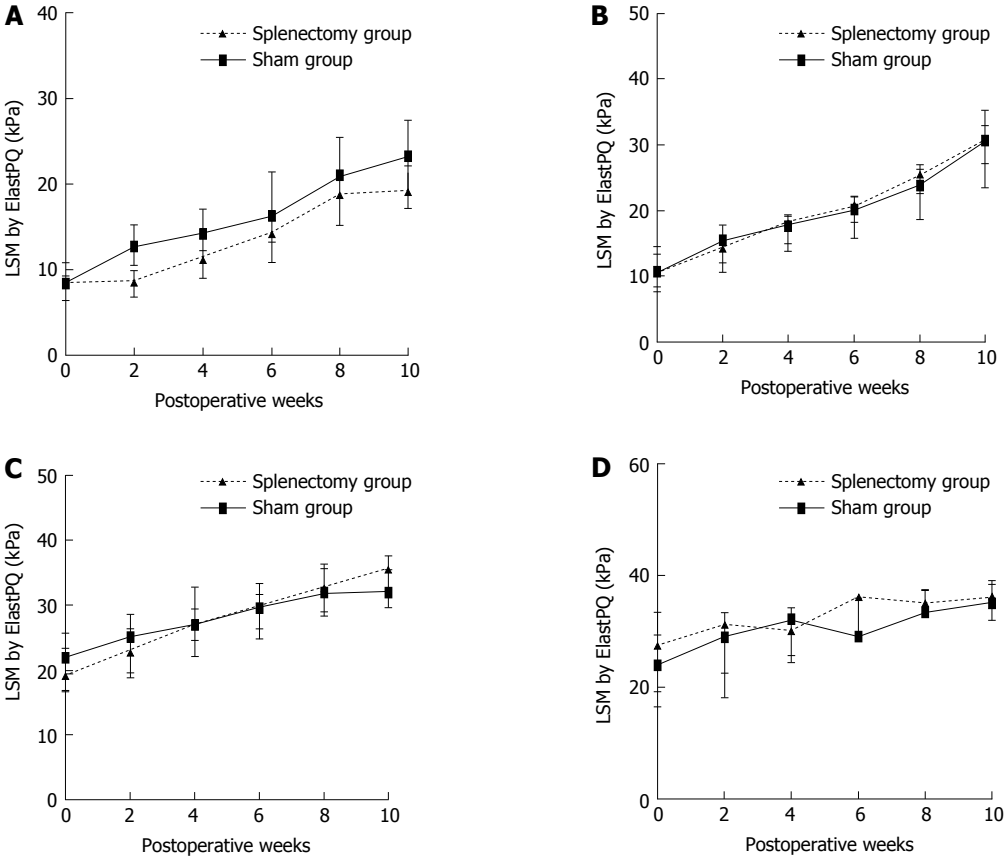


Figure 5 Dynamic changes in liver stiffness measurement by elastography point quantification after surgery in rabbits with F1 (A), F2 (B), F3 (C), and F4 (D) liver fibrosis (sham group *vs* splenectomy group). LSM: Liver stiffness measurement; ElastPQ: Elastography point quantification.

sham operation in Experiment 2 are shown in Tables 6 and 7. For the nine rabbits with F1 liver fibrosis (five in the splenectomy group *vs* four in the sham group), the increase in ElastPQ values was delayed in the splenectomy group compared with that in the sham group during the following the operations (Figure 5A), while the changes in other laboratory parameters, including AST, ALT, albumin, and TB levels, indicated otherwise (Figure 6, Table 7). For the rabbits with F2, F3, and F4 liver fibrosis, no favorable change in parameters, including the ElastPQ, AST, ALT, albumin, and TB levels, was detected in the splenectomy group compared with the sham group over the 10-wk period after the operations (Figure 5B-D, Figure 6 and Table 7).

Table 6 Longitudinal changes in liver stiffness measurement (kPa) *via* elastography point quantification between splenectomy and sham groups

		POW 0	POW 2	POW 4	POW 6	POW 8	POW 10
F1	Group S (n = 5)	8.46 (6.63-8.50)	8.74 (6.97-8.91)	11.34 (9.13-12.89)	14.32 (11.45-15.45)	18.89 (16.43-19.23)	19.24 (17.78-21.09)
	Group L (n = 4)	8.59 (7.12-10.13)	12.79 (11.01-14.62)	14.35 (12.24-16.70)	16.30 (13.25-20.07)	21.01 (16.11-25.45)	23.34 (20.66-26.15)
F2	Group S (n = 4)	10.81 (8.34-13.48)	14.45 (12.53-15.70)	18.28 (16.72-18.56)	20.73 (18.09-22.26)	25.50 (23.10-25.89)	30.79 (27.29-33.31)
	Group L (n = 4)	10.50 (9.31-11.96)	15.39 (13.55-16.92)	17.90 (16.01-19.27)	20.25 (18.92-21.51)	23.89 (23.15-25.24)	30.60 (28.54-32.29)
F3	Group S (n = 4)	19.19 (17.77-21.25)	22.90 (20.70-25.17)	26.99 (25.13-28.88)	29.98 (26.14-33.23)	32.38 (29.06-35.89)	35.79 (33.79-37.31)
	Group L (n = 4)	22.08 (18.24-25.35)	25.17 (21.79-27.59)	27.21 (24.38-30.23)	29.72 (28.27-30.70)	31.89 (29.91-33.98)	32.22 (30.70-34.71)
F4	Group S (n = 3)	27.56 (23.40-28.34)	31.23 (26.84-32.22)	30.12 (27.89-32.12)	36.12 (32.39-36.34)	35.10 (34.12-36.27)	36.12 (34.06-37.28)
	Group L (n = 3)	23.98 (20.21-28.66)	29.12 (23.68-31.19)	32.00 (28.17-33.00)	29.00 (28.56-32.56)	33.43 (33.32-35.32)	35.30 (35.25-37.21)

POW: Postoperative weeks; Group S: Rabbits received splenectomy plus liver biopsy; Group L: Rabbits received liver biopsy only.

Table 7 Longitudinal changes in liver function between splenectomy and sham groups

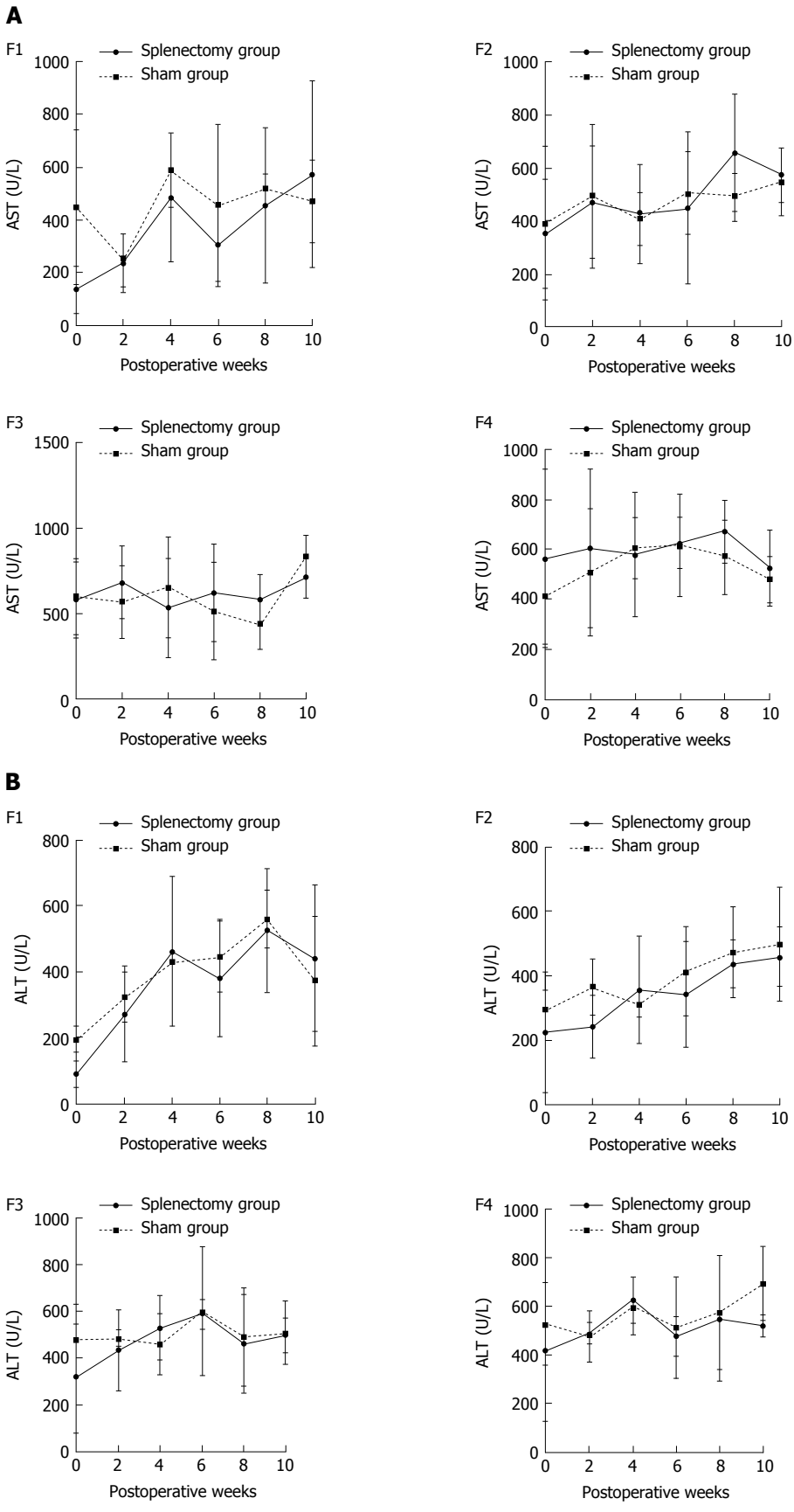
		POW 0	POW 2	POW 4	POW 6	POW 8	POW 10
AST (IU/L)							
F1	Group S (n = 5)	138.0 ± 87.3	236.7 ± 110.6	484.3 ± 244.2	307.3 ± 138.9	454.0 ± 291.3	574.3 ± 350.6
	Group L (n = 4)	447.0 ± 292.7	245.5 ± 98.3	589.5 ± 140.7	455.0 ± 309.7	517.5 ± 55.9	470.0 ± 155.6
F2	Group S (n = 4)	353.6 ± 208.4	473.4 ± 212.1	426.0 ± 186.4	448.6 ± 286.4	658.2 ± 221.9	575.6 ± 104.4
	Group L (n = 4)	392.0 ± 290.5	493.5 ± 269.0	407.5 ± 100.4	506.3 ± 156.8	494.5 ± 88.8	550.0 ± 125.6
F3	Group S (n = 4)	578.3 ± 219.5	681.3 ± 208.9	534.0 ± 292.2	622.7 ± 286.2	584.7 ± 144.5	713.3 ± 122.8
	Group L (n = 4)	598.8 ± 219.5	569.8 ± 208.9	651.5 ± 292.2	513.0 ± 286.2	435.5 ± 144.5	831.8 ± 122.8
F4	Group S (n = 3)	562.2 ± 356.6	604.2 ± 314.3	578.4 ± 247.7	623.8 ± 101.1	672.6 ± 122.6	525.6 ± 153.2
	Group L (n = 3)	412.6 ± 190.9	508.7 ± 253.8	604.0 ± 121.8	616.7 ± 203.1	571.0 ± 145.7	480.3 ± 91.4
ALT (IU/L)							
F1	Group S (n = 5)	88.7 ± 39.2	270.3 ± 144.9	460.7 ± 227.5	379.0 ± 179.2	526.7 ± 185.8	440.7 ± 220.4
	Group L (n = 4)	195.0 ± 39.6	322.0 ± 76.4	430.5 ± 2.1	445.0 ± 108.9	560.5 ± 87.0	372.0 ± 198.0
F2	Group S (n = 4)	224.6 ± 187.8	240.8 ± 96.7	355.8 ± 165.8	342.6 ± 165.1	436.8 ± 74.9	458.0 ± 92.4
	Group L (n = 4)	291.8 ± 65.9	364.3 ± 86.5	310.3 ± 35.8	414.5 ± 136.8	473.3 ± 140.5	498.3 ± 174.0
F3	Group S (n = 4)	315.7 ± 232.7	434.0 ± 171.8	528.3 ± 137.7	588.0 ± 61.6	461.3 ± 208.2	497.3 ± 73.6
	Group L (n = 4)	477.8 ± 151.7	484.0 ± 34.4	459.3 ± 132.5	601.0 ± 274.9	488.8 ± 210.2	507.0 ± 133.2
F4	Group S (n = 3)	416.8 ± 286.2	490.6 ± 43.3	626.2 ± 93.0	478.0 ± 83.3	550.6 ± 252.5	519.2 ± 44.9
	Group L (n = 3)	529.0 ± 167.0	475.8 ± 106.7	600.4 ± 116.3	512.6 ± 207.8	576.8 ± 233.1	695.4 ± 150.6
Albumin (g/L)							
F1	Group S (n = 5)	41.6 ± 2.4	40.5 ± 1.8	39.4 ± 2.3	40.5 ± 3.4	40.0 ± 2.0	39.8 ± 1.7
	Group L (n = 4)	41.3 ± 4.9	40.2 ± 3.0	40.3 ± 1.8	39.3 ± 0.6	39.3 ± 2.0	40.7 ± 1.3
F2	Group S (n = 4)	39.0 ± 1.9	38.3 ± 1.9	38.3 ± 1.5	38.9 ± 2.0	38.9 ± 1.6	38.9 ± 0.8
	Group L (n = 4)	39.6 ± 1.6	39.6 ± 1.7	38.0 ± 1.5	39.5 ± 0.8	39.0 ± 1.4	38.9 ± 1.9
F3	Group S (n = 4)	30.7 ± 4.3	30.3 ± 3.9	29.8 ± 3.3	31.0 ± 3.0	30.2 ± 3.8	30.5 ± 4.1
	Group L (n = 4)	31.1 ± 5.7	30.8 ± 5.1	31.8 ± 4.6	30.6 ± 5.2	30.9 ± 4.9	30.3 ± 4.2
F4	Group S (n = 3)	35.8 ± 5.6	36.0 ± 4.7	34.5 ± 4.1	34.2 ± 4.8	35.1 ± 5.1	35.5 ± 3.6
	Group L (n = 3)	35.3 ± 6.6	34.9 ± 5.8	35.4 ± 6.1	34.8 ± 3.4	34.8 ± 4.9	35.0 ± 5.6
TB (μmol/L)							
F1	Group S (n = 5)	1.08 ± 0.10	1.30 ± 0.16	1.60 ± 0.16	1.58 ± 0.18	1.71 ± 0.23	1.70 ± 0.22
	Group L (n = 4)	1.34 ± 0.16	1.38 ± 0.08	1.39 ± 0.08	1.65 ± 0.20	1.51 ± 0.19	1.58 ± 0.25
F2	Group S (n = 4)	2.01 ± 0.72	2.35 ± 0.58	2.45 ± 0.61	2.65 ± 0.47	2.76 ± 0.51	2.81 ± 0.54
	Group L (n = 4)	2.08 ± 0.23	2.45 ± 0.51	2.62 ± 0.45	2.67 ± 0.23	2.99 ± 0.15	2.68 ± 0.40
F3	Group S (n = 4)	2.36 ± 0.64	2.52 ± 0.64	2.62 ± 0.51	2.66 ± 0.29	2.97 ± 0.19	3.09 ± 0.10
	Group L (n = 4)	2.59 ± 0.76	2.87 ± 0.42	2.88 ± 0.48	2.94 ± 0.33	3.13 ± 0.20	3.09 ± 0.12
F4	Group S (n = 3)	2.30 ± 0.56	2.58 ± 0.54	2.73 ± 0.22	2.88 ± 0.18	3.05 ± 0.26	3.03 ± 0.38
	Group L (n = 3)	2.35 ± 0.84	2.49 ± 0.52	2.82 ± 0.67	2.96 ± 0.13	3.11 ± 0.48	3.08 ± 0.51

POW: Postoperative weeks; Group S: Rabbits received splenectomy plus liver biopsy; Group L: Rabbits received liver biopsy only.

DISCUSSION

In the current study, after four to twenty weeks of fibrosis induction, the LSM increased from 7.88 kPa to 5.86-39.12 kPa in all rabbits with different proven fibrosis stages. Two specific serum markers of liver fibrogenesis (type Ⅳ collagen and hyaluronic acid) were selected to reflect the progression of CCl_4-induced liver fibrosis, and both markers showed a step-wise correlation with liver fibrosis stages compared to the LSM *via* ElastPQ, reinforcing that ElastPQ can reflect the severity of liver fibrosis. However, a comparison of the baseline values of liver stiffness in different studies cannot be performed mainly due to variations in the modeling methods and species.

Although there was a significant increase in the LSM *via* ElastPQ with increased fibrosis stage, there was a degree of overlap between consecutive stages. In


A
F1
Splenectomy group
Sham group
AST (U/L)
Postoperative weeks
F2
F3
F4
B
ALT (U/L)

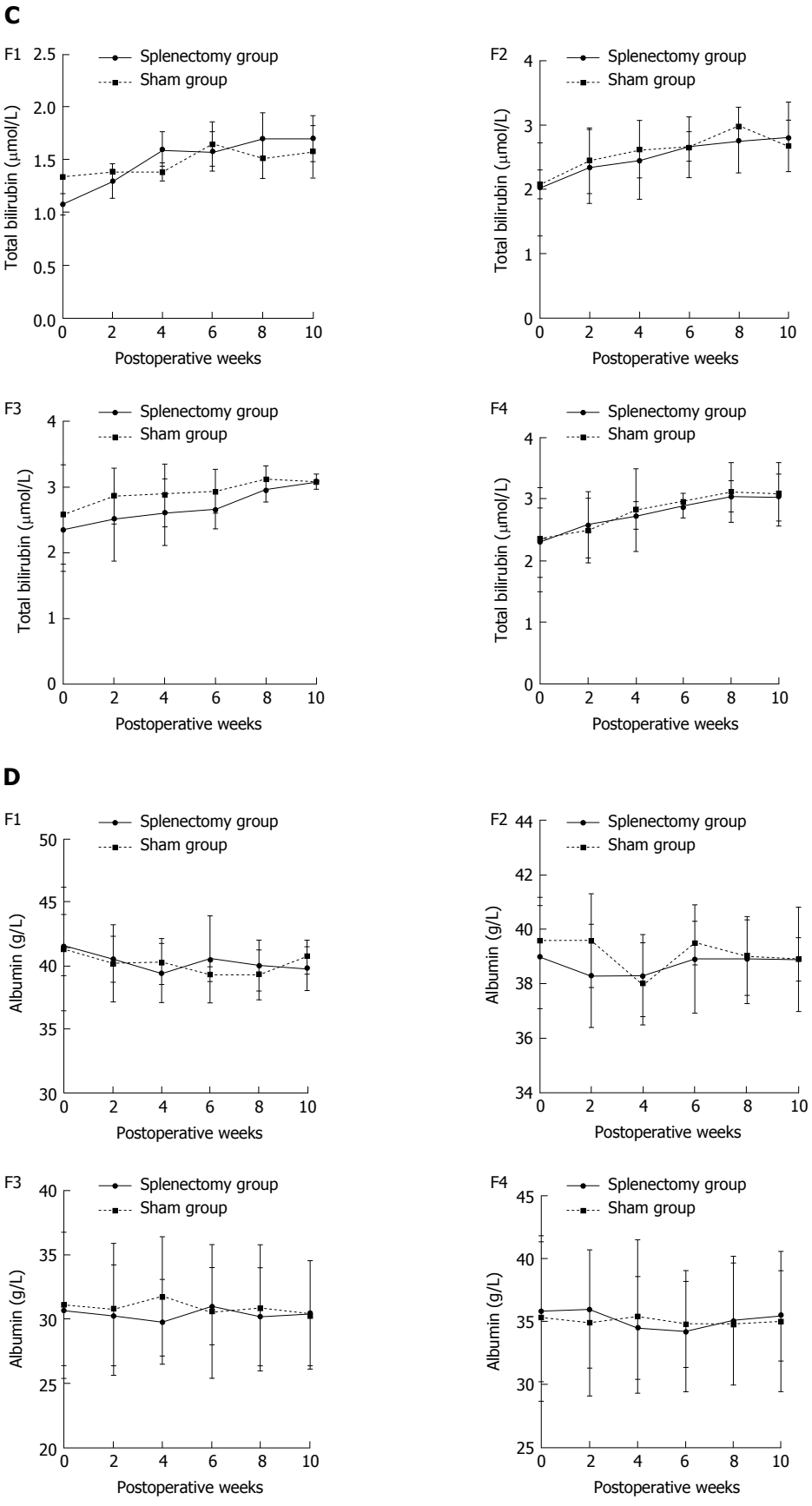


Figure 6 Longitudinal changes in liver function following splenectomy *vs* sham operation at different liver fibrosis stages. A: Changes in AST for rabbits with F1-F4 liver fibrosis; B: Changes in ALT for rabbits with F1-F4 liver fibrosis; C: Changes in total bilirubin for rabbits with F1-F4 liver fibrosis; and D: Changes in albumin for rabbits with F1-F4 liver fibrosis. AST: Aspartate aminotransferase; ALT: Alanine aminotransferase.

this study, for F0-F2 liver fibrosis categories, the LSM was 7.88 (6.60-8.46), 8.46 (6.22-10.35), and 10.89 (8.09-14.46), respectively, which may have been due to an insufficient number of animals with F0-F2. A similar concern was reported in a previous study on the ARFI for assessing liver fibrosis[39]. The ElastPQ cutoff values for minimal fibrosis (F0-F1 *vs* F2-F4), moderate fibrosis (F0-F2 *vs* F3-F4), and cirrhosis (F0-F3 *vs* F4) were defined. As shown in Table 5, the ElastPQ cutoff values for predicting different stages of fibrosis can be clearly distinguished, which may be due to the relatively uniform distribution of rabbits with different stages.

The areas under the ROC curves were compared for ElastPQ, hyaluronic acid and type Ⅳ collagen. The ElastPQ prediction of minimal fibrosis was comparable to that for hyaluronic acid and type Ⅳ collagen, while the ElastPQ prediction of moderate fibrosis and cirrhosis was significantly superior to hyaluronic acid. This outcome demonstrates that this non-invasive technique could have clinical utility.

With continuing basic research, the concept of liver fibrosis has changed from static and progressive to dynamic and bidirectional, especially when the causes of liver damage have been removed. In addition, because there is a significant correlation between the ElastPQ values and liver fibrosis stages, it is theoretically possible to use ElastPQ to non-invasively assess the effect of anti-fibrosis treatments. Indeed, previous studies have reported on the clinical application of TE for dynamically monitoring fibrosis regression during antiviral treatment in chronic hepatitis B and C patients, indicating that the TE values seem to decrease during antiviral therapy[40,41].

Although splenectomy is performed for patients with hypersplenism in some institutions[21,42], hypersplenism in most patients should be considered a laboratory abnormality that does not require treatment or further consideration[43]. However, a previous well-designed study indicated that splenectomy attenuated murine liver fibrosis[22]. Therefore, splenectomy remains controversial for patients with hypersplenism. In the present study, splenectomy was only used to group rabbits and then determine whether splenectomy at different liver fibrosis stages will delay or reverse the progression of liver fibrosis. A previous study indicated that the spleen plays an important regulatory role in rat liver cirrhosis induced by a choline-deficient L-amino acid-defined diet[44]. In this study, a trend suggesting that splenectomy can delay the progression of early liver fibrosis (especially F1) was detected. A previous study using a rat liver fibrosis model indicated that spleen-derived TGF-β1 is involved in the development of liver fibrosis such that decreasing the TGF-β1 level by splenectomy could inhibit hepatic stellate cell activation and then improve liver fibrosis[45]. However, in the present study, splenectomy did not seem to improve late liver fibrosis (especially types F3 and F4); therefore, there may be another potential mechanism for improving early liver fibrosis following splenectomy.

Although activated HSCs have a great impact on liver fibrogenesis, recent studies have suggested that monocytes and their progeny macrophages are also responsible for liver fibrosis[46,47]. Based on a study of monocytes derived from BM[13] as well as studies by Swirski *et al*[14] and other researchers[15,16], there are numerous monocytes in the spleen that could be mobilized in pathological states. As a result, the spleen can be considered a monocyte reservoir. An interesting previous study demonstrated that the spleen is a site for storing and rapidly deploying monocytes involved in inflammation regulation[14]. Therefore, performing splenectomy in the early stages of liver fibrosis would block the rapid deployment of monocytes to the liver, which may alleviate the inflammatory reaction and delay liver fibrosis. In contrast, performing splenectomy in the late stage would not help to postpone liver fibrosis, which may be explained by the hypothesis that during the late stage, monocytes from BM play a predominant role in liver fibrosis. However, it should be further determined whether the diversity of monocyte origin influences the different stages of liver fibrosis.

In summary, ElastPQ is an available, convenient, objective and non-invasive technique for assessing liver stiffness in rabbits with CCl_4-induced liver fibrosis, and paves the way for its clinical application. Additionally, liver stiffness measurements with ElastPQ can dynamically monitor the changes in liver stiffness in rabbit models, or patients, after splenectomy. However, the underlying mechanism by which early splenectomy can decelerate liver fibrosis should be further studied.

COMMENTS

Background

Elastography point quantification (ElastPQ) is a non-invasive technique for assessing tissue stiffness and was used in this study. Splenectomy is a surgical intervention for liver cirrhosis patients with hypersplenism. However, no evidence of changes in fibrotic liver stiffness after splenectomy at different pathological stages using ElastPQ has previously been available.

Research frontiers

Liver biopsy is the reference standard for staging fibrosis. Nevertheless, because of its invasive nature, liver biopsy is difficult to perform in patients who require repeated examination to monitor liver fibrosis progression. An acoustic radiation force impulse (ARFI) technique, ElastPQ, has been developed for measuring tissue stiffness. In this study, the author used a liver fibrosis animal model to demonstrate that ElastPQ is an available, convenient, objective and non-invasive technique for assessing liver stiffness in rabbits with CCl_4-induced liver fibrosis. In addition, the changes in liver stiffness in rabbit models, or patients, after splenectomy can be dynamically monitored by ElastPQ.

Innovations and breakthrough

This is the first animal experiment to confirm that liver stiffness measurements with ElastPQ could be used to dynamically monitor the changes in liver stiffness. The results provide good news for liver fibrosis patients who are in need of long-term follow-up.

Applications

Based on this study, ElastPQ could dynamically monitor the changes in liver

stiffness after interventions.

Terminology

ElastPQ is an ARFI technique that can non-invasively measure tissue stiffness.

Peer-review

This is a well-supported paper presenting a study on utilizing CCl_4-induced liver fibrosis to evaluate liver stiffness measurement approaches.

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ORIGINAL ARTICLE

Basic Study

Hepatoprotective and antioxidant effects of lycopene on non-alcoholic fatty liver disease in rat

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Abstract

AIM

To evaluate the hepatoprotective effect of lycopene (Ly) on non-alcoholic fatty liver disease (NAFLD) in rat.

METHODS

A rat model of NAFLD was first established by feeding a high-fat diet for 14 wk. Sixty-five rats were randomly divided into normal group, model group and Ly treatment groups. Alanine aminotransferase (ALT), aspartate aminotransferase (AST), triglycerides (TG), total cholesterol (TC) in serum and low density lipoprotein-cholesterol (LDL-C), high density lipoprotein-cholesterol (HDL-C), free fatty acid (FFA), malondialdehyde (MDA), superoxide dismutase (SOD), glutathione (GSH) in liver tissue were evaluated, respectively. While the hepatoprotective effect was also confirmed by histopathological analysis, the expression levels of TNF-α and cytochrome P_{450} (CYP) 2E1 in rat liver were determined by immunohistochemistry analysis.

RESULTS

A significant decrease was observed in the levels of serum AST (2.07-fold), ALT (2.95-fold), and the blood lipid TG (2.34-fold) and TC (1.66-fold) in the dose of 20 mg/kg Ly-treated rats ($P < 0.01$), compared to the model group. Pretreatment with 5, 10 and 20 mg/kg of Ly significantly raised the levels of antioxidant enzyme SOD in a dose-dependent manner,

to 90.95 ± 9.56, 109.52 ± 11.34 and 121.25 ± 10.68 ($P < 0.05$, $P < 0.01$), as compared with the model group. Similarly, the levels of GSH were significantly increased ($P < 0.05$, $P < 0.01$) after the Ly treatment. Meanwhile, pretreatment with 5, 10 and 20 mg/kg of Ly significantly reduced MDA amount by 30.87, 45.51 and 54.49% in the liver homogenates, respectively ($P < 0.01$). The Ly treatment group showed significantly decreased levels of lipid products LDL-C ($P < 0.05$, $P < 0.01$), improved HDL-C level and significantly decreased content of FFA, compared to the model group ($P < 0.05$, $P < 0.01$). Furthermore, the Ly-treated group also exhibited a down-regulated TNF-α and CYP2E1 expression, decreased infiltration of liver fats and reversed histopathological changes, all in a dose-dependent manner ($P < 0.05$, $P < 0.01$).

CONCLUSION

This study suggests that Ly has a protective effect on NAFLD, down-regulates expression of TNF-α, and that CYP2E1 may be one of the action mechanisms for Ly.

Key words: Lycopene; Antioxidant; Hepatoprotective; Non-alcoholic fatty liver; Cytochrome P450 2E1

Core tip: Lycopene (Ly), a phytochemical belonging to the carotenoid family, is a red-colored pigment, apolar and acyclic carotenoid. The present study was designed to evaluate the possible hepatoprotective effect of Ly on non-alcoholic fatty liver disease (NAFLD) in rat. This study represents the first examination of the effects of Ly on the therapy of NAFLD, and showed down-regulated expression of TNF-α and indicated that CYP2E1 may be one of the action mechanisms for Ly.

Jiang W, Guo MH, Hai X. Hepatoprotective and antioxidant effects of lycopene on non-alcoholic fatty liver disease in rat. *World J Gastroenterol* 2016; 22(46): 10180-10188 Available from: URL: http://www.wjgnet.com/1007-9327/full/v22/i46/10180.htm DOI: http://dx.doi.org/10.3748/wjg.v22.i46.10180

INTRODUCTION

Non-alcoholic fatty liver disease (NAFLD) is one of the causes of fatty liver, occurring when fat is deposited (steatosis) in the liver due to causes other than excessive alcohol use. NAFLD is considered to cover a spectrum of liver diseases, including simple steatosis, non-alcoholic steatohepatitis (NASH), liver fibrosis, liver cirrhosis and hepatocellular carcinoma (HCC)[1,2]. Ninety percent of patients with NAFLD show close relation with one or more of the following risk factors: hypertension, dyslipidemia, elevated triglyceride (TG) levels, obesity, insulin resistance, metabolic syndrome, type 2 diabetes mellitus and cardiovascular disease[3]. Currently, the percentage of people with NAFLD is approximately 20% worldwide and 25% in Western countries, making it one of the most dominant causes of chronic liver disease affecting both adults and children[4]. NAFLD is more common in patients with severe diabetes and obesity, mortality and disease evolution to liver fibrosis or liver cirrhosis is increased in old people with NAFLD[5]. Recently, the "two-hit" theory has arisen as a popular mechanism, although the cause of NAFLD remains to be clearly elucidated[6]. Furthermore, there is not any specific drug available for NAFLD, and no drug has yet to be tested in clinical phase Ⅲ trials. Therefore, no specific therapy can be firmly recommended to the patients with NAFLD[7].

Lycopene (Ly), a phytochemical belonging to carotenoid family, is a red-colored pigment, acyclic and apolar carotenoid[8]. It is abundantly found in red-colored vegetables and fruits, such as tomatoes, papaya, gac fruit, pink grape-fruit, pink guava, carrots and watermelon, with the concentrations ranging from 9 to 42 mg/kg depending on the variety[9]. Ly displays a range of unique and distinct biological properties owing to its acyclic structure, hydrophobicity and large array of conjugated double bonds. Recently, diverse studies have been reported that lycopene exerts powerful antioxidant activity both *in vitro* and *in vivo* against the oxidation of proteins, lipids and DNA, and also has the potential of quenching singlet oxygen 100 times more efficiently than vitamin E and 125 times more than glutathione (GSH)[10]. Furthermore, even at low oxygen tension, it can also scavenge peroxyl radicals, inhibiting the process of lipid peroxidation[11]. It is the most efficient quencher of singlet oxygen among all naturally occurring carotenoids[11], and recently it has been in great demand as a food additive and a natural antioxidant. Additionally, Ly also exhibited potent neuroprotective, anti-inflammatory, anti-proliferative, maintenance of normal cell metabolism, cognition enhancing properties, regulating blood lipid metabolism and so on[12-16]. Therefore, with this background, we aimed to investigate the possible beneficial effects and the possible action mechanism of Ly on NAFLD using a rat model system.

MATERIALS AND METHODS

Materials and reagents

Alanine aminotransferase (ALT), aspartate aminotransferase (AST), TG, total cholesterol (TC), low density lipoprotein-cholesterol (LDL-C), high density lipoprotein-cholesterol (HDL-C), free fatty acid (FFA), malondialdehyde (MDA), superoxide dismutase (SOD) and GSH kits were obtained from Nanjing Jiancheng Bioengineering Institute (Nanjing, China). Protein assay kit was from Zhongshan Institute of Biotechnology (Beijing, China).

Mouse anti-TNF-α, rabbit anti-cytochrome P_{450}2E1 (CYP2E1), horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG and HRP-conjugated goat anti-

rabbit IgG antibodies were provided by Proteintech Group, Inc. (Chicago, IL, United States).

Ly (> 95%) was purchased from North China Pharmaceutical Co., Ltd. (Shijiazhuang, China).

The high-fat diet (HFD: 88% basic feed + 10% lard + 2% cholesterol) was prepared in our lab.

Animals

Male Wistar rats (body weight 150 ± 10 g) were obtained from the Experimental Animal Center of Harbin Medical University (China). Six rats were kept in each single polyacrylic cage and were quarantined for 1 wk before the experiments. All animals were housed under standard controlled conditions (temperature: 24 ± 1 ℃, humidity: 50% ± 5% and 12 h light/dark cycle), with free access to food and water, and received humane care according to National Institutes of Health Guidelines of the United States (National Research Council of United States, 1996) and the related ethical regulations of Harbin Medical University. Animals were fasted for 12 h before sampling of material.

Experimental design

After acclimatization for 1 wk, 65 Wistar male rats were randomized into 2 groups. Group 1 (normal group) was raised with normal feed (n = 12), and Group 2 (model group) was raised on HFD (n = 53) for consecutive 8 wk. From the 9th wk, all the surviving rats in the model group were further randomly divided into 1 model group and 3 Ly treatment groups, which were given Ly at a dose of 5, 10 or 20 mg/kg/d (n = 12), respectively. The model group was continued on the HFD for 6 wk as before, and the Ly groups were administered orally and continued on the HFD for 6 wk as before.

Rats were sacrificed by cervical dislocation at the end of the experiment, and blood samples of all rats were harvested for serum biochemical markers assay. The fresh liver obtained was weighed to calculate liver coefficient (% = liver weight/body weight × 100). The right liver lobe was fixed in 10% formalin to prepare paraffin sections, and the rest was stored at -80 ℃ for the other assays.

Serum biochemical markers assay

Serum was collected from blood after centrifugation at 3000 rpm for 10 min at 4 ℃. Serum ALT, AST, TG and TC were detected using commercial kits according to the manufacturer's instructions and using a multifunctional biochemistry analyzer (AU600; Olympus, Tokyo, Japan). The absorbance of ALT and AST was read at 505 nm and the enzyme activity was calculated as U/L. The absorbance of TG and TC was read at 510 nm and the content was calculated as mmol/L.

Measurement of MDA formation in lipid peroxidation

Liver homogenate (10%, w/v) was prepared by homogenizing the liver tissue in 150 mmol/L Tris-HCl buffered saline (pH 7.2) with a polytron homogenizer. The level of MDA in liver tissues was measured at 532 nm with a spectrophotometer (U-2001 Hitachi Ltd., Tokyo, Japan) following the kit protocol from Jiancheng Biological Engineering Institute. The data are expressed as nmol/mg protein of liver tissue.

Measurement of antioxidant and antioxidant enzyme activity

SOD and GSH activity were determined by commercial kit from Jiancheng Biological Engineering Institute following the protocol provided by the manufacturer. The absorbance of the SOD reaction was read at 550 nm and the data are expressed as U/mg protein, while the GSH reaction was read at 420 nm and the enzyme activity was calculated as mg/g protein.

Measurement of liver LDL-C, HDL-C and FFA activity

LDL-C, HDL-C and FFA in liver tissue were measured by commercial kit from Jiancheng Biological Engineering Institute following the protocol provided by the manufacturer. The absorbance of LDL-C, HDL-C and FFA reaction was read at 546 nm and the data are expressed as mmol/L.

Histopathological observation

Liver specimens were fixed overnight in 10% formaldehyde buffer, then embedded in paraffin and cut into 5 μm thickness sections according to the routine procedure. The sections were stained with hematoxylin and eosin (HE) for routine histopathological examination, and examined under a light microscope (BX-50; Olympus) at 200 × magnification for the degree of hepatic steatosis and photographed.

Immunohistochemistry analysis of hepatic TNF-α and CYP2E1

Paraffin-embedded sections (5 μm) were mounted on glass slides, then deparaffinized, incubated in 3% H_2O_2 for 10 min to quench endogenous peroxidase activity. The sections were stained with mouse anti-TNF-α antibody and rabbit anti-CYP2E1 antibody at 4 ℃ overnight respectively, after blocking with normal goat serum for 20 min. Then, the sections were incubated with HRP-conjugated goat anti-mouse and HRP-conjugated goat anti-rabbit antibody at 37 ℃ for 30 min, respectively. The immunoreactive antibodies were visualized by incubation with DAB-H_2O_2 at room temperature for 10 min. Images were taken at original magnification of 200 × (Olympus BX-50 Microscope and a Leica DMI; Leica Microsystems, Wetzlar, Germany).

Statistical analysis

Data were expressed as mean ± SD and all statistical comparisons were made by means of a one-way ANOVA test followed by Dunett's t-test. $P < 0.05$ and < 0.01 were considered statistically significant.

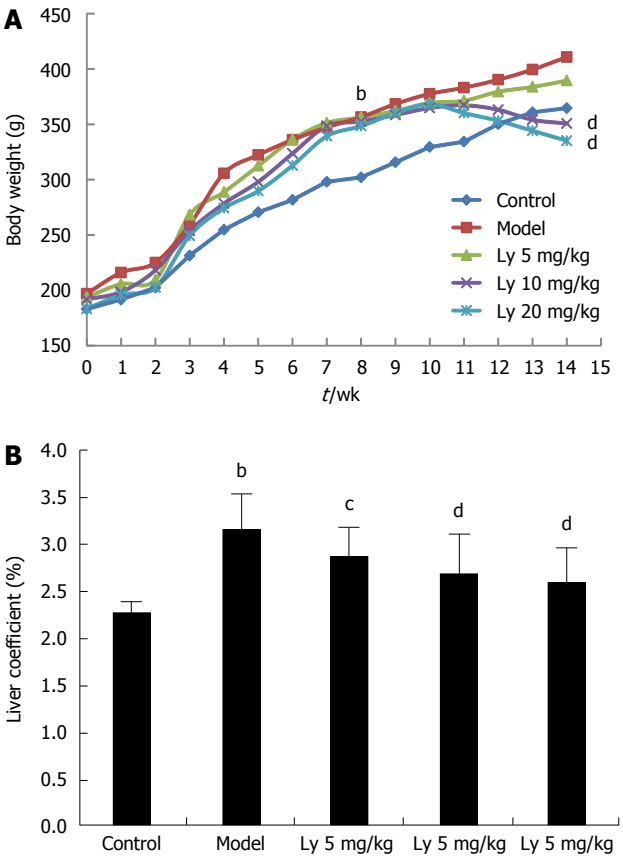

Figure 1 Effects of lycopene on body weight (A) and liver coefficient (B). $^{b}P < 0.01$ *vs* control group; $^{c}P < 0.05$, $^{d}P < 0.01$ *vs* model group. Ly: Lycopene.

RESULTS

Effects of Ly on body weight and liver coefficient

After 8 wk of HFD feeding, the body weights of rats in the model group were significantly increased compared to that of rats in the control group ($P < 0.01$, Figure 1A). Meanwhile, after Ly treatment for 6 wk, the gain in body weight for the rats in the 10 and 20 mg/kg Ly-treated groups was lower than that for the rats in the model group ($P < 0.01$, Figure 1A), which indicated that Ly treatment could inhibit the occurrence of obesity in HFD-administrated rats. Furthermore, consistent with these modifications, the liver coefficient was also reduced markedly in the Ly-treated rats ($P < 0.05$, $P < 0.01$, Figure 1B), compared to the control group.

Effect of Ly on serum ALT and AST levels

Serum levels of AST and ALT indirectly reflects the failure of liver function. As shown in Table 1, serum AST (2.67-fold) and ALT (3.66-fold) activities were significantly increased after the administration of HFD, as compared with the normal group ($P < 0.01$). Compared with the model group, the levels of AST and ALT were significantly decreased in a dose-dependent manner after Ly treatment (5, 10 and 20 mg/kg) ($P < 0.05$, $P < 0.01$, Table 1).

Table 1 Effect of lycopene on serum liver function markers and blood lipid levels

Group	ALT (IU/L)	AST (IU/L)	TG (mmol/L)	TC (mmol/L)
Control	16.72 ± 2.62	60.65 ± 6.28	0.52 ± 0.04	0.81 ± 0.06
Model	61.25 ± 13.55^{b}	162.17 ± 35.53^{b}	1.38 ± 0.21^{b}	3.04 ± 0.72^{b}
Ly 5 mg/kg	30.90 ± 3.84^{c}	95.91 ± 13.65^{c}	1.02 ± 0.10	2.31 ± 0.24
Ly 10 mg/kg	26.33 ± 2.06^{d}	88.53 ± 9.18^{d}	0.75 ± 0.06^{c}	2.00 ± 0.12^{c}
Ly 20 mg/kg	20.77 ± 3.52^{d}	78.44 ± 9.79^{d}	0.59 ± 0.03^{d}	1.83 ± 0.15^{d}

Data are expressed as mean ± SD (n = 12) for each group. $^{b}P < 0.01$ *vs* control group; $^{c}P < 0.05$, $^{d}P < 0.01$ *vs* model group. Ly: Lycopene; ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; TG: Triglycerides; TC: Total cholesterol.

Table 2 Effect of lycopene on liver antioxidant enzyme-specific activities, antioxidant and lipid peroxidation levels

Group	SOD (U/mgprot)	GSH (mg/gprot)	MDA (nmol/mgprot)
Control	131.42 ± 16.24	6.76 ± 1.54	3.46 ± 1.11
Model	77.70 ± 7.63^{b}	2.55 ± 0.78^{b}	7.58 ± 3.10^{b}
Ly 5 mg/kg	90.95 ± 9.56^{c}	2.68 ± 1.26	5.24 ± 1.46^{d}
Ly 10 mg/kg	109.52 ± 11.34^{d}	3.76 ± 0.91^{c}	4.13 ± 1.13^{d}
Ly 20 mg/kg	121.25 ± 10.68^{d}	4.79 ± 1.51^{d}	3.45 ± 1.39^{d}

Data are expressed as mean ± SD (n = 12) for each group. $^{b}P < 0.01$ *vs* control group; $^{c}P < 0.05$, $^{d}P < 0.01$ *vs* model group. Ly: Lycopene; SOD: Superoxide dismutase; GSH: Glutathione; MDA: Malondialdehyde.

Effects of Ly on blood lipid levels

HFD-induced NAFLD provoked a marked incremental change in TC and TG levels compared with those in the normal group ($P < 0.01$, Table 1), which indicates the successful establishment of the NAFLD model in rats. However, after Ly exposure, the concentrations of both TC and TG in blood were remarkably decreased in dose-dependent manners, as compared to the NAFLD model group ($P < 0.05$, $P < 0.01$, Table 1). All of these findings indicate that Ly exerts obvious lipid-lowering effects against NAFLD.

Effects of Ly on liver tissue SOD, GSH and MDA levels

The levels of liver antioxidant activities of SOD and GSH were measured due to the oxidative stress exhibited in the development of NAFLD[17]. SOD and GSH are capable of scavenging the lipid hydroperoxides, lipid peroxide radicals and other products which are toxic metabolites of NAFLD. Therefore, our study measured the contents of SOD, GSH and MDA in liver tissue of rats. From Table 2, we can clearly see the significant differences between the HFD-treated model group and the normal group for the levels of SOD and GSH, which were largely decreased ($P < 0.01$, Table 2) in the HFD-treated group compared with that of normal group. However, pretreatment with 5, 10 and 20 mg/kg of Ly significantly raised the levels of the antioxidant enzyme SOD in a dose-dependent manner, to 90.95 ± 9.56, 109.52 ± 11.34 and 121.25 ± 10.68 respectively ($P < 0.05$, $P < 0.01$, Table 2), as compared with the model

Table 3 Effect of lycopene on low density lipoprotein-cholesterol, high density lipoprotein-cholesterol and free fatty acid levels in liver tissue

Group	LDL-C (mmol/L)	HDL-C (mmol/L)	FFA (mmol/L)
Control	0.34 ± 0.08	0.98 ± 0.10	0.82 ± 0.13
Model	2.48 ± 0.13[b]	0.55 ± 0.02[b]	2.03 ± 0.15[b]
Ly 5 mg/kg	1.32 ± 0.10[c]	0.70 ± 0.04[c]	1.73 ± 0.12
Ly 10 mg/kg	0.95 ± 0.05[d]	0.80 ± 0.05[d]	1.56 ± 0.10[c]
Ly 20 mg/kg	0.62 ± 0.08[d]	0.87 ± 0.05[d]	1.34 ± 0.08[d]

Data are expressed as mean ± SD (n = 12) for each group. [b]P < 0.01 *vs* control group; [c]P < 0.05, [d]P < 0.01 *vs* model group. Ly: Lycopene; LDL-C: Low density lipoprotein-cholesterol; HDL-C: High density lipoprotein-cholesterol; FFA: Free fatty acid.

group. Similarly, the levels of GSH were significantly increased by treatment with 10 and 20 mg/kg of Ly (P < 0.05, P < 0.01, Table 2).

MDA, an end-product of the breakdown of poly-unsaturated fatty acids and related esters, is an important index of lipid peroxidation in many organ homogenates[17]. Administration of HFD caused a significant increase in MDA concentration (2.19-fold), as compared with the normal group (P < 0.01, Table 2). However, pretreatment with 5, 10 and 20 mg/kg of Ly significantly reduced the MDA amount by 30.87, 45.51 and 54.49% in the liver homogenates respectively (P < 0.01, Table 2).

Effects of Ly on LDL-C, HDL-C and FFA levels in liver tissue

Levels of lipid products were significantly increased after 8 wk of HFD feeding in the model group compared to the control group (P < 0.01, Table 3). Results showed that LDL-C was significantly increased in the model group compared with the normal group (P < 0.01, Table 3) and dramatically decreased in the Ly-treatment group, as compared with that in the model group (P < 0.05, P < 0.01, Table 3). In contrast, HDL-C level was significantly decreased at the end of the experiment, and Ly treatment significantly improved the HDL-C level, as compared with that in the model group (P < 0.05, P < 0.01, Table 3). Similarly, the concentration of FFA was remarkably increased after HFD administration, and pretreatment with Ly significantly decreased the content of FFA in a dose-dependent manner (P < 0.05, P < 0.01, Table 3).

Histopathological changes in the liver tissue

Observed with the naked eye, the livers of the control group were deep red, moist, glossy and resilient (Figure 2AⅠ), while those of the model group showed yellow necrotic foci, grey-red color, loss of luster and tumescent (Figure 2AⅡ). However, in Ly-treated rats, the liver injury was attenuated dramatically in a dose-dependent manner (Figure 2AⅢ-Ⅴ).

HE-stained sections are shown in Figure 2B. Under the photomicroscope, liver sections from the normal control group showed normal lobular architecture, liver cells with well-preserved cytoplasm and well-defined nucleus (Figure 2BⅠ). Meanwhile liver sections from the model group showed full fat vacuoles in lobule cells, infiltration of inflammatory cells, cell swelling and lipid degeneration in the central region of the lobules (Figure 2BⅡ). Furthermore, in the liver sections of the Ly-treated group, inflammatory response and lipid degeneration were remarkably alleviated, as compared with the model group, and the liver cell volume became smaller, the fat droplet number was reduced and the hepatic lobules were clearly delineated (Figure 2BⅢ-Ⅴ).

Effect of Ly on immunohistochemistry analysis of hepatic TNF-α and CYP2E1

The immunohistochemistry (IHC) analysis of liver tissue showed no TNF-α expression in the normal group (Figure 3AⅠ), but increased expression of TNF-α in the HFD-model group (Figure 3AⅡ). After pretreatment with Ly (5, 10 and 20 mg/kg), TNF-α expression decreased in a dose-dependent manner, but remained higher than that in the normal group (Figure 3AⅢ-Ⅴ). Quantification of the positive expression of TNF-α is shown in Figure 3AⅥ. The results are presented as mean ± SD of (n = 12). [b]P < 0.01 was significantly different from the normal group; [c]P < 0.05 and [d]P < 0.01 were significantly different from the model group, respectively.

In Figure 3B, the normal liver expressed the lowest amount of CYP2E1 (Figure 3BⅠ). The HFD-model group showed significantly higher expression of CYP2E1, as compared with the controls (P < 0.01, Figure 3BⅡ). Meanwhile the Ly-treated group (5, 10 and 20 mg/kg) showed markedly decreased CYP2E1 expression (Figure 3BⅢ-Ⅴ). Quantification of the positive expression of CYP2E1 is shown in Figure 3BⅥ. The results are presented as mean ± SD (n = 12). [b]P < 0.01 was significantly different from the control group; [c]P < 0.05 and [d]P < 0.01 were significantly different from the model group, respectively.

DISCUSSION

NAFLD is defined by hepatic fat deposition in the absence of excessive alcohol intake, which is also associated with the insulin resistance (IR) and metabolic syndrome[18-20]. Generally, NAFLD is defined as a concentration of hepatic TG exceeding 5% liver weight, and often exhibits a histological spectrum ranging from simple steatosis to NASH. NASH is characterized by hepatocellular damage, fibrogenesis and lobular necro-inflammation[21,22], which may evolve to hepatic cirrhosis and HCC[23,24]. Although HFD-induced NAFLD animal models need a lengthy feeding period, they are more close to human NAFLD in pathophysiology, including induced obesity, IR and hepatic steatosis in mice or rats[25]. Emotional

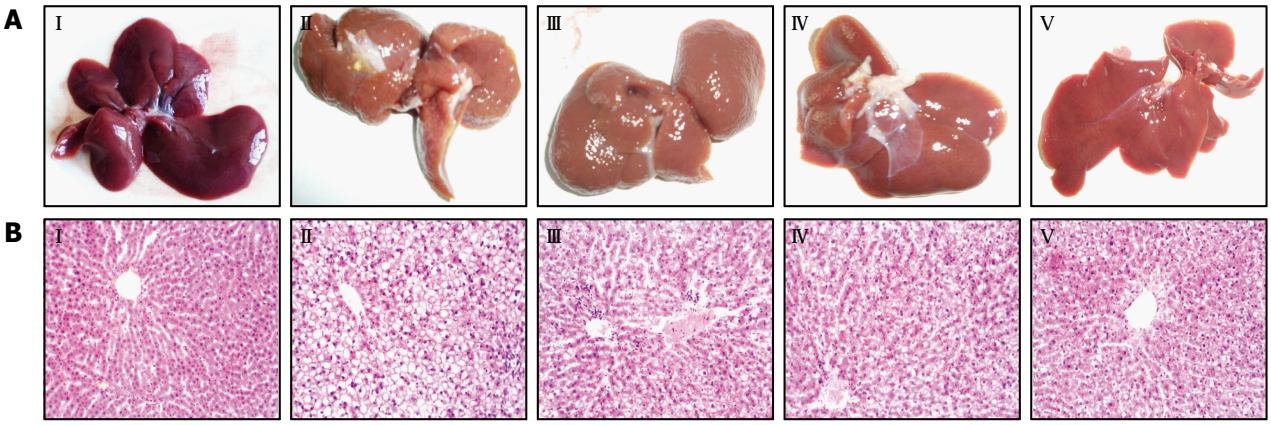

Figure 2 Appearance of rat liver tissue (A) and histopathological examination by HE (B, 200 ×). Ⅰ: Control group; Ⅱ: Model group; Ⅲ: Ly 5 mg/kg group; Ⅳ: Ly 10 mg/kg group; Ⅴ: Ly 20 mg/kg group. Ly: Lycopene.

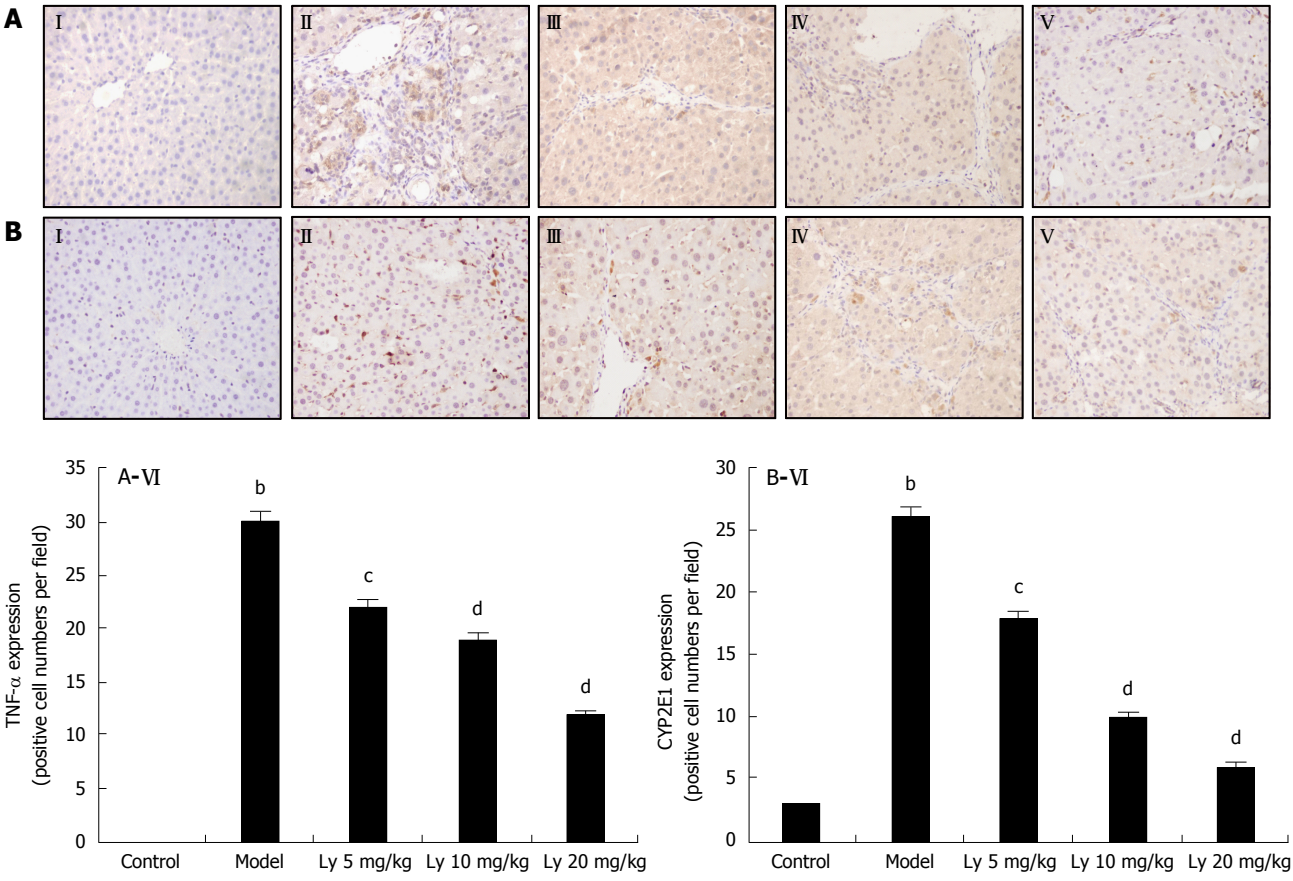

Figure 3 Representative photographs of immunological histological chemistry examination (200 ×). A: TNF-α; B: CYP2E1. Ⅰ: Control group; Ⅱ: Model group; Ⅲ: Ly 5 mg/kg group; Ⅳ: Ly 10 mg/kg group; Ⅴ: Ly 20 mg/kg group; Ⅵ: Quantification of TNF-α (A-Ⅵ) and CYP2E1 (B-Ⅵ) stained cells. The results are expressed as mean ± SD of 12 rats. $^{b}P < 0.01$ *vs* control group; $^{c}P < 0.05$, $^{d}P < 0.01$ *vs* model group. Ly: Lycopene.

disorders or poor diet with the key points of blood stasis and phlegm is regarded as the etiology of NAFLD, and these etiologies are related to the organs of liver, spleen and kidney, according to the traditional medicine theory[26]. Thus, promoting blood circulation to remove meridian obstruction, reducing phlegm, removing dampness and liver-kidney-tonifying are an effective approach to treatment of NAFLD. However, at present, although tremendous effort has made in prevention of NAFLD by clinicians and researchers alike, there are no approved treatment drugs for NAFLD. Hence, developing and exploring a novel agent to delay or reverse the pathogenesis progression in NAFLD are very important objectives.

Ly is a natural pigment, synthesized by plants and microorganisms. Red fruits and vegetables are the most common sources of Ly, which exhibits the highest antioxidant activity among all dietary carotenoids.

Furthermore, the Mediterranean dietary pattern, which includes proportionally high consumption of vegetables and fruits with Ly, has shown notable benefits for NAFLD patients[27,28]. Therefore, nowadays, the potential role of Ly in human health is beginning to be recognized, and the most important health benefits are hypothesized to occur through their ability to protect against oxidative damage[29,30]. *In vitro* studies have demonstrated that Ly is an effective antioxidant and radical scavenger[31,32]. Ly is the most potent singlet oxygen quencher among natural carotenoids, due to its high number of conjugated dienes[33], and recent studies have shown that Ly is at least two times as active as β-carotene in protecting lymphocytes for NO_2· radical-induced membrane damage[34,35], which indicates that Ly is the most potent scavenger of ROS among other major dietary carotenoids[36,37]. In addition, Ly was shown to protect human LDL against photosensitized oxidative damage[32]. Thus, based on the benefits of Ly, the aim of the present study was to explore the effect of Ly in prevention of HFD-induced NAFLD in a rat model. To the best of our knowledge, this is the first time research has attempted to explore the potent effects of Ly on HFD-induced NAFLD rats.

In the present study, compared to a normal control group, it was demonstrated that the liver coefficient and the levels of serum ALT, AST, TG and TC were significantly increased, the levels of LDL-C and FFA in liver were markedly increased, and HDL-C was markedly reduced in HFD-induced NAFLD model rats. Pretreatment with Ly showed that Ly is able to inhibit the incremental changes in ALT and AST, to decrease the TG, TC, LDL-C and FFA levels, and to increase the HDL-C level. In addition, the histopathological changes from microscopy observation correlated with the examination of liver function. The centrilobular hepatic necrosis, ballooning degeneration, fatty change and infiltrating lymphocytes were observed in NAFLD model group. Treatment with Ly prevented these histopathological changes in rats induced with HFD. Thus, these results suggested that the inhibition of the elevation of liver function markers, obvious lipid-lowering and liver damage may related to the protective effect of Ly against HFD-induced NAFLD. Moreover, Ly enhanced the activities of SOD, increased GSH and diminished MDA against the HFD-induced NAFLD in these animals, suggesting that the activity of antioxidants may play a role in the mechanism of its hepatoprotective effects.

TNF-α is a central proinflammatory cytokine, which is associated with a variety of physiological and pathological conditions, including cytotoxicity, growth stimulation, immune-modulation and pro-inflammatory activity. In addition, TNF-α is produced predominantly by the monocyte macrophage lineage in liver, and the main population of this lineage is Kupffer cells. Thus, increased TNF-α production by activated Kupffer cells may be responsible for NAFLD. Furthermore, the most current studies have indicated that inhibition of TNF-α could decrease the content of hepatic fatty storage in the HFD-induced NAFLD model[38]. In our study, the effects of TNF-α in damaged liver was evaluated by IHC. Compared to the normal group, rats treated with HFD showed up-regulated expression of TNF-α, while pretreatment with Ly led to down-regulated expression of TNF-α compared to the HFD-model group.

The isoform 2E1 of CYP is one of the most potent microsome cytochromes to generate ROS, and it is involved in the metabolism of isoniazid and the mediation of its hepatotoxicity[39], which has been exhibited to be invariably increased in the livers of NAFLD patients[40]. In this study, the expression of CYP2E1 in the HFD-model group was observed to be increased, while the Ly treatment group showed a significant down-regulation of its expression, especially in the high-dose Ly-treated group.

In conclusion, oral administration of Ly improved lipid profiles and remarkably decreased the levels of serum AST, ALT, TG and TC, alleviated the levels of liver LDL-C and FFA, increased the activities of antioxidant enzymes (GSH, SOD) and reduced the lipid peroxides in liver (MDA) in NAFLD model rats. Further, the Ly-treated group also showed down-regulated expression of TNF-α and CYP2E1, decreased liver fats infiltration and improved histopathological changes, all in dose-dependent manners. The increased antioxidant enzyme levels and the decreased lipid peroxides contents are suggested to be important mechanisms of Ly in preventing the development of liver damage induced by HFD.

COMMENTS

Background

Non-alcoholic fatty liver disease (NAFLD) is one of the causes of fatty liver, which encompasses a spectrum of liver diseases, including simple steatosis, non-alcoholic steatohepatitis (NASH), liver fibrosis, liver cirrhosis and hepatocellular carcinoma (HCC). Until now, there is not any specific drug available, and no drug has currently been tested in clinical phase Ⅲ trials. Lycopene (Ly), a phytochemical belonging to the carotenoid family, is a red-colored pigment, apolar and acyclic carotenoid. Ly exhibits a range of distinct and unique biological properties owing to its acyclic structure, hydrophobicity and large array of conjugated double bonds. A recent report showed that the Mediterranean dietary pattern, which includes proportionally high consumption of vegetables and fruits with Ly, has notable benefits for NAFLD patients. Thus, with this background, we aimed to investigate the possible beneficial effects and the possible action mechanism of Ly on NAFLD in a rat model system.

Research frontiers

No specific drug has been tested in clinical phase Ⅲ trials for NAFLD to date, and there are few research studies of the hepatoprotective effects of Ly.

Innovations and breakthroughs

This study represents the first investigation of the effects of Ly as a therapy of NAFLD, and showed that down-regulated expression of TNF-α and CYP2E1 may be one of the action mechanisms for Ly.

Applications

This study suggests that Ly has a protective effect on NAFLD, which is very important for the future development of a potent NAFLD drug.

Terminology

NAFLD is one of the causes of fatty liver, defined as biopsy-proven hepatic steatosis. It covers a spectrum of liver diseases, including simple steatosis, NASH, liver fibrosis, liver cirrhosis and HCC. Recently, many NAFLD drug research studies have focused on the traditional Chinese medicines.

Peer-review

This is a meaningful study, in which the effects of "lycopene" were examined on an NAFLD rat model. The results are very important and suggest that Ly exerts a protective effect on NAFLD through down-regulation of TNF-α and CYP2E1 expression.

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ORIGINAL ARTICLE

Case Control Study

Use of a saline-coupled bipolar sealer open liver resection for hepatic malignancy: Medical resource use and costs

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Author contributions: Nichols CI and Vose JG designed the research; Nichols CI performed data analyses; Nichols CI and Vose JG analyzed the data; and Nichols CI and Vose JG wrote the paper and revisions.

Institutional review board statement: Given this study used de-identified patient data, it was not subject to Institutional Review Board approval. The study dataset and full study tables are available from the corresponding author.

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Abstract

AIM

To evaluate outcomes associated with use of a saline coupled bipolar sealer during open partial liver resection.

METHODS

This retrospective analysis utilized the United States Premier™ insurance claims database (2010-2014). Patients were selected with codes for liver malignancy and partial hepatectomy or lobectomy. Cases were defined by use the saline-coupled bipolar sealer; controls had no use. A Propensity Score algorithm was used to match one case to five controls. A deviation-based cost modeling (DBCM) approach provided an estimate of cost-effectiveness.

RESULTS

One hundred and forty-four cases and 720 controls were available for analysis. Patients in the case cohort received fewer transfusions *vs* controls (18.1% *vs* 29.4%, $P = 0.007$). In DBCM, more patients in the case cohort experienced "on-course" hospitalizations (53.5% *vs* 41.9%, $P = 0.009$). The cost calculation showed an average savings in total hospitalization costs of $1027 for cases *vs* controls. In multivariate analysis, cases had lower odds of receiving a transfusion (OR = 0.44, 95%CI: 0.27-0.71, $P = 0.0008$).

CONCLUSION

Use of a saline-coupled bipolar sealer was associated with a greater proportion of patients with an "on course" hospitalization.

Key words: Liver resection; hepatocellular carcinoma; costs

Core tip: This study evaluated outcomes associated with use of a saline coupled bipolar sealer during open partial liver resection. Using US Premier insurance claims data, Cases with use of the saline-coupled bipolar sealer (SCBS) were propensity-score matched to controls with no use. A deviation-based cost modeling (DBCM) approach provided an estimate of cost-effectiveness. Results demonstrated that use of the SCBS in open partial liver resection for hepatic malignancy is associated with reduction in the need for transfusion, and is cost-effective in a DBCM analysis. This technology provides an alternative solution for bleeding control in partial liver resection compared to traditional methods.

Nichols CI, Vose JG. Use of a saline-coupled bipolar sealer open liver resection for hepatic malignancy: Medical resource use and costs. *World J Gastroenterol* 2016; 22(46): 10189-10197 Available from: URL: http://www.wjgnet.com/1007-9327/full/v22/i46/10189.htm DOI: http://dx.doi.org/10.3748/wjg.v22.i46.10189

INTRODUCTION

Liver resection remains the only curative treatment for primary and metastatic liver malignancy. However, despite advances in surgical technique over the past two decades, blood transfusions are still required in a proportion of patients undergoing liver resection (3.3%-59%), varying by the extent of the procedure and device combinations used[1-5]. Predictors of transfusion include factors related to the operative procedure (resection technique, extent of resection, tumor size, need for other major resections during the same hospitalization) as well as patient-specific characteristics (pre-operative hemoglobin and albumin levels, pre-operative biliary drainage, and diagnosis of a primary liver tumor, coronary artery disease, or cirrhosis)[6-9].

The most serious complication associated with transfusion, beyond simple transfusion-related reactions or immunomodulatory effects, is the increased risk of tumor recurrence[6,8-10]. In a meta-analysis of 22 studies evaluating the impact of perioperative allogenic blood transfusion on long term outcomes following hepatocellular carcinoma (HCC) resection, authors found the risk of tumor recurrence was significantly higher among patients with a transfusion at one (OR = 1.70, 95%CI: 1.38-1.10), three (OR = 1.22, 95%CI: 1.08-1.38), and five years (OR = 1.16, 95%CI: 1.08-1.24) post-resection compared with patients with no transfusion. This finding was confirmed in a Cochrane meta-analysis evaluating the risk of cancer recurrence following surgery for colorectal cancer among patients with *vs* without receipt of a transfusion[11]. These studies suggest that transfusion may result in immunosuppression in the early postoperative period, which could allow for the progression of residual carcinoma and influence survival[12].

Prior research has demonstrated the effects of surgical technique, peri-operative blood management protocols, and use of surgical technologies on the risk of transfusion[1-5]. Peri-operatively, studies have examined autologous blood donation, intravenous iron therapy, and strict transfusion protocols. Intraoperatively, other studies have examined the effects of clamping the hepatic artery and portal vein (*i.e.,* Pringle's Maneuver), topical hemostatic agents, and use of technologies such as the cavitron ultrasonic surgical aspirator (CUSA), saline-coupled bipolar sealer (SCBS), argon beam coagulation (ABC), harmonic scalpel, bipolar scissors, vessel sealers, cell saver systems, and hydrodissector[13]. The majority of these studies examined clinical outcomes alone, with few examining the total cost of the procedure or incremental costs associated with complications. Two prior high-quality cost studies applied a novel methodology, deviation-based cost modeling (DBCM), however the primary comparison was of open *vs* laparoscopic approach rather than specific surgical technologies utilized during the procedure[14,15].

Given that few studies to date summarize total direct hospitalization costs by choice of surgical technology during hepatic resection, we sought to examine the resource use and costs by technology choice. Specifically, in the present study we evaluated the clinical and economic outcomes associated with the SCBS during open partial liver resections, using real-world data from a nationally representative US claims database.

MATERIALS AND METHODS

Data source and patient population

This retrospective database analysis reviewed recent healthcare insurance claims data from the Premier Perspective™ database (Premier Inc., Charlotte, NC, United States). Data were analyzed over the period 01/2010 to 06/2014. The database includes information on patient demographics, diagnosis and procedure codes, and cost information for over 2000 hospitals and 300 million patient encounters. This database is limited to the inpatient period, with no ability to track patients longitudinally in follow-up. The Premier database allows for tracking of total hospitalization cost information on a per-patient basis. However, the inherent tradeoff of working with retrospective claims data is the reliance on ICD-9 diagnosis and procedure codes to identify liver resections - with the codes providing no information on the specific number of segments, lobes, or tissue volume resected. Given this study used de-identified patient data, it was not subject to Insti-

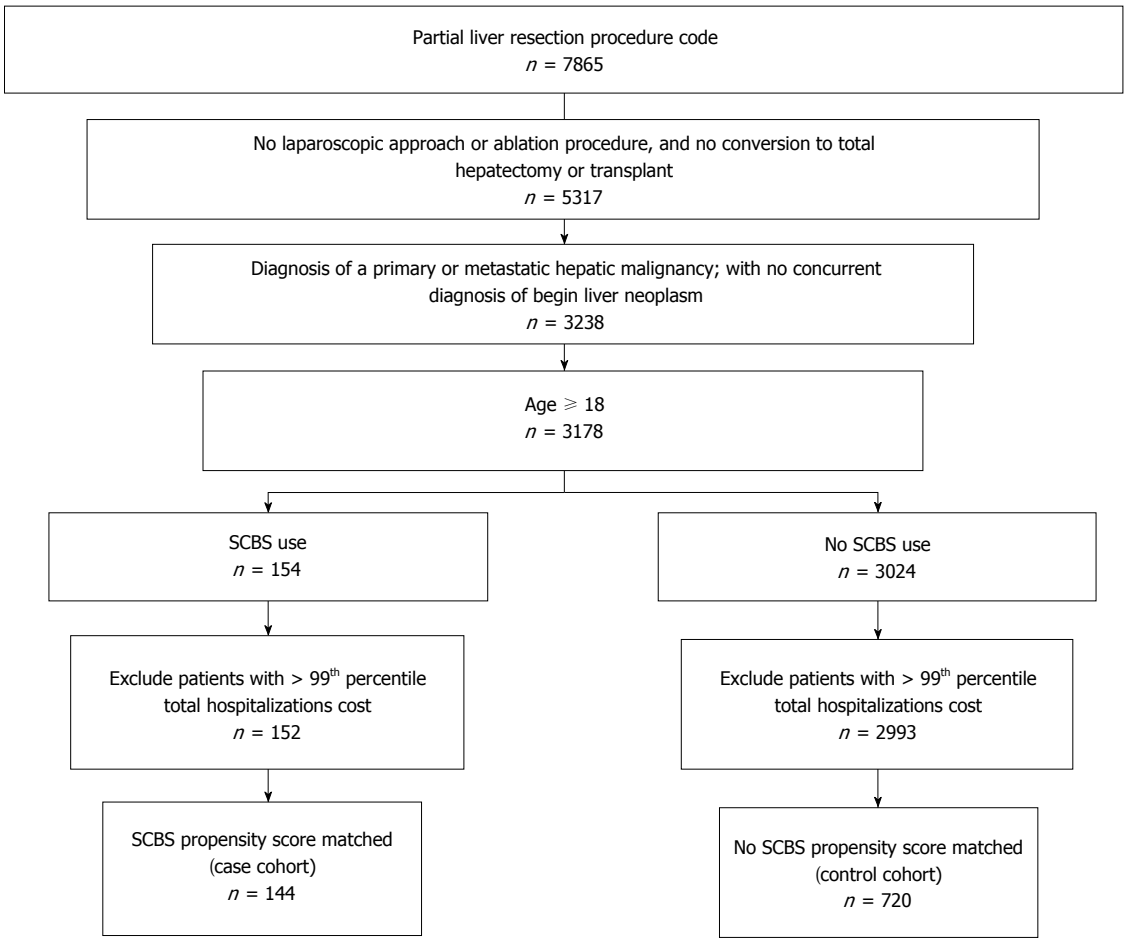

Figure 1 Patient selection. SCBS: Saline-coupled bipolar sealer.

tutional Review Board approval. The study dataset and full study tables are available from the corresponding author.

Patients aged 18 and older with records that included International Classification of Diseases (ICD-9-CM) or Current Procedural Technology (CPT) procedure codes for liver resection during a hospitalization episode (50.22 - partial hepatectomy or 50.3x - lobectomy), accompanied by a diagnosis code for primary malignant neoplasm of the liver (155.0x) or metastatic neoplasm of the liver (197.7x), were selected. Those with benign neoplasms (211.5x) were excluded to reduce the potential confounding effects of different liver pathology and bleeding risk. Total liver resection and transplant procedures were excluded. Operations using ablation procedures or laparoscopic approaches (as identified by ICD-9-CM codes and key terms in Premier Chargemaster records) were excluded due to the high cost of these procedures and to better isolate the effects of SCBS use. Open SCBS device use was identified by the hospital Chargemaster file; laparoscopic SCBS models were excluded.

The "case" cohort was defined as any hospitalization episode meeting all inclusion criteria listed above, where the SCBS was used. The "control" cohort was defined as cases in which the SCBS was not used. Similar to prior cost analyses[16], patients in the top one percent of total hospitalization cost within each cohort were excluded from analysis in order to reduce the effects of extreme outliers (> $87262 among cases and > $153428 among controls). Figure 1 provides a summary of patient selection.

Study measures

Study measures included patient demographic, clinical, hospital, and surgeon characteristics, transfusion procedures and other complications during index hospitalization, hospital length of stay (LOS) and costs. Comorbidity status was evaluated with diagnoses recorded during the one year prior to admission (baseline period) through the index hospitalization episode. The Charlson Comorbidity Index (CCI) score, a composite measure of physical health status commonly used in studies of medical claims and chronic disease[17,18] was calculated for each patient. For this study, malignancy, metastatic solid tumor, and mild or moderate liver disease were excluded from the CCI calculation as these were present for most patients.

Propensity score matching

In order to address selection bias and ensure demographic and hospitalization characteristics were similar

Table 1 Definition of deviation mix for deviation-based cost modeling

Deviation	LOS	Complication group[1]
On course	≤ 50th percentile	No complication
Minor deviation	> 50th percentile	No complication
	≤ 50th	Minor complication, no moderate or major
Moderate deviation	> 50th percentile	Minor complication, no moderate or major
	Any LOS	Moderate no major
Major deviation	Any LOS	Major

[1]Minor complication: Transfusion, urinary tract infection, hemorrhage/hematoma, wound disruption, or transfusion complications; Moderate complication: Deep vein thrombosis, pulmonary embolism, pneumonia, infection, or bile leak; Major Complication: Acute respiratory failure, acute kidney injury, or acute liver failure. LOS: Length of stay.

across the case and control cohorts, a propensity-score matching algorithm was applied. Each case was matched to five controls based on age group, gender, race, region, primary payor, procedure type, indicating diagnosis, other comorbid liver-related conditions, CCI, surgeon specialty, and the proportion of surgeons with history of at least one liver procedure performed in the prior year. These matching covariates were chosen both based on significant differences observed in unmatched cohorts (*P* values < 0.05), and on the basis of clinical and demographic factors that may have impacted surgeon choice of technology use. Matching was applied using the nearest neighbor approach, with a caliper width of 0.10 of the standard deviation of the logit of the propensity score.

Hospital resource use and deviation-based cost modeling

Transfusion procedures were identified by ICD-9 (V58.2, 99.00-99.04) or CPT codes (36430, P9010, P9011) or presence of the term "blood transfusion" in the hospital Chargemaster file. Topical hemostat use was identified by any mention of "hemostat" or "sealant" in the Chargemaster file under the "Medical Surgical Supplies" category.

A DBCM approach was employed to account for variation in resource use associated with different hospital LOS categories and severity of complications[14,15,19]. Vanounou *et al*[15,19] originally developed this approach in analyses evaluating the economic impact of pancreaticoduodenectomy procedures and a comparison of laparoscopic *vs* open liver resection. This methodology measures the frequency and severity of deviations from an "expected" postoperative course and calculates the economic consequences of hospitalizations that do not follow expected outcomes. The benefits of this approach are the incorporation of complications, LOS, and costs into one measure, providing a single outcomes-based metric that provides more information than simply clinical or cost data alone[14,15]. Data on LOS and complications were combined to create four deviation classes: on-course, minor, moderate, and severe. Definitions for each class are listed in Table 1. Once deviation groups were defined, a weighted average mean cost (WAMC) was calculated by multiplying the percentage of patients in each category by the mean cost of that category.

Data analyses

Analyses were performed using the Instant Health Data Suite (Boston Health Economics, Inc., Boston, MA) and SAS software (Version 9.2, SAS Institute, Cary, NC, United States). All costs were inflation-adjusted to 2014 USD using the medical care component of the Consumer Price Index. Statistical significance testing was performed with the Chi-square (χ^2) test for categorical variables (or Fisher's Exact with cell frequencies < 10) and Wilcoxon-Mann-Whitney test for non-normal continuous variables. Predictors of topical hemostat use and transfusion, controlling for demographic and clinical characteristics, provider specialty and experience, and study cohort, were evaluated using logistic regression analysis.

RESULTS

Demographic and clinical characteristics

Between January 2010 and June 2014, 152 cases and 2993 unmatched controls were available for analysis after applying all sample selection criteria, with procedures performed at 284 hospitals nationally. Following application of the propensity score algorithm 144 cases and 720 controls were available for matched analyses (Table 2). Post-match, differences between cohorts were removed, with all clinical characteristics statistically similar.

Inpatient complications

In matched analysis, patients in the case cohort had lower incidence of transfusions *vs* the control cohort, with an absolute risk reduction of 11.3% and relative risk reduction of 38.7% (18.1% *vs* 29.4%, *P* = 0.007). Additionally, patients in the case cohort had fewer cases of acute kidney failure occurring during the same hospitalization episode (3.5% *vs* 8.8%, *P* = 0.048). All other inpatient complications were statistically similar across cohorts, including infection, urinary tract infection, acute respiratory failure, pneumonia, deep vein thrombosis, hemorrhage or hematoma, wound disruption, and bile leak. One patient (0.694%) in the case cohort had evidence of bile leak *vs* eight patients in the control cohort (1.11%), however this difference was not significant (*P* = 1.00). No patients in the case cohort experienced acute liver failure, pulmonary embolism, or transfusion-related complications, while 1.8%, 1.0%, and 0.6% of control patients developed these complications during the inpatient visit (all *P* > 0.05).

Overall, 25.0% of the case cohort showed evidence of topical hemostat use during the liver resection procedure, while 17.2% of the control cohort showed

Table 2 Patient demographics *n* (%)

	Unmatched		*P* value	Matched		*P* value
	SCBS	No SCBS		SCBS	No SCBS	
n	152	2993		144	720	
Age, mean (SD)	62 (12.5)	61.58 (12.1)	0.683	61.49 (12.5)	62.14 (12.1)	0.568
Age group[1]						0.960
18 to 44	10 (6.6)	262 (8.8)	0.868	10 (6.9)	49 (6.8)	
45 to 54	29 (19.1)	536 (17.9)		28 (19.4)	143 (19.9)	
55 to 64	44 (28.9)	914 (30.5)		44 (30.6)	198 (27.5)	
65 to 74	46 (30.3)	862 (28.8)		41 (28.5)	220 (30.6)	
75 plus	23 (15.1)	419 (14.0)		21 (14.6)	110 (15.3)	
Race			0.148			0.692
Black	18 (11.8)	368 (12.3)		18 (12.5)	87 (12.1)	
Caucasian	83 (54.6)	1788 (59.7)		76 (52.8)	407 (56.5)	
Hispanic	0 (0)	41 (1.4)		0 (0)	0 (0)	
Other	51 (33.6)	796 (26.6)		50 (34.7)	226 (31.4)	
Region			< 0.001			0.892
Midwest	31 (20.4)	304 (10.2)		26 (18.1)	112 (15.6)	
Northeast	52 (34.2)	959 (32.0)		49 (34.0)	260 (36.1)	
South	41 (27.0)	1321 (44.1)		41 (28.5)	206 (28.6)	
West	28 (18.4)	409 (13.7)		28 (19.4)	142 (19.7)	
Sex						
Female	71 (46.7)	1315 (43.9)	0.556	67 (46.5)	308 (42.8)	0.461
Payor			0.577			0.903
Commercial	49 (32.2)	1166 (39.0)		49 (34.0)	234 (32.5)	
Medicare	21 (13.8)	362 (12.1)		21 (14.6)	111 (15.4)	
Medicaid	73 (48.0)	1302 (43.5)		65 (45.1)	341 (47.4)	
Other	9 (5.9)	163 (5.5)		9 (6.3)	34 (4.7)	

[1]Excluding primary malignancy, metastatic solid tumor, mild liver disease, moderate or severe liver disease. *P* values were calculated with the χ^2 test (or Fisher's Exact where cell frequencies < 10), *t*-test (or Wilcoxon Mann-Whitney test for skewed distributions). SCBS: Saline-coupled bipolar sealer.

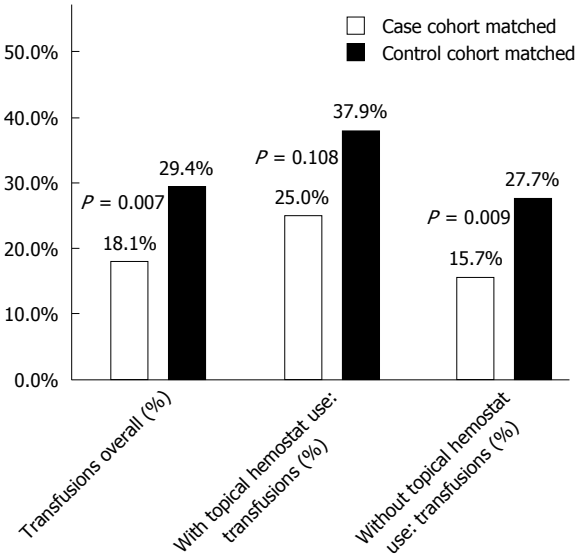

Figure 2 Transfusions and topical hemostat use.

evidence of topical hemostat use (*P* = 0.038). Among patients with topical hemostat use, the incidence of transfusions was lower in the case cohort, however the difference was not statistically significant (25.0% *vs* 37.9%, *P* = 0.108), Figure 2. When a topical hemostat was not used, the case cohort had lower incidence of transfusion compared to the control cohort (15.7% *vs* 27.7%, *P* = 0.009).

DBCM analysis

Length of stay was shorter in the case cohort, however the difference was not statistically significant (7.38 d *vs* 8.18 d, *P* = 0.210; Table 3); the median LOS was six days for each cohort. A greater proportion of patients in the case cohort had an on-course hospitalization *vs* the control cohort (53.5% *vs* 41.9%, *P* = 0.013; Table 4). The proportion in other deviation classes was statistically similar across cohorts. Mean total hospitalization costs were greater among those with an on-course hospitalization in the case cohort *vs* controls ($18000 *vs* $16813, *P* = 0.029); costs in other deviation classes were not statistically different. Overall, accounting for the distribution of patients in each deviation class and mean cost by deviation class, the WAMC for the case cohort was $25503 *vs* $26530 for controls. This represents an average savings of $1027 in the total hospitalization cost per patient when the SCBS was used.

Predictors of topical hemostat use and incidence of transfusion

In logistic regression analysis of predictors of topical hemostat use, patients residing in the South were at greater odds of topical hemostat use compared to those in the Northeast, while patients with the surgery performed by a surgical oncologist were at lower odds of hemostat use compared to general surgeons (Table 5). Patients in the case cohort were at higher odds of

Table 3 Clinical characteristics *n* (%)

	Unmatched		*P* value	Matched		*P* value
	SCBS	No SCBS		SCBS	No SCBS	
n	152	2993		144	720	
Procedure Type						
Partial hepatectomy	99 (65.1)	2061 (68.9)	0.308	98 (68.1)	478 (66.4)	0.772
Lobectomy	53 (34.9)	964 (32.2)	0.552	46 (31.9)	251 (34.9)	0.564
Indicating diagnosis						
Primary hepatobiliary malignancy	63 (41.5)	850 (28.4)	0.001	56 (38.9)	300 (41.7)	0.599
Metastatic liver neoplasm	89 (58.6)	2143 (71.6)	0.001	88 (61.1)	420 (58.3)	0.599
Comorbid liver diagnoses						
Alcoholic cirrhosis	1 (0.66)	33 (1.1)	0.908	1 (0.69)	4 (0.56)	1.000
Non-alcoholic cirrhosis	23 (15.1)	287 (9.6)	0.036	20 (13.9)	100 (13.9)	1.000
Hepatitis A	0 (0)	6 (0.2)	1.000	0 (0)	0 (0)	N/A
Hepatitis B	18 (11.8)	193 (6.4)	0.015	16 (11.1)	96 (13.3)	0.556
Hepatitis C	17 (11.2)	271 (9.1)	0.457	17 (11.8)	71 (9.9)	0.580
Charlson score group[1]			0.518			0.704
0	76 (50)	1637 (54.7)		74 (51.4)	370 (51.4)	
1	45 (29.6)	816 (27.3)		43 (29.9)	196 (27.2)	
⩾ 2	31 (20.4)	540 (18.0)		27 (18.8)	154 (21.4)	
Provider specialty						
Surgical oncology	59 (38.8)	531 (17.7)	< 0.001	56 (38.9)	285 (39.6)	0.950
General surgery	79 (52.0)	1993 (66.6)	< 0.001	74 (51.4)	369 (51.3)	1.000
Other	14 (9.2)	469 (15.7)	0.041	14 (9.7)	66 (9.2)	0.958
Surgeon experience						
⩾ 1 liver procedure in prior year	125 (82.2)	1991 (66.6)	< 0.001	117 (81.3)	595 (82.6)	0.780

[1]Excluding primary malignancy, metastatic solid tumor, mild liver disease, moderate or severe liver disease. *P* values were calculated with the χ^2 test (or Fisher's Exact where cell frequencies < 10), *t*-test (or Wilcoxon Mann-Whitney test for skewed distributions). SCBS: Saline-coupled bipolar sealer.

topical hemostat use *vs* controls (OR = 2.56, 95%CI: 1.70-3.86, *P* < 0.001).

In a regression evaluating predictors of a transfusion during the hospitalization (Table 5), patients aged 75 or older (*vs* ages 18 to 44), Black race (*vs* Caucasian), and patients residing in the South (*vs* Northeast), and patients operated on by an other surgical specialist (*vs* general surgeons) were at higher odds of receiving a transfusion. Patients undergoing a lobectomy (*vs* partial hepatectomy) were at higher odds, as were patients whose diagnosis was a primary malignancy (*vs* metastatic). Controlling for topical hemostat use, patients in the case cohort were at lower odds of transfusion *vs* controls (OR = 0.44, 95%CI: 0.27-0.71, *P* = 0.0008)

DISCUSSION

This retrospective database analysis evaluated the use of the SCBS in open partial liver resection for hepatic malignancy. After matching, patients treated with the SCBS had a lower incidence of transfusions (18.1% *vs* 29.4%, *P* = 0.007). Controlling for topical hemostat use, the reduction of transfusion incidence in univariate analysis was confirmed in multivariate analysis, with SCBS use associated with a lower odds of transfusion *vs* no use (OR = 0.44, 95%CI: 0.27-0.71). Overall, DBCM analyses indicated an average cost savings of $1027 among cases when accounting for the proportion within each "hospital deviation" class, with significantly more patients in the SCBS cohort with an "on course" hospitalization (defined as no complications and a LOS less than the median). We believe this study, despite the lack of clinical detail on number of lobes resected, provides information on "real-world" practice outside of a controlled prospective study or randomized controlled trial.

This study adds to a growing body of research evaluating the safety and efficacy of SCBS in liver resections. Authors at the University of Pittsburgh Starzl Transplant Institute performed a single-arm study evaluating the safety of the SCBS (formerly of "TissueLink Medical") in 170 open liver resection procedures performed between 2001 and 2004[20]. Overall, 3.5% of patients were transfused and 2.4% developed a postoperative bile leak. There were no cases of postoperative hemorrhage, hepatic failure, liver abscess, or reoperation. The authors concluded the SCBS was effective in achieving intraoperative hemostasis in hepatic resection. The observed transfusion rate was much lower than in our present study, however this is likely due to comparing outcomes from a single high-volume hospital *vs* our present study, which includes data from 284 hospitals.

In a prospective single-arm study in Italy, the incidence of early surgical complications (including bleeding, biliary leakage, and abscess development) following 12 partial hepatectomies with the SCBS was evaluated[21]. Mean blood loss was 20 mL (range 5 to 80 mL), with no transfusions and a mean LOS of six days[21]. This LOS is similar to the 7.4 d observed in the case cohort of our study.

Table 4 Length of stay, deviation mix and weighted average mean cost (propensity-matched cohorts)

Characteristic	SCBS	No SCBS	*P* value
Length of stay (LOS), d			
mean (SD)	7.38 (5.18)	8.18 (7.27)	0.210
25th percentile	4	4	
Median	6	6	
75th percentile	8	8	
Deviation mix, *n* (%)			
On course	77 (53.5)	302 (41.9)	0.013
Minor deviation	30 (20.8)	187 (26.0)	0.208
Moderate deviation	28 (19.4)	150 (20.8)	0.821
Major deviation	9 (6.3)	81 (11.3)	0.074
mean (SD) total hospital cost			
On course	18000 (5746)	16813 (6588)	0.029
Minor deviation	28379 (14863)	25452 (11186)	0.454
Moderate deviation	36558 (17777)	35261 (19390)	0.571
Major deviation	45717 (19045)	49082 (35303)	0.568
WAMC total hospitalization cost	$25503	$26530	
WAMC difference	$1027		

P values were calculated with the Wilcoxon Mann-Whitney test for LOS and total hospitalization cost; and the χ^2 test for hospital deviation mix classes. SCBS: Saline-coupled bipolar sealer; WAMC: Weighted average mean cost.

Table 5 Logistic regressions of predictors of topical hemostat use and transfusion

Parameter	Predictors of topical hemostat use			Predictors of transfusion		
	Odds ratio	95%CI	*P* value	Odds ratio	95%CI	*P* value
Age group (*vs* 18 to 44)						
75 plus	1.03	0.50-2.12	0.421	4.55	1.95-10.59	< 0.0001
Race (*vs* caucasian)						
Black	1.21	0.75-1.97	0.275	1.97	1.21-3.19	0.017
United States geographic region (*vs* northeast)						
South	3.67	2.38-5.65	0.0004	1.87	1.19-2.96	0.001
Partial hepatectomy (*vs* lobectomy)	1.25	0.91-1.73	0.175	1.62	1.16-2.27	0.005
Primary malignancy (*vs* metastatic)	0.80	0.53-1.20	0.281	1.54	1.00-2.38	0.050
Provider specialty (*vs* general surgery)						
Surgical oncology	0.30	0.20-0.46	< 0.0001	0.65	0.42-1.01	0.005
Other specialty	0.68	0.40-1.16	0.402	1.48	0.85-2.57	0.023
Case cohort (*vs* matched controls)	2.56	1.70-3.86	< 0.0001	0.44	0.27-0.71	0.0008
Topical hemostat use	N/A	N/A	N/A	1.87	1.33-2.64	0.0004

Only covariates that were significant in at least on model ($P < 0.05$) are listed here. Full model covariates included: age group, sex, race, geographic region, resection type, malignancy type, diagnosis of non-alcoholic cirrhosis, hepatitis b, or hepatitis C, provider specialty, study cohort, and topical hemostat use.

Lastly, two studies have examined the combined use of the SCBS and CUSA. In the largest study of SCBS use in liver resection to date, authors at four hepatopancreaticobiliary units in Europe evaluated the safety and efficacy of combined use of SCBS plus CUSA during 114 minor and 199 major hepatectomies. Authors reported a transfusion rate of 10.5% and two postoperative deaths (0.6%), concluding the combined method is associated with decreased blood loss[9]. A similar Japanese study also evaluated the combined use of CUSA and SCBS ($n = 55$) *vs* CUSA with traditional bipolar electrosurgery ($n = 54$)[22]. The SCBS and CUSA cohort demonstrated significantly lower total blood loss (677 mL *vs* 1076 mL, $P = 0.0486$), shorter transection time (81 min *vs* 115 min, $P = 0.0025$) and fewer ties required (13.1 *vs* 22.8, $P < 0.001$) *vs* the traditional electrosurgery and CUSA cohort[22]. While the combined use of SCBS and CUSA is evaluated in these studies, other device combinations or techniques may provide equivalent outcomes at lower cost. This is an area for future research.

Although it was observed in the present study that a greater proportion of the case cohort had concurrent use of topical hemostats during the procedure (25.0% *vs* 17.2%, $P = 0.038$), it appears hemostat use was reserved for the most severe cases. We infer this due to the incidence of transfusion being greater in both univariate and multivariate analyses among those with topical hemostat use *vs* no use, regardless of SCBS. However, there is likely an unmeasured confounder that is not readily observed in insurance claims data that may have influenced surgeon selection of both the SCBS and a hemostat. Nonetheless, incidence of transfusion remained numerically lower in the case cohort *vs* controls both when topical hemostats were used during the procedure and when they were not.

Limitations of this study center on the lack of detailed clinical detail in the insurance claims dataset used for analysis, which included only diagnosis and procedure codes, and items listed in the hospital Chargemaster. Therefore, we could not evaluate the number of liver segments resected, the relative complexity of the procedure, pre- and post-operative hemoglobin levels, the Hg level triggering a transfusion, or number of units of blood transfused. Also, as noted, specific line-item costs for blood were not available for approximately two-thirds of patients. However, blood costs were captured in the next level roll-up of cost reporting under OR costs. During patient selection we did not attempt to query the Chargemaster file to evaluate concurrent devices used with the SCBS, as the only comparison in this study was at the highest level of use *vs* no use. Given the array of device choices during hepatic resection, and the variance of names listed in the Chargemaster file, we did not attempt to compare concurrent device use. Future studies may address the question of device synergy in influencing clinical outcomes (*e.g.,* SCBS plus CUSA). Finally, while we observed a reduction in the incidence of transfusion associated with use of SCBS in the present study, the SCBS is not designed to provide hemostasis in the event of bleeding from large vessels - thus additional technology or techniques to control bleeding that cannot be accounted for may have been present.

This retrospective database analysis demonstrated that use of the SCBS in open partial liver resection for hepatic malignancy is associated with reduction in the need for transfusion, and is cost-effective in a deviation-based cost modeling analysis. This technology provides an alternative solution for bleeding control in partial liver resection compared to traditional methods.

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COMMENTS

Background

Despite advances in surgical technique over the past two decades, blood transfusions are still required in a proportion of patients undergoing liver resection, varying by the extent of the procedure and device combinations used. Predictors of transfusion include factors related to the operative procedure as well as patient-specific characteristics.

Research frontiers

Prior research has demonstrated the effects of surgical technique, perioperative blood management protocols, and use of surgical technologies on the risk of transfusion. The majority of these studies examined clinical outcomes alone, with few examining the total cost of the procedure or incremental costs associated with complications. Two prior cost studies applied a novel methodology, deviation-based cost modeling (DBCM), however the primary comparison was of open *vs* laparoscopic approach rather than specific surgical technologies utilized during the procedure.

Innovations and breakthroughs

This retrospective database analysis evaluated the use of a saline-coupled bipolar sealer (SCBS) in open partial liver resection for hepatic malignancy. After matching, patients treated with the SCBS had a lower incidence of transfusions (18.1% *vs* 29.4%, $P = 0.007$). Controlling for topical hemostat use, the reduction of transfusion incidence was confirmed in multivariate analysis, with SCBS use associated with a lower odds of transfusion (OR = 0.44, 95%CI: 0.27-0.71). Overall, DBCM cost analyses indicated an average cost savings of $1027 among cases when accounting for the proportion falling into each "hospital deviation" class, with significantly more patients in the SCBS cohort with an "on course" hospitalization (defined as no complications and a length of stay less than the median).

Applications

This analysis demonstrated that use of the SCBS in open partial liver resection for hepatic malignancy is associated with reduction in the need for transfusion, and is cost-effective in a deviation-based cost modeling analysis. This technology provides an alternative solution for bleeding control in partial liver resection compared to traditional electrosurgical methods.

Terminology

A DBCM approach was employed in this study. This methodology measures the frequency and severity of deviations from an "expected" postoperative course and calculates the economic consequences of hospitalizations that do not follow expected outcomes. The benefits of this approach are the incorporation of complications, length of stay, and costs into one measure, providing a single outcomes-based metric that provides more information than simply clinical or cost data alone.

Peer-review

This is an interesting paper and well written. The current results will be great helpful to the surgical fields when evaluating the benefits and costs of alternative blood-management technologies during liver resection.

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ORIGINAL ARTICLE

Case Control Study

Nodular lymphoid hyperplasia: A marker of low-grade inflammation in irritable bowel syndrome?

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Informed consent statement: Consent was not obtained, given the retrospective nature of the study, but the presented data are anonymized and risk of identification is low.

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Abstract

AIM

To evaluate the prevalence of nodular lymphoid hyperplasia (NLH) in adult patients undergoing colonoscopy and its association with known diseases.

METHODS

We selected all cases showing NLH at colonoscopy in a three-year timeframe, and stratified them into symptomatic patients with irritable bowel syndrome (IBS)-type symptoms or suspected inflammatory bowel disease (IBD), and asymptomatic individuals undergoing endoscopy for colorectal cancer screening.

Data collection included medical history and final diagnosis. As controls, we considered all colonoscopies performed for the aforementioned indications during the same period.

RESULTS

One thousand and one hundred fifty colonoscopies were selected. NLH was rare in asymptomatic individuals (only 3%), while it was significantly more prevalent in symptomatic cases (32%). Among organic conditions associated with NLH, the most frequent was IBD, followed by infections and diverticular disease. Interestingly, 31% of IBS patients presented diffuse colonic NLH. NLH cases shared some distinctive clinical features among IBS patients: they were younger, more often female, and had a higher frequency of abdominal pain, bloating, diarrhoea, unspecific inflammation, self-reported lactose intolerance and metal contact dermatitis.

CONCLUSION

About 1/3 of patients with IBS-type symptoms or suspected IBD presented diffuse colonic NLH, which could be a marker of low-grade inflammation in a conspicuous subset of IBS patients.

Key words: Inflammatory bowel diseases; Functional gastrointestinal diseases; Irritable bowel syndrome; Colonoscopy; Inflammation

Core tip: This study sheds light on colonic nodular lymphoid hyperplasia (NLH) in terms of prevalence, gender-distribution and association with known diseases. Our most relevant result is the identification of NLH as a putative marker of low-grade inflammation in a conspicuous subset of irritable bowel syndrome (IBS) cases. Further studies are required to understand the etiopathogenetic mechanisms underlying NLH in IBS, its association with metal contact allergies and its clinical implications.

Piscaglia AC, Laterza L, Cesario V, Gerardi V, Landi R, Lopetuso LR, Calò G, Fabbretti G, Brisigotti M, Stefanelli ML, Gasbarrini A. Nodular lymphoid hyperplasia: A marker of low-grade inflammation in irritable bowel syndrome? *World J Gastroenterol* 2016; 22(46): 10198-10209 Available from: URL: http://www.wjgnet.com/1007-9327/full/v22/i46/10198.htm DOI: http://dx.doi.org/10.3748/wjg.v22.i46.10198

INTRODUCTION

Colonoscopy allows direct visualization of the mucosa of the lower gastrointestinal tract and it is a useful tool to investigate symptoms of lower bowel diseases. However, there are conditions in which symptomatic patients might have a normal colon appearance on colonoscopy, although their intestinal mucosa shows signs of microscopic inflammation at histological examination[1]. In the last years, the introduction of advanced imaging techniques has ameliorated the characterization of mucosal lesions and has permitted to detect minimal mucosal changes that might be missed with standard white-light (WL) colonoscopy[2]. Among such techniques, narrow band imaging (NBI) uses optical filters in front of the light source, to narrow the wavelength of the projected light, to enable visualization of micro-vessel morphological changes and to enhance the visibility of both neoplastic and inflammatory mucosal lesions[3,4].

In our daily practice, we have noted that some patients undergoing colonoscopy showed multiple slightly raised whitish areas, usually < 5 mm in diameter, closely spaced, difficult to see at WL endoscopy and easier to recognize with NBI. When biopsied, these areas always corresponded to clusters of ⩽ 10 lymphoid nodules, composed of hyperplastic benign lymphoid tissue, named "nodular lymphoid hyperplasia" (NLH)[5-9]. Sometimes NLH had a reddish outline, the so-called "red ring sign" (RRS), due to hypervascularization at the base of the follicles, associated with granulocyte infiltrate[6].

Little is known about the epidemiology, pathogenesis, and clinical implications of NLH. NLH is commonly seen in the terminal ileum and colon during paediatric endoscopies, and it has been classically considered a paraphysiologic phenomenon in children[10]. However, there have been reports of NLH in children associated with refractory constipation, viral infection, juvenile idiopathic arthritis, connective tissue disease, immunodeficiency, cow's milk protein hypersensitivity, familial mediterranean fever, and the so-called "autistic enterocolitis"[9,11-18].

In adults, NLH can be asymptomatic, or more rarely presents with gastrointestinal symptoms, like abdominal pain, chronic diarrhoea, and bleeding[18]. NLH can mimic familial polyposis[19,20] and it has been reported in association with inflammatory bowel disease (IBD), celiac disease (CeD), lymphoma, dysgammaglobulinemia, Ehlers-Danlos syndrome, diversion colitis and food allergies[21-28].

Adult NLH is considered a rare finding[29]. Published literature includes case reports and small series of patients; whether this relates to endoscopy underreporting or to the true rarity of the condition is unclear[30]. Indeed, NLH frequency in adults might be largely underestimated because it is hard to recognize at WL endoscopy. Kagueyama *et al*[31], demonstrated that 39% of adult patients with chronic diarrhoea and a normal colonoscopy had NLH at histological examination of serial biopsies taken from the terminal ileum, ascending colon and rectum. In 2010, Krauss *et al*[6] evaluated the significance of lymphoid hyperplasia in the lower gastrointestinal tract in a cohort

of consecutive adult patients and concluded that the presence of colonic NLH is not rare and it may represent a mucosal response to antigenic stimulation, like allergens or pathogens.

Most of the NLH cases that we have found in our daily practice, underwent colonoscopy for irritable bowel syndrome (IBS)-type symptoms, or suspected IBD, while a minority of them was asymptomatic. Based on this clinical observation, the aim of the present study is to evaluate the prevalence of NLH in adults undergoing colonoscopy in San Marino Republic, and its association with known diseases.

MATERIALS AND METHODS

Patients

We evaluated all colonoscopies performed by a single endoscopist (ACP) from January 2012 to January 2015, at the Endoscopy Unit of the State Hospital of San Marino Republic.

We selected all cases showing lesions compatible with NLH, for which biopsies from multiple sites (ileum, ascending, transverse, sigmoid colon, and rectum) were taken. NLH cases were divided into two groups: asymptomatic subjects (a-NLH), who underwent colonoscopy for colorectal cancer screening or family history of colorectal cancer; and symptomatic patients (s-NLH), in which colonoscopy was prescribed for IBS-type symptoms (abdominal pain and/or altered bowel habits in the absence of alarm signs or symptoms) or suspected IBD [abdominal pain and/or altered bowel habits with haematochezia and/or weight loss and/or positive family history of IBD and/or fever and/or increased faecal calprotectin and/or C-reactive protein, and/or anaemia]. Symptomatic patients were further divided according to their final diagnosis into *organic* and *functional* bowel disorders; the latter subset was stratified into IBS [with prevalent diarrhoea (IBS-D), or constipation (IBS-C), or mixed bowel habit (IBS-M)], chronic functional diarrhoea and chronic constipation[32].

In order to measure the prevalence of NLH in asymptomatic subjects and symptomatic patients, we considered all colonoscopies performed by the same endoscopist for the same clinical indications (colorectal cancer screening, IBS-type symptoms, suspected IBD), in the same timeframe (January 2012 to January 2015).

In case of repeated colonoscopies on the same patient, only the first examination was included in the analysis. Other exclusion criteria were incomplete exam, poor bowel preparation, patient's age < 18 or > 75 years, history of colon surgery, prior diagnosis of colorectal cancer, IBD, or CeD.

The study protocol was approved by the local Ethical Committee and conformed to the ethical guidelines of the Declaration of Helsinki (2013).

Data collection: Endoscopy, histology, clinical characteristics and inflammation biomarkers

All colonoscopies were performed under deep sedation with Midazolam and Propofol, with anaesthesiologist's assistance. For bowel preparation, all patients received low-volume polyethylenglycol-based medication (Lovol Esse®). NLH was macroscopically evaluated using Olympus endoscopes CF-HQ190 (for EVIS EXERA III Video System Center CV-190) or CF-Q180A (for EXERA II Video System Center CV-180), in white light and NBI.

Biopsies were fixed in formalin and embedded in paraffin. Four-μm tissue sections were stained with haematoxylin and eosin and evaluated by two expert pathologists (Fabbretti G, Brisigotti M). Each tissue section was observed on light microscopy using × 20 objective lenses and × 10 eyepiece.

We reviewed patients' charts to investigate about bowel habits, self-reported lactose intolerance, metal contact dermatitis, histological confirmation of NLH and final diagnosis.

Statistical analysis

Data were collected in an Excel database. Summary statistics were calculated for each outcome of interest. Continuous data were summarized with mean and standard deviation, while categorical data were summarized with frequency distributions. We used *t*-test and χ^2 test to compare patients' subgroups. A P value < 0.05 was considered statistically significant. Data analysis was generated using Microsoft Excel software (Microsoft Corporation, Redmond, WA, United States) and Real Statistics Resource Pack software (Release 3.5; Copyright 2013-2015 Charles Zaiontz; www.real-statistics.com).

RESULTS

Figure 1 summarizes the study design. 2226 colonoscopies were assessed for eligibility. Of those, 1076 met the exclusion criteria and were ruled out. NLH was observed in 124 of the 1150 cases under analysis (global NLH prevalence about 10%). Of note, NLH was found in 101 of 315 symptomatic patients (32%) and in only 23 of 835 asymptomatic subjects (3%).

Symptomatic patients

The main symptomatic patients' characteristics are summarized in Table 1. Collectively, out of 315 symptomatic patients, 110 were diagnosed with organic disorders (35%), whereas the remaining 65% had functional disease. NLH was found in 32% of symptomatic patients and it was always histologically confirmed (Figure 2). NLH was equally distributed among organic (34%) and functional (31%) conditions.

Symptomatic patients with NLH

In most symptomatic patients with NLH (s-NLH) cases,

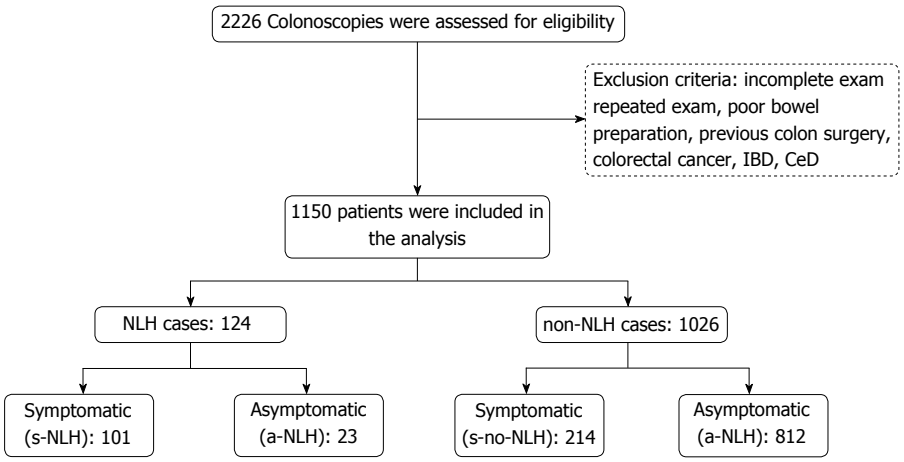

Figure 1 Study design. NLH: Nodular lymphoid hyperplasia; s-no-NLH: Symptomatic patients without NLH; a-NLH: Asymptomatic subjects; s-NLH: Patients with nodular lymphoid hyperplasia; IBD: Inflammatory bowel disease; CeD: Celiac disease.

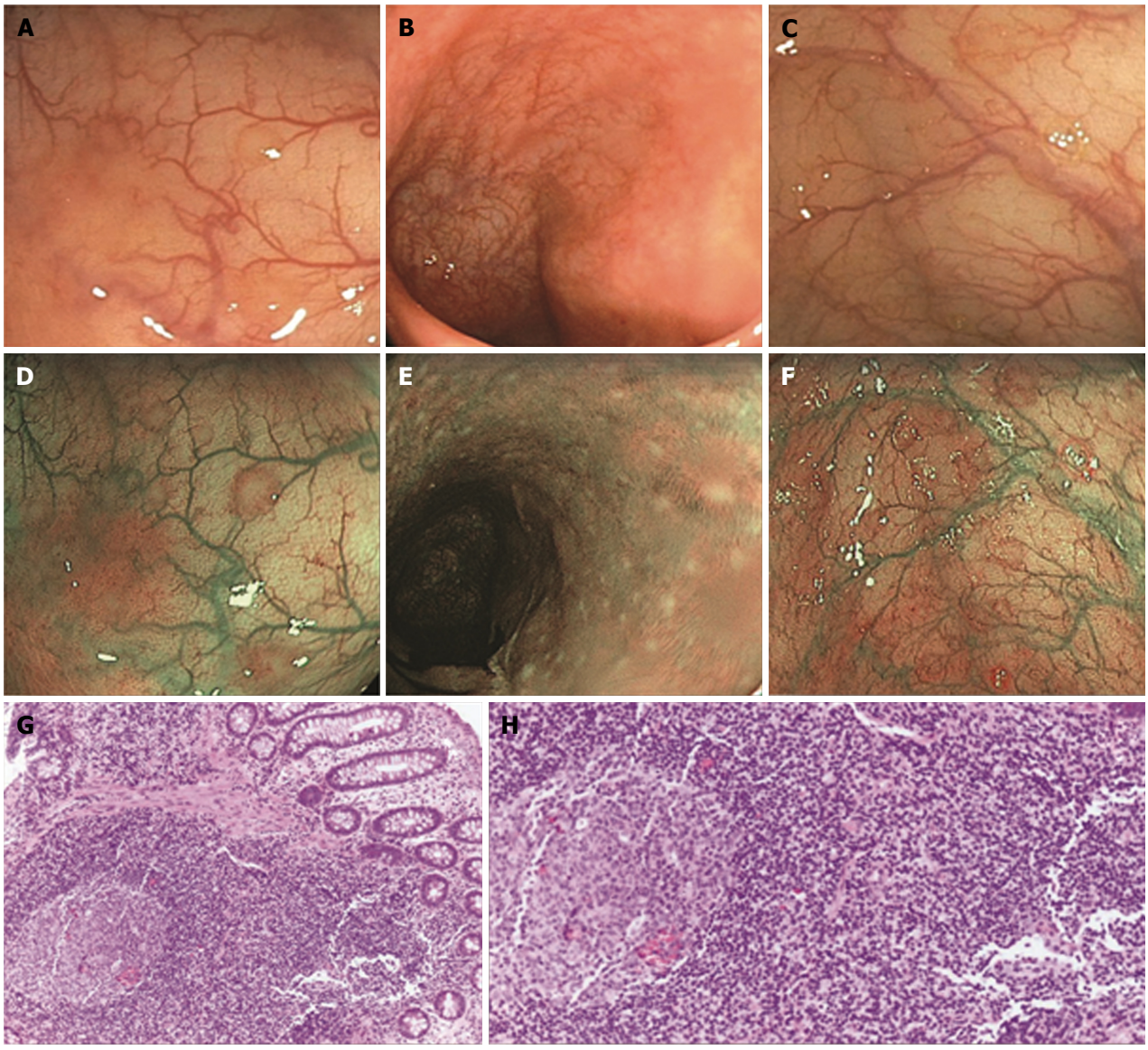

Figure 2 Endoscopic and histological features of nodular lymphoid hyperplasia. A-F: Three typical cases of nodular lymphoid hyperplasia (NLH), as observed at white light (WL) standard endoscopy (A-C) and narrow band imaging (NBI) endoscopy (D-F), respectively. NLH appear as slightly raised whitish areas, usually < 5 mm in diameter, closely spaced, difficult to recognize at WL, and easier to observe by NBI; G and H: Show the histological appearance of NLH (hematoxylin-eosin staining), as clusters of $\leqslant$ 10 lymphoid nodules, composed of hyperplastic benign lymphoid tissue.

Table 1 Symptomatic patients' characteristics

	All patients			s-NLH			s-no-NLH			p (s-NLH *vs* s-no-NLH)	
	s-NLH	s-no-NLH	p (s-NLH *vs* s-no-NLH)	F	O	p (F *vs* O)	F	O	p (F *vs* O)	F	O
Average years of age (mean ± SD)	41 ± 14	53 ± 12	< 0.05[1]	40 ± 13	42 ± 16	NS[1]	49 ± 12	60 ± 10	< 0.05[1]	< 0.05[1]	< 0.05[1]
Female sex	85%	68%	NS[2]	92%	73%	< 0.05[2]	70%	64%	NS[2]	< 0.05[2]	NS[2]
Abdominal pain	91%	83%	NS[2]	91%	92%	NS[2]	80%	89%	NS[2]	< 0.05[2]	NS[2]
Bloating	72%	37%	< 0.05[2]	78%	63%	NS[2]	30%	49%	< 0.05[2]	< 0.05[2]	NS[2]
Diarrhoea	65%	26%	< 0.05[2]	63%	70%	NS[2]	26%	26%	NS[2]	< 0.05[2]	< 0.05[2]
Constipation	17%	36%	< 0.05[2]	19%	14%	NS[2]	38%	34%	NS[2]	< 0.05[2]	< 0.05[2]
Mixed bowel habits	18%	34%	NS[2]	19%	16%	NS[2]	37%	27%	NS[2]	< 0.05[2]	NS[2]
Polyps	24%	45%	< 0.05[2]	23%	24%	NS[2]	42%	52%	NS[2]	< 0.05[2]	< 0.05[2]
Haematochezia	23%	40%	NS[2]	18%	32%	NS[2]	41%	37%	NS[2]	< 0.05[2]	NS[2]
Inflammation	45%	28%	NS[2]	39%	54%	< 0.05[2]	16%	49%	< 0.05[2]	< 0.05[2]	NS[2]
Metal contact dermatitis	74%	6%	< 0.05[2]	66%	77%	NS[2]	7%	5%	NS[2]	< 0.05[2]	< 0.05[2]
Lactose intolerance	63%	28%	< 0.05[2]	38%	77%	< 0.05[2]	26%	32%	NS[2]	< 0.05[2]	< 0.05[2]

Patients were divided into two main groups: s-NLH (with nodular lymphoid hyperplasia, 1st column) and no-NLH (without nodular lymphoid hyperplasia, 2nd column). Each group was further stratified into functional disease (F, 4th and 7th column respectively) and organic disorder (O, 5th and 8th column respectively). *P* values obtained by means of *t*-test ([1]) or χ^2 test ([2]) between the various patients' subsets are indicated in columns 3, 6, 9, 10 and 11. NS: Non significant.

NLH was present in all colonic segments (94%); 21% of patients also showed NLH in the terminal ileum; NLH was observed in the right or left colon alone in 1% and 5% of cases, respectively.

As for clinical presentation, all s-NLH patients complained of altered bowel habits, mainly chronic diarrhoea; most cases also reported abdominal pain and bloating; 23% had haematochezia. In 39% of patients, colonoscopy revealed concomitant macroscopic inflammation (RRS, diffuse hyperaemia, erosions, and ulcers); in 69% of cases, the inflammation was patchy and confined to the left colon and/or rectum; a patchy right-sided colitis was observed in 5% of s-NLH patients; in 8% of cases, the inflammation involved the terminal ileum. Colon polyps were found and removed in 21% of s-NLH patients.

A history of delayed hypersensitivity reactions and in particular of metal contact dermatitis was self-reported by 74% of s-NLH patients, but only a minority of them (9%) had a previous patch test-based diagnosis of nickel (Ni) allergy. Moreover, 63% of patients with NLH self-reported lactose intolerance.

The final diagnosis for s-NLH was of organic disease in 37% of cases and of functional disorder for the remaining 63% (Figure 3). In particular, among patients with organic disorders, we found 16 cases of IBD - 8 ulcerative colitis (UC) and 8 Crohn's disease (CD); 9 parasitic or bacterial infections; 7 diverticular diseases; 1 microscopic colitis; 1 colorectal cancer, 1 CeD and 1 ischemic colitis. Ninety-one percent of patients with functional disorders fulfilled Rome III criteria for IBS, mainly IBS-D. Noteworthy, most of NLH patients with IBD and almost 20% of patients with IBS showed NLH with RRS (Figure 4).

Statistically significant differences between organic and functional conditions associated with s-NLH were found for sex (more women in the functional subset) and self-reported lactose intolerance (more common in organic disorders). On the contrary, there were no statistically significant differences in frequency of bowel habit alterations and metal contact dermatitis between organic and functional s-NLH patients. Also, we did not found a statistically significant correlation between NLH distribution and clinical symptoms (data not shown).

Symptomatic patients without NLH

Symptomatic patients who underwent colonoscopy and did not show NLH [symptomatic patients without NLH (s-no-NLH)] were 214. Many of them complained of altered bowel habits (mostly constipation or mixed), and abdominal pain; 40% also reported haematochezia. In 28% of cases, colonoscopy revealed macroscopic inflammation

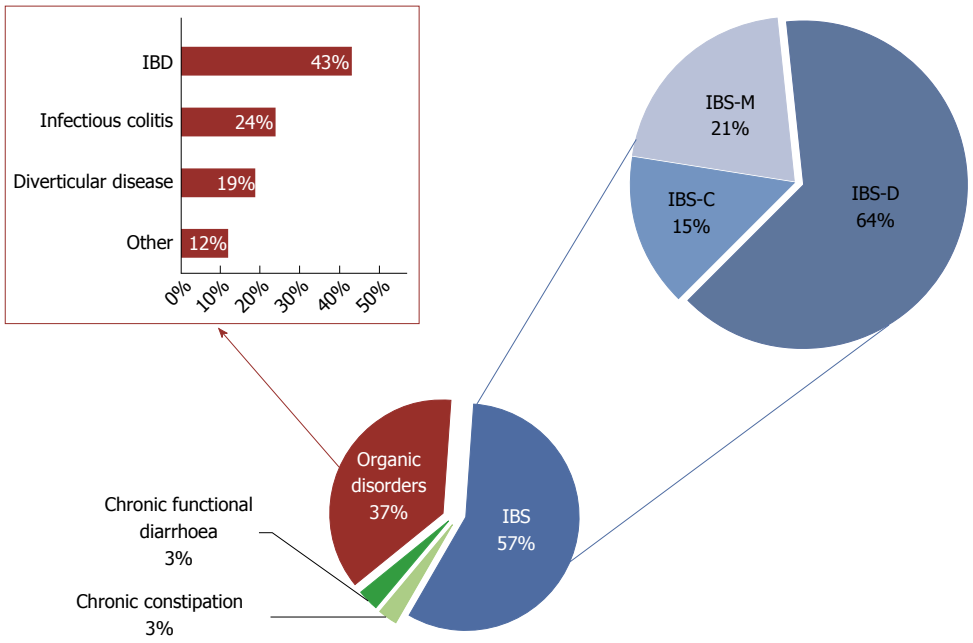

Figure 3 Final diagnosis for symptomatic patients with nodular lymphoid hyperplasia. Patients with nodular lymphoid hyperplasia (s-NLH) patients were divided into organic and functional bowel disorders; the latter subset was further stratified into IBS with prevalent diarrhoea (IBS-D), or constipation (IBS-C), or mixed bowel habit (IBS-M), chronic functional diarrhoea, and chronic constipation, according to the Roma III criteria. Among organic conditions associated with NLH, the most frequent was IBD, followed by infections and diverticular disease. IBS: Irritable bowel syndrome; IBD: Inflammatory bowel disease.

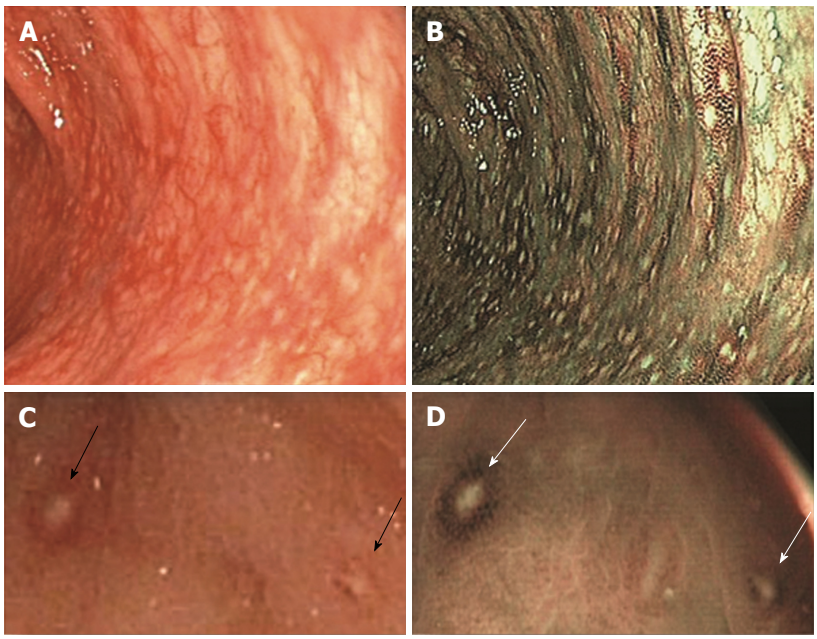

Figure 4 Endoscopic features of nodular lymphoid hyperplasia with red ring sign, due to hypervascularization at the base of the follicles, associated with granulocyte infiltrate. A and B: A typical case of nodular lymphoid hyperplasia (NLH) with red ring sign (RRS) and diffuse macroscopic inflammation, in white light (WL) and narrow band imaging (NBI), respectively: RRS appears as a red (WL) or brown (NBI) outline surrounding NLH foci; C and D: A particular of two NLH foci with RRS, in WL and NBI, respectively (black and white arrows).

(hyperaemia, erosions and ulcers) and biopsies were taken; none of the samples exhibited NLH at histological examination. Colon polyps were found and removed in 45% of s-no-NLH patients.

Regarding the final diagnosis, 34% of s-no-NLH patients were affected by organic diseases, while the remaining 66% had a functional condition (Figure 5). In particular, among patients with organic disorders, we found 5 cases of IBD (1 UC and 4 CD); 2 infectious colitis; 55 diverticular diseases; 12 abdominal adhesions; 3 colorectal cancers. Among patients with functional disorders, 80% fulfilled Rome III criteria for IBS, mostly IBS-C or IBS-M. No differences were found in terms of distribution of self-reported lactose

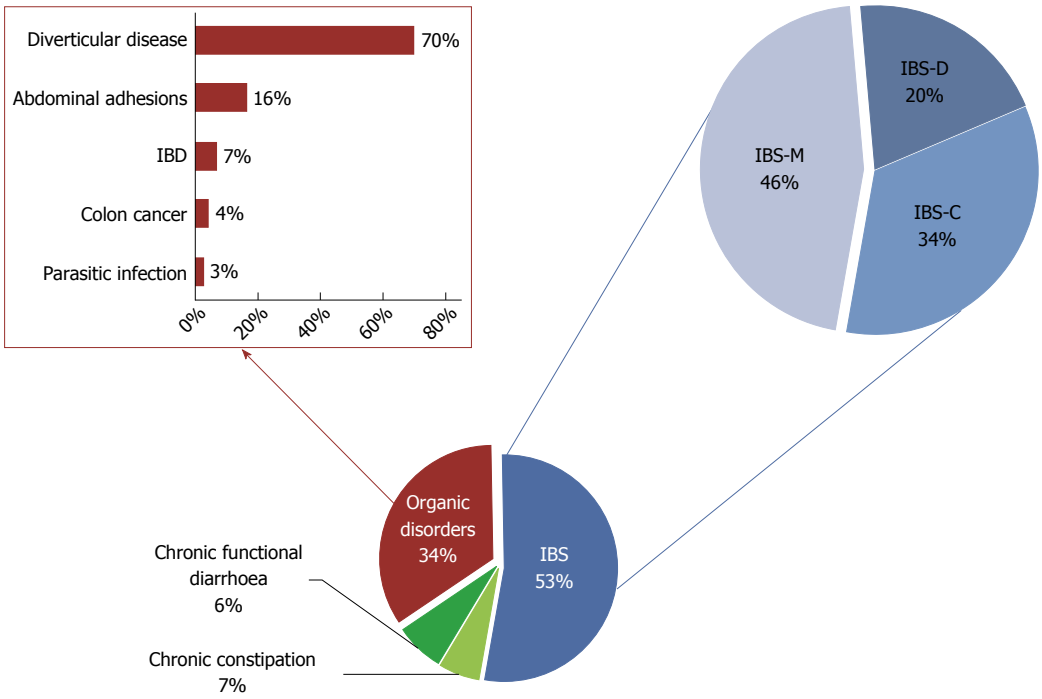

Figure 5 Final diagnosis for symptomatic patients without nodular lymphoid hyperplasia. IBS: Irritable bowel syndrome; IBS-M: IBS mixed bowel habit; IBS-C: IBS with prevalent constipation; IBS-D: IBS with prevalent diarrhoea; IBD: Inflammatory bowel disease.

Table 2 Final diagnosis in symptomatic patients with organic disorders

Organic disorders	s-no-NLH	s-NLH	*P* value
Diverticular disease	70%	19%	< 0.05
Colon cancer	4%	3%	NS
Infections	3%	24%	< 0.05
IBD	7%	43%	< 0.05
Abdominal adhesions	16%	0%	< 0.05
Others	0%	12%	< 0.05

No-NLH: Patients without evidence of nodular lymphoid hyperplasia; NLH: Patients with evidence of nodular lymphoid hyperplasia; IBD: Inflammatory bowel disease; NS: Not significant. The last column enlists the *P* value obtained from the comparison between no-NLH and NLH cases, by means of *t*-test.

intolerance and contact dermatitis between s-no-NLH patients with organic and functional conditions. Noteworthy, we found a statistically significant difference in mean age, being patients with organic disorders older than those with functional conditions. On the contrary, no difference for sex distribution was noted between the two subsets. Patients with functional disorders showed less frequently bloating and macroscopic inflammation at endoscopy.

Comparison between symptomatic patients with and without NLH

Patients with NLH were significantly younger, had more often diarrhoea and bloating and less frequently constipation and colonic polyps *vs* patients without NLH. Moreover, NLH patients reported more frequently metal contact dermatitis and lactose intolerance *vs* patients without NLH, regardless the final diagnosis of functional or organic disorders. Stratifying all symptomatic subjects according to the final diagnosis, among those diagnosed with functional conditions, patients showing NLH were younger, more often female, and had a higher frequency of abdominal pain, bloating, diarrhoea, inflammation, metal contact dermatitis and self-reported lactose intolerance; on the contrary, they showed less frequently constipation, haematochezia, mixed bowel habits and colon polyps, compared with patients without NLH. Among patients with organic diseases, those showing NLH were also significantly younger, had more often diarrhoea, contact dermatitis and lactose intolerance, less frequently constipation and fewer polyps. No differences were noted for sex, abdominal pain, bloating, endoscopic signs of inflammation or haematochezia. Finally, NLH patients with organic conditions were more often diagnosed with IBD and infections, while those without NLH had a higher frequency of diverticular disease and abdominal adhesions; no differences were noted for colorectal cancer frequency (Table 2). We did not found a statistically significant difference in terms of clinical or endoscopic disease activity between IBD patients with or without NLH.

Asymptomatic subjects

Among the 835 individuals who underwent colonoscopy for colorectal cancer screening, 23 had NLH. Thus, the prevalence of NLH in asymptomatic adults was significantly lower compared to the frequency of NLH

in symptomatic patients (3% *vs* 32%, $P < 0.05$).

Most a-NLH patients were female (78%), with a mean age of 57 ± 9 years. NLH was mainly diffuse in all colonic segments (87%) and only 1 case showed involvement of the terminal ileum. Polyps were detected and removed in 48% of patients. A mild asymptomatic diverticular disease was observed in 35% of cases; in only 2 patients with diverticula we noted signs of macroscopic inflammation. Metal contact dermatitis was reported by 44% of a-NLH. One of the cases complained of lactose intolerance. Comparing the a-NLH group with the remaining 812 asymptomatic individuals who underwent screening colonoscopy and did not show NLH (a-no-NLH), the latter ones were older (mean age 61 ± 9 years, $P < 0.05$), more frequently male (female 43%, $P < 0.05$) and had a statistically significant lower prevalence of self-reported contact dermatitis (5%, $P < 0.05$). The polyp detection rate did not significantly differ between the two groups (48% *vs* 64%, $P > 0.05$).

DISCUSSION

The present study assessed the frequency and gender distribution of colonic NLH, observed at WL and NBI endoscopy and histologically confirmed, in adults undergoing colonoscopy in a three-year period. The association between NLH and known diseases was also investigated.

The global prevalence of NLH was 10%. NLH was rare in asymptomatic subjects (3%). In particular, NLH was found mostly in asymptomatic women, who were younger than the relative control population and more often reported metal contact dermatitis. Conversely, NLH was a frequent finding in symptomatic patients, undergoing colonoscopy for IBS-type symptoms or suspected IBD (32%). Also among the symptomatic patients' group, NLH was more frequent in young women, who often complained of metal contact allergies and lactose intolerance. As for clinical presentation, diarrhoea and bloating were more common, while constipation was rarer in NLH *vs* no-NLH patients. Diverticular disease and abdominal adhesions were more frequent in no-NLH cases, while NLH patients more often suffered from IBD or colonic infections and had fewer polyps, likely because of the younger age at presentation.

Overall, our results demonstrate that NLH of the lower gastrointestinal tract is a common endoscopic finding in symptomatic patients, in whom it might reflect a state of enhanced immunological activity. Indeed, it has been postulated that lymphoid hyperplasia results from a chronic activation of the gut immune system by antigenic triggers (*i.e.*, allergens, pathogens, toxins), that lead to repetitive stimulation and eventual hyperplasia of lymphoid follicles. NLH might also develop in conditions of deregulation of the immune system, like in autoimmune diseases[17] or in immunodeficient subjects[18].

The association between food allergies and NLH has been already documented[12,33,34]. Conversely, to the best of our knowledge, there are no published data on the relationship between NLH and allergic contact allergy. Metal allergens, notably Ni, account for a significant proportion of contact sensitization[35]. It has been reported that about 15% of women and 2%-3% of men living in industrialized countries are Ni sensitive and may develop allergic contact dermatitis (ACD), a T cell-mediated inflammatory process of the skin induced by cutaneous absorption of an allergen in a previously sensitized individual. This gender difference is due to different rates of exposure of skin (from jewellery, leathers, *etc.*) to this substance. About 20%-30% of ACD patients also experiences systemic (headache, asthenia, itching), and gastrointestinal (bloating, abdominal pain, diarrhoea) symptoms after eating Ni-rich foods. This condition is known as "Systemic Contact Dermatitis" or "Systemic Ni Allergy Syndrome" (SNAS)[36]. Di Gioacchino *et al*[37], demonstrated that oral challenge with Ni, in women with SNAS, stimulates the immune system, inducing a maturation of T lymphocytes from virgin into memory cells, which accumulate in the intestinal mucosa. An increased frequency of delayed type hypersensitivity to metals has been reported in patients with connective tissue disease and fibromyalgia and it has been speculated that metal-specific T cell reactivity might be an etiological factor in the development of chronic immune-mediated disorders[38,39]. Noteworthy, Cazzato *et al*[40] demonstrated a higher prevalence of lactose intolerance in patients affected by SNAS *vs* controls (74.7% *vs* 6.6%, respectively); the authors argued that the Ni-induced pro-inflammatory status could temporary impair the brush border enzymatic functions, resulting in hypolactasia.

In our study, we observed a very high frequency of self-reported lactose intolerance and metal contact dermatitis in NLH patients. We can suppose that metal allergens might play a pivotal role in the development of lymphoid hyperplasia and hypolactasia. This could also account for the higher observed prevalence of NLH in women, given the increased frequency of Ni-sensitivity in female *vs* male sex.

Another interesting result of our study is the association between NLH and IBD. It is well known that IBD are characterised by an abnormal immunological response to environmental antigens, especially the enteric bacterial flora, in genetically susceptible individuals. Lymphoid follicles represent the main portal of entry for potential pathogens, and it has been suggested that aphthous ulceration in CD and UC originates in follicle-associated epithelium over the lymphoid follicles. In 2010, Krauss *et al*[6], compared the morphology of lymphoid follicles in CD, UC and control patients, in correlation to histological and immunohistochemical findings. In 15 out of 17 patients

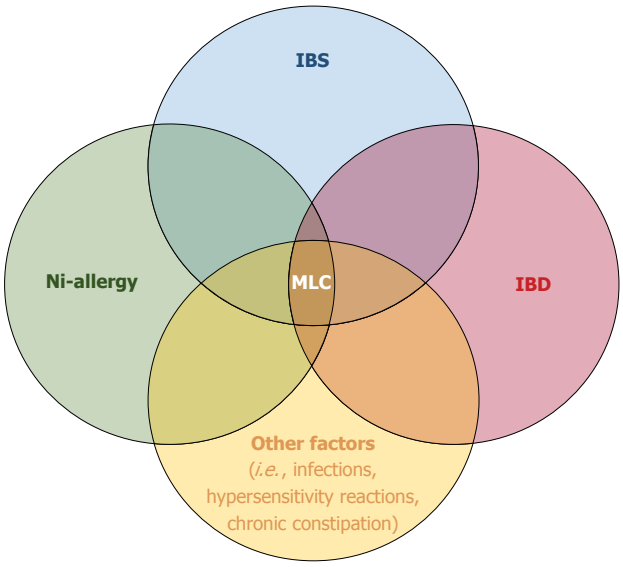

Figure 6 Colonic nodular lymphoid hyperplasia in symptomatic patients constitutes a "minimal lesions colitis" that might be triggered by Nickel or other factors (such as immunodeficiency states, infections, hypersensitivity reactions, chronic constipation). We speculate that minimal lesions colitis (MLC) could represent a distinct pathologic entity in a subset of irritable bowel syndrome (IBS) and systemic Ni allergy syndrome (SNAS) patients, and it might prelude to the development of inflammatory bowel disease (IBD).

with the first manifestation of CD, they documented NLH with RRS. In some NLH with RRS early aphthous ulcers were seen. The authors concluded that lymphoid follicles with RRS probably represent an early sign of aphthous ulcers in CD and, thus, may be considered as early markers of first manifestation and flares in CD.

In our study, 43% of s-NLH were diagnosed as affected from IBD. Interestingly, in most cases of IBD, NLH was associated with RRS.

Finally, we observed for the first time an association between NLH and IBS[41]. Even if multiple advances have been made in the knowledge of IBS pathogenesis, its aetiology remains unknown. Probably, IBS is an "umbrella term", which includes multiple conditions with common gastrointestinal symptoms, but different etiopathogenesis. Among the putative factors involved in the development of IBS, low-grade inflammation has raised growing interest in the last years. Indeed, IBS is more common in gastrointestinal diseases characterized by inflammation, such as CeD, IBD or after severe acute gastroenteritis[42-44]. Mucosal and systemic immune activation has been widely documented in patients with IBS, even in the absence of a previous major gastroenteritis event[45]. Mucosal inflammation is linked to increased mucosal permeability, enterochromaffin cell hyperplasia and higher tissue availability of serotonin, a key factor involved in the control of gut sensorimotor functions[46-48]. Furthermore, the possible link between low-grade inflammation and IBS has been suggested by the observation that adoptive transfer of mucosal biopsy supernatants evoked activation of sensory pain pathways[49,50] and abnormal enteric nervous system responses in recipient rodents[51]. Interestingly, these responses were reduced to a large extent by antagonism of immune-related factors[49-52]. The origin of low-grade inflammation in patients with IBS remains undetermined, but it is likely to be multifactorial, involving genetic predisposition[53,54], stress[55], atopy[56], abnormal intestinal microbiota[57], and higher mucosal permeability[46]. These data confirm the heterogeneity of IBS patients and point toward the necessity to find an objective biomarker of low-grade inflammation, to select those patients who could benefit most from anti-inflammatory therapy.

Our results suggest that NLH could be such a marker of low-grade inflammation in a conspicuous subset of IBS cases, in which a "minimal lesions colitis" (MLC) characterized by diffuse colonic NLH can be found. Notably, patients with NLH have some distinctive features within the IBS population: they are younger, more often female, and have a higher frequency of metal contact dermatitis, abdominal pain, bloating, diarrhoea, and unspecific inflammation. Moreover, 19% of patients with MLC had NLH associated with RRS; we might speculate that in these cases MLC could share common features with IBD.

Our work presents some limitations. Firstly, since this is a retrospective study, the asymptomatic population is not matched with the symptomatic population, because people undergoing colonoscopy for screening purposes are more often male and older, compared to patients with IBS-like symptoms or suspected IBD, who showed a higher prevalence of female and younger people. Moreover, we collected information about metal contact allergies and lactose intolerance from patients' charts; such data were not available for all cases. Additionally, regarding the association between NLH and metal contact dermatitis, only a minority of subjects had performed patch tests and therefore we based our analysis on self-reported history of delayed hypersensitivity reactions to metals. Finally, only a minority of non-NLH patients underwent biopsy sampling during colonoscopy, while in most cases NLH was excluded on the base of endoscopic findings.

In conclusion, colonic NLH is rare in asymptomatic subjects, while it is a frequent finding in symptomatic patients, in whom it might reflect a state of enhanced immunological activity. We can speculate that colonic NLH represents an objective biomarker of low-grade inflammation in a subset of IBS patients, which might be triggered by metal contact reactions; moreover, colonic NLH with RRS might share common features with IBD, supporting the hypothesis that IBS and IBD might be part of the spectrum of the same disease (Figure 6).

Further studies are required to understand the

etiopathogenetic mechanisms underlying colonic NLH in organic and functional conditions, its clinical implications and its possible link with IBD.

COMMENTS

Background

Colonic nodular lymphoid hyperplasia (NLH) is considered a rare finding in adults. NLH can be asymptomatic or more rarely presents with gastrointestinal symptoms, like abdominal pain, chronic diarrhoea and bleeding and it has been reported in association with inflammatory bowel disease (IBD), celiac disease, lymphoma, dysgammaglobulinemia, Ehlers-Danlos syndrome, diversion colitis and food allergies.

Research frontiers

Published literature includes case reports and small series of patients; whether this relates to endoscopy underreporting or to the true rarity of the condition is unclear.

Innovations and breakthroughs

This study sheds light on colonic NLH in adults, in terms of prevalence, gender-distribution and association with known diseases. The most relevant result of our study is the identification of NLH as a putative marker of low-grade inflammation in a subset of irritable bowel syndrome (IBS) cases.

Applications

Diffuse colonic NLH could be a marker of low-grade inflammation in a conspicuous subset of IBS patients and could constitute a link between a subset of IBS cases and IBD.

Peer-review

The authors conclude that colonic NLH could be a marker of low-grade inflammation in a subset of patients with IBS. The point of view is interesting.

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ORIGINAL ARTICLE

Case Control Study

Effect of switching from treatment with nucleos(t)ide analogs to pegylated interferon α-2a on virological and serological responses in chronic hepatitis B patients

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Author contributions: Ye XG designed the research and critically reviewed the manuscript for important intellectual content; He LT performed the search, analyzed the data and wrote the manuscript; Zhou XY collected the cases.

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Abstract

AIM

To investigate the efficacy of switching to pegylated interferon-α-2a (PegIFNα-2a) treatment in nucleos(t)ide analog (NA)-treated chronic hepatitis B (CHB) responder patients.

METHODS

A 48-wk prospective and retrospective treatment trial of NA-treated CHB patients who had received entecavir (ETV) for at least 48 wk and had serum hepatitis B virus (HBV)-DNA < 500 IU/mL, serum hepatitis B envelope antigen (HBeAg) < 100 S/CO, serum alanine aminotransferase, and aspartate aminotransferase levels < 2 × the upper limit of normal of 40 IU/L was performed. The effects on virological and serological responses and adverse reactions to 0.5 mg daily ETV for 48 wk *vs* switching to PegIFNα-2a were compared. Forty-four patients were randomized to be switched from NA treatment to the PegIFNα-2a group, and 44 patients were simultaneously randomized to the ETV group.

RESULTS

After 48 wk of therapy, the decrease in hepatitis B surface antigen (HBsAg) levels was greater in the PegIFNα-2a group than in the ETV group (3.1340 $\log_{10}$ IU/mL *vs* 3.6950 $\log_{10}$ IU/mL, $P = 0.00$). Seven patients who were anti-HBs-positive at baseline achieved HBsAg loss when switched to PegIFNα-2a (15.91% *vs* 0%,

P = 0.018). The HBeAg serological conversion rate was higher in the PegIFNα-2a group than in the ETV group; however, the difference was not significant because of the small sample sizes (34.38% *vs* 21.88%, *P* = 0.232). In the PegIFNα-2a group, patients with HBsAg levels < 1500 IU/mL at baseline had higher HBeAg seroconversion and HBsAg loss rates at week 48 than those with HBsAg levels ⩾ 1500 IU/mL (HBeAg seroconversion: 17.86% *vs* 62.5%, *P* = 0.007; HBsAg loss: 41.67% *vs* 6.25%, *P* = 0.016). Moreover, patients with HBsAg levels < 1500 IU/mL at week 24 had higher HBsAg loss rates after therapy than those with HBsAg levels ⩾ 1500 IU/mL (36.84% *vs* 0%, *P* = 0.004). However, there were no statistically significant differences in HBeAg seroconversion rates (47.06% *vs* 25.93%, *P* = 0.266).

CONCLUSION

NA-treated CHB patients switched to sequential PegIFNα-2a achieved highly potent treatment termination safely.

Key words: Chronic hepatitis B; Entecavir; pegylated interferon-α-2a; Sequential therapy; Effect

Core tip: It is necessary to achieve termination safely with minimal risk of long-term resistance in nucleos(t)ide analog (NA)-treated chronic hepatitis B (CHB) patients. We studied NA-treated CHB patients who stopped NAs safely and achieved sustained virological and immunological responses after treatment. We clarified the efficacy and safety of sequential 48-wk pegylated interferon-α-2a (PegIFNα-2a) in NA-treated CHB patients during and after treatment termination. Patients were selected based on the initial serum hepatitis B surface antigen (HBsAg) level. PegIFNα-2a was adjusted based on HBsAg levels at 24 wk of treatment, an important and significant factor in achieving treatment termination safely with immune control.

He LT, Ye XG, Zhou XY. Effect of switching from treatment with nucleos(t)ide analogues to pegylated interferon α-2a on virological and serological response in chronic hepatitis B patients. *World J Gastroenterol* 2016; 22(46): 10210-10218 Available from: URL: http://www.wjgnet.com/1007-9327/full/v22/i46/10210.htm DOI: http://dx.doi.org/10.3748/wjg.v22.i46.10210

INTRODUCTION

Hepatitis B virus (HBV) infection is a significant clinical problem globally: it is estimated that approximately 240 million individuals are chronically infected with HBV worldwide[1]. The prevalence of HBV varies markedly among regions. China is an intermediate endemic area. According to a national epidemiological survey in China in 2006, among those aged 1-59 years, 7.18% are hepatitis B surface antigen (HBsAg)-positive[2]. There are approximately 100 million individuals living with chronic hepatitis B (CHB) virus infection in China, including approximately 2 million patients. Approximately 20%-30% of chronically infected persons will develop cirrhosis and/or hepatocellular carcinoma (HCC). The World Health Organization (WHO) estimates that 0.65 million deaths annually are attributable to complications from hepatitis B, including cirrhosis and HCC, which are strongly associated with hepatitis B envelope antigen (HBeAg) positivity and serum HBV DNA replication[1]. Additionally, patients who are persistently HBeAg-positive are at higher risk of developing liver cirrhosis (3.5% per year)[1]. Therefore, standardized antiviral treatment is required to improve the prognosis of CHB. Current anti-HBV drugs are divided into two types. One of these is nucleoside analogs (NAs), a large class of direct antiviral drugs. In clinical practice, the duration of treatment of CHB with NAs is unclear. The role of NAs is to inhibit replication of the HBV DNA and reduce the amount of HBV in the blood to achieve therapeutic improvement. However, NAs have a single target and replace the nucleoside during HBV polymerase extension, resulting in termination of chain extension during the viral replication process, thus inhibiting viral replication[3,4]. Therefore, treatment with NAs greatly inhibits viral replication and relieves inflammation but does not eliminate the virus completely nor produces enduring HBeAg seroconversion or HBsAg clearance. Most importantly, NAs almost always produce drug resistance and relapse after discontinuation of therapy. Therefore, to reduce the risk of liver function decompensation, liver cirrhosis and HCC progression in patients with hepatitis B, a long-term antiviral treatment to inhibit HBV is required.

NAs are used widely (about 90%) in CHB treatment in China. However, not all patients are willing to continue taking NAs continuously, despite concerns regarding relapse after treatment, and hope to be able to stop taking the medicine safely. Realizing these hopes represents a tremendous challenge for NA-treated CHB patients.

Interferon (IFN) is another type of drug that has antiviral activity and acts as an immune regulator by inducing host cytokines to inhibit multiple aspects of viral replication. The European Association for the Study of the Liver (EASL)[5] has indicated that IFN therapy is the preferred treatment option for HBeAg-positive patients who achieve stable HBeAg seroconversion and for HBeAg-negative patients who achieve sustained response after therapy. An advantage of IFN is that the duration of IFN anti-HBV treatment has a clear treatment course, which is widely used for the clinical treatment of CHB. Therefore, recent research has focused on a combination therapy

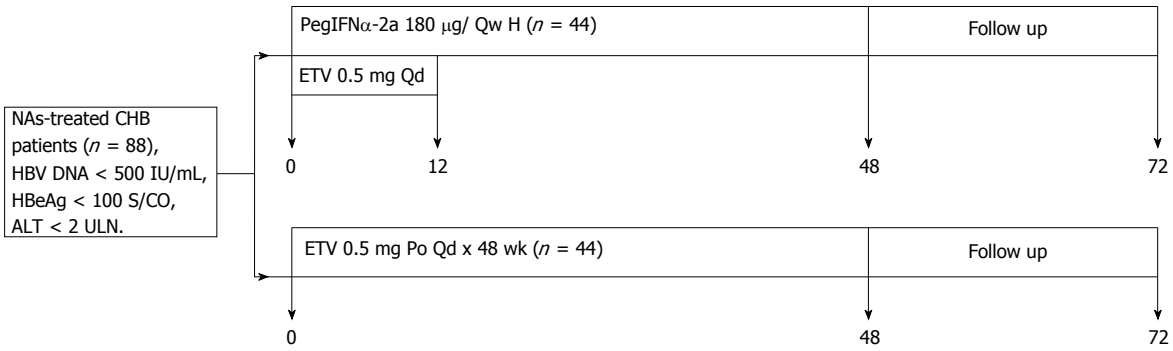

Figure 1 Trial design. HBV: Hepatitis B virus; CHB: Chronic hepatitis B; HBeAg: Hepatitis B envelope antigen; ULN: Upper limit of normal; ALT: Alanine aminotransferase; PegIFNα-2a: Pegylated interferon-α-2a; ETV: Entecavir; Qd: Quaque die; Po: Peros.

of IFN and NAs to exploit the antiviral and immune regulation effects of these drugs. The combination of NAs with IFN improves interferon tolerance, inhibits covalently closed circular DNA (cccDNA) transcription, improves the initial response rate, prevents or delays the generation of NA-resistant mutations and prevents the generation of multidrug-resistant mutations[6]. Therefore, a clinical treatment regimen with a shorter course that allows CHB patients to stop NA treatment safely might be feasible. To ensure that CHB patients who were treated with NAs can safely stop taking NAs and obtain lasting immune control, the Expert Meeting of China in 2013[7] suggested that CHB patients treated with NAs should switch to pegylated interferon (Peg-IFN) or pursue a combined treatment. NA-treated CHB patients who switch to IFN have been reported to achieve higher rates of sustained virological and serological responses than those continuing with NA monotherapy[8,9]. However, supporting medical evidence from clinical trials or clinical, real-life data are lacking.

To help NA-treated CHB patients stop NAs safely and achieve sustained virological and immunological responses after treatment, we investigated the efficacy and safety of switching NA-treated CHB patients to sequential 48-wk PegIFNα-2a by observing the virological response, HBsAg or HBeAg seroconversion rates, and other indicators.

MATERIALS AND METHODS

Study population

This study was a 48-wk prospective and retrospective treatment trial comparing the efficacy and safety of 0.5 mg entecavir (ETV, Baraclude, Bristol-Myers Squibb) daily for 48 wk compared to switching to pegylated interferon alpha-2a (PegIFNα-2a, F. Hoffmann-La Roche Ltd, Basel, Switzerland). All patents were followed up for 24 wk (Figure 1). Patients assigned to PegIFNα-2a received 180 μg/wk for 48 wk, with the first 12 wk overlapping with 0.5 mg daily ETV. Patients assigned to the ETV group continued with ETV monotherapy. A total of 88 patients who had received ETV treatment for at least 48 wk were recruited from the Second Hospital affiliated with Guangzhou Medical University between January 1, 2013, and December 31, 2015. Patients were randomized to receive PegIFNα-2a 180 μg/wk or continue 0.5 mg daily ETV for 48 wk. Eligible patients were HBsAg-positive, had serum HBV-DNA < 500 IU/mL, serum HBeAg < 100 S/CO, and serum alanine aminotransferase (ALT) and aspartate aminotransferase (AST) levels < 2 × the upper limit of normal (ULN) of 40 IU/L. Patients with decompensated cirrhosis and HCC were excluded; as were patients co-infected with hepatitis A, C, or D; those who had been pre-treated with other antivirals; and patients with a history or evidence of other chronic liver diseases, including autoimmune hepatitis or alcohol liver disease.

Curative effect

HBeAg seroconversion was defined as HBeAg loss (HBeAg < 1.0 S/CO) and HBeAb positivity (HBeAb > 1.0 S/CO). HBsAg loss was defined as HBsAg < 0.05 IU/L. HBsAg seroconversion was defined as HBsAg loss and HBsAb positivity (HBsAb >10.0 IU/L).

Observation methods

Clinical examination and routine laboratory tests were performed at the beginning of therapy, at 4, 8, 12, 24, 36, and 48 wk during antiviral therapy and at follow-up at 12 and 24 wk after therapy. Biochemical [serum AST, ALT, creatinine (Cr), and glucose (Glu)] and virological parameters (HBeAg, HBAb, HBcAb status and HBV DNA levels) were measured at each visit. Serum HBV DNA was detected using either a standard generic HBV DNA assay (Da An Gene, normal level of HBV DNA < 500 IU/mL) or the COBAS TaqMan HBV Test (Roche Molecular Diagnostics, Pleasanton, CA, United States). HBeAg, HBeAb, HBcAb status was detected using chemiluminescence measurements. The laboratory technicians were unaware of the trial. The PegIFNα-2a group was divided into an HBsAg < 1500 IU/mL group and an HBsAg ⩾ 1500 IU/mL group based on the HBsAg level at baseline and after 24 wk of therapy.

Table 1 Patient demographics and baseline characteristics

	PegIFNα-2a	ETV	*P* value
Age (yr), mean	35.41 (95%CI: 32.68-39.03)	35.43 (95%CI: 32.42-38.43)	0.832[1]
Male	62.86%	68.57%	0.615[2]
ALT (U/L), mean	34.60 (95%CI: 30.31-38.89)	33.06 (95%CI: 30.15-35.96)	0.745[1]
HBsAg (IU/mL), mean	6168.8630 (95%CI: 3841.12-8496.60)	5879.4557(95%CI: 3643.06-8115.85)	0.960[1]
HBeAg (+)	29/44 (65.91%)	(27/44) 61.36%	0.658[2]

α = 0.05. [1]Non-parametric Wilcoxon test; [2]Pearson's χ^2 test. ALT: Alanine aminotransferase; HBsAg: Hepatitis B surface antigen; HBeAg: Hepatitis B envelope antigen; PegIFNα-2a: Pegylated interferon-α-2a; ETV: Entecavir.

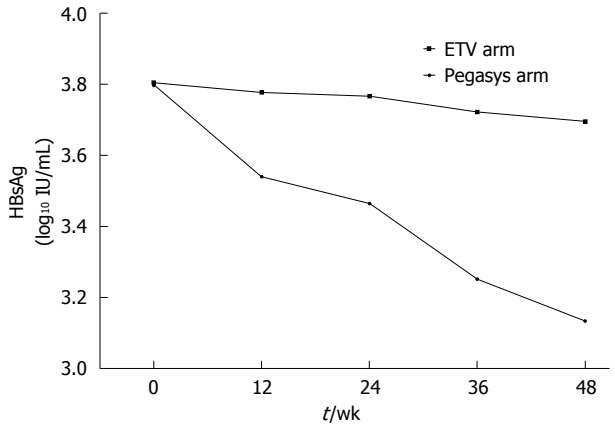

Figure 2 Hepatitis B surface antigen levels in patients in the PEGylated interferon α-2a group and the entecavir group across therapy. HBsAg: Hepatitis B surface antigen; ETV: Entecavir.

Statistical analysis

Statistical analysis was conducted using SPSS version 13.0 software. Quantitative data were analyzed by a *t*-test or non-parametric Wilcoxon test as appropriate, and qualitative data were analyzed by Pearson's χ^2 test and Fisher's exact test.

RESULTS

Baseline in the PegIFNα-2a and ETV groups

There were 88 patients in the trial (PegIFNα-2a, *n* = 44; ETV, *n* = 44), and all patients accepted the regular 48 wk of treatment and 24 wk of follow-up. The mean age was 35.41 years (95%CI: 32.68-39.03) in the PegIFNα-2a group and 35.43 years (95%CI: 32.42-38.43) in the ETV group. There were no statistically significant differences in age, gender or serum biochemical data between the two groups (Table 1).

Efficacy

HBsAg levels in patients in the PegIFNα-2a group (Figure 2): During therapy, HBsAg levels were 3.7902, 3.5405, 3.4661, 3.2511, and 3.1340 $\log_{10}$ IU/mL at baseline and weeks 12, 24, 36, and 48 of therapy, respectively. However, the changes were small in the ETV group. After 48 wk of therapy, the decrease in HBsAg levels was greater in the PegIFNα-2a group than in the ETV group (3.1340 $\log_{10}$ IU/mL *vs* 3.6950 $\log_{10}$ IU/mL, *P* = 0.00, Table 2).

Table 2 Patient demographics after 48 wk of therapy

	PegIFNα-2a	ETV	*P* value
HBsAg levels ($\log_{10}$ IU/mL)	3.1340	3.6950	0.00[1]
HBsAg loss rate (%)	15.91	0.0000	0.018[2]

α = 0.05. [1]Non-parametric Wilcoxon test; [2]Pearson's χ^2 test. HBsAg: Hepatitis B surface antigen; PegIFNα-2a: PEGylated interferon-α-2a; ETV: Entecavir.

Serological response (Table 2): In the PegIFNα-2a group, seven of the 44 patients achieved HBsAg loss, and one patient exhibited HBsAg seroconversion. By contrast, no patients in the ETV group achieved HBsAg loss or HBsAg seroconversion after 48 wk of therapy. More patients attained HBsAg loss in the PegIFNα-2a group (15.91%) than in the ETV monotherapy group (15.91% *vs* 0%, *P* = 0.018). There were five and two individuals who remained HBeAg-positive and -negative at baseline, respectively. During the NA treatment period, both the PegIFNα-2a and ETV groups experienced HBeAg seroconversion. The HBeAg serological conversion rate was higher in the PegIFNα-2a group than in the ETV group, although the difference was not significant because of the small sample sizes (34.38% *vs* 21.88%, *P* = 0.232).

As the treatment time increased, the decrease in HBsAg levels became more obvious. Significantly more patients (Figures 3 and 4) in the PegIFNα-2a group had HBsAg levels of <100 IU/mL after treatment than before treatment (50.00% *vs* 9.09%, *P* = 0.00). Among patients with HBsAg levels < 1500 IU/mL, the percent changes were 72.73% *vs* 25.00%, *P* = 0.00, and in patients with HBsAg levels < 3000 IU/mL, the percent changes were 86.36% *vs* 36.36%, *P* = 0.00.

Early HBsAg decline predicted the response at week 48. The highest rates of HBeAg seroconversion and HBsAg loss were observed in patients with an HBsAg level < 100 IU/mL at week 24 (Figures 5 and 6). In the PegIFNα-2a group, patients with an HBsAg level < 100 IU/mL, an HBsAg level < 1500 IU/mL, or an HBsAg level < 3000 IU/mL achieved 50%, 42.2%, and 40.74% HBeAg seroconversion at week 48,

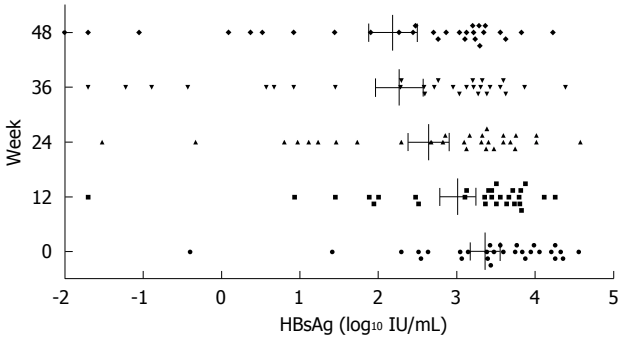

Figure 3 Change in hepatitis B surface antigen levels during PEGylated interferon α-2a sequential therapy. HBsAg: Hepatitis B surface antigen.

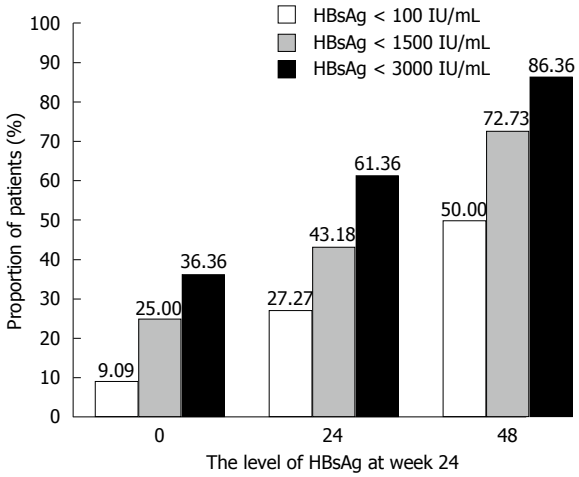

Figure 4 Proportion of patients in different hepatitis B surface antigen level groups during pegylated interferon α-2a sequential therapy. HBsAg: Hepatitis B surface antigen.

respectively.

In the PegIFNα-2a group, patients with an HBsAg level < 100 IU/mL, an HBsAg level < 1500 IU/mL, or an HBsAg level < 3000 IU/mL achieved 50%, 36.84% and 25.93% HBsAg loss at week 48, respectively.

In the PegIFNα-2a group, patients with an HBsAg level < 1500 IU/mL at baseline had higher HBeAg seroconversion and HBsAg loss rates at week 48 compared with those with an HBsAg level ⩾ 1500 IU/mL (HBeAg seroconversion: 62.5% *vs* 17.86%, $P < 0.05$; HBsAg loss: 41.67% *vs* 6.25%, $P < 0.05$). Moreover, those with an HBsAg level < 1500 IU/mL at week 24 had higher HBsAg loss rates after therapy compared with those with an HBsAg level ⩾ 1500 IU/mL (36.84% *vs* 0%, $P < 0.05$). However, the differences in HBeAg seroconversion between the groups were not significant (47.06% *vs* 25.93%, $P > 0.05$) (Table 3).

Off-treatment follow-up: Week 24 responders

During the 24-wk follow-up, all the patients who switched to PegIFNα-2a maintained HBV-DNA negative status and normal serum AST and ALT.

Thirty-five patients, including two who were HBeAg-positive after 48 wk of treatment with PegIFNα-2a, experienced HBeAg seroconversion during 24 wk of follow-up. The cumulative HBeAg seroconversion rate was 41.46%, whereas no patients achieved HBeAg seroconversion in the ETV group. However, the difference between the groups was not significant (41.46% *vs* 21.95%, $P = 0.058$).

The HBsAg level of one patient who lost HBsAg with PegIFNα-2a treatment for 48 wk was 0.67 IU/mL at the 24th wk of follow-up, even though the patient maintained HBeAg seroconversion, HBV-DNA negative status and normal serum ALT and AST.

One patient who received PegIFNα-2a developed a complication of hyperthyroidism during week 39, and the patient was not discontinued due to methimazole treatment.

Adverse events, including headache, dry mouth, weakness and decreases in leucocytes, erythrocytes, and platelets, occurred in the majority of patients. One of the 35 patients in the PegIFNα-2a group had these adverse events, which were mild and had no effect on treatment progress. However, a minority of patients in the ETV group had the above-mentioned adverse events.

DISCUSSION

In our study, regardless of the baseline levels or HBeAg positivity or negativity of the two groups of CHB patients, HBV DNA was fully suppressed by 1-5 years of NA treatment. Despite this suppression, these patients remained HBeAg-positive, albeit at lower levels. Compared with ETV monotherapy, the addition of PegIFNα-2a for 48 wk, based on HBsAg-titer monitoring produced higher HBeAg seroconversion, greatly decreased HBsAg levels, and achieved HBsAg loss and even HBsAg seroconversion with no relapse after 24 wk of follow-up. These results are similar to Ouzan's report[10].

The NEPTUNE study[11] indicated that 14% of patients treated with PegIFN for one year had deferred HBeAg seroconversion, and 86% of the patients achieved HBeAg seroconversion during the therapy. In this study, the rate of HBeAg seroconversion was 21.95%, which lower than the rate after PegIFNα-2a treatment (36.59% *vs* 21.95%, $P = 0.145$). This result is consistent with previous research[12] on ETV monotherapy. Moreover, we observed that PegIFNα-2a was effective even after treatment was terminated. The OSST study[9] confirmed higher HBeAg seroconversion rates in the PegIFNα-2a group compared with the ETV group (14.9% *vs* 6.1%, $P = 0.0467$). The differences in HBeAg seroconversion between the two groups were not significant, possibly because of the small sample size in our study.

HBsAg loss is considered the ultimate long-term goal of antiviral therapy by the Asian Pacific

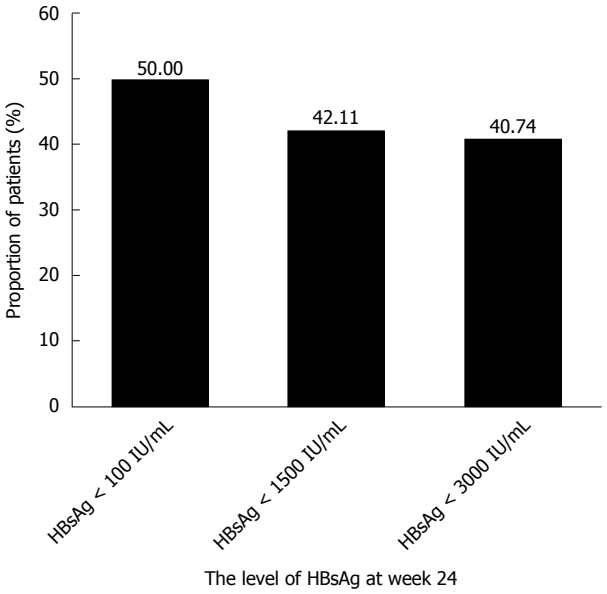

Figure 5 The hepatitis B envelope antigen seroconversion proportion after treatment baseline at different levels of hepatitis B surface antigen at week 24 during pegylated interferon α-2a sequential therapy. HBsAg: Hepatitis B surface antigen.

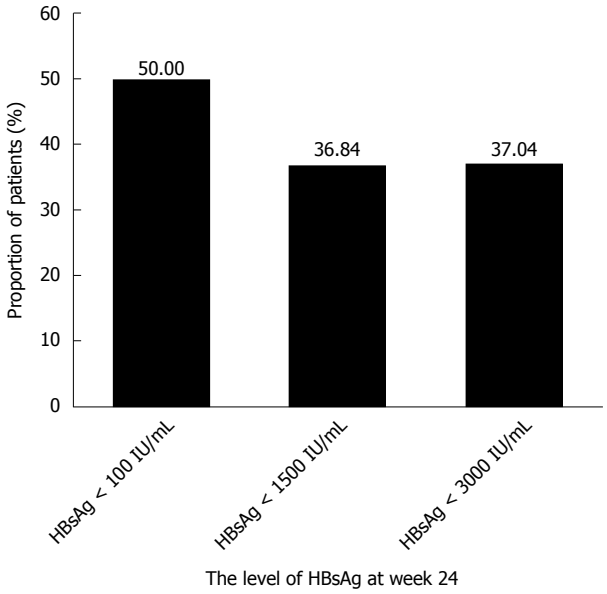

Figure 6 The proportion of hepatitis B surface antigen loss after treatment with pegylated interferon α-2a at different levels of hepatitis B surface antigen at week 24. HBsAg: Hepatitis B surface antigen.

Association for the Study of the Liver, EASL, and the American Association for the Study of the Liver[5,13,14]. However, achieving HBsAg loss and sustained virological and serological responses is difficult with general treatment using NAs. The median number of years of NA treatment required for HBsAg loss is 52.2 years (interquartile range: 30.8-142.7)[15]. In our study, 50% of patients exhibited a reduction of HBsAg levels to less than 100 IU/mL after PegIFNα-2a therapy, and 86.36% of patients had HBsAg levels < 1500 IU/mL. The level of serum HBsAg was predominantly and closely associated with intrahepatic cccDNA levels[16]. HBsAg levels < 100 IU/mL at the end of the treatment indicated a sustained response to NA-induced HBeAg seroconversion[17]. At the 3-year post-treatment follow-up, 52% of the patients with HBsAg levels < 10 IU/mL at the end of treatment achieved HBsAg loss[18]. Moreover, IFN has both antiviral and immunomodulatory effects, and thus decreases the amount of cells containing the HBV intrahepatic cccDNA molecule, which is required for sustained, chronic HBV infection[6]. Therefore, patients switching to PegIFNα-2a might achieve permanent HBeAg seroconversion and even achieve HBsAg loss to reach the ideal endpoint of therapy. Experts have suggested that to resolve long-term medication problems, and achieve higher HBeAg seroconversion, HBsAg loss and sustained response after treatment termination, NA-treated CHB patients should receive the combination therapy or switch to PegINF[7].

Shouval *et al*[19] demonstrated that after 48 wk of ETV treatment alone and 24 wk of follow-up, the virological relapse rate was 97%, and 39% of patients had serum ALT of less than 1 × ULN. Additionally, Seto *et al*[20] indicated that after 24 and 48 wk of entecavir treatment, 74.2% and 91.4% of patients suffered recurrent viremia. Chaung *et al*[21] reported that 90% of patients experienced virological relapse once they discontinued NA therapy. In the present study, during the 24-wk follow-up, none of the patients who switched to PegIFNα-2a became HBV-DNA positive or had abnormal serum AST or ALT.

During the follow-up period, one of the patients in the PegIFNα-2a group who exhibited HBsAg loss at the end of the 48 wk of treatment became HBsAg-positive and exhibited an increased level of HBsAg. During the 24-wk follow-up period, this patient maintained normal hepatic function, and the level of HBV-DNA was below the detection limit of 0.67 IU/mL cccDNA remaining in the liver cells of patients who undergo HBsAg loss[22]. Additionally, in HBsAg-loss patients, the median interval between HBV DNA measurements was 48 mo. The viral load in the extrahepatic reservoir decreases with time[22]. Therefore, we considered the patient to be at a persistent low HBsAg level and closely monitored the patient's liver function and HBV DNA levels.

HBeAg seroconversion and lower HBsAg levels can reduce the incidence of liver cirrhosis and liver cancer[23,24]. Compared with ETV monotherapy, PegIFNα-2a not only increased the serological conversion rate, but also produced an ideal effect after treatment termination, which has a persistent influence on the immune function of patients who achieved HBeAg seroconversion. Additionally, IFN prevents the formation of HBV proteins and depletes the intrahepatic cccDNA pool, which results in further HBsAg loss compared with ETV alone[10].

Therefore, the results indicated that the application

Table 3 Predictors of response to PEGylated interferon-α-2a using baseline parameters and hepatitis B surface antigen levels at week 24

	HBsAg loss at week 48		HBeAg seroconversion at week 48	
	n	*P* value	*n*	*P* value
HBsAg level < 1500 IU/mL at baseline	5/12 (41.67%)	0.016[1]	10/16 (62.5%)	0.007[1]
HBsAg level ≥ 1500 IU/mL at baseline	2/32 (6.25%)		5/28 (17.86%)	
HBeAg-positive at baseline	5/29 (17.24%)	1.000[1]	-	-
HBeAg-negative at baseline	2/15 (13.33%)		-	
HBsAg level < 1500 IU/mL at week 24	7/19 (36.84%)	0.004[1]	8/17 (47.06%)	0.266[1]
HBsAg level ≥ 1500 IU/mL at week 24	0/25 (0)		7/27 (25.93%)	

[1]Continuity correction, α = 0.05. HBsAg: Hepatitis B surface antigen; HBeAg: Hepatitis B envelope antigen.

of PegIFNα-2a induces a strong cccDNA decline and low serum levels of HBsAg, thus reducing the relapse rate of CHB patients after treatment termination and improving immune control and safe treatment termination.

Santantonio *et al*[25] and Marcellin *et al*[26] reported that HBsAg loss and seroconversion rates did not differ significantly between lamivudine monotherapy and combined PegIFNα-2a therapy in patients who were HBeAg-negative. Janssen *et al*[27] also indicated that HBeAg loss or seroconversion rates were similar after lamivudine monotherapy and combined PegIFNα-2a therapy in HBeAg-positive patients. However, these reports did not focus on NA-treated CHB patients. Therefore, additional clinical cases must be analyzed to determine if INF monotherapy directly, or the combined NA and interferon treatment, is superior for NA-treated CHB patients.

The level of HBsAg in the patients who were HBeAg-negative or HBeAg-positive was not related to the curative effect in the PegIFNα-2a group ($P > 0.05$). The lower the HBsAg level at baseline, the higher the HBeAg seroconversion and HBsAg loss rates at week 48. We speculated that the efficacy of 48 wk of treatment based on HBsAg levels at the 24th wk of therapy would produce a HBsAg loss rate of up to 36.84% ($P < 0.05$) in patients with a serum HBsAg level < 1500 IU/mL, and the efficacy was not determined by HBeAg seroconversion ($P > 0.05$).

The HBsAg loss rate of the patients with serum HBsAg levels < 1500 IU/mL in our trial was obviously higher than that observed in the OSST study[9] (44.44% *vs* 25%), which may be related to the lower baseline HBV DNA levels (< 500 IU/mL) and longer ETV combination to ensure persistent virus inhibition. There are no unified clinical recommendations on how long NAs and PegIFNα-2a therapy should administered. The benefits of prolonged treatment with ETV or extended PegIFNα-2a treatment in patients with higher serum HBsAg levels at baseline require further clinical observation.

This study has some limitations, such as the small sample size, which prevented deeper analysis of the relationship between HBsAg levels and curative effect after 48 wk of therapy. CHB is a chronic disease; therefore, the follow-up period of only 24 wk was relatively short. The prognosis of patients requires longer follow-up times and further observation.

In conclusion, brief treatment of NA-treated CHB patients with a combination of NAs and PegIFNα-2a could achieve highly potent treatment termination safely, with a minimal risk of long-term resistance. Based on the initial serum HBsAg level in NA-treated CHB patients, we could select superior patients to switch to PegIFNα-2a and, according to the levels of HBsAg at 24 wk of treatment, adjust the treatment to continue with PegIFNα-2a or switch to NAs. This protocol has an important and significant effect on achieving treatment termination safely and with immune control.

COMMENTS

Background

Hepatitis B virus (HBV) infection is a significant clinical problem globally: it is estimated that approximately 240 million individuals are chronically infected with HBV worldwide. Therefore, standardized antiviral treatment, including nucleos(t)ide analogues (NAs) and interferon (IFN) is required to improve the prognosis of chronic hepatitis B (CHB). NA-treated CHB patients who switch to IFN have been reported to achieve higher rates of sustained virological and serological responses than those continuing with NA monotherapy. However, supporting medical evidence from clinical trials or clinical, real-life data are lacking.

Research frontiers

Recent research has focused on combination therapy with IFN and NAs to exploit the antiviral and immune regulation effects of these drugs. However, there are very few clinical studies about this combination worldwide, especially in China. This research investigated the efficacy of switching to IFN in NA-treated CHB patients.

Innovations and breakthroughs

NAs are used widely in CHB treatment in China. Not all patients are willing to continue taking NAs continuously. The European Association for the Study of the Liver indicated that IFN therapy is the preferred treatment option for CHB patients who achieve a sustained response after therapy. This research focused on the efficacy of a combination therapy with IFN and NAs. The authors analyzed different monitoring methods for NA-treated CHB patients switching to IFN, which has an important and significant effect on choosing suitable patients and estimating the risk of long-term resistance in treatment termination.

Applications

Medical evidence from clinical trials helps clinicians choose different types of standardized antiviral treatment for different CHB patients. The present

research showed that brief treatment of NA-treated CHB patients with a combination of NAs and pegylated interferon-α-2a (PegIFNα-2a) could achieve highly potent treatment termination safely, with a minimal risk of long-term resistance.

Terminology

Currently, standardized antiviral treatment includes nucleoside NAs and IFN. Treatment of CHB has a clear treatment course with IFN. However, CHB patients need to take NA for a long and undefined time because NAs cannot eliminate the virus completely. CHB patients treated with Entecavir (ETV) have a chance of HBeAg seroconversion at least 48 wk and show decreased serum alanine aminotransferase and aspartate aminotransferase levels. However, ETV cannot decrease the HBsAg level nor sustain virological and serological responses after therapy, luckily, IFN helps to fill this gap.

Peer-review

In this paper, the authors investigated the efficacy and safety of switching CHB patients successfully treated with Entecavir to PegIFNα-2a NA-treated. The topic is of great interest. In fact, in recent years several attempts have been performed to transform a "long-life" treatment with NAs to a treatment of a "finite" duration. The paper is well written and can be considered for publication after minor revisions.

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ORIGINAL ARTICLE

Retrospective Study

Incidence of hepatocellular carcinoma in outpatients with cirrhosis in Brazil: A 10-year retrospective cohort study

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Abstract

AIM

To determine the incidence of hepatocellular carcinoma (HCC) and the impact of HCC surveillance on early diagnosis and survival of cirrhotic outpatients.

METHODS

In this retrospective cohort study, cirrhotic outpatients undergoing HCC surveillance between March 2005 and March 2014 were analyzed. Exclusion criteria were HIV coinfection; previous organ transplantation; diagnosis of HCC at first consultation; missing data in the medical chart; and less than 1 year of follow-up. Surveillance was carried out every six months using ultrasound and serum alpha-fetoprotein determination. Ten-year cumulative incidence and survival were estimated through Kaplan-Meier analysis.

RESULTS

Four hundred and fifty-three patients were enrolled, of which 57.6% were male. Mean age was 55 years. Hepatitis C virus and heavy use of alcohol were the main etiologic agents of cirrhosis. HCC was diagnosed in 75 patients (16.6%), with an estimated cumulative incidence of 2.6% in the 1st year, 15.4% in the 5th year, and 28.8% in the 10th year. Median survival was estimated at 17.6 mo in HCC patients compared to 234 mo in non-HCC patients (P < 0.001). Early-stage HCC was more often detected in patients who underwent

surveillance every 6 mo or less (P = 0.05). However, survival was not different between patients with early stage *vs* non-early stage tumors [HR = 0.54 (0.15-1.89), P = 0.33].

CONCLUSION

HCC is a frequent complication in patients with cirrhosis and adherence to surveillance programs favors early diagnosis.

Key words: Liver cirrhosis; Hepatocellular carcinoma; Epidemiology; Surveillance; Survival

Core tip: This retrospective cohort chart review study provides novel data regarding the incidence of hepatocellular carcinoma (HCC) in the South of Brazil. Of 453 patients with cirrhosis attending a specialized reference clinic between March 2005 and March 2014, 75 (16.6%) developed HCC, with a cumulative incidence of 2.6%, 15.4% and 28.8% in the 1st, 5th, and 10th year respectively. Early-stage HCC was more often detected in patients undergoing strict surveillance every 6 mo. Results from this study highlight the need for strict surveillance programs favoring early diagnosis and, probably, a better prognosis.

Appel-da-Silva MC, Miozzo SAS, Dossin IA, Tovo CV, Branco F, Mattos AA. Incidence of hepatocellular carcinoma in outpatients with cirrhosis in Brazil: A 10-year retrospective cohort study. *World J Gastroenterol* 2016; 22(46): 10219-10225 Available from: URL: http://www.wjgnet.com/1007-9327/full/v22/i46/10219.htm DOI: http://dx.doi.org/10.3748/wjg.v22.i46.10219

INTRODUCTION

Liver cancer is the second leading cause of cancer death worldwide; it is also the fifth most common cancer in men and the ninth in women. In 2012, an estimated 782000 new cases of liver cancer occurred in the world, with report of 745000 deaths[1]. Among primary liver malignancies, hepatocellular carcinoma (HCC) accounts for 70%-85% of cases, and is associated with chronic liver disease and/or cirrhosis in 70%-90% of cases[2,3].

The burden of HCC varies with geographic location, especially when associated with cirrhosis[3]. Around 80% of cases occur in developing countries, and 55% in China alone[4]. In highly endemic areas, such as sub-Saharan countries and Asia, the annual incidence rate is around 30/100000 population[4-6]. Mediterranean countries (Italy, Spain and Greece) report intermediate incidence rates, with 10-20 cases/100000/year. An increase in the burden of HCC in low-incidence areas (Australia, North America, South America, and United Kingdom), with fewer than 5 cases/100000/ year, has also been recently noted. In these areas, the growing prevalence of hepatitis C virus (HCV) infection, alcohol consumption, and nonalcoholic fatty liver disease (NAFLD) are the main causes underlying the increasing number of HCC cases[2,3,7-11].

In Latin America, limited data are available on the incidence and population characteristics of patients with HCC[12]. In Brazil, a national epidemiological survey sponsored by the Brazilian Society for Hepatology[13] evaluated 1405 patients with HCC in 29 centers across the country. Using the Barcelona Clinic Liver Cancer (BCLC) staging classification[14], 43% of the individuals were diagnosed with early stage tumors; 35% with intermediate stage tumors; and 22% with advanced stage tumors. Also, 98% had cirrhosis, which was caused by HCV in 39% and heavy use of alcohol in 14%. In the South of Brazil, HCV has been identified as the main etiologic factor of cirrhotic outpatients[15].

Screening and surveillance of HCC using abdominal ultrasound have been shown to detect tumors at an earlier stage, increasing the odds of treatment and the adherence of health care services to current practice guidelines[16-19]. Nevertheless, epidemiological studies in the United States have shown that only 12% to 78.8% of patients receive routine surveillance[20,21]; possible barriers to screening and surveillance include socioeconomic factors and the lack of specific health policies for HCC[22].

The objective of the present study was to determine the incidence of HCC and the impact of HCC surveillance on early diagnosis and survival of cirrhotic outpatients attending a tertiary hospital clinic in the South of Brazil.

MATERIALS AND METHODS

We carried out a retrospective cohort chart review study including all patients aged 18 years or older diagnosed with cirrhosis attending a specialized reference clinic (Complexo Hospitalar Santa Casa, Porto Alegre, Brazil) between March 2005 and March 2014. Exclusion criteria were HIV coinfection, previous organ transplantation, diagnosis of HCC at the first clinic appointment, incomplete medical records, or follow-up of less than 1 year. The diagnosis of cirrhosis was based on clinical, laboratory, and on ultrasonographic and/or upper GI endoscopic features. Those patients whose diagnosis remained inconclusive, percutaneous liver biopsy were carried out.

All patients underwent screening and surveillance for HCC, with abdominal ultrasound and serum alpha-fetoprotein (AFP) determination every 6 mo. Computed tomography (CT) or abdominal magnetic resonance imaging (MRI) with contrast were performed in all patients with evidence of nodular lesion measuring ⩾ 1 cm in diameter on ultrasound[23].

HCC diagnosis was based on typical findings on contrast-enhanced CT or abdominal MRI - early arterial phase enhancement followed by rapid washout at the

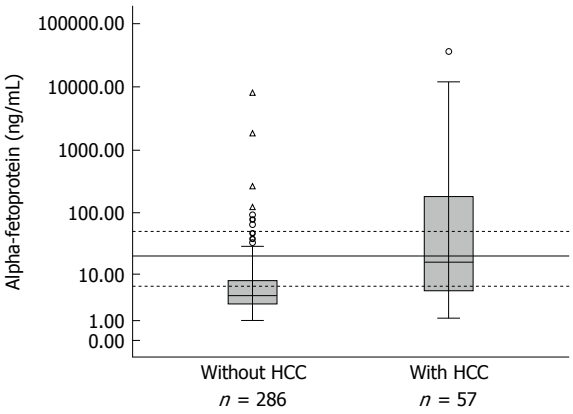

Figure 1 Log-transformed alpha-fetoprotein values at the end of the study in patients with and without hepatocellular carcinoma.

late portal/venous phase. Inconclusive cases were referred for biopsy and histological evaluation[23]. Patients with a diagnosis of HCC were classified according to BCLC criteria[14].

All charts were reviewed for selection of study variables and outcomes during the study period, considering the data available for the first and the last consultations. The following variables were analyzed: age, sex, etiology of liver disease, Child-Turcotte-Pugh[24] score, Model for End-Stage Liver Disease score[25], use of statins, and serum levels of AFP.

The establishment of alcohol consumption was made through self-report of regular drinking, in a daily basis. Heavy use of alcohol was considered when alcohol consumption was greater than 40 g per day for men and women.

All patients received specialized treatment according to the etiology of liver disease and risk factors identified. Obese and/or NAFLD patients were referred to a Clinical Nutrition outpatient clinic. Those with alcohol dependency were headed for a public specialized psychiatric service and encouraged to attend support groups to stop drinking.

The patients were divided into two groups: with or without HCC. To compare the groups in terms of continuous variables with normal distribution, Student's *t* test was used. Mann-Whitney's test was used for comparison of variables with non-Gaussian distribution. For the comparison of categorical variables, the χ^2 test and Fisher's exact test were used. To evaluate the performance of AFP as a diagnostic tool, in patients with HCC, sensitivity, specificity, post-test probability, and likelihood ratio were calculated for different serum level ranges. These data were also represented as ROC curves and box plots generated with log-transformed values. Kaplan-Meier analysis was performed to examine cumulative incidence and survival in the 10-year follow-up period, with statistical significance calculated using the log-rank test. HR with 95%CI was calculated using a Cox regression model. Significance level was set at $\alpha = 5\%$.

Microsoft® Office Excel 2010 was used to store data, and the Statistical Package for the Social Sciences v. 22.0 (IBM® SPSS) was used for analysis of results. The normality of data distribution was determined using the Kolmogorov-Smirnov test. Quantitative variables with normal distribution were expressed as mean and standard deviation; variables with non-normal distribution were expressed as median and interquartile range. Simple and relative frequencies were used for categorical variables.

The research protocol was approved by the Research Ethics Committee at Universidade Federal de Ciências da Saúde de Porto Alegre (protocol 367511/2011, approval report 14/2014).

RESULTS

Of 738 eligible patients, the following were excluded: 105 with incomplete medical records, 88 with non-cirrhotic portal hypertension, 54 who were lost to follow-up, and 14 with HIV co-infection. Of the remaining 477 cirrhotic patients, 24 were diagnosed with HCC at the first clinic appointment and were thus excluded from the study. Thus, the final sample included 453 patients.

During follow-up, 75 patients (16.6%) were diagnosed with HCC. Median follow-up for this group was 15.7 mo. Among the 378 patients who did not develop HCC, median follow-up was 58.4 mo. Table 1 shows demographic and clinical data of the groups with and without HCC.

AFP levels were available for 343 patients, of which 57 had a diagnosis of HCC (16.7%). Baseline and end-of-study AFP levels were significantly different between patients with and without HCC. Stratification of serum AFP levels into four ranges (Figure 1 and Table 2) revealed a trend for AFP > 20 ng/mL to predict HCC. The highest diagnostic probability was observed for AFP levels ⩾ 50 ng/mL (Table 2). Accuracy of AFP was measured by the area under the ROC curve, whose value was 0.769 (95%CI: 0.70-0.84).

The 10-year cumulative incidence of HCC was analyzed using a Kaplan-Meier curve (Figure 2). During this 10-year period, 453 patients were followed-up. The estimated incidence of HCC was 2.6% in the 1st year, 15.4% in the 5th year, and 28.8% in the 10th year.

Among 419 patients who reported not using statins, 73 (17.4%) had HCC, *vs* only 1 patient among 34 using statins (2.9%), $P = 0.028$.

Survival analysis showed median survival of 234 mo (19.5 years) for the group without HCC and 17.6 mo (1.5 year) for patients with HCC. At the end of 10 years, none of the HCC patients were alive, whereas 55.8% of the patients without HCC were still living ($P < 0.001$, Figure 3).

BCLC staging of HCC at the time of diagnosis showed early stage tumors in 40 (53.3%) patients, intermediate stage tumors in 26 (34.6%) patients, and advanced tumors in 9 (12%) patients. Only 50.7% of individuals with HCC had undergone ultrasound surveillance every

Table 1 Demographic and clinical characteristics of cirrhotic outpatients attending a hospital clinic in the South of Brazil *n* (%)

Characteristic	HCC *n* = 75	Without HCC *n* = 378	*P* value
Age (yr)	54.9 ± 10.7	53.2 ± 12.2	0.23
Male sex	44 (58.7)	217 (57.4)	0.90
Cirrhosis etiology			0.27
HCV	35 (46.7)	132 (34.9)	
Alcohol	16 (21.3)	93 (24.6)	
HCV + alcohol	15 (20.0)	74 (19.6)	
HBV	2 (2.7)	3 (0.8)	
HBV + alcohol	0 (0.0)	5 (1.3)	
NAFLD	1 (1.3)	7 (1.8)	
Cryptogenic	1 (1.3)	12 (3.2)	
Other	5 (6.7)	52 (13.8)	
Baseline Child-Pugh	*n* = 74	*n* = 377	0.81
A	45 (60.8)	229 (60.7)	
B	22 (29.7)	119 (31.6)	
C	7 (9.5)	29 (7.7)	
End-of-study Child-Pugh	*n* = 75	*n* = 367	0.38
A	30 (40.0)	168 (45.8)	
B	25 (33.3)	127 (34.6)	
C	20 (26.7)	72 (19.6)	
Baseline MELD	*n* = 60	*n* = 292	
	11.2 (6; 25)	12.0 (6 ;27)	0.12
End-of-study MELD	*n* = 71	*n* = 330	
	13.4 (6; 31)	13.1 (6; 45)	0.65
Baseline AFP, ng/mL	*n* = 69	*n* = 261	
	6.1 (3.7; 19.0)	4.0 (1.5; 8.0)	0.01
End-of-study AFP, ng/mL	*n* = 57	*n* = 286	
	16 (4.9; 187.0)	4.0 (2.5; 7.8)	< 0.001

Other, autoimmune hepatitis, primary biliary cholangitis, hemochromatosis, primary sclerosing cholangitis, alpha-1 antitrypsin deficiency; MELD and AFP expressed as median and interquartile range (25%-75%). HCV: Hepatitis C virus; HBV: Hepatitis B virus; NAFLD: Nonalcoholic fatty liver disease; MELD: Model for End-Stage Liver Disease; AFP: Alpha-fetoprotein.

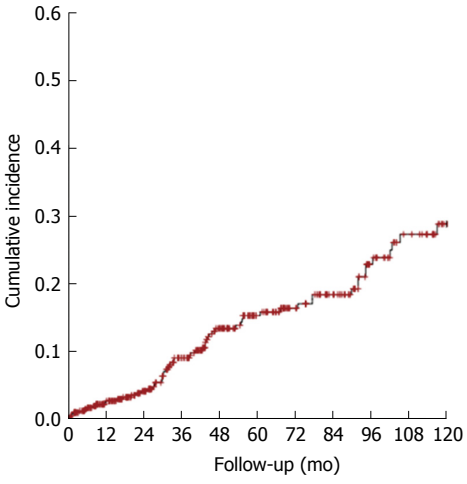

Figure 2 Ten-year cumulative incidence of hepatocellular carcinoma in cirrhotic outpatients.

6 mo. The analysis of tumor staging (early *vs* non-early) according to frequency of ultrasound surveillance showed a higher number of cases diagnosed with early stage tumors in patients with surveillance every 6 mo or less (P = 0.05). However, survival was not different between patients with early stage *vs* non-early stage tumors [HR = 0.54 (0.15-1.89), P = 0.33].

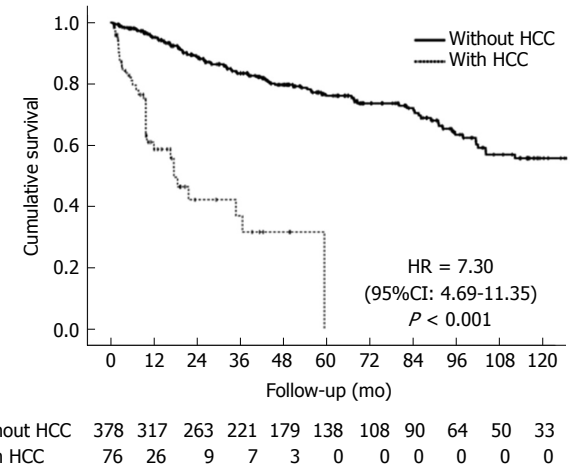

Figure 3 Kaplan-Meier cumulative survival curve in patients with hepatocellular carcinoma and 10-yr follow-up. HCC: Hepatocellular carcinoma.

DISCUSSION

Given the impact of HCC incidence on patients with cirrhosis, as well as the scarcity of data regarding this population in Latin America, we set out to determine the incidence of HCC and the role of a surveillance program in a cohort of cirrhotic patients attending an outpatient clinic in the South of Brazil, region predominantly composed by European descendants.

In this study, 75 of 453 (16.6%) patients developed HCC over 10 years - a higher incidence than the 8.1% observed in a cohort followed-up in the Southeast of Brazil[26]. Data from other countries also reveal higher incidences in various populations, such as 17.5% in the United States[27] and 27% in an Italian cohort[28]. Because Brazil is a country of continental proportions, the higher incidence detected in the South may be explained by geographic and/or racial heterogeneity, as well as specificities related to risk factors and access to health care services for screening, diagnosis, and follow-up. The predominance of the male sex and the mean age at diagnosis were similar to those described in other national[13,29,30] and international[12,31,32] studies.

In the present study, the etiology of liver disease was similar in patients with or without HCC, with HCV and alcohol being the main etiologic agents. In Brazil, chronic HCV infection and alcohol consumption are a major public health problem[33,34]; nevertheless, in some regions HBV is still an important cause of cirrhosis and HCC[35]. Llovet *et al*[36] have shown that in Europe and North America, HCV and alcohol are more frequently associated with HCC than HBV, differently than what occurs in Asia and Africa.

The establishment of surveillance programs for patients with chronic liver disease gained momentum after the study by Zhang *et al*[37], which showed that

Table 2 Pre-test probability, likelihood ratio, post-test probability, sensitivity, and specificity of alpha-fetoprotein ranges to predict hepatocellular carcinoma

AFP level (ng/mL)	Pre-test probability	LR+	Post-test probability	Sensitivity	Specificity
< 6.0	16.60%	0.50	9.1%	66.7%	66.3%
6-19.9	16.60%	1.00	16.6%	45.6%	89.3%
20-50	16.60%	1.31	20.8%	35.1%	96.1%
> 50	16.60%	10.03	66.8%	35.1%	96.1%

LR: Likelihood ratio; AFP: Alpha-fetoprotein.

performing abdominal ultrasound and AFP testing every 6 mo was capable of identifying patients in earlier stages of the disease, increasing survival in up to 37% of cases.

A major objective of follow-up of patients with cirrhosis is the screening and surveillance of HCC according to various consensus statements and guidelines[31,38-40]. Brazilian Society for Hepatology[41] has recently recommended the performance of abdominal ultrasounds every 6 mo, with measurement of AFP strictly in sites where physicians who are experienced in ultrasound are not available.

AFP was recognized in the 1970s as a tumor marker for diagnosis of HCC. This biomarker lost ground after many studies showed low sensitivity and specificity for detection of early stage tumors, leading to the exclusion of AFP dosing from the main consensus statements[31,38,40]. Despite the debate, the Asian Pacific Association for the Study of the Liver and the Japan Society of Hepatology kept the recommendation for serial AFP measurement, based on the understanding that this information could complement ultrasound surveillance[39,42]. In any case, it is well recognized that AFP may play an important prognostic role in the follow-up of these patients, since high AFP levels may signal more aggressive, multifocal tumors associated with venous portal thrombosis and/or metastases[43].

In the present study, serum AFP levels were higher in patients with HCC than in those without HCC. Nevertheless, the absence of a cutoff point with satisfactory sensitivity and specificity to detect HCC compromises the usefulness of this test. We believe that AFP dosing is more valuable to establish HCC prognosis than HCC diagnosis [44].

The incidence of HCC has been increasing globally, especially in the West, as a consequence of the obesity epidemic and of the growing number of patients with chronic liver disease[45]. In our cohort, cumulative HCC incidence was 2.6%, 15.4%, and 28.8% in the 1st, 5th, and 10th year respectively, which is similar to the data reported for other cirrhotic cohorts[27,46].

We observed that more patients were diagnosed with early stage HCC, as determined by BCLC criteria, in the presence of ultrasound monitoring at 6-mo intervals, even if survival was similar in this group, as compared to the group submitted to surveillance ultrasound at broader intervals. The difficulty in demonstrating increased survival associated with surveillance programs involves ethical issues relating to the performance of randomized, controlled trials. In this cohort, despite the lower survival of HCC patients *vs* those with cirrhosis and without HCC, there was no difference between those who underwent strict surveillance and those who did not. Sangiovanni *et al*[28] successfully demonstrated increased survival in cirrhotic patients with HCC undergoing surveillance between 1985 and 2011.

Interestingly, we observed a negative association between use of statins and development of HCC. Even though this might be a chance finding, given the low number of patients using this medication, previous studies have reported an effect of statins on patients with chronic liver disease[47-54]. All these previous works have described a protective effect. In fact, Chiu *et al*[48] described a reduction of 38% in the risk of HCC in patients from a surveillance program.

In conclusion, the findings of the present study underscore the high incidence of HCC in individuals with cirrhosis, highlighting the importance of stimulating the adherence of health care services and patients to surveillance programs.

COMMENTS

Background

Liver cancer is the second leading cause of cancer death worldwide and, among primary liver malignancies, hepatocellular carcinoma (HCC) accounts for 70%-85% of cases, and is associated with chronic liver disease and/or cirrhosis in 70%-90% of cases.

Research frontiers

All patients with chronic liver diseases are advised and guided to programmed screening and surveillance for HCC in order to allow early detection of nodular lesion.

Innovations and breakthrough

This study presents the incidence and impact of HCC in patients with cirrhosis in the South of Brazil and demonstrates that the adherence to surveillance programs are indeed effective for early diagnosis.

Applications

The present study underscore the high incidence of HCC in individuals with cirrhosis, highlighting the importance of stimulating the adherence of health care services and patients to surveillance programs.

Terminology

Screening and surveillance programs are usually done through periodic

abdominal ultrasound every 6 mo and may be associated with serum alpha-fetoprotein. Computed tomography or abdominal magnetic resonance imaging with contrast were performed in all patients with evidence of nodular lesion measuring ⩾ 1 cm in diameter on ultrasound.

Peer-review

This retrospective cohort chart review study does a good job regarding the incidence of HCC in the South of Brazil and displays the need for strict surveillance programs favoring early diagnosis and prognosis. It is very well-written and the Discussion interprets the findings in view of the results obtained in this and in past studies on this topic. The study gives significant information and it may possibly help clinicians to develop further studies.

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ORIGINAL ARTICLE

Prospective Study

Genotype specific peripheral lipid profile changes with hepatitis C therapy

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Author contributions: Seetharam AB designed the study; Pedersen MR, Patel A and Backstedt D performed the data collection; Choi M analyzed the data; Pedersen MR wrote the paper; and Seetharam AB revised the manuscript for final submission.

Institutional review board statement: This study was reviewed and approved by the Banner University Medical Center - Phoenix Institutional Review Board.

Informed consent statement: The need for informed consent for the prospective study was waived by our institutional review board.

Conflict-of-interest statement: Anil Seetharam has served as a speaker and accepted speaker's fees for Gilead and Janssen.

Data sharing statement: No additional data are available.

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Abstract

AIM

To evaluate magnitude/direction of changes in peripheral lipid profiles in patients undergoing direct acting therapy for hepatitis C by genotype.

METHODS

Mono-infected patients with hepatitis C were treated with guideline-based DAAs at a university-based liver clinic. Patient characteristics and laboratory values were collected before and after the treatment period. Baseline demographics included age, ethnicity, hypertension, diabetes, hyperlipidemia, treatment regimen, and fibrosis stage. Total cholesterol (TCHOL), high density lipoprotein (HDL), low density lipoprotein (LDL), triglycerides (TG), and liver function tests were measured prior to treatment and ETR. Changes in lipid and liver function were evaluated by subgroups with respect to genotype. Mean differences were calculated for each lipid profile and liver function component (direction/magnitude). The mean differences in lipid profiles were then compared between genotypes for differences in direction/magnitude. Lipid profile and liver function changes were evaluated with Levene's test and student's *t* test. Mean differences in lipid profiles

were compared between genotypes using ANOVA, *post hoc* analysis *via* the Bonferroni correction or Dunnett T3.

RESULTS

Three hundred and seventy five patients enrolled with 321 (85.6%) achieving sustained-viral response at 12 wk. 72.3% were genotype 1 (GT1), 18.1% genotype 2 (GT2), 9.7% genotype 3 (GT3). Baseline demographics were similar. Significant change in lipid profiles were seen with GT1 and GT3 (ΔGT1, p and ΔGT3, p), with TCHOL increasing (+5.3, P = 0.005 and +16.1, P < 0.001), HDL increasing (+12.5, P < 0.001 and +7.9, P = 0.038), LDL increasing (+7.4, P = 0.058 and +12.5, P < 0.001), and TG decreasing (-5.9, P = 0.044 and -9.80 P = 0.067). Among genotypes (ΔGT1 v. ΔGT2 v. ΔGT3, ANOVA), significant mean differences were seen with TCHOL (+5.3 v. +0.1 v. +16.1, P = 0.017) and HDL (+12.3 v. +2 v. +7.9, P = 0.040). Post-hoc, GT3 was associated with a greater increase in TCHOL than GT1 and GT2 (P = 0.028 and P = 0.019).

CONCLUSION

Successful DAA therapy results in increases in TCHOL, LDL, and HDL and decrease in TG, particularly in GT1/GT3. Changes are most pronounced in GT3.

Key words: Hepatitis C genotypes; Lipids; Metabolic syndrome

Core tip: Different genotypes of the hepatitis C virus (HCV) are associated with differing levels of hepatic steatosis, with genotype 3 (GT3) having the strongest direct association. In this investigation, change in peripheral lipid panels during direct-acting antiviral therapy were assessed in a large HCV treatment cohort with respect to genotype. Total cholesterol in patients with GT3 increased significantly during treatment compared to other genotypes. Associated steatosis and differing lipid kinetics may influence response rates to direct acting therapy and may also influence genotype specific risks of hepatic and systemic complications.

Pedersen MR, Patel A, Backstedt D, Choi M, Seetharam AB. Genotype specific peripheral lipid profile changes with hepatitis C therapy. *World J Gastroenterol* 2016; 22(46): 10226-10231 Available from: URL: http://www.wjgnet.com/1007-9327/full/v22/i46/10226.htm DOI: http://dx.doi.org/10.3748/wjg.v22.i46.10226

INTRODUCTION

Chronic hepatitis C virus (HCV) infection is associated with hepatic steatosis and hypocholesterolemia[1]. HCV utilizes peripheral lipid metabolism pathways including hepatocyte very-low-density lipoprotein for viral assembly and requires several apolipoproteins for production of infective particles[2,3]. Chronic HCV increases levels of hepatic steatosis independent of other classical risk factors for non-alcoholic fatty liver disease[1]. The magnitude of this effect varies by genotype. Genotype 3 (GT3) in particular is associated with a primary hepatic steatosis that appears to correlate directly with viral load while genotype 1 (GT1) and 2 (GT2) have less pronounced secondary steatosis related to increased insulin resistance and body mass index[1-3].

Successful clearance of HCV viremia with immunomodulatory therapy (pegylated interferon and ribavirin) has been associated with a rise in serum total cholesterol (TCHOL) and low density lipoprotein (LDL)[4]. In the post-interferon era, Meissner *et al*[5] demonstrated that patients with chronic HCV GT1 treated with sofosbuvir and ribavirin had increases in their serum LDL and decrease in serum triglyceride (TG).

Peripheral lipid profile changes during treatment for non-genotype 1 infection with DAA therapy are thus far uncharacterized. The purpose of this study was to examine effects of DAA therapy on serum TCHOL and peripheral lipid components and evaluate differences in responses among HCV genotypes.

MATERIALS AND METHODS

Ethical considerations

This study was reviewed and approved by the Banner University Medical Center - Phoenix Institutional Review Board. While data was collected prospectively, all patients were monitored in accordance with American Association for the Study of Liver Disease and Infectious Diseases Society of America hepatitis C guidelines. As there was no deviation from standard of care, need for informed consent for the prospective study was waived by the institutional review board.

Study design

We performed a prospective cohort study of consecutively-enrolled mono-infected HCV patients achieving sustained virologic response at 12 wk (SVR12) treated at Banner University of Arizona Medical Center in Phoenix, Arizona from January 2014 to November 2015. After institutional review board approval, outpatient medical records were reviewed and variables of interest tabulated.

Treatment regimens

All patients were treated according to the American Association for the Study of Liver Diseases and the Infectious Diseases Society of America guidelines active at the time of treatment initiation. Consecutively enrolled subjects received one of the following treatment regimens: pegylated Interferon alfa 2a + sofosbuvir + ribavirin; sofosbuvir + ribavirin; sofosbuvir + simeprevir; or ledipasvir + sofosbuvir. When applicable, ribavirin was dosed by weight, 1000 mg total daily dose if weight < 75 kg or 1200 mg total daily dose if > 75 kg.

Baseline demographics

Baseline demographics were recorded prior (within 30 d prior) to regimen initiation including: age, ethnicity, treatment regimen, and fibrosis stage as well as the presence of hypertension, diabetes, and hyperlipidemia prior to treatment. Liver function tests [including alanine aminotransferase (ALT), total bilirubin, and albumin] as well as the protime/International Normalized Ratio (INR) were recorded before and after treatment. Fibrosis stage was assessed *via* FibroSure serum testing (Laboratory Corporation of American, Herdon, Virginia) or liver biopsy. Presence of hypertension, diabetes, and hyperlipidemia prior to treatment was determined based on documentation of formal diagnosis in the medical record and concomitant medications regimens indicative of the diagnosis (*e.g.*, insulin use was considered indicative of diabetes). Medication lists were monitored prior to and after the end of treatment for any new or discontinued medications.

Metabolic measurements

Per protocol in the Liver Clinic at Banner University of Arizona Medical Center in Phoenix, Arizona, fasting lipid profiles, including TCHOL, high density lipoprotein (HDL), LDL, and TG low density were measured prior to treatment and at the end of treatment (within one month). Testing was performed *via* commercially available assays with Laboratory Corporation of America (Phoenix, Arizona) and Sonora Quest Laboratories (Tempe, Arizona). Metabolic variables were measured at two different time points: (1) prior to treatment (start); and (2) completion of treatment regimen (end of treatment response-ETR).

Response to treatment

End of treatment response and SVR12 were biochemically defined by an undetectable or below the lower limit of quantification HCV RNA PCR quantitative assay (Laboratory Corporation of America, Phoenix, Arizona and Senora Quest Laboratories, Tempe, Arizona). Liver enzyme and function tests (including ALT, total bilirubin, and albumin) as well as the protime/INR were recorded before and after treatment using standardized assays at these same laboratories.

Statistical analysis

Baseline demographics by genotype were compared using descriptive statistics including chi square analysis for categorical variables and one-way ANOVA for continuous variables. Patients not achieving SVR12 were excluded. Changes in lipid profile and liver function tests were evaluated for significance with Levene's test of equal variances and the paired *t* test.

Mean differences were calculated for each component of the lipid profile within each genotype from treatment start to end. Mean differences were compared among genotypes for differences in direction/magnitude by total population and patients with cirrhosis and non-cirrhosis independently using ANOVA. When significant differences were present, post-hoc analysis was performed using the Bonferroni correction (when equal variances assumed) or Dunnett T3 (when equal variances not assumed) to determine the significantly different pairs. Significance was set at $P < 0.05$. Subgroup analysis of the changes in lipid profiles was performed separately for cirrhotics and non-cirrhotics by genotypes.

SPSS software (Statistical Product and Services Solutions, version 22, Chicago, IL, United States) was used for statistical analyses. All authors had access to the study data and had reviewed and approved the final manuscript.

RESULTS

Study population

A total of 375 patients were enrolled, of which 321 (85.6%) achieved SVR12 and were included in the study. Of these, 232 (72.3%) had G1, 58 (18.1%) had G2, and 31 (9.7%) had G3. Baseline demographics (Table 1) were similar, including prevalence of diabetes, hypertension, and hyperlipidemia. Incidence of cirrhosis was significantly higher in the G2 group (56.9%) than the G3 group (45.2%). During DAA therapy, serum albumin increased and ALT decreased across all genotypes (all $P < 0.01$). Serum INR improved only in G2 ($P < 0.001$) (Table 2).

Changes in peripheral lipid profiles during DAA therapy stratified by cirrhosis

On analysis by genotype, significant changes in lipid profiles were seen with GT1 and GT3. In GT1, TCHOL increased from 156.9 to 162.2 mg/dL ($P = 0.005$), LDL increased from 80.2 to 87.6 mg/dL ($P = 0.058$), HDL increased from 51.6 to 63.6 mg/dL ($P < 0.001$), and TG decreased from 114.6 to 108.7 mg/dL ($P = 0.044$). In GT3, TCHOL increased from 141.5 to 157.6 mg/dL ($P < 0.001$), HDL increased 45.4 to 53.3 mg/dL ($P = 0.038$) and LDL increased from 81.4 to 93.9 mg/dL ($P < 0.001$). No significant changes were seen for GT2. These trends were consistent irrespective of the presence or absence of cirrhosis (Table 3). In the total population, absolute pre-treatment TCHOL was lowest in GT3 ($P = 0.032$), however similar between all three groups at the end of treatment ($P = 0.81$).

Differential effects in peripheral lipid profile based on genotype

On post-hoc comparison of the mean differences in lipid profiles between genotype (GT1 *vs* GT2 *vs* GT3, p), significant changes were seen in the total population with TCHOL (+5.3 mg/dL *vs* +0.1 mg/dL *vs* +16.7 mg/dL, $P = 0.017$) and HDL (+12.3 mg/dL *vs* +2 mg/dL *vs* +7.9 mg/dL, $P = 0.049$) (Table 4). GT3 was associated with a greater increase in TCHOL than both GT1 ($P = 0.028$) and GT2 ($P = 0.019$). There was no significant difference in HDL changes between paired

Table 1 Baseline demographics *n* (%)

	All	Genotype 1	Genotype 2	Genotype 3	*P* value
Number of patients	321 (100)	232 (72.3)	58 (18.1)	31 (9.7)	
Age (yr)	57.7 ± 10.3	58.9 ± 9.5	55.5 ± 12.1	54.6 ± 10.8	0.009
Gender (male)	221 (68.8)	160 (59.4)	41 (61.2)	20 (64.5)	0.845
Ethnicity					
Caucasian	229 (71.3)	171 (73.7)	40 (70.0)	18 (58.1)	0.018
African American	19 (5.9)	18 (7.8)	1 (1.7)	0 (0.0)	
Hispanic	55 (17.1)	32 (13.8)	13 (22.4)	10 (32.3)	
Asian	16 (5.0)	9 (3.9)	4 (6.9)	3 (9.7)	
Other	2 (0.6)	2 (0.9)	0 (0.0)	0 (0.0)	
Diabetes	79 (24.6)	59 (25.4)	14 (24.2)	6 (19.4)	0.593
Hypertension	132 (41.1)	105 (45.3)	22 (37.9)	5 (16.1)	0.086
Hyperlipidemia	59 (18.4)	44 (19.0)	12 (20.7)	3 (9.7)	0.453
Cirrhotic	150 (46.7)	103 (44.4)	33 (56.9)	14 (45.2)	< 0.001
Treatment					
IFN + SOF + RBV		25 (10.8)	0 (0.0)	0 (0.0)	
SOF + RBV		57 (24.6)	58 (100)	31 (100)	
SOF + SMV		42 (19.0)	0 (0.0)	0 (0.0)	
SOF + LDV		140 (60.3)	0 (0.0)	0 (0.0)	

IFN: Pegylated interferon; SOF: Sofosbuvir; RBV: Ribavirin; SMV: Simeprevir; LDV: Ledipasvir.

Table 2 Changes in liver function tests

	Genotype 1			Genotype 2			Genotype 3		
	Start Tx	End Tx	*P* value	Start Tx	End Tx	*P* value	Start Tx	End Tx	*P* value
Albumin (g/dL)	3.5 ± 0.6	3.8 ± 0.6	< 0.001	3.5 ± 0.5	3.7 ± 0.6	< 0.002	3.4 ± 0.5	3.7 ± 0.6	< 0.001
ALT (U/L)	70.2 ± 58.3	31.7 ± 38.7	< 0.001	68.7 ± 48.3	26.4 ± 17.8	< 0.001	101.9 ± 61.6	30.7 ± 30.3	< 0.001
Total Bilirubin (mg/dL)	1.0 ± 0.8	0.9 ± 0.8	0.008	1.0 ± 0.9	0.9 ± 0.9	0.202	1.1 ± 0.7	1.1 ± 0.8	0.904
INR	1.1 ± 0.2	1.1 ± 0.3	0.112	1.1 ± 0.1	1.0 ± 0.1	< 0.001	1.1 ± 0.2	1.1 ± 0.3	0.509

ALT: Alanine transaminase; INR: International Normalized Ratio.

Table 3 Changes in lipid profile by genotype in patients with cirrhosis and non cirrhosis

	Total population			Non-cirrhotics			Cirrhotics		
	Start Tx	End Tx	*P* value	Start Tx	End Tx	*P* value	Start Tx	End Tx	*P* value
Genotype 1									
T Chol	156.9 ± 36.4	162.2 ± 41.0	0.005	169.9 ± 33.2	175.7 ± 38.2	0.046	146.4 ± 35.6	151.4 ± 40.0	0.052
HDL	51.6 ± 18.5	63.9 ± 32.8	< 0.001	54.4 ± 19.5	72.7 ± 36.4	< 0.001	49.3 ± 17.2	56.9 ± 27.6	0.002
LDL	80.2 ± 28.8	87.6 ± 62.7	0.058	87.1 ± 28.1	91.8 ± 31.6	0.091	74.6 ± 28.2	84.2 ± 79.1	0.15
TG	114.6 ± 56.0	108.7 ± 56.0	0.044	117.3 ± 55.1	111.6 ± 57.6	0.27	112.5 ± 56.6	106.4 ± 56.5	0.070
Genotype 2									
T Chol	162.9 ± 35.8	163.0 ± 32.9	0.99	174.2 ± 29.8	173.7 ± 27.5	0.91	148.2 ± 37.7	148.9 ± 34.1	0.90
HDL	52.8 ± 18.6	54.8 ± 20.0	0.39	54.9 ± 17.4	55.3 ± 17.4	0.88	50.0 ± 19.7	54.0 ± 23.0	0.28
LDL	86.7 ± 34.0	87.4 ± 30.2	0.82	95.3 ± 31.8	96.4 ± 27.0	0.82	75.4 ± 33.4	75.7 ± 30.0	0.93
TG	114.3 ± 64.1	112.4 ± 65.8	0.73	120.6 ± 68.4	124.0 ± 74.2	0.72	106.1 ± 59.7	97.1 ± 48.6	0.078
Genotype 3									
T Chol	141.5 ± 38.4	157.6 ± 34.4	< 0.001	161.4 ± 35.5	181.5 ± 23.5	0.001	125.1 ± 32.5	137.9 ± 29.1	0.025
HDL	45.4 ± 15.1	53.3 ± 16.6	0.038	49.3 ± 14.0	53.2 ± 14.3	0.37	42.2 ± 15.3	53.3 ± 18.2	0.065
LDL	81.4 ± 32.1	93.9 ± 34.9	< 0.001	92.5 ± 26.1	110.4 ± 21.4	0.003	72.2 ± 33.6	80.3 ± 37.9	0.045
TG	108.6 ± 47.5	98.8 ± 41.2	0.047	119.0 ± 58.6	112.6 ± 47.1	0.36	100.1 ± 33.4	87.5 ± 31.2	0.12

All values expressed as mean ± SD. All units are in mg/dL. T Chol: Total cholesterol; HDL: High density lipoprotein; LDL: Low density lipoprotein; TG: Triglycerides; Start Tx: Prior to treatment; End Tx: End of treatment.

genotypes on post-hoc analysis. In non-cirrhotics, the trends were similar, with changes in TCHOL (+5.8 mg/dL *vs* -0.4 mg/dL *vs* +12.8 mg/dL, $P = 0.066$) and in HDL (+18.2 mg/dL *vs* +0.5 mg/dL *vs* +11.1 mg/dL , $P = 0.008$). GT3 was associated with a greater increase in TCHOL than GT2 ($P = 0.048$). Additionally, GT1 was associated with a greater increase in HDL than GT2 ($P = 0.012$). In cirrhotics, there were no differences seen in the changes in lipid profiles between genotype (Table 4).

DISCUSSION

Chronic hepatitis C infection is closely linked to lipid metabolism *via* shared use of the classical secretory

Table 4 Differential effect in lipid profile by genotype in patients with cirrhosis and non-cirrhosis

	All patients				Non-cirrhotics				Cirrhotics			
	GT1	GT2	GT3	*P* value	GT1	GT2	GT3	*P* value	GT1	GT2	GT3	*P* value
T Chol	5.3	0.1	16.1	0.017	5.8	-0.4	12.8	0.066	5.0	0.7	20.1	0.39
HDL	12.3	2.0	7.9	0.049	18.2	0.5	11.1	0.008	7.6	4.0	4.0	0.69
LDL	-5.9	-1.1	-11.9	0.550	4.6	1.0	8.1	0.150	9.7	0.3	17.9	0.82
TG	7.4	2.1	21.2	0.130	-5.7	3.4	-12.6	0.650	-6.1	-9.0	-6.4	0.76

All values expressed as mean ± SD. All units are in mg/dL. T Chol: Total cholesterol; HDL: High density lipoprotein; LDL: Low density lipoprotein; TG: Triglycerides; Start Tx: Prior to treatment; End Tx: End of treatment.

pathway[2,6-8]; however, specific viral/host protein interactions require further elucidation[9]. The link between lipid metabolism and HCV was of particular interest during the era of interferon-based treatment, when components of the metabolic syndrome were identified as negative predictors of achieving SVR[10]. However, medications aimed to optimize metabolic syndrome conditions prior to antiviral treatment, such as the PPAR-γ agonist pioglitazone and the 3-hydroxy-3-methylglutaryl-coenzyme A reductase inhibitor fluvastatin, yielded no significant improvement in SVR rates with pegylated-interferon based therapy[11-15].

Increase in serum cholesterol levels during HCV infection has been demonstrated with successful HCV treatment. Previously identified changes in peripheral lipid profiles included increases in TCHOL and LDL[4,16-18]. It has also been associated with increases in HDL[16,19], though not in all studies[4,17]. In our study, we found that treatment with DAA resulted in increases in TCHOL, LDL, HDL as well as a decrease in TGs in GT1, and increases in TCHOL, HDL, and LDL in GT3. Changes occurred irrespective of established pre-treatment cirrhosis. GT2 did not have any significant changes in lipid particle concentration. Meissner *et al*[5], in his 2015 study of 55 GT1 patients treated sofosbuvir and ribavirin without interferon, also reported an increase in LDL and decrease in TG, however did not find any significant changes in TCHOL or HDL.

Research on the differences in lipid metabolism between genotypes of hepatitis C have focused mainly on steatosis. A review of 14 studies from 1997 through 2004 estimated the prevalence of steatosis in patients with chronic HCV to be 40%-86% (mean approximately 55%), compared to a baseline of approximately 20%-30% of patients in the United States and other western countries without the virus[20]. GT3 in particular is associated with an increased incidence and severity of hepatic steatosis, estimated at 73% in this same study[20]. This steatosis, in contrast to GT1, is independent of any co-existing insulin resistance or obesity[1-3]. Hypocholesterolemia has been found to various extents across genotypes and is also known to be more pronounced in patients with GT3[21,22]. This was also seen in our study. While the exact mechanism is unknown, it likely relates to alterations of the distal cholesterol synthesis pathway[23].

Relatively little is known about the different effect of genotypes on the magnitude of lipid profile changes from start to end of treatment. One study identified that only GT3 treatment responders, but not non-responders or any GT1 patients, demonstrate an increase in serum cholesterol[24]. A more recent investigation found significant post-therapeutic increases in TCHOL, LDL, HDL, and TGs, but greater increases in HDL in patients with GT2[16]. All of the aforementioned analyses primarily included subjects treated with interferon and ribavirin.

Our study is the first to compare mean differences in lipid profile components between GT1, GT2, and GT3 after treatment with DAAs. We found patients with GT3 to have the most profound changes in lipid profile, characterized by a significantly greater increase in TCHOL than both GT1 and GT2 across the entire population. This was also reflected in our cirrhotic and non-cirrhotic subgroups. On the other hand, the lipid profiles of patient with GT2 were relatively unaffected by treatment in the cirrhotics, non-cirrhotics, and combined analysis, though the reason for this is unclear. Nonetheless, GT2 experienced improvement in synthetic function congruent with GT1 and GT3, as noted by an increase in albumin, and perhaps even better than GT1 and GT3 as evidenced by the significant improvement in INR not seen in the other two genotypes.

Unfavorable lipid changes are most pronounced in those with HCV GT3 infection who are successfully treated with DAAs. This may be a reflection of the more severe hypocholesterolemia that affects this group prior to treatment. Here, the increase in TCHOL brought it into a range that was not significantly different than either GT1 or GT2. The long term effect of the change in lipid profiles on cardiovascular risk and mortality is unknown, though it has been demonstrated that that patients successfully treated for HCV have lipid profiles may return to levels that potentially increase coronary disease risk[4]. Long term follow-up of these patients is warranted to correlate these changes with clinical outcomes.

COMMENTS

Background

Different genotypes of the hepatitis C virus (HCV) are associated with differing levels of hepatic steatosis, with genotype 3 having the strongest direct association.

Innovations and breakthroughs

In this investigation, change in peripheral lipid panels during direct-acting antiviral therapy were assessed in a large HCV treatment cohort with respect to genotype. Total cholesterol in patients with genotype 3 increased significantly during treatment compared to other genotypes. Associated steatosis and differing lipid kinetics may influence response rates to direct acting therapy and may also influence genotype specific risks of hepatic and systemic complications.

Peer-review

The authors deal with a very interesting topic concerning the genotype specific peripheral lipid changes during daa therapy are uncharacterized. The manuscript is well written and although only four markers are used to support the scientific hypothesis data are promising.

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ORIGINAL ARTICLE

Prospective Study

Long-term prognostic impact of circulating tumour cells in gastric cancer patients

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Author contributions: Ito H conceived and designed the experiments, collected blood samples, performed the experiments, managed clinical examination, performed treatment of individual patients, delivered clinical data, and performed the statistical analysis and data interpretation; Sato J, Tsujino Y, Gohda K, and Murakami K analysed the blood cells in the peripheral blood samples from the patients; Yamaguchi N and Onimaru M managed clinical examination, performed treatment of individual patients, and delivered clinical data; Kimura S participated in the study design, maintained the equipment, including the test tubes, and performed data interpretation; Ohmori T and Ishikawa F participated in the study design and performed data interpretation; Inoue H participated in the study design, performed treatment of individual patients, and performed data interpretation; all authors have read and approved the final manuscript.

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Abstract

AIM

To analyse the long-term prognostic impact of circulating tumour cells (CTCs) in gastric cancer patients who underwent surgery.

METHODS

A 7.5-mL peripheral vein blood sample was obtained from each patient with treatment-negative gastric adenocarcinoma before surgery. OBP-401, a telomerase-specific, replication-selective, oncolytic adenoviral agent carrying the green fluorescent protein gene, was used to label CTCs. Correlations between the number of CTCs and clinical end points were evaluated.

RESULTS

The median follow-up period of the surviving patients with gastric cancer was 60 mo. The CTC number tended to increase concomitantly with disease progression. The overall survival of patients with more than five CTCs in 7.5-mL of peripheral blood was lower than that of patients with five or less CTCs, although the difference was not significant (P = 0.183). A significant difference in relapse-free survival was found between patients with more than five and those with five or less CTCs (P = 0.034).

CONCLUSION

A lower number of CTCs was correlated with higher relapse-free survival rates in patients. Detection of CTCs using OBP-401 may be useful for predicting prognosis in gastric cancer.

Key words: Circulating tumour cells; Gastric cancer; Surgery; Telomerase; Prognosis

Core tip: We show the long-term prognostic impact of circulating tumour cells (CTCs) in 65 patients with gastric cancer in this report. OBP-401, a telomerase-specific, replication-selective, oncolytic adenoviral agent carrying the green fluorescent protein gene, was used to label CTCs. A lower number of CTCs was correlated with higher relapse-free survival rates in patients with gastric cancer.

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INTRODUCTION

The presence of circulating tumour cells (CTCs) in peripheral blood indicates a systemic disease stage in various malignancies, as CTCs are thought to be the source of haematogenous metastasis[1]. Detection of CTCs in peripheral blood is useful for prognosis, monitoring of disease progression, and evaluation of treatment efficacy in breast[2], lung[3], prostate[4], skin[5], colon[6], gastric[7], and esophageal cancer[8,9]. Although various methods have been developed to detect CTCs, the most commonly used techniques for their enrichment and characterisation are density gradient separation[10], immunomagnetic separation[11], flow cytometry[12], direct enrichment by filtration[13], and microchip technology[14]. The CellSearch System (Veridex, LLC, Raritan, NJ, United States), which is based on immunomagnetic cell enrichment, is one of the most widely used techniques for automated enrichment and detection of CTCs[15,16]. The advantage of immunomagnetic cell separation is that CTCs can be directly visualised under a microscope. In the CellSearch assay, cells detected with antibodies against epithelial markers (*e.g.*, epithelial cell adhesion molecules, or EpCAMs) are classified as CTCs. During the epithelial-mesenchymal transition (EMT), an important process that occurs in CTCs[17], expression of epithelial surface markers is reduced. Thus, systems that rely on epithelial markers may fail to detect CTCs undergoing EMT[18]. Methodologies based on direct enrichment by filtration may circumvent this issue to some extent, although cells detected in this manner often lack tumourigenicity.

Increased telomerase activity is a common characteristic of malignant tumours, and telomerase plays important roles in carcinogenesis and disease progression[19]. OBP-401 (TelomeScan, Oncolys BioPharma, Tokyo, Japan) is a telomerase-specific, replication-selective modified viral agent in which the human telomerase reverse transcriptase (*TERT*) gene promoter is inserted into the E1 region, and the green fluorescent protein (*GFP*) gene is placed under the control of the cytomegalovirus promoter in the E3 region as a marker of viral replication[20]. Thus, OBP-401 only proliferates in viable cells with high telomerase activity and provides a fluorescent label that allows tumour cells to be labelled, regardless of their epithelial marker expression profiles. We previously used OBP-401 to detect cells with high telomerase activity in blood samples of healthy and treatment-negative gastric cancer patients before surgery. We took 7.5-mL peripheral blood samples from cancer patients before surgery and healthy volunteers. We detected viable GFP-positive CTCs in the blood samples after incubation with OBP-401. This revealed that in patients with gastric cancer, a greater proportion of "high telomerase activity" cells was associated with a significantly poorer prognosis[21]. In this report, we describe the final long-term results (median follow-up time of five years) of this initial study, which demonstrate that the OBP-401-dependent CTC assay has clinical utility in patients with gastric cancer.

MATERIALS AND METHODS

Patients and healthy volunteers

This report was the final analysis of our prospective preliminary study on CTCs from 65 patients with treatment-negative gastric adenocarcinoma who underwent surgery at the Digestive Disease Center of the Showa University Northern Yokohama Hospital between April 2010 and May 2011, and from whom we extracted peripheral blood samples before treatment. The inclusion criteria were: (1) histologically proven adenocarcinoma of the stomach by endoscopic biopsy; (2) clinical solitary tumour; (3) no prior endoscopic resection, chemotherapy, or radiotherapy; (4) aged 20-80 years; (5) Eastern Cooperative Oncology Group performance status (Oken *et al*[22], 1982) of 0 or 1; (6) sufficient organ function; and (7) written informed consent. The exclusion criteria were: (1) synchronous or metachronous malignancy; (2) pregnant or breast-feeding women; (3) active or chronic viral hepatitis; (4) active bacterial or fungal infection; (5) diabetes mellitus; (6) systemic administration of corticosteroids; and (7) unstable hypertension. The pathologic stage of the disease was determined according to the seventh edition of the American Joint Committee on Cancer/ International Union Against Cancer TNM classification system[23]. The depth of the tumour invasion in four patients without gastrectomy and the regional lymph node status of seven patients without sufficient lymphadenectomy were surgically diagnosed.

All of the patients were checked regularly in our hospital every 3 mo for the first 3 years post-operation, and every 6 mo for the following two post-operative years. The patients also underwent endoscopy and computed tomography at least once a year, according to their disease stage and course. Healthy volunteers were also recruited as controls. All healthy volunteers were employees of Sysmex Corporation, which included seven men (mean age, 31.4 years; range, 24-39 years) and three women (mean age, 33.7 years; range, 26-48 years). All volunteers underwent medical check-ups upon employment and annually; check-ups included medical interviews, auscultation, chest radiography, and blood and urine analyses. In addition, individual interviews were conducted before sample collection; any volunteer who was currently receiving medical treatment, pregnant, or breast-feeding or who had donated blood within the past month was excluded.

Telomerase-specific viral agent

OBP-401, a telomerase-specific, replication-selective adenoviral agent in which the *TERT* promoter element drives the expression of the *EIA* and *EIB* genes and into which the *GFP* gene is integrated, was used. The sensitivity and specificity of the assay using OBP-401 have been reported previously[24]. Viral samples were stored at -80 ℃.

Sample preparation and immunostaining

Details of sample preparation and assay were described in our previous report[21]. A 7.5-mL peripheral vein blood sample was obtained from each patient before surgery and from each healthy volunteer. The samples were drawn into tubes containing citric acid, phosphoric acid, and dextrose, and stored at 4 ℃. The assay was started within 48 h of sample collection. The samples were centrifuged for 5 min at 540 × *g*, and the plasma phase was removed. The cells were then washed four times with phosphate-buffered saline (PBS) and twice with Roswell Park Memorial Institute medium. The samples were infected with 4×10^8 plaque-forming units of OBP-401 virus by incubation in the medium for 24 h at 37 ℃. Dead cells were stained with the red-fluorescent reactive dye L23102 (Life Technologies, Carlsbad, CA, United States), OBP-401 was inactivated, and the cells were fixed with 2% paraformaldehyde for 20 min at room temperature. The samples were treated with a surface-active agent (Emalgen 2025G; Kao Chemicals, Tokyo, Japan) for 10 min at 40 ℃ to degrade red blood cells. Phycoerythrin-labelled anti-human CD45 antibody (BioLegend, San Diego, CA, United States) was diluted 1:5, and Pacific Blue-labelled anti-human CD326 (EpCAM) antibody (BioLegend) was diluted 1:10 in PBS containing 2% foetal bovine serum. Cells were incubated with the diluted antibodies for 30 min at 25 ℃. After being washed with PBS containing 2% foetal bovine serum, the cells were mounted on two glass slides for microscopic analysis.

GFP fluorescence intensity of cultured cancer cell lines

Approximately 30000 cultured cells were added into 7.5-mL blood samples from healthy volunteers, which were mixed with various cancer cell lines: A549 (lung carcinoma), HepG2 (hepatocellular carcinoma), HEC-1 (endometrial carcinoma), KATO-III (gastric carcinoma), SBC-3 (small cell lung carcinoma), LNCaP (prostate adenocarcinoma), MDA-MB-468 (breast carcinoma), and OVCAR-3 (ovarian carcinoma). The cell lines were cultured according to the vendor's specifications.

Determination of GFP fluorescence intensity and cell size threshold

The threshold for GFP fluorescence intensity and cell size (diameter) were set based on the values from samples of healthy volunteers and the patients with gastric cancer by using receiver operating characteristic (ROC) analysis. The blood samples were subjected to the CTC detection assay, and GFP-positive cells were scored by fluorescence microscopy.

Cell counting and analysis

All detectable GFP-positive cells on the two slides were analysed under a computer-controlled fluorescence microscope (IX71, Olympus, Tokyo, Japan); the observer was blinded to the sample details. Cells with fluorescence intensities and diameters exceeding the threshold were scored as GFP-positive. Both EpCAM-positive and EpCAM-negative subpopulations

Table 1 Patient characteristics and clinical findings

Variable		Number of patients
Sex	Male	46
	Female	19
Age (yr; mean, range)		58.8; 33-76
Gastrectomy	Distal	29
	Total	32
	None	4
Surgical curability	R0	57
	R1	0
	R2	8
Clinical course	Survival without relapse	47
	Survival after relapse	2
	Survival after non-curative surgery	1
	Decease	15
Recurrence site (including overlap)	None	47
	Remnant stomach	1
	Hematogenous	5
	Lymphatic	4
	Peritoneal dissemination	5
	Non-curative surgery	8
Postoperative chemotherapy	None	37
	Adjuvant chemotherapy	11
	Adjuvant and therapeutic chemotherapy	9
	Therapeutic chemotherapy after non-curative surgery	8
TNM stage	I	40
	II	6
	III	10
	IV	9
Depth of tumour invasion	T1	36
	T2	8
	T3	9
	T4	12
Lymph node metastasis	N0	39
	N1	5
	N2	6
	N3	15
Distant metastasis	M0	56
	M1	9
Main histological type	Differentiated	25
	Undifferentiated	40
Lymphatic invasion	L0	35
	L1	26
	LX	4
Venous invasion	V0	35
	V1-2	26
	VX	4

were found in these cells, consistent with the finding that tumour cells undergoing EMT can be EpCAM-negative[18].

Institutional review board statement and clinical trial registration

The study was approved by the Institutional Review Board of the Showa University, Northern Yokohama Hospital (No. 0903-03). This study was registered with the University Hospital Medical Information Network in Japan, UMIN000004026.

Informed consent statement

The study protocol was explained to the patients and volunteers before written informed consent was obtained.

Statistical analysis

All statistical analysis was performed using JMP Pro 12.2.0 (SAS Institute, Cary, NC, United States). Non-parametric comparisons were performed using the Wilcoxon signed-rank test, with a normal approximation. ROC analysis was performed to examine the difference between *GFP* fluorescence intensity and cell size in the blood samples of patients versus those in healthy volunteers. Cox proportional hazards analysis was used to investigate risk factors for survival, and to calculate overall and relapse-free survival rates. $P \leqslant 0.05$ was considered statistically significant.

RESULTS

Patient characteristics and pathological findings

The clinicopathological characteristics of 65 patients (46 men and 19 women; mean age 60.7 years; range 33-76 years) are summarised in Table 1. The median follow-up period for the surviving patients was 60 mo. Fifty-seven of the 65 patients underwent pathological curative surgery, and of these patients, 10 experienced disease recurrence. Fifteen patients died. Twenty-nine patients had distal gastrectomy, 32 had total gastrectomy, and four had exploratory laparotomy. Twenty of the 57 patients that underwent curative surgery also received adjuvant chemotherapy, and nine of these 20 patients received therapeutic chemotherapy after disease recurrence.

Gallery of GFP-positive cancer cell lines after OBP-401 infection

After OBP-401 infection, GFP-positive cancer cell lines were detected (Figure 1A).

Comparison of GFP fluorescence intensity between cell lines and blood cells

The *GFP* fluorescence intensity [mean equivalent fluorochrome (MEFL)] of the cell lines and the GFP-positive cells detected in the peripheral blood samples are shown in Figure 1B. MEFL was higher in cell lines than in the GFP-positive cells in the peripheral blood samples from either healthy volunteers or patients with gastric cancer. In turn, MEFL was higher in GFP-positive cells from patients with gastric cancer than in the corresponding cells from healthy volunteers.

Comparison of GFP fluorescence intensity and cell diameter between patients and volunteers

The *GFP* fluorescence intensity and diameter of cells isolated from the peripheral blood samples are

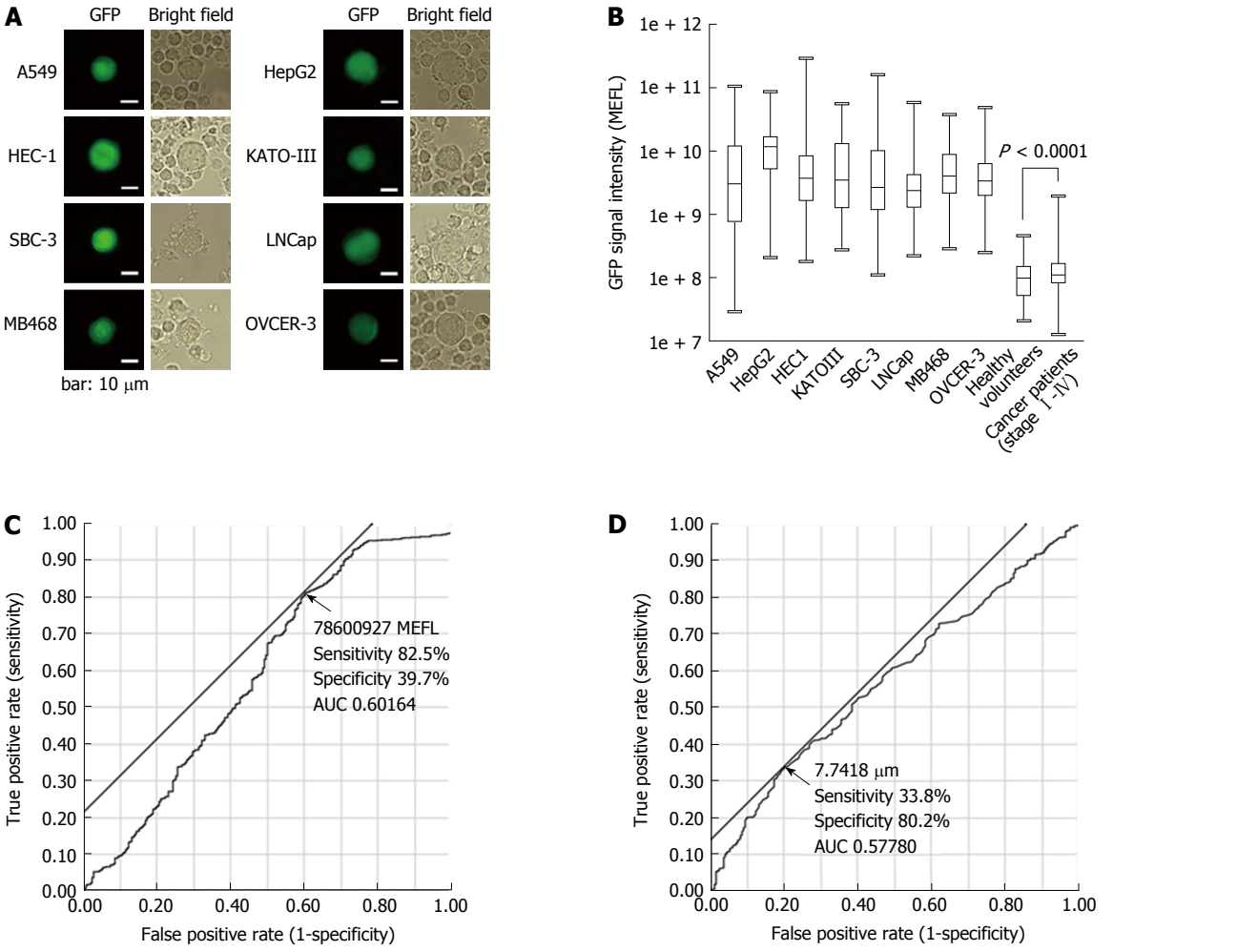

Figure 1 Comparison of green fluorescent protein fluorescence intensity and cell diameter. A: Gallery of GFP-positive cell lines. The cell lines used were A549, HepG2, HEC-1, KATO-III, SBC-3, LNCaP, MDA-MB-468, and OVCAR-3; B: Comparison of GFP fluorescence intensity of cultured cell lines and blood cells from healthy volunteers and gastric cancer patients. The bottom and top of the box represent the lower and upper quartiles, and the band across the box shows the median. The lower and upper bars at the ends of the whiskers show the lowest data point and the highest data point, respectively. The cell lines used were A549, HepG2, HEC-1, KATO-III, SBC-3, LNCaP, MDA-MB-468 and OVCAR-3; C: Comparison of GFP fluorescence intensity between patients and healthy volunteers; D: Comparison of cell diameter between patients and healthy volunteers. To determine the cut-off line, we used ROC analysis to compare the GFP fluorescence intensity and diameter of cells from gastric cancer patients with those of healthy volunteers. Cells from the patients with gastric cancer had higher GFP intensities than those from the healthy volunteers (cut-off 78600927 MEFL sensitivity 82.5 %, specificity 39.7 %, and AUC 0.602). The diameters of GFP-positive cells in samples from patients with gastric cancer were higher than those in samples from healthy volunteers (cut-off 7.7418 μm, sensitivity 33.8 %, specificity 80.2 %, and AUC 0.578). GFP: Green fluorescent protein.

shown in Figure 1C and D. Based on ROC analyses, we defined cells with 78600927 MEFL or higher GFP fluorescence intensity and 7.7418 μm or larger diameter as the CTCs.

Association of CTCs with pathological findings

An increased number of CTCs was associated with disease progression. There was statistically significant difference in the number of CTCs between samples from patients with Stage Ⅰ and those from patients with Stage Ⅲ disease (P = 0.0460, Figure 2A). The number of CTCs also tended to increase concomitantly with progression of the primary tumour, as there was a statistically significant difference in the number of CTCs between samples from patients with T1 and those from patients with T4 tumours (P = 0.0335, Figure 2B). There was also a statistically significant difference in the number of CTCs between samples from patients with N0 and those with N2 lymph node spread status (P = 0.0381, Figure 2C). However, there was no significant difference in the number of CTCs between samples from patients with distant metastases and those in which distant metastasis was absent (P = 0.4667, Figure 2D). The number of CTCs was also higher in samples from patients with lymphatic invasion, although there was no significant difference compared to patients without this clinical feature (P = 0.1297, Figure 2E). Similarly, although the number of CTCs in samples from the patients with venous invasion was higher than those in samples without this complication, the difference was not significant (P = 0.0558, Figure 2F). Finally, we observed no significant difference in the number of CTCs in samples from patients with differentiated tumours when compared to

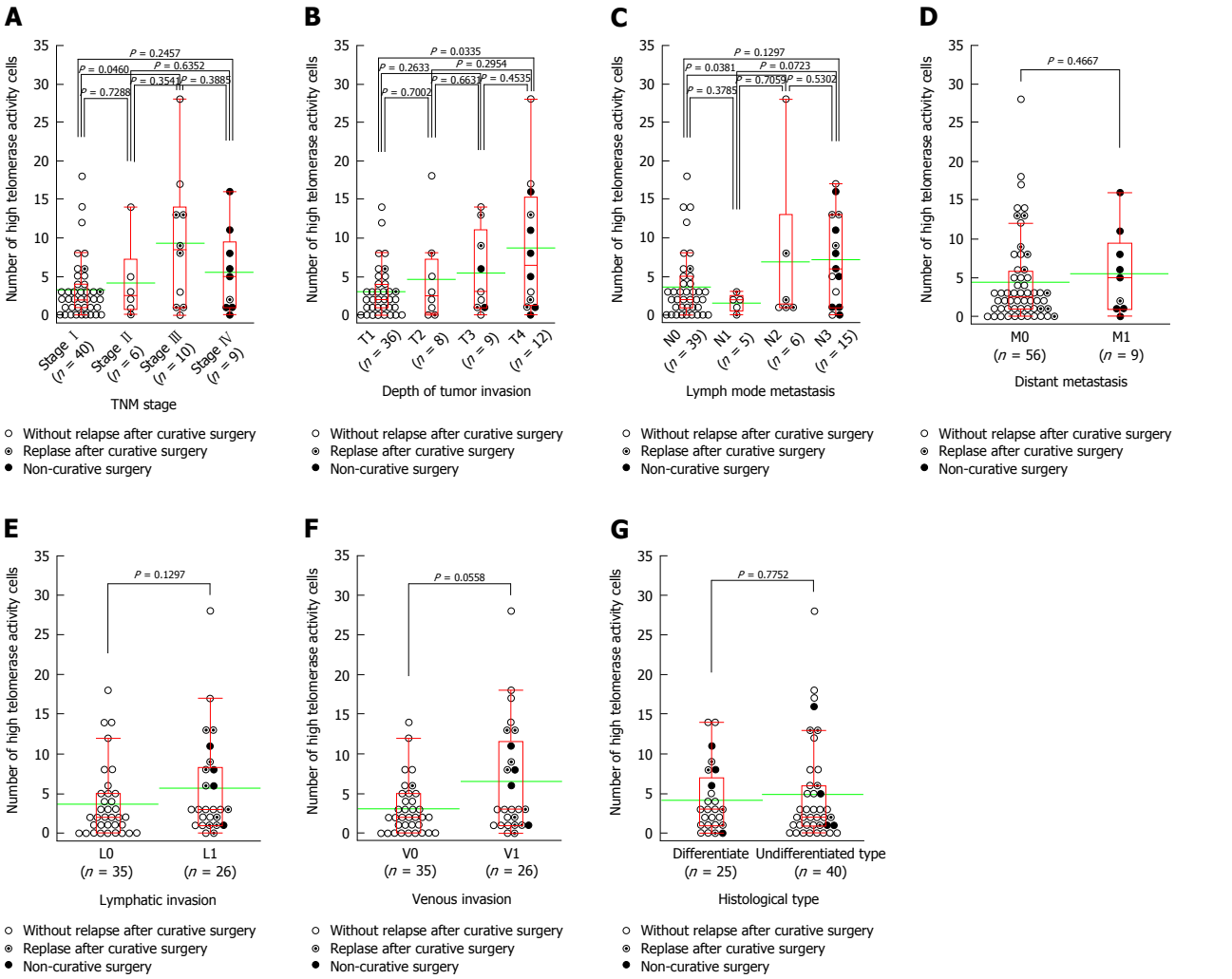

Figure 2 Relationship between circulating tumour cell number and pathological findings. Dots indicate the numbers of CTCs in each patient blood sample. The bottom and top of the box represent the lower and upper quartiles, and the band across the box shows the median. The lower and upper bars at the ends of the whiskers show the lowest data point within 1.5 interquartile ranges of the lower quartile, and the highest data point within 1.5 interquartile ranges of the upper quartile, respectively. The green bar shows the average. A: Disease stage (Stages 1-4 indicate disease progression); B: Depth of tumour invasion (T1-T4 indicate increasing depth); C: Lymphatic metastasis (N0-3 indicate number of metastatic lymph nodes); D: Distant metastasis (M0 = negative, M1 = positive); E: Lymphatic invasion (L0 = negative, L1 = positive); F: Venous invasion (V0 = negative, V1-V2 = positive); G: Histological type (differentiated and undifferentiated types). A: Relationship between CTC number and disease stage; B: Relationship between CTC number and T category. The number of CTCs tended to increase with progression of primary tumour. There was a statistically significant difference in the number of CTCs between samples from patients with T1 and those from patients with T4 status (P = 0.0335); C: The relationship between CTC number and N category. There was a statistically significant difference in the number of CTCs between samples from patients with N0 and those from patients with N2 classification (P = 0.0381); D: Relationship between CTC number and M category. There was no significant difference in the number of CTCs between samples from patients with distant metastases and those from patients without distant metastasis (P = 0.4667); E: Relationship between CTC number and lymphatic invasion. The number of CTCs in samples from patients with lymphatic invasion was higher than that in patients without invasion. However, this result did not reach the level of significance (P = 0.1297); F: Relationship between CTC number and venous invasion. The number of CTCs in samples from patients with venous invasion was higher than that in samples from patients without this pathology. However, this result did reach the level of significance (P = 0.0558); G: Relationship between CTC number and histological type. There was no significant difference in the number of CTCs between samples from patients with differentiated tumours and those from patients with undifferentiated tumours (P = 0.7752). CTCs: Circulating tumour cells.

those with undifferentiated malignancies (P = 0.7752, Figure 2G).

Overall and relapse-free survival

The overall survival rate of patients who had more than five CTCs (66.2%) was lower than that of patients who had five or less CTCs (80.5%); however, this difference was not significant (P = 0.183, Figure 3A). The relapse-free survival rate of patients who had more than five CTCs (64.3%) was significantly lower than that of patients who had five or less CTCs (88.3%) (P = 0.034, Figure 3B).

Prognostic factor for survival

We investigated prognostic factors related to patient survival by using Cox proportional hazards analysis. Univariate analysis showed that fStage was, in some cases, a significant factor (fStage Ⅱ, P = 0.196; fStage Ⅲ, P = 0.0003; fStage Ⅳ, P < 0.0001). In contrast, the presence of more than five CTCs was not a significant factor (P = 0.183). Multivariate analysis including these two factors showed fStage to be the

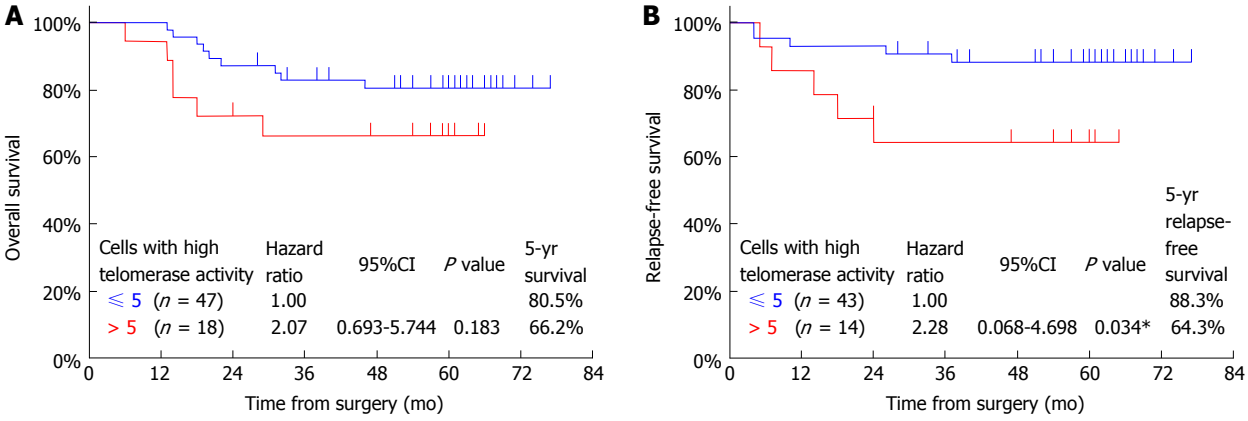

Figure 3 Overall and relapse-free survival. A: The overall survival rate of 65 patients was compared using Cox proportional hazards analysis. Although there was no significant difference, the overall survival rate of the patients with more than 5 CTCs was lower than that of patients with 5 or less CTCs (hazard ratio, 2.07; 95%CI: 0.693-5.744; $P = 0.183$); B: The relapse-free survival rates of 57 patients with different CTC levels who underwent curative surgery were compared using Cox proportional hazards analysis. Relapse-free survival was significantly lower in patients with more than 5 CTCs (hazard ratio, 2.28; 95% CI: 0.068-4.698; $P = 0.034$). CTCs: Circulating tumour cells.

Table 2 Risk factors for prognosis of patients ($n = 65$)

Variable	Univariate analysis			Multivariate analysis		
	Hazard ratio	95%CI	*P* value	Hazard ratio	95%CI	*P* value
Number of high telomerase activity cells						
≤ 5	1.0			1.0		
> 5	2.069	0.693-5.744	0.183	0.900	0.294-2.591	0.847
fStage						
fStage Ⅰ	1.0			1.0		
fStage Ⅱ	7.097	0.281-179.3	0.196	7.106	0.281-179.6	0.182
fStage Ⅲ	25.18	4.053-482.6	0.0003[b]	26.17	4.017-510.6	0.0004[b]
fStage Ⅳ	83.57	14.86-1567	< 0.0001[b]	85.76	14.91-1623	< 0.0001[b]

[b]$P < 0.01$.

only significant factor (fStage Ⅱ, $P = 0.182$; fStage Ⅲ, $P = 0.0004$; fStage Ⅳ, $P < 0.0001$), and the number of CTCs (more than five) to be non-significant ($P = 0.847$) (Table 2).

Risk factor for relapse after curative surgery

We also investigated factors for increased risk of relapse by Cox proportional hazards analysis. Univariate analysis showed that certain fStages were significant risk factors (fStage Ⅱ, $P = 0.337$; fStage Ⅲ, $P = 0.0001$; fStage Ⅳ, $P = 0.005$). However, the presence of more than 5 CTCs had no significant influence on relapse rates ($P = 0.052$). Multivariate analysis including these two factors showed fStage to be the only significant factor (fStage Ⅱ, $P = 0.343$; fStage Ⅲ, $P = 0.001$; fStage Ⅳ, $P = 0.004$), whereas the number of CTCs was non-significant ($P = 0.350$, Table 3).

DISCUSSION

Here, we used a telomerase-specific adenoviral agent to detect CTCs to avoid relying on the heterogeneous expression of epithelial markers in CTCs undergoing EMT. The enumeration of CTCs is particularly important in gastric cancer, which is the second leading cause of cancer-related death worldwide. Our current data indicate that detection of CTCs may indeed be a useful prognostic indicator for use in patients with gastric cancer, and are consistent with previous reports[25,26].

Our previous preliminary study showed that the number of CTCs isolated from cancer patients was related to surgical and pathological disease progression. Specifically, there were more CTCs in samples from patients with Stage Ⅲ than in those with Stage Ⅰ disease. The CTC count was also higher in patients with tumour depth T4 than in those with T1, and in individuals with lymph node metastasis status N2 versus those with N0. In addition, we found that the number of CTCs was associated with disease stage and relapse after curative surgery in gastric cancer patients. In the current study, the relapse-free survival rate of patients who had more than five CTCs was significantly lower than that of patients who had five or less. The overall survival rate of patients with more than five CTCs tended to be lower than that of the patients with five or less; in this case, however, the difference was not statistically significant. The number of CTCs was not an independent risk factor for either

Table 3 Risk factors for relapse of patients who underwent curative surgery (n = 57)

Variable	Univariate analysis			Multivariate analysis		
	Hazard ratio	95%CI	*P* value	Hazard ratio	95%CI	*P* value
Number of high telomerase activity cells						
≤ 5	1.0			1.0		
> 5	3.566	0.988-12.88	0.052	1.971	0.471-8.861	0.350
fStage						
fStage Ⅰ	1.0			1.0		
fStage Ⅱ	3.635	0.169-37.96	0.337	3.576	0.166-37.35	0.343
fStage Ⅲ	17.78	4.065-121.8	0.0001[b]	13.75	2.806-100.7	0.001[b]
fStage Ⅳ	239.6	7.943-7785	0.005[b]	289.4	9.331-9733	0.004[b]

[b]$P < 0.01$.

overall or relapse-free survival. However, we suggest this may be due to the relatively small sample size we studied, and that examination of larger cohorts might reveal a more significant impact of CTC number on these clinical parameters. Cancer stem cells (CSCs) in the blood of cancer patients are increasingly viewed as important determinants of cancer metastasis[27,28] and prognosis[29]. Given that CSCs have high telomerase activity, and share many of the molecular hallmarks of EMT[30], we suggest that our CTC assay could be used to detect both CSCs and CTCs during EMT.

One limitation of our study is that we could not achieve maximal sensitivity and specificity with regard to CTC detection. The definition of CTCs in this study was based on the threshold of GFP fluorescence intensity and cell diameter. However, these criteria resulted in a significant overlap between the data of healthy volunteers and those of cancer patients. More studies that compare healthy individuals with a larger population of patients with different cancer types are needed to clarify the suitability of CTC detection for clinical use. Another limitation of our study was that we did not determine the metastatic potential of the CTCs that we detected. Ideally, the functions of CTCs should be analysed after cell sorting, and CTCs with metastatic potential could be identified using additional tools such as DNA ploidy analysis[31,32]. Furthermore, gene expression profiling of CTCs, primary tumours, and metastatic tumours will also provide important insight into the mechanisms responsible for cancer metastasis. In summary, CTCs are useful predictors of disease progression in gastric cancer patients, but they do not constitute an independent prognostic factor.

In conclusions, CTC number tended to increase with surgical and pathological disease progression. Although not an independent risk factor, a higher number of CTCs was significantly correlated with disease relapse in gastric cancer after curative surgery. However, our study analysed only a small number of participants, and whether all the CTCs we detected have true metastatic potential was not determined. Further studies with a larger number of participants are therefore required to confirm the findings of this study.

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COMMENTS

Background

Detection of circulating tumour cells (CTCs) in peripheral blood is useful for prognosis, monitoring disease progression, and evaluation of treatment efficacy in malignancies, and various methods have been developed to detect CTCs.

Research frontiers

Most CTC detection systems that rely on epithelial markers may fail to detect CTCs undergoing the epithelial-mesenchymal transition.

Innovations and breakthroughs

Because OBP-401 is a telomerase-specific adenoviral agent, the OBP-401 assay does not depend on the expression of surface epithelial markers.

Applications

In this study, viable CTCs with high telomerase activity were detected using OBP-401 in blood samples from patients with gastric carcinoma. The authors showed that a lower number of CTCs correlated with higher relapse-free survival rates in patients with gastric cancer.

Terminology

The authors believe that the OBP-401 assay for detection of CTCs is clinically useful for patients with gastric carcinoma.

Peer-review

The authors presented a novel technique for detection of CTCs that does not depend on surface epithelial markers. The authors showed that a lower number of CTCs was correlated with higher relapse-free survival rates in patients with gastric cancer.

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ORIGINAL ARTICLE

Randomized Controlled Trial

Randomized controlled study of the safety and efficacy of nitrous oxide-sedated endoscopic ultrasound-guided fine needle aspiration for digestive tract diseases

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Author contributions: Wang CX, Wang J and Chen YY performed the majority of experiments; Wang CX and Wang JN provided vital reagents and analytical tools and were also involved in writing and revising the manuscript; Yu X and Yang F provided the collection of all the human material; and Sun SY designed the study and wrote the manuscript.

Institutional review board statement: This study was approved by the Institutional Review Board and Ethics Committee of China Medical University (clinical trial registration number: ChiCTR-OCC-15005853).

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

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Abstract

AIM

To evaluate the efficacy and safety of nitrous oxide-sedated endoscopic ultrasound-guided fine needle aspiration.

METHODS

Enrolled patients were divided randomly into an experimental group (inhalation of nitrous oxide) and a control group (inhalation of pure oxygen) and heart rate, blood oxygen saturation, blood pressure, electrocardiogram (ECG) changes, and the occurrence of complications were monitored and recorded. All patients and physicians completed satisfaction questionnaires about the examination and scored the process using a visual analog scale.

RESULTS

There was no significant difference in heart rate, blood oxygen saturation, blood pressure, ECG changes, or complication rate between the two groups of patients ($P > 0.05$). However, patient and physician satisfaction were both significantly higher in the nitrous oxide

compared with the control group ($P < 0.05$).

CONCLUSION

Nitrous oxide-sedation is a safe and effective option for patients undergoing endoscopic ultrasound-guided fine needle aspiration.

Key words: Endoscopic ultrasonography; Nitrous oxide; Sedation; Fine needle aspiration

Core tip: Endoscopic ultrasonography (EUS) has been widely used in the diagnosis and treatment of gastrointestinal tract and pancreaticobiliary diseases. However, EUS-guided fine needle aspiration (EUS-FNA) is a time-consuming procedure associated with pain and discomfort. Nitrous oxide, also known as laughing gas, is a colorless, short-acting inhaled agent that can produce anesthetic, analgesic, and anxiolytic effects. Safety and efficacy of nitrous oxide-sedated EUS-FNA. However, inhaled nitrous oxide has no effect on heart or lung function and patients remain awake, and this thus represents a feasible mode of sedation for EUS. The current study aimed to establish the safe.

Wang CX, Wang J, Chen YY, Wang JN, Yu X, Yang F, Sun SY. Randomized controlled study of the safety and efficacy of nitrous oxide-sedated endoscopic ultrasound-guided fine needle aspiration. *World J Gastroenterol* 2016; 22(46): 10242-10248 Available from: URL: http://www.wjgnet.com/1007-9327/full/v22/i46/10242.htm DOI: http://dx.doi.org/10.3748/wjg.v22.i46.10242

INTRODUCTION

Endoscopic ultrasonography (EUS) has been widely used in the diagnosis and treatment of gastrointestinal tract and pancreaticobiliary diseases. However, EUS-guided fine needle aspiration (EUS-FNA)[1-6] is a time-consuming procedure associated with pain and discomfort. Painless EUS can reduce patient suffering and thus make patients more likely to accept the examination. Intravenous anesthetic agents including benzodiazepine-opioid drugs and propofol are commonly used, but their respiratory-inhibitory effects limit their application[7]. Furthermore, most EUS procedures require water injection into the digestive tract, which increases the risk of aspiration during anesthesia. Nitrous oxide, also known as laughing gas, is a colorless, short-acting inhaled agent that can produce anesthetic, analgesic, and anxiolytic effects. However, inhaled nitrous oxide has no effect on heart or lung function and patients remain awake, and this thus represents a feasible mode of sedation for EUS[7]. The current study aimed to establish the safety and efficacy of nitrous oxide-sedated EUS-FNA.

MATERIALS AND METHODS

Study subjects

The inclusion criteria for the study were patients who required EUS-FNA and agreed to sedation with nitrous oxide. Patients were excluded if they exhibited any of the following contraindications to nitrous oxide sedation or EUS: (1) intending to get pregnant or in the first trimester of pregnancy; (2) coma; (3) within 1 wk of gas cerebral angiography; (4) diving diseases or a recent history of diving activities; (5) middle ear diseases; (6) pneumothorax, pulmonary cystic fibrosis, or chronic debilitating weakness due to other respiratory disorders; (7) intestinal obstruction; (8) history of gastrointestinal surgery; (9) history of sinus or nasal-septum surgery; (10) need for endoscopic treatment; (11) American Society of Anesthesiology (ASA) grade > 3; and (12) blood oxygen saturation < 95% and systolic blood pressure < 90 mmHg as displayed on the monitor.

This study was approved by the Institutional Review Board and Ethics Committee of China Medical University (clinical trial registration number: ChiCTR-OCC-15005853). All patients voluntarily provided written informed consent for their participation in this study. The operator performing the EUS-FNA procedure in this study was familiar with the technique.

Equipment

The following equipment was used: a nitrous oxide sedation system (AII 5000C; Shenzhen Security Technology Co., Ltd., China) (Figure 1); a patient monitor (PM-7000; Mindray); an ultrasound scanner (EUB 6500, Hitachi, Tokyo, Japan); a linear array echo-endoscope (Pentax EG3830UT, Japan); and a 22-gauge needle (EUS N-22-T, Wilson-Cook, United States).

Study design

This was a prospective, randomized, controlled clinical study. The patients were divided into an experimental group and control group using a random-number table. The experimental group received nitrous oxide inhalation, with the inspiratory flow of nitrous oxide adjusted according to the depth of sedation (range of nitrous oxide concentrations 30%-70%). The control group received oxygen inhalation at a concentration of 100% and flow rate of 2-3 L/min. Patients were placed in the left lateral recumbent position during the endoscopic procedure and EUS-FNA was performed using a linear array echo-endoscope (Figure 2). This was a single-blind study and the patients were unaware of the identity of the inhaled gas. Patients with unsuccessful EUS were excluded from the current study.

Patient monitoring

This study trained auxiliary nurses who adjusted the inhalation flow of nitrous oxide during the endoscopic operation, under the guidance of a physician, and

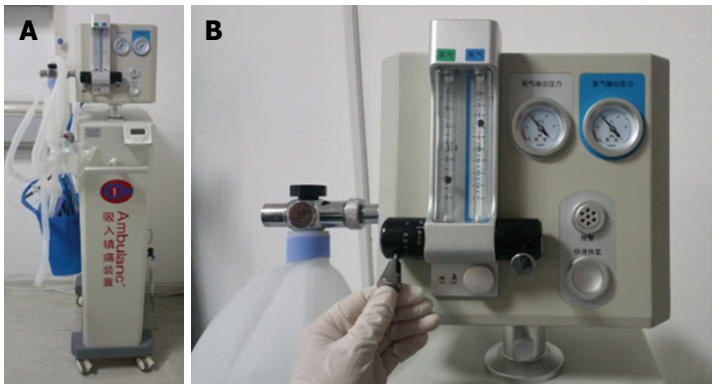

Figure 1 Nitrous oxide sedation system (All 5000C; Shenzhen Security Technology Co., Ltd., China).

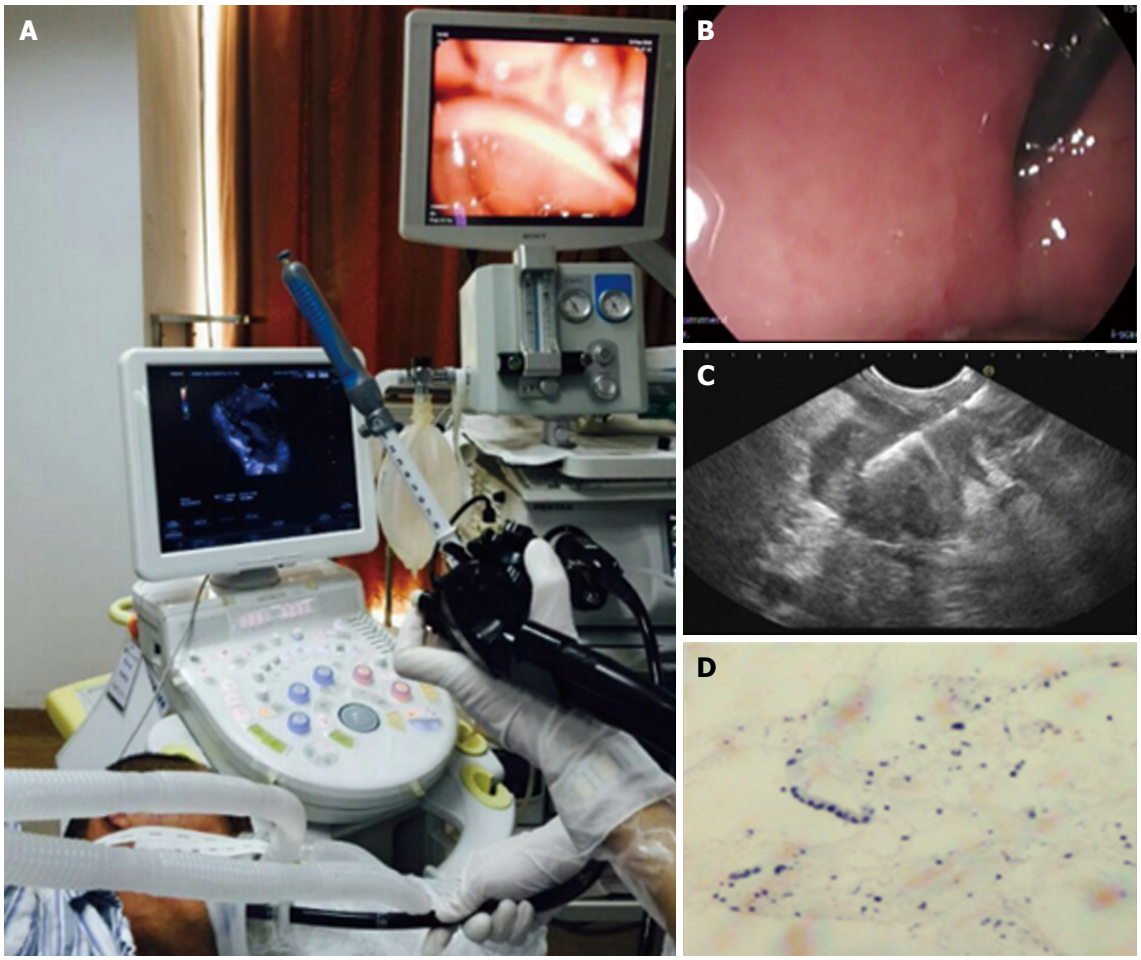

Figure 2 Patients were placed in the left lateral recumbent position during the endoscopic procedure and endoscopic ultrasonography-guided fine needle aspiration was performed using a linear array echo-endoscope. A: Patients were placed in the left lateral recumbent position during the endoscopic procedure and endoscopic ultrasonography-guided fine needle aspiration was performed using a linear array echo-endoscope; B: A 22G needle was used to puncture the pancreatic lesion; C: The lesion was in the body of the pancreas; D: The diagnosis of histology was adenocarcinoma.

who provided nursing care for the patients. All the physicians in this study were trained in cardiopulmonary resuscitation and tracheal intubation, ensuring that patients received timely basic cardiac life support. Patients' blood pressure, oxygen saturation, and heart rates were monitored closely, and the monitor set off an alarm if the oxygen saturation dropped to < 95% or the heart rate decreased to < 50 beats/min (bpm). Blood pressure was measured automatically every 3 min, and the monitor alarm went off if the systolic blood pressure was < 90 mmHg. Continuous electrocardiogram (ECG) monitoring was performed in all patients. The auxiliary nurse helped to observe patients' thoracic movements and respiratory rates, and assisted endoscopic physicians to adjust the nitrous oxide inhalation flow promptly according to the depth of sedation, the

specific circumstances of the patients, and examination during endoscopic operation. The auxiliary nurses were also responsible for monitoring the patients until 10 min after the termination of nitrous oxide inhalation to ensure that their vital signs were stable.

Safety evaluation

Patients were monitored closely and the following negative events were recorded: oxygen desaturation (oxygen saturation < 95%, but ⩾ 90%), hypoxia (oxygen saturation < 90%, but ⩾ 85%), severe hypoxemia (oxygen saturation < 85%), hypotension (systolic blood pressure < 90 mmHg), bradycardia (heart rate < 50 bpm), and tachycardia (heart rate > 120 bpm).

Patients and endoscopists completed questionnaires regarding their degree of satisfaction with the examination process, and scored them on a visual analog scale (VAS) scale. The following questions were included: (1) evaluation of the operation by the endoscopist: (smooth, ordinary, not smooth); (2) patient discomfort during the operation process (slight, moderate, severe); (3) patient tolerance with the examination process (good, medium, and low); and (4) willingness to receive the same examination again if needed (yes, no).

Treatment of complications

The endoscopy procedure was suspended and the inhalation of nitrous oxide in the experimental group was reduced or suspended and replaced by inhalation of pure oxygen if the patients experienced blood oxygen saturation < 95%, systolic blood pressure < 90 mmHg, or a heart rate < 50 bpm. If the above parameters were immediately restored to normal levels, the operation was continued and patients in the experimental group were given inhalational nitrous oxide at a slightly reduced flow rate than before. However, if the above-mentioned parameters persisted for > 1 min, the operation was terminated. If patients in the experimental group showed signs of excessive inhalation, nitrous oxide was reduced or terminated and replaced by oxygen inhalation. Signs of excessive inhalation of nitrous oxide included the following: disappearance of original signs of comfort and relaxation; new or sudden intolerance, dizziness, vertigo, agitation, or irritability; repeated or ambiguous words and poor response to verbal commands; fixation of eyes and unresponsiveness; sleepiness and difficulty keeping eyes open, or drowsy; dreaming or fantasizing; uncontrolled laughter; stopping breathing; or nausea and vomiting.

Statistical analysis

All the measured data were presented as means. All analyses were performed using SPSS 16 statistical software.

RESULTS

A total of 2877 patients required EUS examinations from March 1 2015 to May 31 2016, of whom 42 patients who required EUS-FNA were enrolled in the study (1.5%) according to the above criteria. There were 21 patients in the control group (pure oxygen group) and 21 patients in the experimental group (nitrous oxide group). There was no significant difference in ASA, age, or sex between the two groups. One patient failed to finish the EUS examination (difficulty in passing through the throat), and was excluded from the current study. The remaining 41 patients (20 in the control group and 21 in the experimental group) completed the examination and the relevant questionnaires, including 16 women and 25 men, average age 42.4 years, (range, 27-69 years). The average time to completion of EUS-FNA was 29 min (range, 14-47 min). The maximal concentration of nitrous oxide used varied among cases and ranged from 30%-70%.

The ECG monitoring results are shown in Table 1. Among the 21 patients in the nitrous oxide group who completed the examination, one patient (4.8%) experienced temporary oxygen desaturation and one experienced hypoxemia (4.8%). The symptoms resolved immediately after termination of nitrous oxide inhalation and inhalation of pure oxygen, with no decline in oxygen saturation after the restart of nitrous oxide inhalation. No patients developed severe hypoxemia, bradycardia (heart rate < 50 bpm), systolic blood pressure < 90 mmHg during the examination process, or tachycardia (heart rate > 120 bpm). There was no significant difference in heart rate, oxygen saturation level, or ECG changes between the two groups.

The results of the questionnaires completed by the physicians and patients are listed in Table 2 and the VAS scores are listed in Table 3. All physicians and patients completed the questionnaires. Physician and patient satisfaction with the examination process was significantly higher in the nitrous oxide group compared with the pure oxygen group (patient scores 87 *vs* 72, $t = 4.702$, $P < 0.05$; physician scores 91 *vs* 70, $t = 10.163$, $P < 0.05$). Among the 21 patients in the nitrous oxide group, 18 (85.7%) were willing to receive the same examination again if required, compared with only 9 of 20 (45%) in the control group.

DISCUSSION

EUS has been widely used in the diagnosis and treatment of digestive tract diseases. It can get closer to the common bile duct and pancreas than transabdominal ultrasound, thus avoiding interference from digestive tract gases and resulting in clearer imaging, and is considered to be preferable to computed tomography and magnetic resonance imaging

Table 1 Electrocardiogram changes in patients during examination

Group	Heart rate		Oxygen saturation			Significant change in electrocardiogram
	> 120 bpm	< 50 bpm	Oxygen desaturation	Hypoxia	Severe hypoxia	
Experimental	0	0	1	1	0	0
Control	2	0	1	2	0	0
P value	> 0.05	> 0.05	> 0.05	> 0.05	> 0.05	> 0.05

Table 2 Results of questionnaire

Group	Evaluation by physicians			Patient discomfort			Patient tolerance		
	Steady	Ordinary	Not steady	Mild	Moderate	Severe	Good	Medium	Poor
Experimental	17	3	1	18	2	1	18	3	0
Control	11	2	8	6	3	12	9	5	7
P value	< 0.05		< 0.05	< 0.05		< 0.05	< 0.05		< 0.05

Table 3 Patient and physician visual analog scale scores for examination process

Group	Patient satisfaction score	Physician satisfaction score	Willing to receive same examination again *n* (%)
Experimental	87	91	18 (85.7)
Control	72	70	9 (45)
P value	< 0.05	< 0.05	< 0.05

for the diagnosis of small pancreatic lesions. Numerous studies have demonstrated unique advantages of EUS for the diagnosis of gastrointestinal submucosal tumors. Furthermore, EUS-FNA is the first choice of diagnostic procedure in many diseases, such as pancreatic, gastrointestinal subepithelial, and mediastinal lesions[1-6]. However, the use of a long hard endoscope means that it is much more uncomfortable than general gastroscopy, and cannot be tolerated by some patients. Although EUS can be performed under intravenous propofol anesthesia, some lesions can only be displayed clearly after gastric infusion of water, which increases the risk of aspiration during intravenous propofol anesthesia. The success rate of EUS could therefore be improved by establishing a method for reducing pain and discomfort and increasing patient tolerance.

Nitrous oxide is an inhaled sedative and analgesic agent, which passes through the blood-brain barrier into the brain and functions by inhibiting excitatory neurotransmitter release and nerve impulse conduction in the central nervous system, and altering the permeability of ion channels. Nitrous oxide does not stimulate the respiratory tract or bind to hemoglobin, and does not cause respiratory depression or damage heart, lung, liver, or kidney function. Nitrous oxide sedation is currently used widely in clinical situations, including in emergency surgery, dentistry, childbirth, abortion and curettage, and pediatrics, and can also be applied for gastrointestinal endoscopic sedation. Nitrous oxide-sedated endoscopy examinations have been shown to be safe and effective[8-22], and nitrous oxide has proven a safe and effective choice for colonoscopy sedation and analgesia[8-10,12,17,19-22]. However, nitrous oxide is rarely used for EUS. Lan *et al*[8] compared the diagnostic accuracy, safety, complications, and patient and examiner satisfaction among different sedation approaches in patients undergoing upper gastrointestinal endoscopy. Patients in the nitrous oxide sedation group reported greater satisfaction with the endoscopy procedure than patients in the conventional group (no sedation), with overall better tolerance and less pain, nausea, and vomiting ($P < 0.05$). A review of 11 studies by Welchman *et al*[9] concluded that nitrous oxide provided comparable analgesia to intravenous sedation for patients undergoing colonoscopy. Wang *et al*[10] first mentioned the use of nitrous oxide in EUS, and concluded that it offered a comfortable, safe and feasible option, especially for procedures requiring irrigation. Michaud and Gottrand[13] showed that the time taken to regain consciousness was short following nitrous oxide sedation, which could effectively meet the sedative requirements for children undergoing gastroscopic examination, thus providing a valuable alternative method of sedation. Michaud *et al*[14] compared the sedative effects of propofol and nitrous oxide in patients undergoing colonoscopy, and showed that both agents had similar sedative and pain-relieving effects, facilitated the operation, and shortened recovery time. In addition, nitrous oxide has demonstrated minimal effects on nerve function and therefore does not affect the patient's ability to drive[15].

In this study, we monitored heart rate, blood oxygen saturation, blood pressure, and ECG in patients undergoing EUS under nitrous oxide sedation. Nitrous oxide had minimal effects on all these parameters, similar to the effects of pure oxygen. Patient and endoscopist satisfaction surveys and VAS scores

indicated that the use of nitrous oxide significantly increased patient tolerance to EUS. Nitrous oxide sedation therefore represents a safe and effective choice in patients undergoing EUS-FNA.

COMMENTS

Background

Endoscopic ultrasonography (EUS) has been widely used in the diagnosis and treatment of gastrointestinal tract and pancreaticobiliary diseases. However, EUS-guided fine needle aspiration (EUS-FNA) is a time-consuming procedure associated with pain and discomfort. Nitrous oxide, also known as laughing gas, is a colorless, short-acting inhaled agent that can produce anesthetic, analgesic, and anxiolytic effects. However, inhaled nitrous oxide has no effect on heart or lung function and patients remain awake, and this thus represents a feasible mode of sedation for EUS. The current study aimed to establish the safety and efficacy of nitrous oxide-sedated EUS-FNA.

Research frontiers

Nitrous oxide, also known as laughing gas, is a colorless, short-acting inhaled agent that can produce anesthetic, analgesic, and anxiolytic effects. However, inhaled nitrous oxide has no effect on heart or lung function and patients remain awake, and this thus represents a feasible mode of sedation for EUS. It is the first time to establish the safety and efficacy of nitrous oxide-sedated EUS-FNA.

Innovations and breakthroughs

Nitrous oxide-sedated endoscopy examinations have been shown to be safe and effective, and nitrous oxide has proven a safe and effective choice for colonoscopy sedation and analgesia. However, nitrous oxide is rarely used for EUS. In this study, the authors monitored heart rate, blood oxygen saturation, blood pressure, and electrocardiogram (ECG) in patients undergoing EUS under nitrous oxide sedation. Nitrous oxide had minimal effects on all these parameters, similar to the effects of pure oxygen. Patient and endoscopist satisfaction surveys and visual analog scale (VAS) scores indicated that the use of nitrous oxide significantly increased patient tolerance to EUS.

Applications

In this study, the authors monitored heart rate, blood oxygen saturation, blood pressure, and ECG in patients undergoing EUS under nitrous oxide sedation. Nitrous oxide had minimal effects on all these parameters, similar to the effects of pure oxygen. Patient and endoscopist satisfaction surveys and VAS scores indicated that the use of nitrous oxide significantly increased patient tolerance to EUS. Nitrous oxide sedation therefore represents a safe and effective choice in patients undergoing EUS-FNA.

Terminology

Nitrous oxide-sedation is a safe and effective option for patients undergoing endoscopic ultrasound-guided fine needle aspiration.

Peer-review

This is an interesting study about the safety and efficacy of nitrous oxide-sedated endoscopic ultrasound-guided fine needle aspiration for digestive tract diseases.

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CASE REPORT

"*En bloc*" caudate lobe and inferior vena cava resection following cytoreductive surgery and hyperthermic intraperitoneal chemotherapy for peritoneal and liver metastasis of colorectal cancer

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Abstract

There are diverse protocols to manage patients with recurrent disease after primary cytoreductive surgery (CRS) with hyperthermic intraperitoneal chemotherapy (HIPEC) for peritoneal carcinomatosis. We describe a case of metachronous liver metastasis after CRS and HIPEC for colorectal cancer, successfully treated with a selective metastectomy and partial graft of the inferior vena cava. A 35-year-old female presented with a large tumour in the cecum and consequent colonic stenosis. After an emergency right colectomy, the patient received adjuvant chemotherapy. One year later she was diagnosed with peritoneal carcinomatosis, and it was decided to carry out a CRS/HIPEC. After 2 years of total remission, an isolated metachronous liver metastasis was detected by magnetic resonance imaging surveillance. The patient underwent a third procedure including a caudate lobe and partial inferior vena cava resection with a prosthetic graft interposition, achieving an R0 situation. The postoperative course was uneventful and the patient was discharged on postoperative day 17 after the liver resection. At 18-mo follow-up after the liver resection the patient

remained free of recurrence. In selected patients, the option of re-operation due to recurrent disease should be discussed. Even liver resection of a metachronous metastasis and an extended vascular resection are acceptable after CRS/HIPEC and can be considered as a potential treatment option to remove all macroscopic lesions.

Key words: Cytoreductive surgery; Liver resection; Hyperthermic intraperitoneal chemotherapy; Colorectal cancer; Liver metastasis

Core tip: Treatment of liver recurrence after a cytoreductive surgery and hyperthermic intraperitoneal chemotherapy is a great challenge. We report here the case of a young patient with metachronous liver metastases who was treated with a limited resection of segment I of the liver and vascular graft interposition of the inferior vena cava achieving a long-term survival. The surgical approach of these patients is extremely complicated and often requires complex major surgical procedures.

Sánchez-Velázquez P, Moosmann N, Töpel I, Piso P. "*En bloc*" caudate lobe and inferior vena cava resection following cytoreductive surgery and hyperthermic intraperitoneal chemotherapy for peritoneal and liver metastasis of colorectal cancer. *World J Gastroenterol* 2016; 22(46): 10249-10253 Available from: URL: http://www.wjgnet.com/1007-9327/full/v22/i46/10249.htm DOI: http://dx.doi.org/10.3748/wjg.v22.i46.10249

INTRODUCTION

Peritoneal carcinomatosis (PC) is the second most common presentation of metastatic colorectal cancer and is diagnosed in up to 4%-6% of these cases. Twenty five percent of these patients have the peritoneum as the only site of disseminated disease[1]. PC was traditionally considered the last stage of the disease and was associated with a poor prognosis so that patients were often relegated to palliative systemic therapies. In recent decades multimodal PC treatment has made great advances; cytoreductive surgery (CRS), hyperthermic intraperitoneal chemotherapy (HIPEC) and systemic chemotherapy have shown promising results and have become standard therapy for PC patients in several countries[2-7]. Various studies have reported that patients undergoing CRS with total macroscopic cytoreduction and HIPEC may achieve prolonged overall survival and potentially even a complete cure in selected patients[4,8,9].

Up to 80% of patients with PC of colorectal origin treated with CRS and HIPEC are likely to recur[10,11]. In selected cases the possibility of a reoperation due to recurrence or even an extensive abdominal surgical procedure can be individually assessed. The data available on this approach show favourable long-term outcomes with similar morbidity and mortality to that of initial CRS/HIPEC[12-15]. Simultaneous or staged combined CRS and liver resections have also been performed with comparable morbidity and long-term results[16]. In this report we describe the case of a young female patient with metachronous liver metastasis after CRS and HIPEC for colorectal cancer successfully treated by a selective liver and vascular resection.

CASE REPORT

A 35-year-old female was referred to our emergency unit in February 2011, presenting with abdominal distension, pain, and vomiting for 3 d. Her medical history was only remarkable for asthma. Abdominal computed tomography (CT) was performed and revealed a large tumor in the cecum with consecutive colonic stenosis. The patient underwent an emergency right colectomy, and an R0 situation was achieved. Pathologic examination showed pT4a pN2a (4/22) cM0 poorly differentiated adenocarcinoma. Between March and August 2011 she received 12 cycles of adjuvant FOLFOX (folinic acid, 5-fluorouracil, and oxaliplatin). In February 2012 a CT scan identified lesions in the peritoneum with suspicions of peritoneal carcinomatosis. Our multidisciplinary tumour board decided on pursuing CRS with HIPEC. Abdominal exploration revealed widespread peritoneal carcinomatosis, especially in the pelvis and a large tumor mass in the left sub-diaphragmatic region with a peritoneal cancer index (PCI) of 14. Total parietal and diaphragmatic peritonectomy, proctocolectomy with an end ileostomy, terminal ileum resection, splenectomy, omentectomy, hysterectomy and bilateral salpingo-oophorectomy (CCR-0) were performed to remove the macroscopic tumor. HIPEC was carried out to treat the microscopic residual with mitomycin C, according to the closed-abdomen technique. The postoperative course was uneventful and the patient was duly discharged from hospital. At this time, no further chemotherapy was recommended as the patient had completed adjuvant chemotherapy after the first operation and a R0 situation was achieved. Adjuvant systemic chemotherapy, according to our institution protocols, is performed only in chemo naïve patients. From February 2012 until June 2014 the patient stayed free from recurrence. In July 2014 abdominal magnetic resonance imaging (MRI) surveillance revealed a solid tumor in segment 1 of the liver (Figure 1) so the patient underwent explorative laparotomy. During the procedure no evidence of a peritoneal recurrence was shown but many adhesions were found from the previous operations. A 4 cm tumor was identified in the caudate hepatic lobe infiltrating the inferior vena cava (IVC). The distal cava was mobilized towards the left renal vein and was divided at a level free of tumor

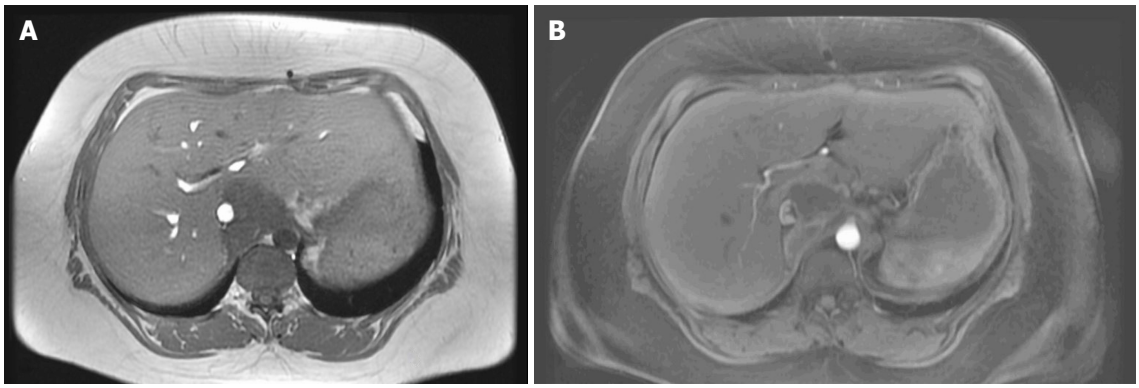

Figure 1 Magnetic resonance imaging shows an isolated liver metastasis in caudate lobe of the liver.

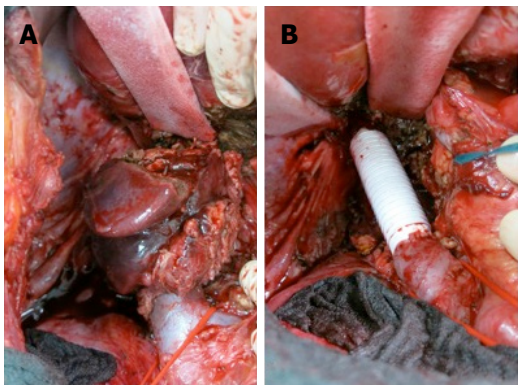

Figure 2 Distal inferior vena cava. A: Tumor mass infiltrating the inferior vena cava (ICV) at the bifurcation of the renal veins (vessel loop); B: Vascular interposition graft after partial resection of the IVC.

(Figure 2A). Liver segment 1 was transected following the clamp-crushing technique and ICV cranial to the tumor was dissected and divided under the major suprahepatic trunks. Vascular continuity was restored using a prosthetic graft interposition sutured with a running suture of prolene 5/0 onto the proximal and distal IVC (Figure 2B). The postoperative course was mainly uncomplicated except for right pleural effusion, initially managed conservatively with diuretic therapy, and later by thoracocentesis. The patient was finally discharged on the 17th postoperative day. Pathological examination revealed a poorly differentiated adenocarcinoma with identical immunohistochemical phenotype as previously. At this point it was decided not to continue with chemotherapy, as a CCR-0 situation had been surgically achieved and the patient had completed the 12-cycle adjuvant treatment in the past (Table 1). Eighteen months after metastasis resection of the liver and 4 years after CRS and HIPEC, the patient shows no evidence of neoplastic disease.

DISCUSSION

After initial CRS and HIPEC, recurrences are mostly intra-abdominal, even if complete cytoreduction is achieved[17]. Protocols differ among the different institutions, thus a number of different treatments are applied in high-volume centres, including chemotherapy, tumour debulking or re-do surgery for intraadominal metastasis.

The presence of synchronous liver metastases (LM) and PC was traditionally a contraindication for cytoreductive surgery. However, it has been shown that selected patients with low PCI and three or fewer LM can achieve prolonged survival if a liver resection is performed simultaneously[16,18]. A recent meta-analysis by Cuba *et al*[19] shows improved overall survival (OS) in patients who were treated with CRS and HIPEC and curative treatment of LM as compared to patients treated with modern systemic chemotherapy alone.

Nevertheless, it remains unclear which approach should be used in patients with isolated metachronous LM, as in our case report. Iterative cytoreductive surgery is feasible in cases of recurrence and appears to be worthwhile in terms of long-term outcomes[20]. The study by Sugarbaker and colleagues was on one of the largest series and included 70 patients with PC of colorectal origin[15]. This study showed that 53% of the patients had at least one reoperation after the initial cytoreduction. The overall survival of patients with repeated surgery approach was significantly longer (39 mo *vs* 20 mo). Brouquet *et al*[13] reported on a cohort of 20 patients with repeat CRS + intraperitoneal chemotherapy (IPC) for isolated peritoneal tumour recurrence of all origins. Five- and 10-year overall survival (OS) rates were 72.5% and 58.1% respectively.

Even though they studied selected groups of patients, the studies by Sugarbaker and Brouquet underline the possibility of highly favourable outcomes and even long-term survival in palliative patients with recurrence of peritoneal carcinomatosis.

In this context, the study by Kianmanesh *et al*[12] included 43 patients with PC of colorectal cancer origin who underwent CRS/HIPEC and specifically evaluated the role of simultaneous liver resection. They concluded that patients with colorectal PC, iterative CRS and HIPEC achieved appreciable long-term survival and that liver metastasis resection did not negatively influence the postoperative outcomes. However, they

Table 1 History and detail surgical treatment of our patient's disease

Time point	Diagnosis	Procedure
2011	Stenotic tumour of the cecum	Right Colectomy
		Adjuvant chemotherapy (12 cycles with folinic acid, 5-fluorouracil, and oxaliplatin)
2012	Peritoneal carcinomatosis	CRS (CCR-0)
	PCI = 14	Total parietal and diaphragmatic Peritonectomy
		Proctocolectomy with end ileostomy
		Terminal ileum resection
		Splenectomy
		Omentectomy
		Hysterectomy
		Bilateral salpingo-oophorectomy
		HIPEC
		43.8 mg Mitomycin C intraperitoneal (1 h)
2014	Liver metastases segment Ⅰ with ⅣC infiltration	Resection of liver segment Ⅰ
		Partial resection of ⅣC with prosthetic graft interposition
		Cholecystectomy
		Partial adrenalectomy
2016	No evidence of neoplastic disease	

PCI: Peritoneal cancer index; CRS: Cytoreductive surgery; HIPEC: Hyperthermic intraperitoneal chemotherapy.

did not specify whether an extended vascular resection was performed or if liver resections were performed in cases of metachronous LM.

Achieving complete cytoreduction in most cases is challenging and implies an aggressive approach combining major surgical procedures not exempt from complications. In selected cases, as in the one presented here, the option of re-do surgery for liver metastasis is feasible. Even extended vascular resection is acceptable after CRS/HIPEC and can be considered as a potentially curative treatment option. Early detection of tumour recurrence through a close follow-up is essential, as well as a multidisciplinary assessment of patient selection. The patients should then be referred to a centre specialized in the treatment of peritoneal and liver metastases. In the present case, staged resection of both metastatic sites achieved long-term survival for a young female patient. To our knowledge, this is this first report on liver resection due to metachronous liver metastases following CRS and HIPEC.

COMMENTS

Case characteristics

A 35-year-old patient with liver recurrent disease after an extended cytoreductive surgery and hyperthermic intraperitoneal chemotherapy (HIPEC) procedure.

Clinical diagnosis

Abdominal magnetic resonance imaging (MRI) diagnosed the liver recurrence in the follow-up.

Differential diagnosis

There is no possible differential diagnosis in this case.

Laboratory diagnosis

All labs were within normal limits.

Imaging diagnosis

MRI showed a solid tumor in segment 1 of the liver.

Pathological diagnosis

pT4a pN2a (4/22) cM0 poorly differentiated adenocarcinoma.

Treatment

Complete surgical excision of lesion.

Related reports

There are currently no other reports of surgical excision of a liver metastasis in the caudate lobe two years after a cytoreductive surgery.

Experiences and lessons

The report is good example of patient tailored treatment in cases where guidelines are missing or suggest only palliative or best supportive care. It is also novel to perform such an extensive surgery after cytoreductive surgery and HIPEC.

Peer-review

The paper is well written.

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CASE REPORT

Response of *BRCA1*-mutated gallbladder cancer to olaparib: A case report

Yuan Xie, Yan Jiang, Xiao-Bo Yang, An-Qiang Wang, Yong-Chang Zheng, Xue-Shuai Wan, Xin-Ting Sang, Kai Wang, Da-Dong Zhang, Jia-Jia Xu, Fu-Gen Li, Hai-Tao Zhao

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Author contributions: Xie Y and Jiang Y contributed equally to this work; Yang XB, Wang AQ, Zheng YC, Wan XS followed up the patient; Wang K, Zhang DD, Xu JJ provided genetic analysis for the variants tested in the patient; Li FG analyzed the genetic data and revised the manuscript; Sang XT and Zhao HT provided financial support for this work; Xie Y and Jiang Y wrote the paper; all authors have read and approved the final manuscript.

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Abstract

Gallbladder cancer (GBC), although considered as a relatively rare malignancy, is the most common neoplasm of the biliary tract system. The late diagnosis and abysmal prognosis present challenges to treatment. The overall 5-year survival rate for metastatic GBC patients is extremely low. *BRCA1* and *BRCA2* are the breast cancer susceptibility genes and their mutation carriers are at a high risk for cancer development, both in men and women. Olaparib, an oral poly ADP-ribose polymerase inhibitor, has been approved by the Food and Drug Administration and the European Commission for the treatment of ovarian cancer with any *BRCA1/2* mutations. The first case of a *BRCA1*-mutated GBC patient who responded to olaparib treatment is reported here.

Key words: *BRCA*; Mutation; Olaparib; Poly ADP-ribose polymerase inhibitor; Gallbladder cancer

Core tip: Gallbladder cancer (GBC) is the most common neoplasm of the biliary tract system. *BRCA1*, the first major breast cancer susceptibility gene, has been widely studied in breast and ovarian cancers. Olaparib, an oral poly ADP-ribose polymerase (PARP) inhibitor, has been approved by the Food and Drug Administration and the European Commission for the treatment of ovarian cancer with any *BRCA1/2* mutations. However, there is no report of a germline *BRCA1* functional mutation in GBC prior to this case. Even further, the GBC with a *BRCA1* mutation responded to the PARP inhibitor olaparib.

Xie Y, Jiang Y, Yang XB, Wang AQ, Zheng YC, Wan XS, Sang XT, Wang K, Zhang DD, Xu JJ, Li FG, Zhao HT. Response of *BRCA1*-mutated gallbladder cancer to olaparib: A case report. *World J Gastroenterol* 2016; 22(46): 10254-10259 Available from: URL: http://www.wjgnet.com/1007-9327/full/v22/i46/10254.htm DOI: http://dx.doi.org/10.3748/wjg.v22.i46.10254

INTRODUCTION

Gallbladder cancer (GBC) derives from the mucosal epithelial lining of the gallbladder and the cystic duct. It is a relatively rare malignancy, but is the most frequent malignant neoplasm of the biliary tract system. Epidemiological studies have demonstrated that the incidence of GBC is characterized by remarkable geographic distribution and ethnic disparities. The incidence is extraordinarily high in American Indians, elevated in Southeast Asia and quite low elsewhere in the Americas[1]. Although GBC limits in Southeast Asia, with increasing global migration, the incidence is also increasing in the west, and spreads worldwide. The prognosis of GBC is dismal and the median survival for locally advanced GBC with non-surgical treatment is about 8 mo[2]. Some patients detected incidentally during routine cholecystectomy for cholelithiasis have a long-term survival, but they only account for 2% of all cases with GBC[3]. Clinically, the adjuvant treatment for GBC is gemcitabine or 5-fluorouracil-based chemotherapy, with or without radiotherapy[4]. Even though the response rate remains low, there is no effective treatment. Here we report that a *BRCA1*-mutated GBC patient responded to the poly ADP-ribose polymerase inhibitor (PARPi) olaparib.

CASE REPORT

A 74-year-old man, with a past history of primary hypertension, atrial fibrillation, coronary disease and cholelithiasis, presented with epigastric pain. The patient underwent a robot-assisted prostate cancer surgery on November 29, 2013, and his mother had died of esophageal cancer. Computed tomography (CT) of the abdomen revealed multiple low-density intrahepatic lesions as well as the gallbladder lesion on May 7, 2015. PET-CT revealed multiple hypermetabolic intrahepatic lesions apart from the porta hepatis on May 14, 2015. A laparoscopic exploration was performed and an intrahepatic biopsy was conducted on May 26, 2015. Histologic examination indicated GBC (Figure 1). Considering the dismal prognosis and his poor physical condition, systemic chemotherapy was not preferred. After having obtained consent from the patient and his family, we tested the tissue. Two specimens from different liver metastases and a blood sample were sent for next generation sequencing panel. We detected all genomic alteration types on over 390 genes commonly associated with cancers and found a somatic *MET* P1086A mutation in one of two liver metastases, but there was no literature to confirm this was a functional mutation. Bioinformatics analysis also suspected *MET* P1086A could have an impact on MET function. However, we also detected a germinal *BRCA1* Q858* mutation in both liver metastases and further Sanger sequencing confirmed this result (Figure 2). Furthermore, the patient's offspring and siblings also had been screened for *BRCA* mutation from their saliva samples, and some family members were also *BRCA1* Q858* mutation carriers

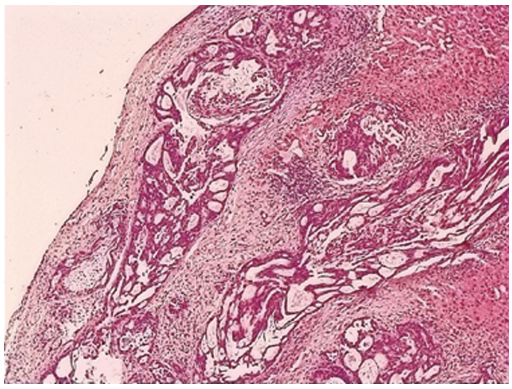

Figure 1 Histologic examination indicated gallbladder cancer with hepatic infiltration.

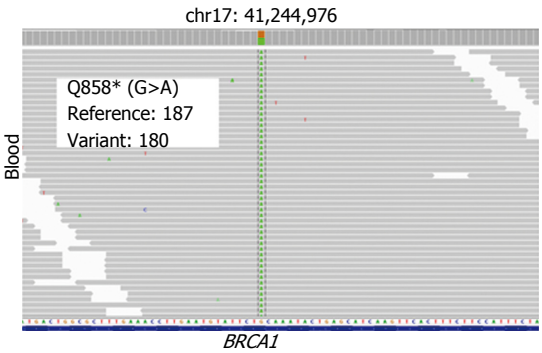

Figure 2 Genomic images from the integrated genome viewer for the alteration in *BRCA1* found in the patient's blood sample. The number of reads for the reference allele and variant allele are shown for each alteration.

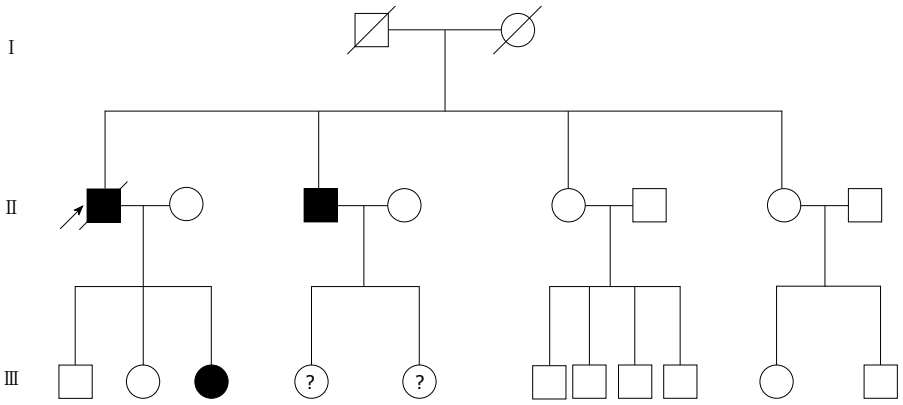

Figure 3 Pedigree of 74-year-old man affected by gallbladder cancer found to be carrier of *BRCA1* gene mutation (indicated with arrow). Black denotes carrier of *BRCA1* mutation.

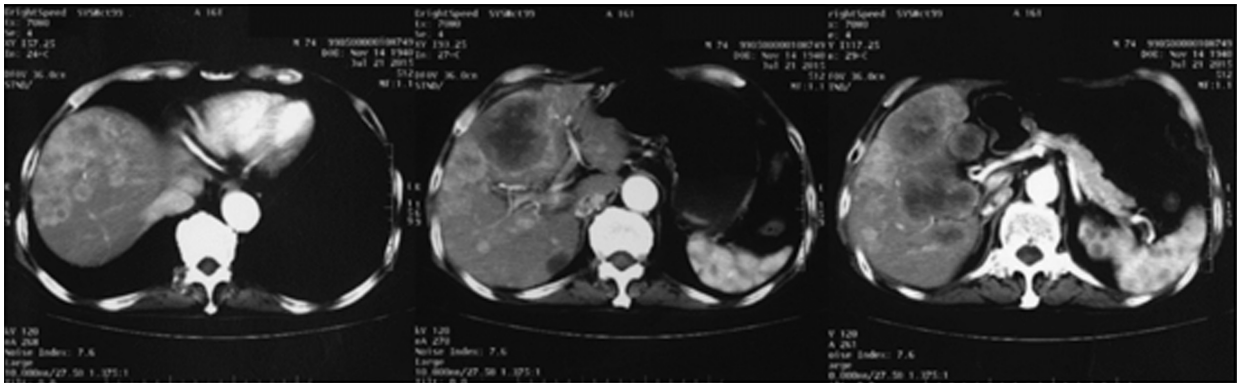

Figure 4 Baseline (July 21, 2015) computed tomography of the abdomen revealed many intra- and extra-hepatic lesions before initiating olaparib treatment.

(Figure 3). The nonsense mutation may lead to the premature termination of BRCA1 protein translation and nonsense-mediated mRNA decay, and the loss-of-function disenables its involvement in transcriptional regulation of gene expression and repair of DNA damage, particularly double-strand breaks[5]. Several studies have demonstrated that *BRCA1* mutations increase the risks of breast, ovarian, prostate and pancreatic cancer[5-7]. Poly ADP-ribose polymerase (PARP) inhibitors have been studied as potential cancer therapeutics by means of inhibiting base excision repair (BER) as well as by trapping PARP[8,9]. A number of clinical trials have shown patients with germline *BRCA1/2* mutations, especially in breast and ovarian cancer, to receive PARP inhibitor olaparib with survival benefit[10-12]. Based on the gene alteration testing report and the clinical trial studies, the patient was started on olaparib 400 mg twice daily on July 21, 2015 (Figure 4). The patient could tolerate the dose, and subsequently his pain was relieved significantly. On August 23, 2015, CT of the abdomen revealed the shrinkage of both intra- and extra-hepatic lesions and some extra-hepatic lesions even appeared to be invisible (Figure 5). The patient responded well to olaparib until the occurrence of obstructive jaundice. On October 9, 2015, CT of the abdomen indicated that intrahepatic lesions had dwindled; nevertheless, extrahepatic lesions became large and progressed (Figure 6). Subsequently, percutaneous transhepatic cholangiodrainage was performed to reduce the serum bilirubin level and the olaparib treatment was suspended from that time. We intended to resume olaparib treatment in combination with platinum agents at a later date. Unfortunately, the patient passed away as a result of severe biliary tract infection on November 25, 2015.

DISCUSSION

Like other cancers, substantial molecular alterations in genes contribute to the pathogenesis of GBC. Hitherto, in GBC, over 1450 single nucleotide variants, 34 deletions have been reported. The most frequent mutations are *TP53* (18%-63%), *KRAS*, *ERRB3* and *ERBB2* (*HER2*)[13]. *BRCA1*, the first major breast cancer susceptibility gene, has been widely studied in breast and ovarian cancers. However, there is no report of germline *BRCA1* functional mutation in GBC prior to this case. Even further, the GBC with a *BRCA1* mutation responded to the PARP inhibitor olaparib.

Association of *BRCA1/2* mutations with susceptibility to breast and ovarian cancer has been investigated for

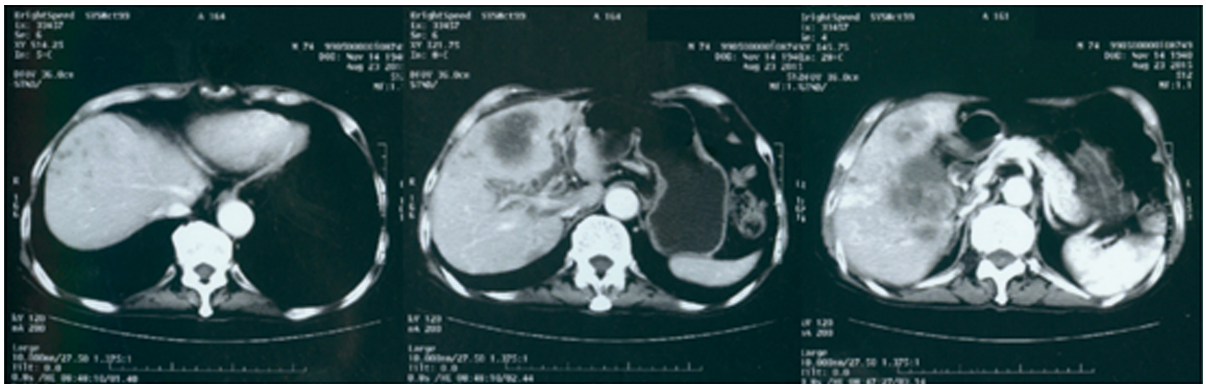

Figure 5 One month post-olaparib treatment (August 23, 2015). Computed tomography of the abdomen revealed shrinkage in both the intra- and extra-hepatic lesions and extra-hepatic lesions even appeared to be invisible.

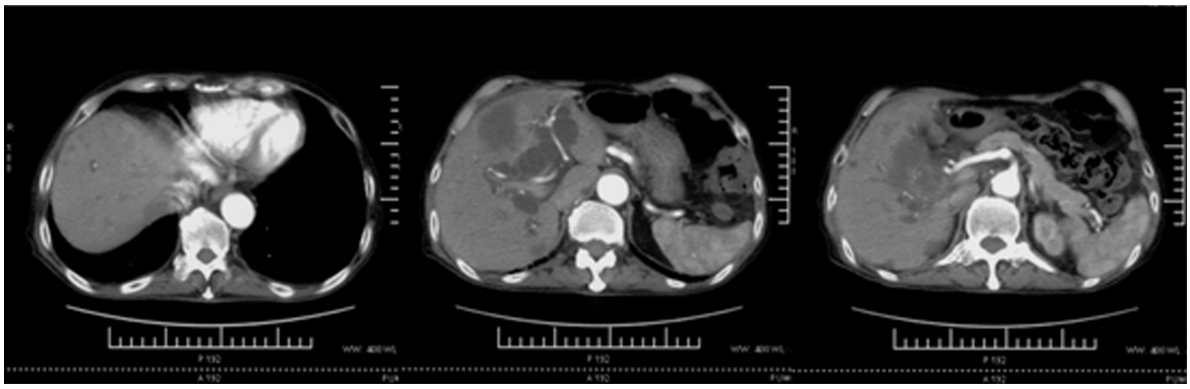

Figure 6 Two and half months post-olaparib treatment (October 9, 2015). Computed tomography of the abdomen indicated that intrahepatic lesions dwindled; nevertheless, extrahepatic lesions became large and progressed.

years. It is estimated that about 60% of women with *BRCA1/2* mutations have developed breast cancer[14]. A woman who carries a germline *BRCA1/2* mutation could be 5 times more likely to develop breast cancer than one who does not carry any BRCA1/2 mutation[15]. Men who have *BRCA1/2* mutations are more likely to have prostate or pancreatic cancers. Men are 3.5 times and 8.6 times more likely to develop prostate cancer for *BRCA1* and *BRCA2* mutation carriers by age 65, respectively[16]. Similar to prostate cancer, *BRCA1/2* poses a risk of pancreatic cancer development. Overall, *BRCA1* mutation increases the risk by 0- to 4.11-fold, while the *BRCA2* mutation increases the risk by 2.13- to 21.7-fold[17].

The BRCA proteins play a pivotal role in repair of double-strand DNA breaks *via* homologous recombination (HR). Due to deficiency in BRCA proteins, *BRCA*-mutated cells are not capable of locating the DNA recombinase RAD51 to damaged DNA and hence are unable to perform HR efficiently. Subsequently, an error-prone DNA repair mechanism, such as non-homologous end joining, is compelled to be used by cells, which often leads to cell death. BER, as one of the single-strand DNA break repair mechanisms, is crucial to address damaged single-strand DNA. Olaparib is an oral PARPi, and it was approved by the Food and Drug Administration and European Commission for the treatment of ovarian cancer with any *BRCA1/2* mutations in 2014. Olaparib, by means of blocking BER, can convert single-strand DNA breaks to double-strand breaks, which gives rise to selective death of HR-deficient tumor cells. Mounting evidence has indicated that *BRCA*-mutated cancers are highly sensitive to PARP inhibitors and platinum agents. Compared with wild-type cells, *BRCA*-mutated cells are 1000-fold and 5-fold more sensitive to PARPi and platinum agents, respectively[18,19].

In this case, we observed that the intrahepatic lesions had a favorable response to olaparib, while the extrahepatic lesions had a progression with the emergence of olaparib resistance. Despite the fact that olaparib holds considerable promise in targeted therapies for *BRCA*-mutated breast or ovarian cancers, drug resistance has became a potential issue. So far, several resistance mechanisms have been proposed. Olaparib-triggered secondary *BRCA* mutations are perhaps considered as the most well-validated mechanism in patients; others include up-regulation of PgP transporter, loss of 53BP1 as well as PARP expression[20-22]. The comprehensive genomic alteration testing may provide novel clinical strategies for personalized therapy in advanced GBC. More

mechanisms regarding chemoresistance are expected to be explored and understood in the future, which will help develop strategies to re-sensitize tumor cells to PARPi and improve the long-term effectiveness.

COMMENTS

Case characteristics

A 74-year-old man, with a past history of primary hypertension, atrial fibrillation, coronary disease and cholelithiasis, presented with epigastric pain.

Clinical diagnosis

The physical examination revealed tenderness of the epigastrium, without rebound tenderness and muscle tonus.

Differential diagnosis

Hepatocellular carcinoma, intrahepatic cholangiocarcinoma, metastatic lesions of non-hepatic origins, gallbladder cancer (GBC).

Laboratory diagnosis

The blood test for tumor markers revealed elevation of carbohydrate antigen 19-9 (4815.0 U/mL) and carcinoembryonic antigen (12.5 ng/mL), while alpha-fetoprotein and prostate specific antigen were within normal limits. The blood test for liver function revealed elevation of total bilirubin (23.0 μmol/L) and direct bilirubin (9.2 μmol/L), while alanine aminotransferase was within normal limits and the test for hepatitis virus was negative.

Imaging diagnosis

Computed tomography (CT) revealed multiple low-density intrahepatic lesions as well as the gallbladder lesion. Positron emission tomography-CT (PET-CT) revealed multiple hypermetabolic intrahepatic lesions apart from the porta hepatis.

Pathological diagnosis

Pathological examination revealed GBC with hepatic infiltration.

Treatment

The patient underwent a laparoscopic exploration and an intrahepatic biopsy. Two specimens from different liver metastases and a blood sample were sent for next generation sequencing panel. A germinal *BRCA1* Q858* mutation in both liver metastases was detected and further Sanger sequencing confirmed this result. Based on the gene alteration testing report and the clinical trial studies, the patient was started on olaparib 400 mg twice daily.

Related reports

There is no report of germline *BRCA1* functional mutation in GBC prior to this case. Even further, the GBC with a *BRCA1* mutation responded to the poly ADP-ribose polymerase (PARP) inhibitor olaparib.

Term explanation

BRCA1, the first major breast cancer susceptibility gene, has been widely studied in breast and ovarian cancers; their mutation carriers are at a high risk for cancer development. Olaparib, an oral PARP inhibitor (PARPi), has been approved by the Food and Drug Administration and European Commission for the treatment of ovarian cancer with any *BRCA1/2* mutations.

Experiences and lessons

This case report describes the response of a germline *BRCA1*-mutated GBC patient to the PARPi olaparib. While the comprehensive genomic alteration testing may provide novel clinical strategies for personalized therapy in advanced GBC, drug resistance has become a potential issue. More discoveries concerning the mechanisms for chemoresistance will help develop strategies to re-sensitize tumor cells to PARPi and improve the long-term effectiveness.

Peer-review

This is a very interesting case report. In this manuscript, the authors reported a 74-year-old man, with a past history of primary hypertension, atrial fibrillation, coronary disease and cholelithiasis, who presented with epigastric pain.

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CASE REPORT

Schwannoma in the hepatoduodenal ligament: A case report and literature review

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Author contributions: Xu SY collected case data and prepared the photos; Sun K proofread the pathologic materials; Xu SY wrote the manuscript; Xie HY, Zhou L, Zheng SS and Wang WL proofread and revised the manuscript; all authors approved the final version to be published.

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Institutional review board statement: The study was reviewed and approved by the Institutional Review Board of the First Affiliated Hospital, School of Medicine, Zhejiang University, Hangzhou, China.

Informed consent statement: Informed consent was obtained from the patient.

Conflict-of-interest statement: The authors declare that there is no conflict of interest related to this report.

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Abstract

Schwannomas are mesenchymal neoplasms with low malignant potential that arise from Schwann cells. They can occur almost anywhere, although the most common

locations are the head, neck and extremities. Primary benign schwannoma of the hepatoduodenal ligament is rare. To date, only three cases have been reported in the English literature. In the present study, we report a case of hepatoduodenal ligament schwannoma in a 43-year-old male, who was admitted to our hospital because of a abdominal mass found by physical examination. It was hard to determine the definitive location and diagnosis of the mass using ultrasound, computed tomography and magnetic resonance cholangiopancreatography. During laparotomy, the mass was found in the hepatoduodenal ligament and close to the cholecystic duct, so we resected the gallbladder and cholecystic duct along with the mass. The gross specimen revealed an 8.5 cm × 5.5 cm × 3.0 cm localized tumor. Microscopic examination showed that the tumor was mainly composed of spindle-shaped cells. Immunohistochemical staining showed a strong positive S-100 protein reaction. Finally, the lesion was diagnosed as a benign schwannoma in the hepatoduodenal ligament. However, one month later, the patient was readmitted to our hospital because of skin and sclera jaundice caused by common bile duct stenosis without common bile duct stone or tumor. The patient recovered well after implantation of a common bile duct stent under endoscopic retrograde cholangiopancreatography. He was followed up for a period of 17 mo, during which he was well with no complications.

Key words: Schwannoma; Hepatoduodenal ligament; Endoscopic retrograde cholangiopancreatography; Laparotomy; Jaundice

Core tip: To date, only three cases of the hepatoduodenal ligament schwannomas have been reported in the English literature. We present the fourth hepatoduodenal ligament schwannoma. It is challenging to determine the location and obtain a precise diagnosis prior to operation. Following complete tumor excision, patients with benign schwannomas generally have a good prognosis. Common bile duct stenosis after resection of the schwannoma in hepatoduodenal ligament has not been reported and we present the first one cured by implanting a common bile duct stent under endoscopic retrograde cholangiopancreatography. We also conduct a literature review so as to deepen the understanding of the subject.

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INTRODUCTION

Schwannomas are neurogenic tumors originating from the Schwann cells in nerve sheaths[1]. They can occur in patients at all ages with no obvious gender difference. As revealed by cytogenetic analysis, most schwannomas showed either monosomy 22 or loss of 22q material[2]. More than 90% of schwannomas are benign and comprise about 5% of benign soft-tissue neoplasms[3,4]. They can arise in almost every location, although the most common sites are the head, neck and extremities[5]. Schwannomas in the hepatoduodenal ligament are uncommon and only three cases have been reported in the English literature[6-8]. Patients with schwannomas in the hepatoduodenal ligament are normally asymptomatic and the tumors are found incidentally. We present a case of hepatoduodenal ligament schwannoma in a 43-year-old male and review the literature. He is believed to be the first patient with subsequent common bile duct stenosis after complete removal of hepatoduodenal ligament schwannoma and to be cured by implantation of a common bile duct stent under endoscopic retrograde cholangiopancreatography (ERCP).

CASE REPORT

On November 21, 2014, a 43-year-old male was admitted to our hospital for physical examination. His abdomen was soft, lax and nondistended without evidence of a palpable mass. His family history had no significant disease. Laboratory results were normal. Ultrasound (US) revealed an 8.3 cm × 5.2 cm, well-defined hypodense lesion, between the pancreatic head and portal vein. No blood flow signal was found within the mass by Color Doppler US. An unenhanced computed tomography (CT) scan showed an 8.2 cm × 5.1 cm well-defined cystic and solid mass above the pancreatic head and adjacent to the common hepatic artery. The pancreaticoduodenal artery was compressed by the mass. On contrast-enhanced CT, the mass showed no obvious enhancement (Figure 1B). Computed tomography angiography showed that the blood supply of the tumor was probably from the branches of the pancreaticoduodenal artery (Figure 1C). Magnetic resonance cholangiopancreatography (MRCP) showed that the mass was inhomogeneous and hyperintense on T2-weighted images and probably located in the pancreatic head, and the middle-low segment of the common bile duct was compressed (Figure 2). According to the imaging examinations, an abdominal mass was primarily considered.

After sufficient preoperative preparation, exploratory laparotomy was performed. We found a mass surrounded by a fibrous capsule in the hepatoduodenal ligament, closely adjacent to the gallbladder, cholecystic duct, common bile duct, portal vein, right hepatic artery, duodenum and postcava, without biliary

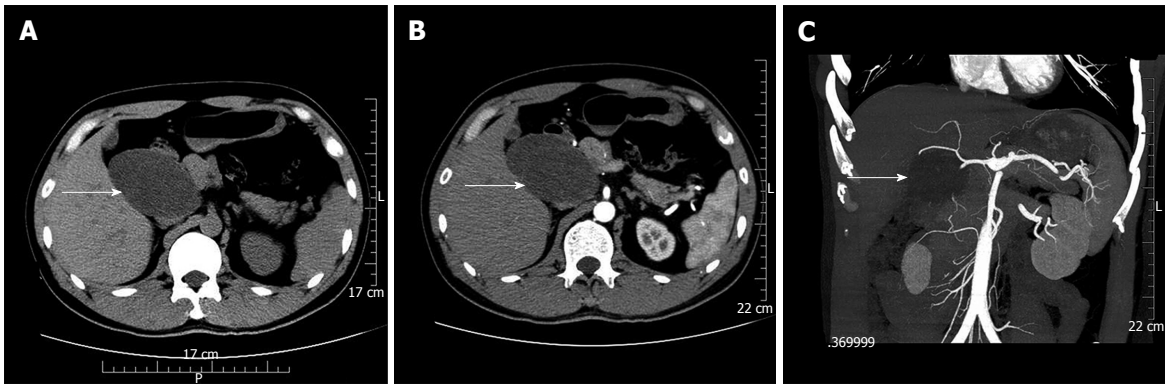

Figure 1 Computed tomography findings. A: An unenhanced computed tomography (CT) scan showed an 8.2 cm × 5.1 cm well-defined cystic and solid mass (arrow) above the pancreatic head and adjacent to the common hepatic artery; B: On contrast-enhanced CT, the mass (arrow) showed no obvious enhancement; C: CT angiography showed that the tumor blood supply (arrow) was probably from branches of the pancreaticoduodenal artery.

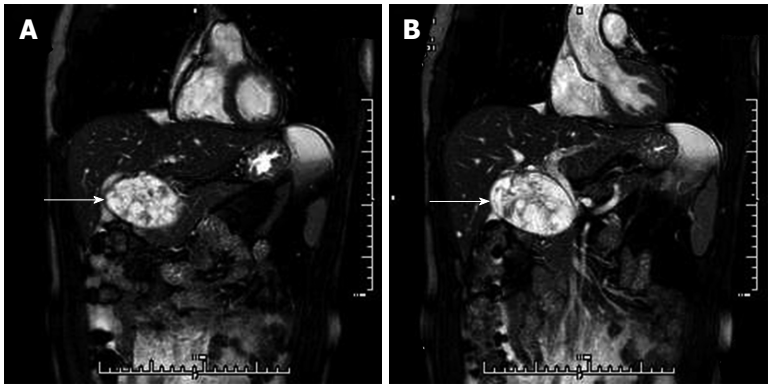

Figure 2 Magnetic resonance cholangiopancreatography findings. A: Magnetic resonance cholangiopancreatography (MRCP) showed that the mass (arrow) was inhomogeneous and hyperintense on T2-weighted images and probably located in the pancreatic head; B: The middle-low segment of the common bile duct was compressed.

duct dilatation. The tumor blood supply was mainly from the surrounding vessels of the duodenum. We carefully separated these tissues around the tumor and ligated the tumor blood vessels. However, the mass and cholecystic duct were too close to separate, so we resected the gallbladder and cholecystic duct along with the mass. Intraoperative frozen-section pathology could not offer an accurate diagnosis and only suggested a soft-tissue tumor.

Macroscopically, there was a mass in the hepatoduodenal ligament 8.5 cm × 5.5 cm × 3.0 cm in size and yellowish-white in color. Microscopically, the tumor had a capsule that was adjacent to the cholecystic duct (Figure 3A) and mainly consisted of spindle-shaped cells with no atypia, compatible with a benign schwannoma with both hypercellular and hypocellular areas visible (Figure 3B). Immunohistochemical investigation showed that protein S-100 was positive (Figure 3C), while CD34 (Figure 3D), CD117 and smooth muscle actin (SMA) were negative. Finally, the tumor was diagnosed as schwannoma in the hepatoduodenal ligament. After surgery, the patient recovered uneventfully and left the hospital 6 d later.

However, one month later, the patient was readmitted to our hospital because of skin and sclera jaundice without abdominal distension, abdominal pain, fever, nausea and vomiting. Laboratory results were: total bilirubin 113 μmol/L (0-21), direct bilirubin 76 μmol/L (0-5), indirect bilirubin 37 μmol/L (3-14), aspartate transaminase 301 U/L (8-40), alanine transaminase 543 U/L (5-35), alkaline phosphatase 452 U/L (40-150), γ-glutamyl transpeptidase (GGT) 441 U/L (11-50) and creatinine 90 μmol/L (45-84). No other abnormal laboratory results were found.

US showed that the intra- and extrahepatic bile ducts were expanded. The diameter of the initial segment of the common bile duct was 1.1 cm with no mass or stones in the duct. MRCP showed that the middle common bile duct segment was narrow and even interrupted, while the higher common bile duct segment and intrahepatic bile ducts were expanded (Figure 4). So, the patient was diagnosed with jaundice caused by common bile duct stenosis. Under ERCP, we implanted a stent into the strictured common bile duct (Figure 5). One day later, the patient recovered well and left our hospital. He was followed up for 17 mo,

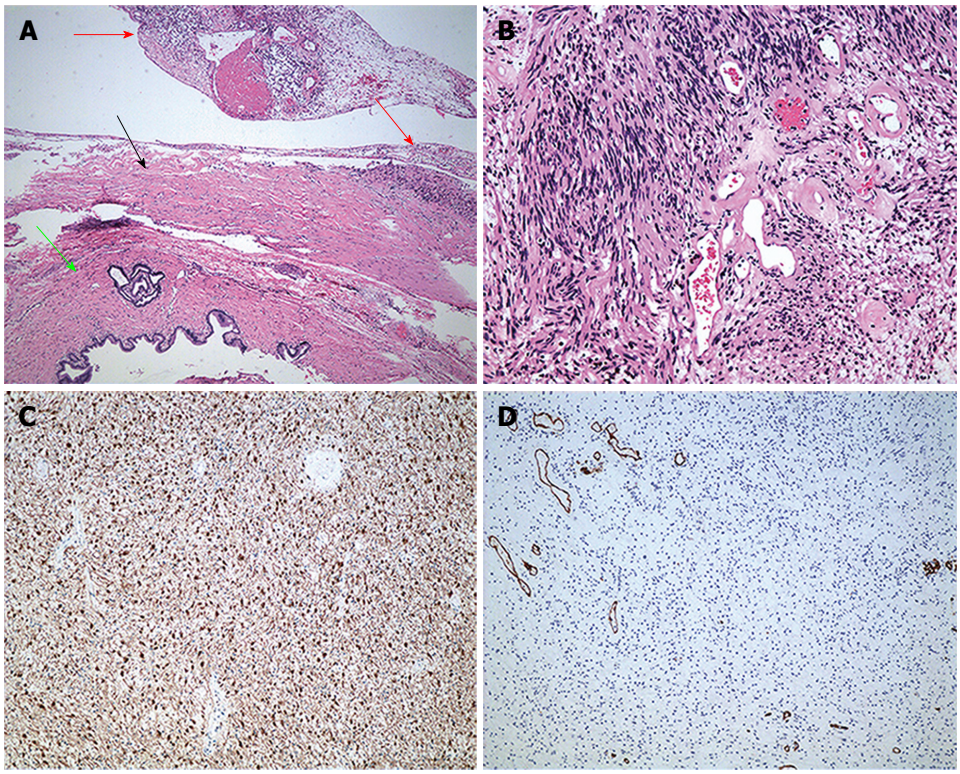

Figure 3 Microscopic examination and immunohistochemical staining. A: Microscopically, the tumor (red arrow) with a capsule (black arrow) was adjacent to the cholecystic duct (green arrow) (HE, × 200); B: The tumor mainly consisted of spindle-shaped cells with both hypercellular and hypocellular areas (HE, × 200). Immunohistochemical investigation showed that the tumor was positive for protein S-100 (C) and negative for CD34 (D) (HE, × 100). HE: Hematoxylin and eosin.

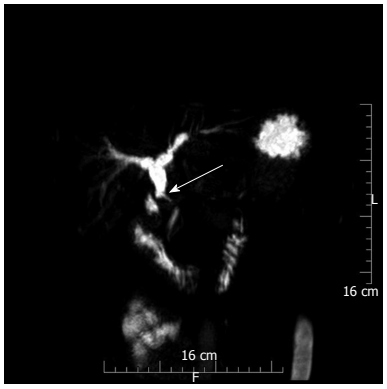

Figure 4 Magnetic resonance cholangiopancreatography findings after surgery. Magnetic resonance cholangiopancreatography showed that the middle common bile duct segment was narrow and even interrupted (arrow), while the higher common bile duct segment and intrahepatic bile ducts were expanded.

during which, he was well with no complications.

DISCUSSION

Schwannomas are neoplasms that originate from Schwann cells of nerve sheaths[9]. More than 90% of schwannomas are benign and comprise only approximately 5% of benign soft-tissue neoplasms[5]. Schwannomas can occur in patients at any age with no significant gender difference, but are most commonly found in patients between 20 and 50 years old[5]. They can arise almost anywhere, although the head, neck and extremities are the most common sites[10]. In the abdominal cavity, the retroperitoneum (6% of primary retroperitoneal tumors)[11] and stomach[12] are the most frequently involved sites. However, schwannomas in the ligaments[13], bowel mesentery[14] and abdominal organs including the gallbladder[15], pancreas[16] and liver[17] are rare. To the best of our knowledge, only three cases of schwannoma in the hepatoduodenal ligament have been reported[6-8]. The clinical characteristics of these cases including the present one are shown in Table 1. One patient was female and the other three patients were male, aged 62, 29, 50 and 43 years, respectively (mean age, 46 years). One patient presented with pain in the right abdomen following trauma and a mass in the hepatoduodenal ligament was occasionally found by imaging. The remaining patients were all asymptomatic and the masses were found by routine physical examination. Although every patient received more than two imaging examinations, none was accurately diagnosed as schwannoma in the hepatoduodenal ligament preoperatively.

Accurate preoperative diagnosis of the tumor is a huge challenge because neither the clinical symptoms nor the radiological characteristics of schwannomas are specific. Definitive diagnosis can only be determined by histopathological and immunohistochemical examinations of surgical specimens. Schwannomas are encapsulated tumors that consist of hypercellular

Table 1 Clinical characteristic of the four patients with benign schwannoma in the hepatoduodenal ligament

Ref.	Year	Sex/age	Symptom	Imaging method	No.	Size (cm)	Preoperative diagnosis	Treatment	Follow-up (mo)	Status
Nagafuchi *et al*[6]	1993	F/62	Asymptomatic	US, CT, ERC, CA	Solitary	9 × 5 × 4.5	NA	Laparotomy	26	Survived
Pinto *et al*[7]	2011	M/29	Asymptomatic	US, endoscopy, US, biopsy, MRI	Solitary	4.5 × 2.9	Spindle cell neoplasia or stromal tumor	Laparotomy	NA	NA
Tao *et al*[8]	2016	M/50	Right abdominal pain	US, CT	Solitary	4.5 × 2.5 × 2.5	Stromal tumor	Laparoscopic surgery	7	Survived
Present case	2016	M/43	Asymptomatic	US, CT, MRCP, CTA, ERCP	Solitary	8.5 × 5.5 × 3.0	Abdominal mass	Laparotomy	17	Survived

NA: Not available; US: Ultrasound; CT: computed tomography; MRI: Magnetic resonance imaging; MRCP: Magnetic resonance cholangiopancreatography; CTA: Computed tomography angiography; ERC: Endoscopic retrograde cholangiography; CA: Celiac angiography; ERCP: Endoscopic retrograde cholangiopancreatography.

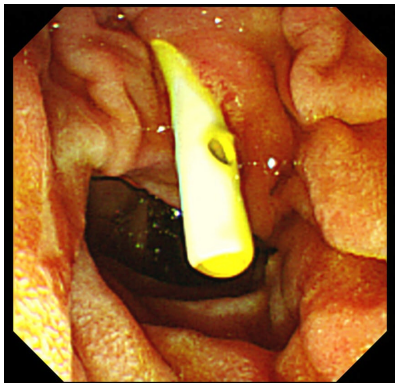

Figure 5 Endoscopic retrograde cholangiopancreatography. Under Endoscopic retrograde cholangiopancreatography, a stent was implanted into the strictured common bile duct.

(Antoni type A) and hypocellular (Antoni type B) areas, with varying amounts of these two histological components[8]. The former is composed of closely packed spindle cells with occasional nuclear palisading, as well as Verocay bodies. The latter consists of loosely arranged tumor cells and abundant myxoid stroma. Occasionally, these may degenerate and become cystic[8]. Immunohistochemically, schwannomas are strongly positive for S-100, and negative for desmin, smooth muscle myosin, SMA, CD34 and CD117[18].

Precise diagnosis of these tumors prior to operation is difficult. Multiple imaging modalities including US, CT and magnetic resonance imaging (MRI) can be performed to establish a probable diagnosis. Schwanommas are usually showed as well-defined hypodense lesions by US and no echoic enhancement is demonstrated by Color Doppler US[8]. On unenhanced CT, schwannomas are usually well-defined hypodense lesions with encapsulation and/or cystic degeneration. Schwannomas with high Antoni A areas appear inhomogeneous due to increased lipid content. Antoni B areas of schwannomas appear cystic and multiseptated and show low density due to loose stroma and low cellularity[2]. On contrast-enhanced CT, Antoni A areas are usually enhancing lesions, whereas Antoni B areas are frequently nonenhancing lesions[8]. On MRI, the schwanommas typically appear hypointense on T1-weighted images and inhomogeneous and hyperintense on T2-weighted images[2,19]. By outlining the degree of vascular involvement of the tumor, MRI is also useful to assess the potential biological behavior of these tumors as benign or malignant[19]. Endoscopic US (EUS) is helpful to clarify the location and nature of the mass[7]. In addition, celiac angiography can be used to indicate the arteries supplying the tumor[6]. EUS-fine needle aspiration (FNA) may contribute to precise preoperative diagnosis. In a case reported by Li *et al*[20], a pancreatic schwannoma was accurately diagnosed preoperatively by EUS-FNA. In another report, three cases of asymptomatic retroperitoneal tumors were diagnosed as benign schwannomas by EUS-FNA, thus avoiding surgical resection[21].

Surgery can demonstrate the tumor site and be curative. In the present case, we found by laparotomy that the mass was located in the hepatoduodenal ligament and adjacent to important tissues and organs including the gallbladder, cholecystic duct, common bile duct, duodenum and postcava. The tumor vascular supply was mainly from the surrounding vessels of the duodenum. We carefully separated these tissues around the tumor and ligated the blood vessels. However, the mass and cholecystic duct was too close to separate, so the gallbladder and cholecystic duct were removed completely along with the tumor. Histopathological and immunohistochemical examinations of surgical specimens showed a schwannoma in the hepatoduodenal ligament. However, 1 mo later, the patient was readmitted to our hospital because of jaundice and diagnosed with obstruction of the common bile duct without a mass or stones in the duct. Fortunately, the patient was cured by implantation of a common bile duct stent under ERCP.

In conclusion, schwannoma in the hepatoduodenal ligament is rare. We have presented the fourth hepatoduodenal ligament schwannoma. It is a challenge to determine the location and obtain a precise diagnosis prior to surgery, although multiple imaging modalities

are used. Following complete tumor excision, patients with benign schwannomas generally have good prognosis. Common bile duct stenosis after resection of schwannoma in the hepatoduodenal ligament has not been reported previously. We have presented the first case to be cured by implantation of a common bile duct stent under ERCP.

COMMENTS

Case characteristics

A 43-year-old man was referred to our hospital because of an abdominal mass found by physical examination.

Clinical diagnosis

The abdomen was soft, lax and nondistended without evidence of a palpable mass.

Differential diagnosis

Abdominal sarcoma, abdominal neurogenic tumor, pancreatic cancer and cholangiocarcinoma.

Laboratory diagnosis

Before surgery, laboratory results were normal.

Imaging diagnosis

Ultrasound (US) revealed an 8.3 cm × 5.2 cm, well-defined hypodense lesion between the pancreatic head and portal vein. No blood flow signal was found within the mass by Color Doppler US. An unenhanced computed tomography (CT) scan showed an 8.2 cm × 5.1 cm well-defined cystic and solid mass above the pancreatic head and adjacent to the common hepatic artery. The pancreaticoduodenal artery was compressed by the mass. On contrast-enhanced CT, the mass showed no obvious enhancement. Computed tomography angiography showed that the blood supply of the tumor was probably from branches of the pancreaticoduodenal artery. Magnetic resonance cholangiopancreatography showed that the mass was inhomogeneous and hyperintense on T2-weighted images and probably located in the pancreatic head, with compression of the middle-low segment of the common bile duct. According to imaging examinations, an abdominal mass was primarily considered.

Pathological diagnosis

Microscopically, the tumor had a capsule and was adjacent to the cholecystic duct (Figure 3A), and mainly consisted of spindle-shaped cells with no atypia, compatible with a benign schwannoma with both hypercellular and hypocellular areas. Immunohistochemical investigation showed that the tumor was positive for protein S-100, but negative for CD34, CD117 and smooth muscle actin. Finally, the tumor was diagnosed as a schwannoma in the hepatoduodenal ligament.

Treatment

The patient underwent complete resection of the gallbladder and cholecystic duct along with the tumor in the hepatoduodenal ligament.

Related reports

Schwannoma in the hepatoduodenal ligament is rare. To date, only four cases have been reported in the English literature, including our case presented in this report.

Experiences and lessons

It is a challenge to determine the location and obtain a precise diagnosis prior to surgery, although multiple imaging modalities are used. Following complete tumor excision, patients with benign schwannomas generally have good prognosis. Common bile duct stenosis after resection of the schwannoma in hepatoduodenal ligament has not been reported and we present the first case to be cured by implantation of a common bile duct stent under endoscopic retrograde cholangiopancreatography.

Peer-review

This study highlights the diagnosis and treatment of a rare schwannoma in hepatoduodenal ligament and the authors also conducted a literature review so as to deepen the understanding of the subject. The information of this paper is valuable to the readers.

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